Developing new methods to identify airborne proteins responsible for causing allergic asthma

by

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A thesis submitted for the degree of Doctor of Philosophy.

March 2019

Abstract

Allergic diseases such as asthma and hayfever are the most common chronic disease in European children and adults and occur in response to substances present in the environment, referred to as allergens. Allergens are present in indoor, outdoor and occupational environments. In most cases, the allergen responsible for allergic symptoms will be identified using diagnostic assessments such as skin prick testing; however, there are examples of individuals experiencing allergic symptoms where the allergen responsible has not yet been identified. This is particularly common in the occupational environment.

In this PhD I aimed to explore methods used to identify proteins highly likely to be allergens and responsible for causing allergic asthma. I conducted a systematic review on asthma outbreaks to describe which laboratory methods have been used to identify airborne allergens. I then developed and optimised my own laboratory methods to identify airborne proteins that could be responsible for causing allergic asthma.

I found that air sampling, skin prick testing and detection of serum specific-IgE binding were the most frequent methods used to identify allergens associated with outbreaks of asthma. Using known allergens from mouse urine, I developed and optimised a method to identify unknown allergens. The method consists of an enhanced chemiluminescence western blot and in-gel excision of serum specific-IgE binding regions, for protein identification by nano liquid chromatography coupled to tandem mass spectrometry. Application of this method identified potentially novel allergenic proteins existing within different occupational settings. In animal testing facilities located in London and Salzburg, allergenic proteins were identified from mouse urine, mouse epithelium and the common fruit fly (*Drosophila melanogaster*). In supermarket scratch bakeries based in the UK allergenic proteins were identified from 'improver' enzymes. I also developed methods for detecting outdoor airborne protein from Teflon and polycarbonate filters used for air sampling.

The registration of allergens on online databases relies on researcher-based identifications and the increase in allergen component-resolved diagnosis means this field is likely to develop rapidly in the coming years. Further work is needed to confirm the allergenicity of the proteins identified in this thesis, that may be responsible for causing allergic asthma.

Declaration of Originality

I declare that the work presented in this thesis is my original work unless otherwise stated or referenced.

Copyright Declaration

Acknowledgements

First and foremost, I would like to thank my supervisors Professor Debbie Jarvis, Dr Meinir Jones and Professor Peter Burney for their unending guidance, support and mentorship throughout my doctoral experience. I am especially grateful to Debbie and Meinir for their unwavering patience in the latter stages of my PhD, to ensure I got my thesis to submission. I would also like to thank my supervisors for enabling me to travel and conduct research in Austria, so I could push the bounds of my professional and personal growth.

A unique and special thank you goes to my laboratory partner – Jennifer Canizales. You have provided me with endless laughter, support and stability during all the highs and lows of my PhD. I can't wait to finally put on those graduation gowns together.

I would also like to thank all the other friends I worked with during my time at EKB. Jennifer Welch for providing an unlimited source of support and entertainment in the laboratory. Hannah, Alex, Laura and Vian for all the absolutely necessary lunch and coffee breaks.

To the allergen proteomics group and Professor Peter Briza in Salzburg, Austria I would like to thank you. I so enjoyed the 3 months I spent with you all in the laboratory and outside work tasting traditional Austrian cuisine and spending time in the beautiful alpine landscape.

I'd like to acknowledge my funders, the MRC-PHE Centre for Environment & Health and the National Heart & Lung Institute for providing me with the opportunity to study at Imperial College London.

Finally, I would like to thank my parents, friends and loved ones and especially my dad. Thank you for encouraging me to begin this and supporting me unconditionally to the finish.

Abbreviations

AHC	Ammonium hydrogen carbonate
ALLOHA	ALLergen + Oxidative particles + Hospitalisation with Airway disease
AP	Alkaline phosphatase
APC	Antigen presenting cell
BCIP	5-bromo-4-chloro-3'-indolyphosphate
BSA	Bovine serum albumin
CID	Collision induced disassociation
CIE	Counterimmunoelectrophoresis
dH_2O	Distilled water
DMF	Dimethylformamide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ESI	Electrospray ionisation
FEIA	Fluorescent enzyme immunoassay
FTICR	Fourier transform ion cyclotron resonance
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HLA	Human leukocyte antigen
HMW	High molecular weight
HRP	Horseradish peroxide
HPLC	High performance liquid chromatography
IEF	Isoelectro-focusing
IgE	Immunoglobulin-E
IOM	Institute of Occupational Medicine
ISAC	Immuno Solid-phase Allergen Chip
IVC	Individually ventilated cage
IUIS	International Union of Immunological Society

LAA	Laboratory animal allergy
LC	Liquid chromatography
LMW	Low molecular weight
MALDI	Matrix assisted laser desorption ionisation
MHC	Major histocompatibility complex
MUP	Mouse urinary proteins
MS	Mass spectrometry
MW	Molecular weight
m/z	Mass-to-charge ratio
NBT	Nitro-blue tetrazolium
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline, Tween-20
PBS-T/BSA	Phosphate-buffered saline, Tween-20/bovine serum albumin
P-K	Prausnitz-Küstner
PM	Particulate matter
ppm	Parts per millions
PRISMA	Preferred reporting items for systematic reviews and meta-analysis
PSM	Peptide-spectrum match
RAST	Radioallergosorbent assay
RIA	Radioimmunoassay
RT	Room temperature
SDAP	Structural Database of Allergenic Proteins
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SIC	Specific inhalation challenge
SPT	Skin prick testing
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline, Tween-20
TBS-T/BSA	Tris-buffered saline, Tween-20/bovine serum albumin
TEMED	Tetramethyl ethylenediamine
TFA	Tetrafluoro-acetic acid

Th	T-helper cell
TMB	Tetramethyl benzidine
TOF	Time-of-flight
UniProtKB	Universal Protein Database
UPW	Ultrapure water

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1 Introduction

Allergic diseases, such as asthma, hay fever and eczema have increased substantially over the last 70 years and represent the most common chronic disease in European children and adults (EAACI Advocacy Manifesto, June 2015).

Allergy arises from a hypersensitivity reaction initiated by the immune system, in response to substances present in the environment coming from, for example, plants and animals (Palm et al., 2012). These substances are referred to as allergens, and in the UK up to one third of the population will react to ubiquitous environmental allergens such as grass pollen (Team, 2010). Allergens are a substantial cause of morbidity in those people who have the propensity to react to them, when exposed in the indoor, outdoor and occupational environments.

Allergens in the indoor and outdoor environments, known to provoke an asthmatic reaction are commonly derived from animals, plants, mould and fungi (Aalberse, 2000). There are now over 400 agents that have been identified as being responsible for eliciting allergic symptoms in adults in the occupational environment, where allergens may be derived from sources such as chemicals, food and animals (Malo and Chan-Yeung, 2009). The burden of occupational asthma is estimated to account for 5-15% of all adult-onset asthma cases in the general population (Soriano et al., 2017).

When children or adults present to their physicians with allergic symptoms, a detailed clinical history is taken and used in combination with diagnostic assessments including skin prick testing (SPT) and blood tests, to identify the causative agent (Sastre-Ibañez and Sastre, 2015). For many cases, the allergen responsible for allergic symptoms will be identified.

However, there are examples of individuals experiencing allergic symptoms where the causative allergen is not included in routine clinical tests or has not yet been identified as being allergenic. This is particularly common in the occupational environment. For example, allergy to natural rubber latex became well-known only after it gained mainstream use in the medical profession, where it was found that an inhalable fraction containing latex particles could elicit occupational asthma (Czuppon et al., 1993).

There are historical examples of epidemics of asthma occurring within communities. When these occurred, researchers and clinicians were convinced there must be an airborne agent responsible for the sudden peaks in severe asthma, but they were unable to immediately identify the causal allergen. The most recent and spectacular example of this was the collective epidemics of asthma related to soybean exposure in Barcelona (Antó et al., 1989). These epidemics were entirely preventable through reducing the release of soybean dust from ships that were unloading in the harbour (Antó et al., 1993).

It has been hypothesised that 'unknown allergens' may also be responsible for asthma occurring in the community, in outbreaks that are too small to be detected by routine health surveillance methods. Burney et al., (2008) showed that asthma patients who had exacerbations were 'allergic' to particulate air pollution that was collected on the day of their asthma attack. This occurred in patients who were not allergic to pollens or moulds (i.e. the allergens we might most expect to be present on outdoor particles). They proposed that the reported associations of asthma exacerbations with particulate air pollution, could be due to allergens from industrial or other sources that could explain smaller 'outbreaks' of asthma. The precise source and type of allergen that might cause such symptoms was unknown.

In the allergy field, there has been little research on how to identify 'new' airborne allergens which are present in communities or the occupational environment, which may be causing disease.

This PhD will explore methods that can be used in the occupational and outdoor environment to identify novel airborne allergens that are responsible for causing unexplained allergic respiratory symptoms.

1.1 Allergy

Respiratory allergy is a type I hypersensitivity reaction, mediated by the immunoglobulin E (IgE) antibody, which follows the inhalation of airborne allergens (Platts-Mills et al., 2011). The tendency for an individual to produce IgE antibodies that are allergen 'specific' is defined as atopy (Johansson et al., 2001). Individuals may produce specific-IgE to one allergen from one source, multiple allergens from one source, or many allergens from different sources – (also known as polysensitisation) (Migueres et al., 2014). This production of specific-IgE is responsible for allergies of the respiratory tract, which include asthma and rhinitis (also known as hay fever) (Platts-Mills & Woodfolk, 2011).

1.1.1 Allergic sensitisation

An individual is said to be sensitised if initial exposure to an allergen has caused their immune system to produce IgE antibodies that are specific for the allergen to which they are sensitised, which may or may not lead to clinical symptoms of allergy (Galli et al., 2008).

On initial exposure by inhalation, the allergen first makes contact with immune cells at the airway epithelium barrier. Here, the allergen will encounter dendritic cells which are a type of antigen presenting cell (APC). The dendritic cells will capture and phagocytose the allergen, breaking it into peptide fragments which are then transported to the major histocompatibility complex II (MHC-II) on the cell surface and presented to naïve T-cells. The MHC-II/allergen complex binds with a specific receptor on the naïve T-cell which activates proliferation and differentiation of T-helper type 1 (Th1) and T-helper type 2 (Th2) cells (Noti, 2018).

Th2 cells are often associated with "type 2 inflammation", now considered an important molecular mechanism of asthma sympotmology (Calzada et al., 2018). Th2 cells regulate the proliferation of inflammatory mediators, secretion of epithelial cytokines and control the mechanism for which B-cells undergo 'isotype switching' to form IgE-positive B-cells. B-cells are a type of white blood cell that are responsible for producing specific immunoglobulins. IgE-positive B-cells mature into plasma cells that secrete specific-IgE, which circulates and binds with very high affinity to mast cells. This is a pre-requisite of allergic sensitisation, as

mast cells are now primed with specific-IgE for future allergen exposure (da Silva et al., 2014). A schematic of allergic sensitisation is shown in Figure 1-1.

Type 2 innate lymphoid cells (ILC2s) have recently been characterised as important effector cells involved in type 2 inflammation. ILC2s are recruited to the upper airways in patients with atopy and asthma in response to a nasal allergen challenge and correlate with known features of type 2 inflammation, such as baseline total IgE, IL-5 secretion as well as eosinophil numbers, suggesting they play a major role in the allergic response following an allergen provocation (Dhariwal et al., 2017). Other important upstream cytokines involved in type 2 inflammation are epithelial-derived IL-33 and thymic stromal lymphopoietin (TSLP). IL-33 has a sensing role; it can be released in response to damaged airway epithelial cells and induces production of other type 2 cytokines, including IL-4, IL-5. TSLP is able to activate dendritic cells which in turn trigger the Th2 inflammatory response (Mitchell and O-Byrne 2017).

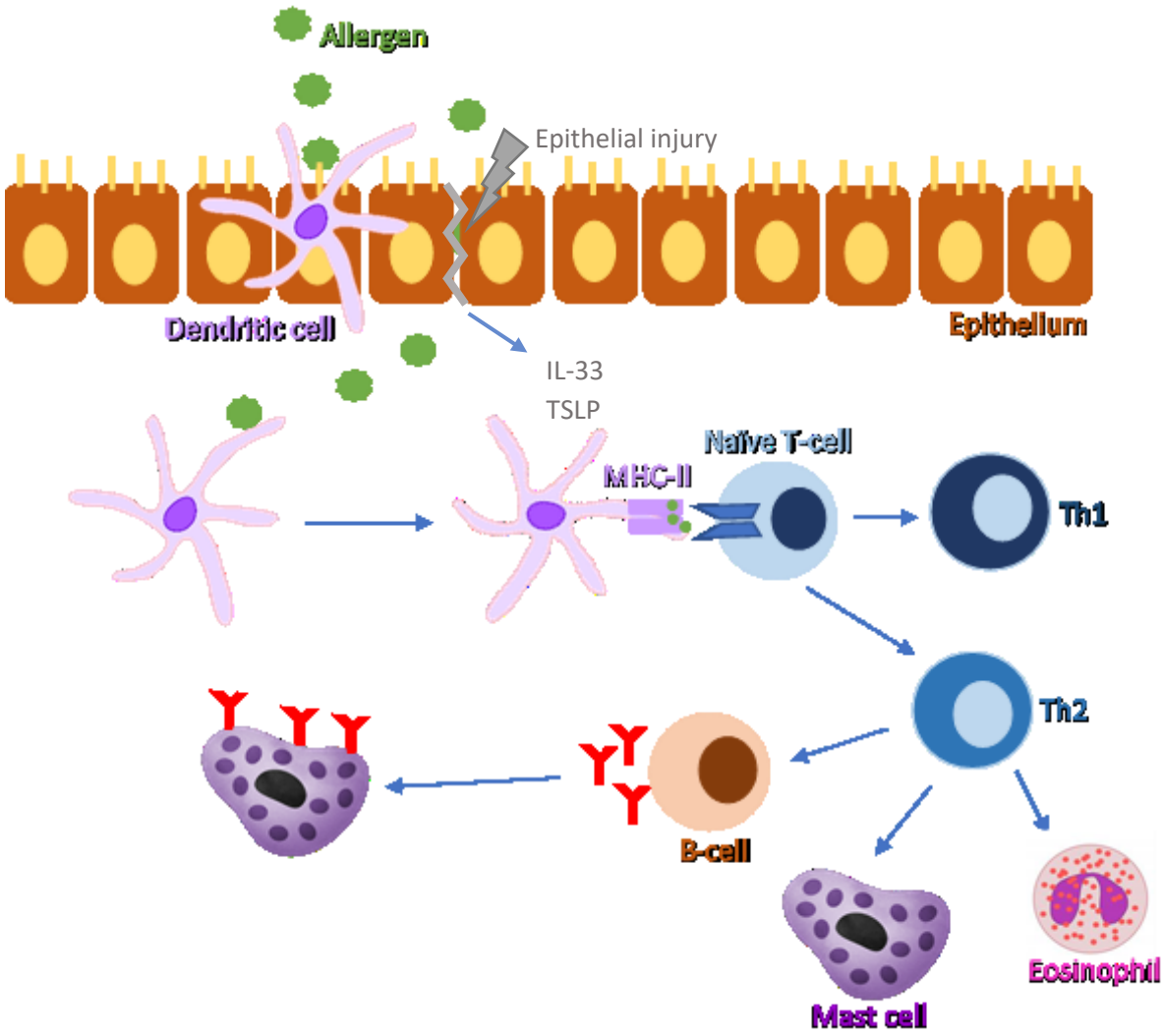


Figure 1-1 Allergic Sensitisation. Allergens will initially make contact with dendritic cells at the epithelial barrier which phagocytose the allergen into peptide fragments. These fragments are transported to the major histocompatibility complex II (MHC-II) on the surface of dendritic cells for presentation to naïve T-cells. Upon binding to a specific receptor on the naïve T-cell, proliferation of T-helper type 1 (Th1) and T-helper type 2 (Th2) is activated. Th2 cells regulate eosinophil and mast cell proliferation and B-cell isotype switching to IgE. B-cells mature into plasma cells which secrete specific IgE (shown in red) which circulates and binds to mast cells which are primed for future allergen exposure. IL: Innate lymphoid cell TSLP: thymic stromal lymphopoietin (Adapted from Oettgen and Broide (2012).

1.1.2 Early and late phase response

On re-exposure, the allergen can bind to specific-IgE that is bound to the surface of mast cells in the blood. The immune system's response on allergen re-exposure is divided into the early phase response and the late phase response.

Within minutes of exposure to an allergen, an acute immediate hypersensitivity reaction can occur. The allergen interacts with specific-IgE bound to mast cells by cross-linkage and specific-IgE undergoes conformational changes which causes degranulation of the mast cell (Janeway et al., 2001). In degranulation, pre-formed mediators including histamine are released by exocytosis. Histamine is capable of mediating some of the fundamental signs of asthma, such as bronchospasm, oedema and mucus secretion (White, 1990). Leukotrienes and prostaglandins are also released by mast cells and have an acute effect on smooth muscle contraction and mucus secretion. This early phase response by mast cell mediators is known to have a critical role in many allergic diseases, such as anaphylaxis, conjunctivitis, urticaria and acute asthma (Golden, 2004).

The late phase response occurs approximately 3-8 hours after allergen exposure and is characterised by infiltration of primarily T-cells and neutrophils, eosinophils, macrophages, cytokines and basophils, to airway epithelial tissues (Gleich, 1982; Oettgen & Broide, 2012). The release of so many inflammatory mediators causes more prolonged and localised inflammation that damages epithelial tissue. If the inflammation occurs frequently then fibrous scar tissue used to repair the area can cause tissue remodelling, resulting in a change of structure and function of the affected area. For example, remodelling of the airways may occur in patients with chronic asthma (Leonardi et al., 2012).

1.1.3 Immunoglobulin E (IgE)

Discovery

The discovery of a serum component responsible for type 1 hypersensitivity reactions was critical in developing our current understanding of allergy. This was achieved by an experiment by Prausnitz & Küstner (1921), who sought to determine whether allergy could be transferred from one individual to another, by the passive transfer of serum. They took serum from Küstner (who was known to develop clinical allergic respiratory symptoms after eating fish) and injected it intracutaneously into Prausnitz's arm, followed by boiled fish extract injected into the same site. They observed an immediate local hypersensitivity reaction, confirming a component within the transferred serum was responsible for the allergic reaction. The test was henceforth termed the 'Prausnitz-Küstner' (P-K) test, or 'Passive-transfer' test and was used to diagnose allergy by the transfer of serum from an allergic individual into another willing and healthy participant. At the time, the uncharacterised serum component causing the reaction was named a 'reaginic' or 'skin-sensitising' antibody. The P-K test has since been abandoned, given concerns around the transmission of diseases through the blood.

Much later, in 1967 the IgE antibody was identified by Kimishige and Teruko Ishikaka. They demonstrated that a positive P-K test could not be ascribed to any of the known antibodies at the time, which were IgM, IgG, IgA or IgD (Ishizaka et al., 1967). At the same time, Johansson and Bennich, (1967) identified an immunoglobulin protein that was able to inhibit the P-K test but was distinct from other known immunoglobulins. These researchers were able to come together and at a WHO International Reference Centre for Immunoglobulins conference in 1968 it was agreed to term the fifth serum immunoglobulin, IgE. These discoveries paved the way for the first structural studies, chemical analysis and measurement of serum IgE (Wide et al., 1967; Platts-Mills et al., 1978; Holowka and Baird, 1983).

Structure and function

Serum IgE is found in all mammalia, has a molecular weight of 180kDa, is heat labile and does not pass the placental barrier. It co-evolved with basophils and mast cells and its original purpose was primarily in host defence against parasites such as helminths and some protozoa (Oettgen and Broide, 2012). However, it is most well-known for its role in the allergic response, with allergy and asthma now representing a far greater threat than parasitic infection, especially in developed Western society (Bell, 1996).

Like other immunoglobulins, IgE is produced from B-cells and has a structure consisting of two heavy (H) and two light (L) chains, each with variable (V) and constant (C) regions, assembled into a tetrameric structure by disulphide bonds. The constant regions on the heavy chain distinguish the various immunoglobulins. For IgE, the heavy chains each have 4 constant regions, and each contain 110 amino acids and one variable region. Each light chain consists of 1 variable and 1 constant region. The binding sites for allergens are located on the variable regions, while constant regions are responsible for the effector functions that bind IgE to mast cells and basophils (Lafaille et al., 2015).

Levels of specific-IgE are often distinguished from levels of total IgE in clinical tests used in allergy diagnosis. Levels of specific-IgE are used to determine allergic sensitisation to specific allergens, whilst total IgE includes all specific and non-specific IgE and high levels are usually associated with general atopy. However, there are other rarer disorders, such as parasitic infections, malignancies and immunodeficiencies which are associated with high levels of total IgE. Therefore, individuals may have a high level of total IgE without suffering from an allergic disorder or alternatively, normal levels of total IgE may be observed in a patient with clinically diagnosed allergy (Grundbacher, 1975; Gergen et al., 2009).

The concentration of total IgE in serum is low in comparison with other immunoglobulins, ranging 17 – 450ng/ml and has a shorter serum half-life of 2-3 days. Atopic individuals often have 10 times the average serum concentration of total IgE found in non-atopic individuals (Mahmoudi, 2016). Table 1-1 shows the variation between the immunoglobulin isotypes (Mahmoudi, 2016: p.7). When bound to mast cells, however, specific-IgE can have a far longer half-life (months) which is particularly relevant in solid organ donations, whereby organ transplants may still harbour specific-IgE coated mast cells (Bhinder et al., 2011).

Table 1-1 The immunoglobulin classes. (Adapted from Mahmoudi, (2016): p.7)

Immunoglobulin (Ig)	Molecular weight (kDa)	Serum concentrations	Serum half-life (days)	Subclasses	Placental transfer	Function
IgA	170 or 350	1.4-4mg/ml	6	IgA1, 2	-	Mucosal immunity
IgD	160	0-0.4mg/ml	3		-	Activates B-cells
IgE	180	17-450ng/ml	2-5		-	Type I hypersensitivity
IgG	160	8-16mg/ml	23	IgG1, 2, 3, 4	+	Type II hypersensitivity
IgM	900	0.5-2mg/ml	5		-	Type II hypersensitivity

1.1.4 Cells in the allergic inflammatory response

As shown, there are a number of cells and inflammatory mediators, such as Th1 and Th2 that are involved in the allergic immune response. There are other T-cell subsets including Th9 and Th17 that are produced by naïve T-cells. Th9 is thought to be involved in mucus production and tissue inflammation (Soroosh and Doherty, 2009) and Th17 has been associated with chronic neutrophilic inflammation (Aujla and Alcorn, 2011). Eosinophils are a source of cysteinyl leukotrienes which cause smooth muscle contraction and airway hyperresponsiveness. Neutrophils can recruit many inflammatory mediators and are often detected in asthma exacerbations and in individuals who die suddenly of severe asthma. Macrophages phagocytose foreign organisms and are often found at the tissue site of allergic inflammation (Bradley et al., 1991; Kay & Corrigan, 1992; Fahy, 2009).

There are currently over 70 cytokines that have been identified, of which a subset is known to be expressed during allergic inflammation. They are extracellular signalling molecules that act through receptors to promote or inhibit inflammation (Ferreira, 2003) . Although all these factors are important for understanding the allergic response they are not investigated within this thesis – which is directed towards the identification of allergens that have not yet been characterised.

1.2 What makes an allergen

An allergen can be broadly defined as an antigen that binds to specific-IgE antibodies. In most cases, allergens are relatively small, water-soluble proteins or glycoproteins with carbohydrate side chains (Woodfolk et al., 2015). Intense research efforts have been made to identify common structural and functional features that determine whether a protein is likely to induce allergy (Stadler and Stadler, 2003; Ladics et al., 2011). While some features are seen to contribute, no single factor has been identified that determines what makes a protein an allergen (Scheureret al., 2015).

1.2.1 Structure, function and homology

There are four common features of allergens that have been identified (enzymatic activity, protein stability, a non-homologous relation to microbial pathogens, toll-like receptor agonist activity and certain biochemical functions), none of which are necessary or sufficient individually or in combination to make a protein allergenic.

Enzymatic activity is frequently described in the literature as important for making proteins allergenic (Huby et al., 2000). House dust mite allergens act as proteases; causing direct damage to the bronchial epithelium and inflammation in the lung (Reithofer and Jahn-Schmid, 2017). This may suggest that protease allergens are more relevant in chronic asthma, but this does not fit with major allergens identified from other pest species (e.g. cockroach). Exposure to cockroach has, in some parts of the world had a significant association with chronic asthma but to date none of the allergens identified have exhibited protease activity (Wünschmann et al., 2005).

Protein stability is also attributed as a common feature amongst allergenic proteins (Pekar et al., 2018). Proteins are more likely to be able to trigger an immune response if they can withstand both thermal processing and gastrointestinal enzymes, that usually break down proteins. This is particularly the case for many seafood allergens, such as the parvalbumin allergen, Gad c 1 from codfish (Kuehn et al., 2014).

The lack of bacterial homology amongst identified allergens has been suggested as a common denominator of proteins that are allergens. Santiago et al., (2012) compared the

amino acid sequence of known allergens with helminths, protozoans, fungi and bacteria and found no homologues of allergens amongst bacteria. Allergens with limited levels of conserved amino acid sequences displayed the highest IgE prevalence. They suggest that for the majority of allergens, any similarity to bacteria would be likely to lead to immunological tolerance, based on the relative “uniqueness” of an antigen being associated with allergenicity.

A majority of defined major allergens are thought to be lipid-binding proteins (Trompette et al., 2009). The major house dust mite allergen, Der p 2, was shown to have structural as well as functional homology with the lipopolysaccharide-binding component of the Toll-like receptor (TLR) 4 signalling complex (Trompette et al., 2009). This suggests that some lipid bindng proteins are able to interact directly with the TLR4 complex to facilitate TLR signalling which can enhance Th2 responses and may well underlie the allergenicity of such proteins.

Comparison of allergen amino acid sequences to determine common properties of allergens was also performed by Radauer et al., (2008). They found that 5% of protein families contained allergens and the most frequent biochemical functions of allergens included hydrolysis of proteins, storage functions and polysaccharides and lipid structures.

However, many allergens do not exhibit these features and they are not reliable for predicting whether a protein is an allergen.

1.2.2 Binding to IgE

The part of an allergen that binds to specific-IgE is known as the IgE binding epitope. The IgE binding epitope is determined by the protein backbone and will have either a conformational or linear structure (Aalberse and Crameri, 2011). In a conformational epitope the amino acids are brought together by folding of the amino acid chain. Allergen epitopes are usually conformational as only some antibodies can bind a linear epitope and usually do so with a far lower affinity (Meno, 2011), making an allergic reaction less likely. An allergen must have two IgE binding epitopes to facilitate cross-linking of specific-IgE; this is necessary to cause mast cell degranulation and dispersion of inflammatory mediators

(Aalberse and Crameri, 2011). The cross-linking of specific-IgE with an allergen is depicted in Figure 1-2.

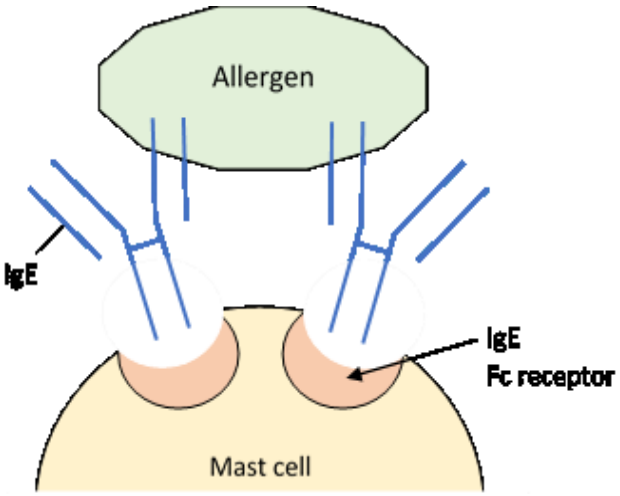


Figure 1-2 Schematic diagram of allergen cross-linkage to cell-bound IgE

1.2.3 Cross-reactivity

The capacity to bind to specific-IgE is a pre-requisite of a molecule being termed an allergen, but not every allergen that binds to specific-IgE will induce allergic sensitisation. These so-called 'non-sensitising allergens' can only induce allergic symptoms if the individual has become sensitised to another allergen that shares structural and functional characteristics. A well-known example of this 'cross-reactivity' is the major birch pollen allergen, Bet v 1, which is cross reactive with the non-sensitising apple allergen Mal d 1. This cross-reactivity means that individuals sensitised to birch may experience allergic symptoms if they eat apple (Ahammer et al., 2017).

1.2.4 Allergen nomenclature

A nomenclature system for allergens was first presented in the 1986 'Bulletin from the World Health Organisation', by an International Union of Immunological Society sub-committee (Marsh et al., 1986). They put forward guidelines for naming allergens which were based on the allergen source and its taxonomic name. This has since become known as the WHO/IUIS approved Allergen Nomenclature.

Their allergen naming convention consists of using the first 3 letters of the genus, 1 letter from the species epithet and a numerical value. For example, the first allergen identified from the European house dust mite, *Dermatophagoides pteronyssinus* was called Der p 1. The information required to assign a protein as an allergen was described by Marsh et al., (1986) and Marsh et al., (1987). This included the molecular weight, isoelectric point, amino acid composition or sequence and immunochemical characterisation (allergen recognition by specific-IgE) of the allergenic protein.

During the 1990's, the technology to generate amino acid sequences became more readily available (Bowie et al., 1990). With this, a revised version of the allergen nomenclature in 1994 stated that the sequence information of the allergen would be required for allergen registration (King et al., 1994; King, 1995) The Allergen Nomenclature sub-committee has continually refined its criteria for the registration of a protein as an allergen.

Currently, the protein must be purified and characterised, it must be shown that exposure induces clinical symptoms of allergy and specific-IgE binding from at least five patients to the characterised protein must be demonstrated (Pomés et al., 2018). The information needed to register an allergen in the WHO/IUIS approved nomenclature is shown in Figure 1-3 (Pomés et al., 2018: p.7).

This allergen nomenclature also considers varying forms of the same allergen, such as isoallergens and variants (isoforms). An isoallergen is defined as an allergen from one species that shares the same biological function, similar molecular weight and ≥67% sequence identity. Whereas an allergen variant (also called an isoform) is ascribed to allergen sequences that only differ by a small number of amino acid substitutions and typically show greater than 90% sequence identity (Chapman, 2008). Birch pollen allergen, Bet v 1, is a prime example of an allergen with multiple isoallergens. It has 31 isoallergens that show between 73-98% sequence identity (Sparholt et al., 1997). In the nomenclature, isoallergens are distinguished by 2 numerals following a dot after the allergen number and variants by 2 subsequent numerals, e.g. Bet v 1.0101 (Chapman et al., 2007).

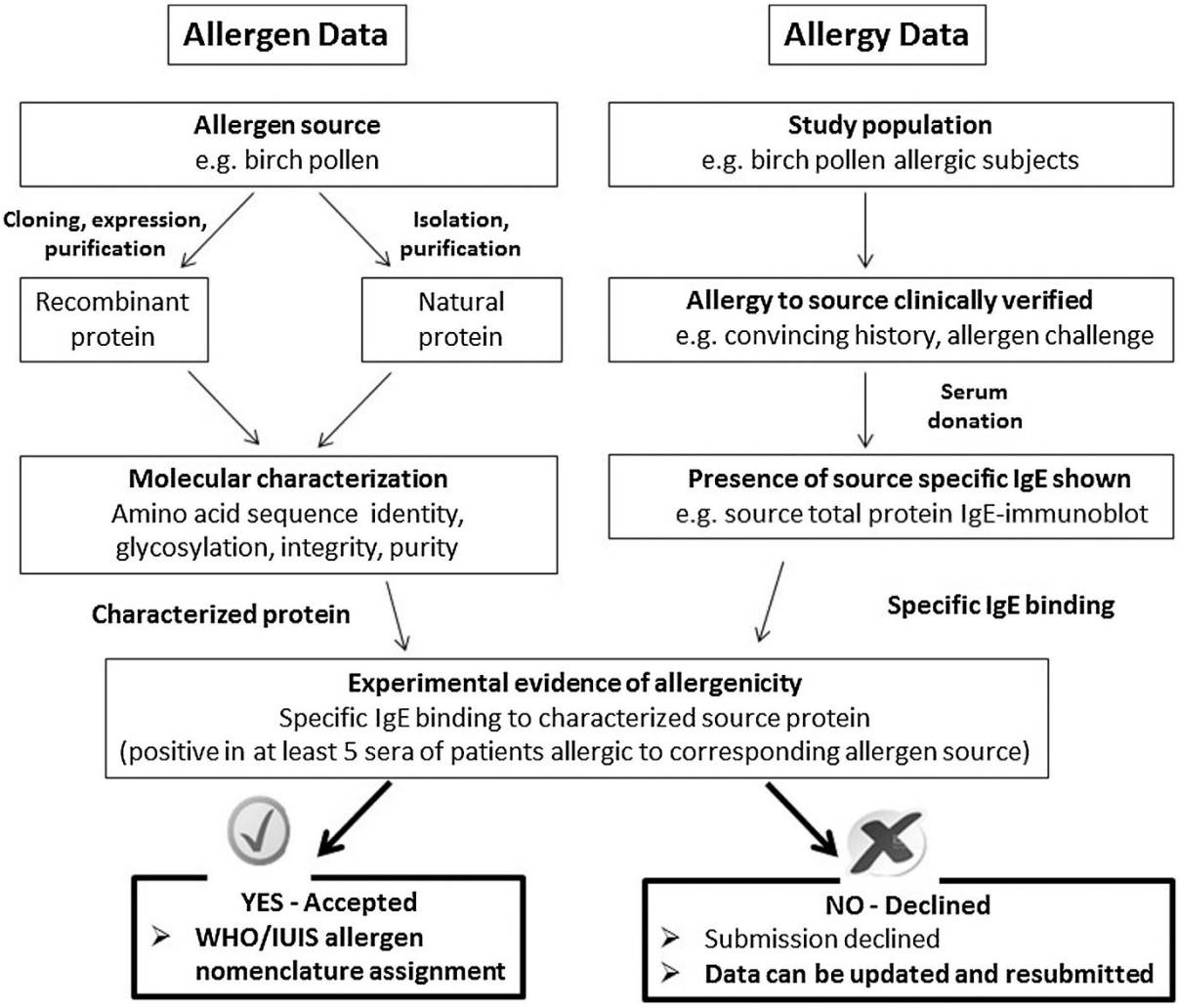


Figure 1-3 Criteria for allergen inclusion in the WHO/IUIS Allergen Nomenclature (Pomés et al., 2018: p.7)

1.2.5 Becoming airborne

For an allergen to be inhaled and enter the mucosal surface of the airways it must be attached to a particle that can become airborne. The size of this particle is important in determining the site for allergen deposition (lower or upper airways), as well as ensuring it passes through the airway epithelial barrier (Woodfolk et al., 2015). Well-known examples of allergen-carrying particles include pollen grains, mould spores, faecal particles, hair and skin flakes. Mite allergens are often associated with large-sized particles (>20µm) that settle rapidly while lipocalin allergens from cat, dog, mouse and rat tend to be carried on small particles (<1-20µm) that stay suspended in the air for much longer periods of time (Platts-Mills et al., 1986; Bush et al., 1998). Table 1-2 (on page 37) lists the source of common airborne allergens related to asthma and the allergen-carrying particles.

1.2.5.1 Indoor allergens

The majority of indoor airborne allergens are derived from animal sources that are usually either household pets or unwanted pests (AAAAI, 2018). Allergens from pests such as cockroach and dust mite are usually found on larger particles, so tend to settle in dust and are found in high concentrations on floors, carpets and other indoor furnishings (Platts-Mills et al., 1986). House dust mites are the most ubiquitous source of indoor allergen that induce allergic asthma, but their presence can vary with the local environment, as their survival depends on the surrounding humidity and temperature (Arlian et al., 1992). Dog and cat allergens are mostly derived and dispersed on small aerosolised particles from their urine or dander and can be measured in ambient air (Hilger et al., 2012).

1.2.5.2 Outdoor allergens

Outdoor pollen and fungi are the most well-documented sources of outdoor allergen exposure (Burge, 2002). Allergy to pollen represents one of the most frequent Type 1 hypersensitivity allergic reactions (de Martinis et al., 2017). The most common allergic disease induced by pollen is allergic rhinitis, as pollen grains can deposit on the nasal mucosa and release allergenic particles (Varshney and Varshney, 2015).

A potential mechanism of pollen-induced asthma was discovered by Suphioglu et al., (1992) who used *in-vitro* tests to show that pollen grains could rupture in rainwater by osmotic shock and release hundreds of starch granules that are small enough (3µm in diameter) to enter the airways. They also measured these starch granules during the pollen season and found a 50-fold increase in their atmospheric concentration on days following rainfall. Atmospheric conditions, especially thunderstorms have been shown to worsen asthma symptoms induced by pollen (Marks and Bush, 2007).

Table 1-2 Common airborne allergens related to asthma. (Adapted from Global Atlas of Allergy, EAACI (2014) and Platts-Mills and Woodfolk, (2011)).

Source	Particles	Allergen	Molecular weight (kDa)	Size in microns
Dust mite 	Faeces 	Der p 1 Der p 2 Derp 10 Der p 11	25 16 33 102	15-30
Cockroach 	Frass (droppings) 	Bla g 1 Bla g 2 Bla g 4 Bla g 5	47 39 21 23	15-40
Cat 	Hair 	Fel d 1 Fel d 2 Fel d 4 Cat IgA	10 69 10 200 (glycosylated)	2-20
Alternaria 	Spores 	Alt a 1 Alt a 2 Alt a 3	17 22 14	20 x 14
Grass 	Pollen 	Lol p 1	27	30-40

1.3 Occupational asthma

Occupational asthma represents the most common respiratory allergic disorder in the workplace, however, occupational allergy can also manifest as rhinitis, dermatitis and hypersensitivity pneumonitis. Of the overall burden of adult asthma, occupational asthma is estimated to account for about 5-15% of cases (Balmes et al., 2003). This is distinguished from 'work-exacerbated asthma' which occurs when pre-existing asthma is aggravated in the workplace, rather than when it is caused by occupational exposures (Henneberger et al., 2011). There are now more than 400 workplace agents that are known to cause occupational allergy (Malo and Chan-Yeung, 2009).

Workplace agents known to cause occupational asthma are traditionally categorised according to their molecular weight, as either low or high molecular weight allergens. High molecular weight compounds act through an IgE-mediated mechanism and low molecular weight compounds are usually chemical sensitizers that act through less clear immunologic mechanisms (Mapp, 2001; Malo and Chan-Yeung, 2009; Tarlo and Lemiere 2014)

1.3.1 High molecular weight (HMW) allergens

The high molecular weight (HMW) allergens are mostly made up of proteins of plant or animal origin. Some of the most well-known sources of allergens in the workplace are laboratory animals, seafood, latex and flour which are briefly discussed below.

Laboratory animal allergy is a well described occupational disease caused by exposure to high molecular weight proteins derived from urine and dander of mammalian species such as mice and rat (Agrup et al., 1986). This occupational disease is described in greater detail in Chapter 4 of this thesis.

Baker's asthma is one of the oldest recognised occupational diseases (Ramazzini 1700) and still one of the most common forms of occupational asthma worldwide. The prevalence of baker's asthma has been estimated at between 15-30% of workers that are exposed (Armentia et al., 1990). Allergic symptoms such as rhinitis and asthma are mostly caused by exposure to flour derived from wheat, rye, barley, buckwheat or soybean (Houba et al., 1998). However, since the 1970's additives or so-called 'improver' enzymes have also been

used in bakeries, to enhance the baking process. Improver enzymes have many functions in the baking industry, this includes increasing the specific volume of bread, breaking down starch, giving a finer crumb structure and improving dough stability. One enzyme in particular – fungal α-amylase, stimulates the growth and fermentation process of yeast and has been implicated in causing symptoms of baker's asthma (Baur et al., 1986). Allergy to fungal α-amylase as well as other improver enzymes used in the baking industry is described further in Chapter 6 of this thesis.

The prevalence of occupational asthma and rhinitis related to seafood processing, shows significant variation; estimated at 5-24% by Moscato et al., (2008) and 7-36% by Jeebhay et al., (2001). The aerosolization of allergenic seafood proteins is thought to occur by processes such as grinding, boiling, degutting and mincing and the variation in prevalence is thought to be due to differential exposure to seafood constituents (Jeebhay et al., 2001). In 1986, SPT was used with extracts of crab to positively diagnose crab-processing workers with occupational asthma (Cartier et al., 1986).

Latex is a natural material that has been used to make many commercial products. It is considered to be the most well-characterised source associated with occupational allergy, with 15 individual allergen components from latex registered on the official WHO/IUIS Allergen Nomenclature (Cabañes et al., 2012). Latex allergy was recognised as a problem in the healthcare industry, due to its use in medical gloves. In 1990, Lagier et al., (1990) proposed that the powder normally used to help donning and removal of gloves (corn-starch), could act as a latex allergen carrier, producing airborne particles loaded with latex protein that could be inhaled and make contact with the respiratory tract. As a result, powdered latex gloves were largely replaced with other synthetic and nitrile gloves and the burden of occupational asthma due to latex allergy was significantly reduced (Wallemacq et al., 2006).

1.3.2 Low molecular weight (LMW) allergens

The low molecular weight (LMW) allergens are usually reactive chemical species, with a molecular weight less that 5kDa, that act via an unclear allergic mechanism. For some LMW agents, such as acid anhydrides, the development of occupational asthma is accompanied

by the production of specific-IgE antibodies. This occurs when LMW allergens function as haptens and bind to carrier proteins forming a hapten-carrier complex. However, for many LMW agents, such as isocyanates, specific-IgE is only detected in a small subset of individuals affected (Maestrelli et al., 2009).

In facilities producing toluene diisocyanate the annual incidence rate of isocyanate-induced asthma was reported as 1.8% (Ott et al., 2000). Acid anhydrides are used in the manufacture of epoxy resins; trimellitic anhydride and phthalic anhydride, which have been responsible for inducing respiratory disease in exposed workers that has manifested as rhinitis, asthma or both (Venables, 1989). In addition, exposure to platinum salts has been shown to cause symptoms of rhinitis and asthma and the rate of sensitisation has recently been recorded as 2.4 per 100-person years of follow-up (Heederik et al., 2016), which is lower than earlier studies (Linnett and Hughes, 1999).

1.4 Clinical tests for diagnosing allergy

The clinical tests available for diagnosing allergy to a specific allergen can be divided into those conducted *in vivo* or *in vitro*. *In vivo* methods include SPT, intradermal tests and inhalation challenges. *In vitro* methods are further divided into allergen specific-IgE assays or basophil degranulation assays. The original allergen specific-IgE assay is known as the radioallergosorbent test (RAST), which has since been replaced by the trademarked ImmunoCAP assay (Bousquet et al., 1990). More recently, a multiplex chip technology has been developed to offer a more comprehensive allergy diagnosis, however it is not widely used (Hamilton, 2017). Basophil degranulation assays which are less commonly used include the basophil activation test and histamine release assay (Stahl et al., 1984; Knol et al., 1991). These clinical tests are described below.

1.4.1 *in vivo* methods for allergy diagnosis

1.4.1.1 Skin prick (SPT) and intradermal testing

Before the discovery of IgE and the development of serum-based immunoassays, the SPT was the principle means of diagnosing allergy (Ebruster, 1959). It remains a useful diagnostic tool, as it is robust, easy to administer and an efficient means of screening a patient for allergy to the tested allergens. A European SPT panel for specific inhalant allergens is commonly used and includes birch, grass mix, *Alternaria*, cat, dog and house dust mite (Heinzerling et al., 2013). Although the results from a SPT should be used in conjunction with a clinical history and/or serum-based immunoassays, its ease of use means it remains a cornerstone in allergy diagnosis.

To conduct a SPT, a medical-practitioner will place a drop of allergen extract onto a clean area of skin, usually on the front of a patient's forearm. A sharp needle or lancet is passed through the allergen extract at a ~50-degree angle to the skin, to form a small break in the skin that is enough for the allergen extract to penetrate. After 15-20 minutes, a 'wheal and flare reaction' is observed for a positive result. The reaction results from inflammatory mediators that are released during mast cell degranulation caused by serum specific-IgE binding to the penetrating allergen. The wheal size (the longest diameter and its orthogonal diameter) will be recorded and any false-negative or false-positive reactions are controlled

for by using a positive control (histamine) and a negative control (saline). There are several definitions of what constitutes a 'positive test' but nearly all utilise measures of the average wheal diameter, the requirement of no reaction to the negative control and a positive reaction to histamine. In addition, the longest wheal diameter from the test allergen extract must usually be 3mm greater than the negative control (Peebles et al., 2012).

The allergen extract can also be injected intradermally; however, the risk of anaphylaxis via this method is greater and it is not as commonly used. Using a hypodermic syringe, a more-dilute allergen extract solution (100-1000 fold less concentrated than used for SPT), is injected under the dermis (Dreborg, 1993). Positive and negative controls are used in the same way as for SPT.

1.4.1.2 Specific inhalation challenge

The specific inhalation challenge (SIC) is a diagnostic tool, considered as the gold standard in diagnosing cases of occupational asthma. It was formally developed in the early 1970's by Pepys and Hutchcroft, (1975) and was initially performed in the UK at the Royal Brompton Hospital.

The technique, although used worldwide, is only conducted in a limited number of centres, which have specialist equipment and properly trained healthcare professionals. During a SIC the patient is exposed to the suspected agent under controlled conditions and with close observation. Their lung function is measured during and following the exposure and compared to their response to a control substance, usually methacholine. This technique is useful for occupational asthma diagnosis as the suspected causative allergen may not be available as a SPT reagent (Aasen et al., 2013; Vandenplas et al., 2014).

1.4.2 *in vitro* methods for allergy diagnosis

1.4.2.1 Allergen specific-IgE assays

Methods for determining the presence of allergen specific-IgE from patients' serum are collectively known as 'allergosorbent' tests, as the allergen used is immobilised to an insoluble matrix (Peebles et al., 2012). Compared with *in vivo* testing, these methods are more standardised and automated, however the results from serum-based assays are not immediate, not as translatable to the patient (SPT's provide visual evidence), more expensive and require venesection (Wood et al., 1999).

1.4.2.2 The radioallergosorbent test (RAST)

The RAST was introduced in the 1960's (Wide et al., 1967) and was marketed in 1974 by Pharmacia Diagnostics in Uppsala. A radioactive isotope, Iodine-125 is used as a detection substrate for determining the quantity of allergen specific-IgE in patient serum. A gamma scintillation counter is used to give a percentage of the total counts of radioactivity observed. Although no agreed upon cut off percentage for a positive result exists, significant laboratory experience has shown that a cut from >2% will identify practically all individuals with specific-IgE sensitisation (Jones et al., 2013; Jones et al., 2017).

Because of the recognised hazard of using radioactive isotopes, this detection method has largely been replaced with the use of fluorophore substrates; however, the basic principles of allergen and specific-IgE binding remain the same. The allergen is linked to a solid phase to which serum is added, specific-IgE in the serum that is specific for the allergen will bind and the remaining unbound serum specific-IgE is washed off. A labelled human anti-IgE antibody is added which will bind to specific-IgE and a detection molecule (usually an alkaline phosphatase (AP) or horseradish peroxidase (HRP) substrate) will then be added and bind to the labelled anti-IgE antibody (Wide et al., 1967; Williams et al., 2008).

1.4.2.3 Singleplex ImmunoCAP assay

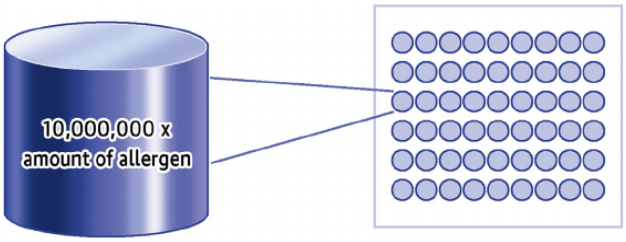
The ImmunoCAP system, developed by Thermo Fisher Scientific in Uppsala is now the most commonly used system to quantify allergen specific-IgE. It is a fully automated fluorescent

enzyme immunoassay (FEIA) that tests for specific-IgE to over 650 allergens. In the assay, allergens are covalently bound to a cellulose solid phase and react with allergen specific-IgE in patient serum. An enzyme-labelled anti-IgE antibody is used to bind to specific-IgE and a developing agent is added which produces fluorescence when bound to the enzyme. The measure of fluorescence represents the quantity of allergen specific-IgE present in the serum. This is measured in the range 0 to 100 kUA/l, where A represents allergen specific-IgE antibodies. The standard cut-off has been 0.35 kUA/l; however, 0.1 kUA/l is being more frequently used as a cut-off value (Hamilton and Oppenheimer, 2015).

The ImmunoCAP assay has several advantages. As the allergen is fixed in excess to the cellulase solid phase, it allows for complete binding of specific-IgE antibodies, resulting in high sensitivity. The assay has good specficity as there is no interference from competitive inhibition by allergen specific-IgG antibodies. However, the ImmunoCAP assay does not distinguish between binding to separate allergen components which may have greater or lesser clinical relevance (van Hage et al., 2017). Due to this, irrelevant positive results may be obtained if allergen specific-IgE binds to proteins in the allergen extract that have no clinical relevance. This is the case for glycoproteins present in plant and insect allergens with an α-1,3-fucose epitope structure, also known as 'cross-reactive carbohydrate-determinants'. These are present in pollens and venoms and are a well-defined cause of false-positive results in allergy diagnosis (Altmann, 2016).

1.4.2.4 Multiplex ImmunoCAP chip technology

For the last decade a multiplex ImmunoCAP specific-IgE assay has been available. This is a microarray chip technology which allows for the simultaneous measurement of specific-IgE from one serum sample to a broad spectrum of individual allergen components from different allergen sources. Trademarked as an ImmunoCAP Immuno Solid-phase Allergen Chip (ISAC), the device contains allergen components immobilised in a polymer-coated microarray (Hamilton, 2017) and uses the same FEIA technique to measure specific-IgE as the ImmunoCAP assay described above. In Figure 1-4 is a comparison of the singleplex ImmunoCAP assay and the multiplex ImmunoCAP ISAC (van Hage et al., 2017).



	ImmunoCAP	ImmunoCAP ISAC
Allergen:	Allergen extracts or molecules	Allergen molecules
Amount of allergen:	1-2 µg per ImmunoCAP	100 fg / spot
Number of allergens:	One per ImmunoCAP (one allergen source/ mix (around 10 allergen sources)/allergen molecule	112 per chip
Amount of serum:	50 µL per ImmunoCAP	40 µL per ISAC chip
Sensitivity:	0.1 kU/L (calibrated to WHO human reference preparation)	0.3 ISU
Reported answer:	One per ImmunoCAP	112 per chip
Interference with IgG:	No	Yes
Coefficient of variation:	Low	Comparably greater

Figure 1-4 Comparison of allergen specific-IgE measurement by singleplex ImmunoCAP or multiplex ImmunoCAP ISAC (van Hage et al., 2017: p.975).

The use of ISAC in clinical diagnosis provides a detailed patient specific-IgE antibody profile to numerous allergen components, using only a minute amount of serum (40µL). ISAC was tested in 23 sites worldwide, and it gave the same results regardless of the different site, laboratory conditions, handler and device used. Although the chip could be used as a screening tool to conduct several measurements simultaneously, its cost (£2500) has limited its potential use (van Hage et al., 2017). Determining a patients specific-IgE profile offers the opportunity for more personalised treatment and immunotherapy against specific allergen components. This is not dissimilar to other areas of the health sector that have chosen to move towards a more precision-based medicine approach as it is advantageous both for cost savings and patient outcome (Heffler et al., 2018). Unfortunately, this advanced technology is not suitable for allergy diagnosis in the occupational setting. The allergen

components included on ISAC are targeted to those affecting the general population and does not include allergens found in many workplace environments.

1.4.3 Other cellular assays

1.4.3.1 Basophil activation test

The basophil activation test was first developed in the 1990's (Knol et al., 1991) and has since been standardised. It is used occasionally in clinical diagnosis of allergy but is more commonly used in the allergy and immunology research sector (Boumiza et al., 2005). The test is also used to monitor the progress and success of allergen immunotherapies (Saini and MacGlashan, 2012). The test relies on specific-IgE bound to the surface of basophils causing granules to be released in the presence of the suspected allergen. Blood is incubated with the allergen and then treated with fluorescently labelled antibodies which will identify basophils as well as specific granule-associated proteins. Flow-cytometry is used to detect the activated basophils and if more than 15% are activated the test is classed as positive (Boumiza et al., 2005).

This method is particularly useful as an additional test in allergy diagnosis, when the patient's clinical history and specific-IgE or SPT produce discordant results, which is more commonly encountered in cases of food allergy (Santos and Shreffler, 2017). The use of flow cytometry also allows for the analysis of a large sample of cells which gives a more accurate result. However, the method can be laborious as the basophils must be identified, then distinguished as being either activated or non-activated from the large number of cells. The method also requires the use of whole blood from the patient (Moneret-Vautrin et al., 2003; MacGlashan, 2013; Hoffmann et al., 2015).

1.4.3.2 Histamine release assay

The histamine release assay similarly relies on the use of whole blood and detecting the release of mediators from activated basophils, in the presence of the suspected allergen. It can be conducted using a glass-fibre fluorometric method, where released histamine binds to the solid phase. All other blood constituents are washed away and trapped histamine is

eluted by increasing the pH. A complex is then formed with o-phthaldialdehyde which can be quantified fluorometrically (Stahl et al., 1984; Larsen et al., 2018). In 2007, Elberling et al., (2007) obtained blood from patients with respiratory symptoms related to perfume as well as healthy volunteers and used the histamine release assay to show an increased basophil reactivity to perfume in those with respiratory symptoms.

1.4.4 Tests for allergy more commonly used in the research sector

1.4.4.1 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay, commonly known as the ELISA is a well-established technique used for the quantitative measurement of specific proteins, such as antigens or antibodies. The ELISA method has some variations and two that are commonly used are the indirect ELISA and the sandwich ELISA (Kindt, 2007).

In an indirect ELISA, antibodies such as specific-IgE from patient serum can be quantified. Serum is incubated in a microwell plate that is coated with the suspected allergen. Specific-IgE is allowed to react with the allergen and any remaining antibody is washed away. An enzyme-conjugated secondary antibody is then added to the plate which can bind with serum specific-IgE, and then an enzyme substrate is added. When the substrate-enzyme reaction occurs, a coloured product is formed and the measure of absorbance determined by spectrophotometric plate readers is used to calculate the quantity of specific-IgE (Wal et al., 1995; Wachholz et al., 2005). Silva et al., (2001) developed an indirect (reverse) ELISA to measure serum specific-IgE to Der p 2, a major house dust mite allergen, showing it was just as sensitive as radioactive techniques. A major drawback of this assay in measuring specific-IgE, is that *all* specific antibodies irrespective of their isotype can become bound to the plate. Specific-IgG antibody is known to compete with specific-IgE for binding to the allergen (Kadooka et al., 2000). The ImmunoCAP and RAST methods do not have this problem as there is a relative excess of allergen in the test, which is sufficient for accurate detection of specific-IgE without competition from specific-IgG.

A sandwich ELISA can be used to quantify a specific allergen. For this technique, an antibody specific for the allergen of interest is coated to the plate. The sample containing the allergen is added and allowed to react with the antibody. After washing the plate, an enzyme-

conjugated antibody that is also specific for the allergen (at a different epitope) is added. This is followed by an enzyme substrate which similarly will react to produce a coloured product if allergen is present, and absorbance is read in the same way to calculate the quantity of allergen present (Wachholz et al., 2005). Sandwich ELISA's for several animal allergens are commercially available from the company, Indoor Biotechnologies. Commercial Fel d 1, Can f 1 and Mus m 1 ELISA kits use purified natural single allergens as the standard, which have been quantified by amino acid analysis (Zahradnik and Raulf, 2014).

In all these tests for allergy that have been described, the methodology is reliant on using extract sources or purified forms of known allergen. For SPT a panel of known allergens is used and in RAST and ImmunoCAP a known allergen is coupled to a solid phase. These methods do not allow for the identification and diagnosis of allergy to potential 'new' allergens. We propose the following method combined with modern proteomic techniques will allow for this.

1.4.4.2 SDS-PAGE and western blot

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a method used to determine the molecular weight of different proteins (Laemmli, 1970). Protein samples are loaded onto a polyacrylamide gel which is placed in an electrophoresis buffer. A voltage is then applied which causes negatively charged molecules to move through the gel in the direction of the positive anode. Smaller proteins will migrate further than larger ones and so proteins become separated based on their molecular weight. To visualise the proteins, there are several different stains that are used; Coomassie is the most common as it is very easy to use whilst silver staining has the highest sensitivity.

A western blot can be used after SDS-PAGE, to detect which of the separated proteins from an allergen extract are able to bind to specific-IgE from sensitised serum. For this method, the allergen extract is separated in different lanes of a gel, the gel is then transferred to a membrane (usually nitrocellulose) and each lane is incubated with a serum sample. Serum is washed off from the membrane and a labelled human anti-IgE antibody is added which will

bind to any specific-IgE bound to allergen components on the membrane (Towbin et al., 1979). Depending on the detection method used a substrate that binds to the labelled anti-IgE antibody will be added and its signal detected to distinguish specific-IgE binding proteins (Ghosh et al., 2014). In addition, as allergen extracts can be separated in sufficient amounts during SDS-PAGE, compared with allergen extracts used in the ELISA, this method can largely avoid the issues of competition with specific-IgG antibodies (Wachholz et al., 2005).

Developments in the field of proteomics mean that the proteins binding to serum specific-IgE that are detected through SDS-PAGE and western blot, can be identified in new ways.

1.5 Proteomics

1.5.1 Early protein sequencing

Protein sequencing is a means of determining the amino acid sequence of all or part of a protein. The first protein that was fully sequenced was insulin by Sanger et al. in 1955 by using chromatographic methods. At a similar time, a cleavage method by Edman, (1949) for partial protein sequencing was published, which has since become known as the 'Edman degradation' method. The method uses a series of chemical reactions that remove and identify the amino acid residue at the N-terminus of the polypeptide chain. It involves a repetition of this process to reveal the amino acid sequence of the protein. It is also referred to as N-terminal sequencing. In the 1970's it was a popular method to identify small to medium sized proteins, but its use began to decrease in the 1990's when superior mass spectrometry (MS) methods of protein sequencing were introduced (Mann, 2016).

1.5.2 Modern mass spectrometry

MS is a powerful analytical technique used to measure the mass-to-charge ratio (*m/z*) of ionic species within a sample. It has become the method of choice for protein identification and quantification (Schuchardt and Sickmann, 2007). For protein identification, an approach known as 'bottom-up proteomics' is commonly used, whereby proteins are separated in a gel prior to MS, and then digested with an enzyme (most commonly trypsin), to generate peptides which are more amenable for MS analysis (Zhang et al., 2013).

MS can be divided into the following steps: (1) pre-separation chromatography (2) ionisation and (3) injection of ions into a mass analyser to generate a mass spectrum of individual peptides based on the *m/z* ratio.

1.5.2.1 Pre-separation chromatography

Prior to ionisation, peptides will almost certainly be separated by a chromatographic method. The two most commonly used are gas chromatography (GC) for volatile compounds and liquid chromatography (LC) for biological samples.

A high-performance version of liquid chromatography (HPLC) is commonplace in MS-based proteomics. It relies on high pressure pumps to pass the peptide sample in a solvent solution through a column with an adsorbent material, typically made of silica particles. It is the interaction between the solvent and silica that determines the pattern of peptide separation (Mitulovic and Mechtler, 2006).

1.5.2.2 Ionisation

The coupling of LC with MS was made possible for mainstream clinical research only after the development of electrospray ionisation (ESI) and matrix assisted laser desorption ionisation (MALDI) (Strathmann and Hoofnagle, 2011). ESI and MALDI are ionisation techniques which convert molecules into ions; a necessary step for peptides to be measured by their *m/z* ratio, to create a mass spectrum.

Developed by Fenn et al., (1989), ESI works by pumping liquid samples containing protein in a solvent and through a needle. The sample is nebulised at the tip to form a fine spray of charged droplets. These droplets are rapidly evaporated using heat and dry nitrogen and charged sample ions are released from the droplets becoming separated from the solvent in the process. The ionised sample is then transferred into a high vacuum and through a small aperture into the mass analyser. Banerjee and Mazumdar, (2012) recently reviewed the use and development of ESI-MS in applications such as chemical imaging, organometallic chemistry and protein identification and characterisation. ESI-MS has been able to successfully identify post-translational modifications that affect protein mass (Mann and Jensen, 2003) and interpret three-dimensional protein conformations (Kaltashov and Abzalimov, 2008).

MALDI, was developed by Tanaka et al., (1988) and is somewhat less used in the proteomics field. Briefly, the liquid sample is mixed with organic material (matrix) and applied to a metal plate positioned in the MS high vacuum source. A pulsed laser beam irradiates the sample, causing its desorption from the plate followed by separation from the solvent. The sample is then ionised by protonation and deprotonation and speeds towards the vacuum for entry into the mass analyser. MALDI based protein identification was used by Cucu et al., (2012)

to identify soybean proteins in food products that would remain after food processing.

1.5.2.3 Mass analyser

A commonly used and trade-marked mass analyser is the Orbitrap, developed by Alexander Makarov (Hu et al., 2005). An Orbitrap consists of an outer barrel-shaped electrode and an inner spindle-like electrode which together trap ions by 'electrodynamic squeezing'. The ions enter at the injection site and oscillate with different rotational frequencies that represent their mass to charge (*m/z*) ratio (Figure 1-5 (Planet Orbitrap, 2018)). After separating ions by their *m/z* ratio (MS_1), precursor ions can be selected for further fragmentation (MS_2). This is known as tandem mass spectrometry (MS/MS).

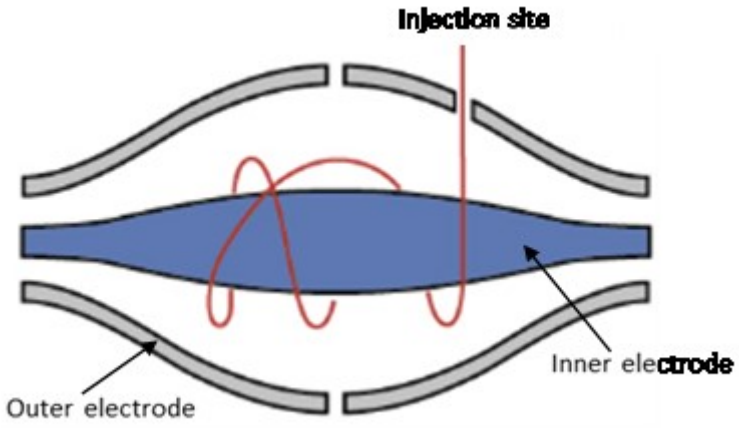


Figure 1-5 Schematic of Orbitrap technology. Ions enter at the injection site and oscillate between the outer and inner electrodes (Adapted from Planet Orbitrap, (2018)).

Collision induced dissociation (CID) is a popular method for fragmentation in tandem MS because of its reproducible fragmentation of the peptide bond (Molina et al., 2008). During CID an inert gas (usually helium or argon) is introduced into the ion trap which induces collision of precursor ions, fragmenting them into N-terminal and C-terminal peptide fragments (termed b and y product ions). The pattern of fragment ions and their mass is known as the mass spectrum, which can be pieced together to ultimately determine the peptide and protein amino acid sequence (Wells and McLuckey, 2005).

There are four other types of mass analysers commonly used in proteomics: quadrupole, ion trap, time of flight (TOF) and fourier transform ion cyclotron resonance (FTICR). They each

have different capabilities in terms of *m/z* range, accuracy, sensitivity and resolution (Hoffmann et al., 2007). These are important parameters in measuring the performance of an instrument. For example, accuracy is normally measured in parts per million (ppm) and represents the ratio between the *m/z* measurement error and *m/z* true value (Mann and Kelleher, 2008).

1.5.2.4 Protein identification

Once acquired, the experimental mass spectrum can be searched against theoretical spectra in protein databases, to determine the best peptide or protein match. The first search algorithm software to perform this was known as Sequest (Eng et al., 1994) and now, together with Mascot (Perkins et al., 1999), is the most widely used search algorithm for protein identifications. It provides every peptide-spectrum match (PSM), with a score based on how well matched the experimental peptide spectrum is with the theoretical peptide spectrum.

Software packages such as 'PEAKS Studio' or 'Scaffold' are used to generate reports showing the protein and peptide matches with data such as the number of peptides and spectra identified, the number of unique peptides and spectra, the match score, amino acid sequence coverage and the protein accession number.

1.5.3 Protein and allergen databases

The most comprehensive resource for obtaining theoretical spectrum data to compare with experimental data is the Universal Protein Knowledge Base (UniProtKB) database (Boutet et al., 2007). It is freely accessible and contains protein sequences with taxonomic and functional information, each denoted with an individual accession number.

The advances in proteomic technologies has allowed for the development of allergen-specific databases. Most of these contain overlapping data, but they vary in the number of allergens included, available data on each allergen, criteria for allergen inclusion, literature references, allergen origin and three-dimensional structures (Ghosh et al., 2011). One of the most widely used allergen databases is the WHO/IUIS Allergen Nomenclature (previously

described in section 1.2.4). Other established databases include Allergome, AllergenOnline and the Structural Database of Allergenic Proteins (SDAP) (Radauer, 2017). A comparison of these databases in shown in Table 1-3.

Table 1-3 Most widely used allergen databases. (Adapted from Radauer, (2017) and Mari et al., (2009)).

Name	Website	Year of public release	Data Source
WHO/IUIS Allergen Nomenclature Database	www.allergen.org	2000	Submission
Allergome platform	www.allergome.org	2005	Literature and sequence databases
AllergenOnline	www.allergenonline.org	2005	Literature and sequence databases
Structural Database of Allergenic Proteins (SDAP)	www.fermi.utmb.edu	2002	Sequence databases

The WHO/IUIS Allergen Nomenclature provides much of the main data found in other databases. The Allergome platform is widely cited by the allergy research community as it provides an exhaustive repository of allergen data, that is not limited by strict inclusion criteria. It gives allergenicity scores based on the extent of sequence characterisation, specific-IgE binding, skin tests, immunologic activity assays and epidemiological data (Mari et al., 2009).

AllergenOnline is a peer-reviewed database and was curated to identify proteins that could pose a risk of allergen cross-reactivity, especially those introduced into genetically modified foods (Goodman et al., 2016). The SDAP is also peer reviewed and provides allergen sequences, structures and epitopes in addition to bioinformatic tools for predicting allergenicity of proteins by analysing for cross-reactivity. Despite the use of prediction models, these databases still rely on experts in molecular allergology to identify novel allergenic proteins (Brusic et al., 2003; Radauer, 2017).

1.6 Mass spectrometry used in allergen identification and characterisation

The use of MS in medical research has already become widespread, with techniques developed for quantifying various biomarkers of disease, monitoring toxic drug metabolites and medical imaging in cancer patients (Che et al., 2015). MS has been used to measure variability between allergen extracts, quantify known allergenic proteins and obtain protein sequences for recombinant allergen production (Swoboda et al., 1995; Koeberl et al., 2014; Spiric et al., 2017; Oeo-Santos et al., 2018).

Erler et al., (2011) used western blot methods together with MS to compare the composition of SPT solutions and commercial allergen extracts for birch pollen from different origins. They observed that amongst the extracts, the isoform composition of Bet v 1 and quantity of allergen varied considerably, as well as finding Bet v 2 and Bet v 7 absent from one extract. The value of MS in investigating the reliability of extracts that are used in allergy diagnosis is also supported by Chapman and Briza, (2012) who discuss the future of complete allergen standardisation by the combined use of immunoassays and MS.

As described in section 1.2.3, allergens from different species may often be cross-reactive because they share similar protein homology. As MS allows for the protein sequence to be determined, sequence homology across species can be investigated to identify possible cross-reactive allergens. Saha et al., (2015) used serum from ten coconut pollen allergic patients and manual de novo sequencing as part of MS analysis to identify a vicilin-like protein as the major allergen from coconut pollen, also reported as a major allergen in many seed species. Specific-IgE binding regions were identified as 11s globulin, enolase and isoflavone reductase, because they shared significant homology with other well-known allergenic proteins.

Kamath et al., (2014) used a combined western blot and MS approach to identify food allergens. Protein extracted from black tiger prawn was separated using SDS-PAGE, and western blot was used to determine specific-IgE reactivity profiles from serum of 16 shellfish allergic patients. LC paired with tandem MS was then used to identify known and potential 'new' food allergens (triose phosphate isomerase, aldolase and titin) that corresponded to the specific-IgE binding regions observed by western blot. This combined approach showed that of the patients reacting to novel allergens, not all were allergic to the major allergens of

shellfish; such as tropomyosin, which is primarily used for diagnosing shellfish allergy. This highlights the need to identify all individual allergenic components from an allergen source, so a positive diagnosis of allergy is not missed.

Allergens from ragweed pollen have also been investigated using a combined western blot and MS approach. Bouley et al., (2015) showed that 50 of 92 patients with ragweed allergy, had specific-IgE for a 37kDa allergen, which was distinct from Amb a 1. The 37kDa allergen was sequenced using MS and its purified form confirmed as an allergen using basophil activation tests. They used three-dimensional modelling to show the novel allergen had significant homology with other cysteine protease allergens, including Der p 1. The allergen has since been recorded in the WHO/IUIS Allergen Nomenclature as Amb a 11.0101.

A research group in India have been using similar methods and published work since I embarked on this PhD. They have identified allergenic components from *Lantana camara*, (an airborne pollen predominant in India) using serum from patients who exhibited allergenicity to *L. camara* by ELISA and histamine assay (Ghosal et al., 2016). They identified pathogenesis-related thaumatin-like protein as the major allergen from this species. Dey et al., (2016) identified 11 allergenic proteins from the aerospore, *Curvularia pallescens*, also found in India, by excising specific-IgE binding regions and applying them to MALDI-tandem MS. MS-proteomics clearly has an important role to play in the future of allergen identification and standardisation. As the diagnosis of allergy is moving towards the use of individual allergen components (as shown with ImmunoCAP ISAC technology), the identification of all allergenic and non-allergenic proteins from an allergen source is required.

In the studies described above, the methodologies used included similar laboratory experiments, such as SDS-PAGE, western blotting and mass spectrometry. However, these were applied to different allergen samples collected via different techniques. For example, Erler et al., (2011) and Bouley et al., (2015) used protein extracted from commercially available pollen extracts, while Ghosal et al., (2016) used protein from freshly collected pollen grains and Dey et al., (2016) extracted protein from airborne fungal spores trapped with an Anderson volumetric sampler and grown on agar.

There is at present no standard method or protocol used for identifying potential 'new' allergens from airborne samples. However, using similar experiments (SDS-PAGE, western blotting and mass spectrometry) reported in these articles, a method could be established whereby airborne proteins are collected and previously unknown allergens are identified. Such a method is of particular relevance when allergic symptoms in communities are unexplained, or in occupational settings where unknown allergens are more commonly encountered.

In the following chapter, I have conducted a systematic review to investigate what laboratory methods have been used previously, to detect and identify allergens responsible for causing unexplained symptoms of allergic asthma.

2 A systematic review of clinical and laboratory tests used for investigating outbreaks of asthma thought to be associated with unusual peaks in outdoor airborne allergens.

2.1 Introduction

Asthma is the most common chronic respiratory disease in children and adults and its prevalence has increased globally over recent decades (EAACI Advocacy Manifesto, June 2015). Attacks of asthma can be triggered by exposure to sources of airborne allergen, such as pollen, mould, mite faeces and animal dander (Platts-Mills et al., 2011). However, individuals can still experience life-threatening hospital admissions of acute sudden severe asthma that go unexplained (Royal College of Physicians, 2014).

It is well recognised that the incidence of asthma attacks shows seasonal fluctuations with spring, summer and autumn peaks attributed to both increases in airborne concentrations of pollens and moulds, and upper respiratory tract viral infections. However, in addition to this seasonal variation there have been reports of geographical clusters or short-term 'epidemics' of acute severe asthma reported in several parts of the world. These have been investigated from an epidemiological, clinical and laboratory perspective, so that the causal agent (hypothesised to be an airborne allergen) can be identified. Such reports have identified previously unrecognised allergen sources and linked some of these episodes to extreme weather events such as thunderstorms.

In a number of asthma outbreaks, the cause was identified as aeroallergen. The most well documented of these were, the point source release of soybean and castor bean and increases in airborne pollen or mould during thunderstorm events.

This systematic review was conducted to establish which laboratory methods have been used to investigate outbreaks of asthma in which an unknown airborne allergen was considered to be a likely cause. A review of this literature has not, to date, been published.

Aim

The overarching aim was to establish which laboratory methods have been used to successfully identify the cause of asthma outbreaks, that is, how investigators have identified whether and what allergen caused the outbreak.

2.2 Methods

The guidelines from 'The Preferred Reporting Items for Systematic Reviews and Meta-Analyses' (PRISMA) Statement were followed. I defined a search strategy to identify all reports on outbreaks, clusters or epidemics of asthma occurring in child or adult populations in which aeroallergen was considered as a likely cause. The eligibility criteria were conceived using specified study characteristics P.I.C.O.S:

- Population: children and adults from the general population.
- Interventions & Comparators: not applicable as the review was a narrative synthesis of events (rather than a randomised control trial).
- Outcome: a. Primary – severe asthma exacerbation as defined by symptoms, treatment, healthcare utilisation and objective clinical measures. b. Secondary – anticipated as nasal symptoms suggestive of allergic rhinitis.
- Study design: all epidemiological study designs investigating this problem included.

The review protocol was written according to the PRISMA-P 2015 Statement and registered on PROSPERO – the International Register of Systematic Reviews. The registered working title "Systematic review of outbreaks of asthma occurring in child or adult populations in which unusual peaks in aeroallergen is identified as a likely cause" was used. Electronic searches were performed on MEDLINE (OVID interface 1946 to March 2016), EMBASE (OVID interface 1947 to March 2016) and Global Health (OVID interface 1973 to March 2016) using the search strategy:

> Asthma$ AND ((castor adj bean) OR soybean OR thunderstorm OR allergen.mp.) AND (epidemic OR outbreak OR cluster OR endemic).

There were no language or date restrictions applied to the search. Articles pre-dating 1946 but known by asthma experts and colleagues as reports of clusters of asthma were also assessed for inclusion into the review. Duplicates were removed and search results were uploaded to reference management software (EndNote). The articles were shared with two review authors, who screened and performed data extraction in parallel and discussed any discrepancies.

A pilot screen of the titles and abstracts was used to define the study selection criteria. Articles were not selected for inclusion if they were conference reviews, animal studies, or had a primary focus on immunotherapy, treatment, infection, parasites, skin or food allergy, phenotypes, or the 'hygiene hypothesis'. Articles reporting on general increases or seasonal fluctuations of asthma were also excluded.

Full-texts were then screened. Full-text articles not freely available were requested on loan from Imperial College London libraries. Articles on seasonal and occupational asthma were excluded. References that were cited in included papers were screened and studies that cited included papers were screened in the same way to include all relevant articles.

The following data were then extracted from each included paper: citation, article type, study design, time(year) & location of epidemic, air sampling methods, skin prick tests, laboratory tests, causal agent, results of laboratory and skin prick tests, all other results and conclusions. As the extracted information was qualitative, no quantitative or meta-analysis was undertaken for this review.

2.3 Results

2.3.1 Search strategy

The electronic search strategy identified 696 records: 146 from MEDLINE, 64 from GLOBAL HEALTH and 481 from EMBASE. An additional three articles, pre-dating the search criteria but known by asthma experts and colleagues were also identified for inclusion and 204 records were removed as duplicates.

After screening titles and abstracts, 414 records were excluded. Of the 81 remaining articles, 53 were selected for inclusion after screening the full-text. Searching reference and citation lists of the 53 articles, identified a further 38 articles for inclusion. In addition, a table of thunderstorm asthma studies was identified (D'Amato et al., 2012), from which 4 articles were selected for inclusion. After discussion between review authors, it was decided to remove reviews and overviews. Figure 2-1 shows the full screening process.

Data were extracted from 85 articles. Of these, 45 papers related to soybean, 31 to thunderstorm events, 7 to castor bean, one further outbreak that was associated with a grain mill but with no causative agent identified (Cowan et al., 1963) and Morrison (1960) who described an overnight outbreak with no associated sudden meteorological changes.

The most common laboratory and clinical methods used in the asthma outbreaks are shown in Table 2-1. The most common broad approaches adopted were air sampling, SPT and RAST.

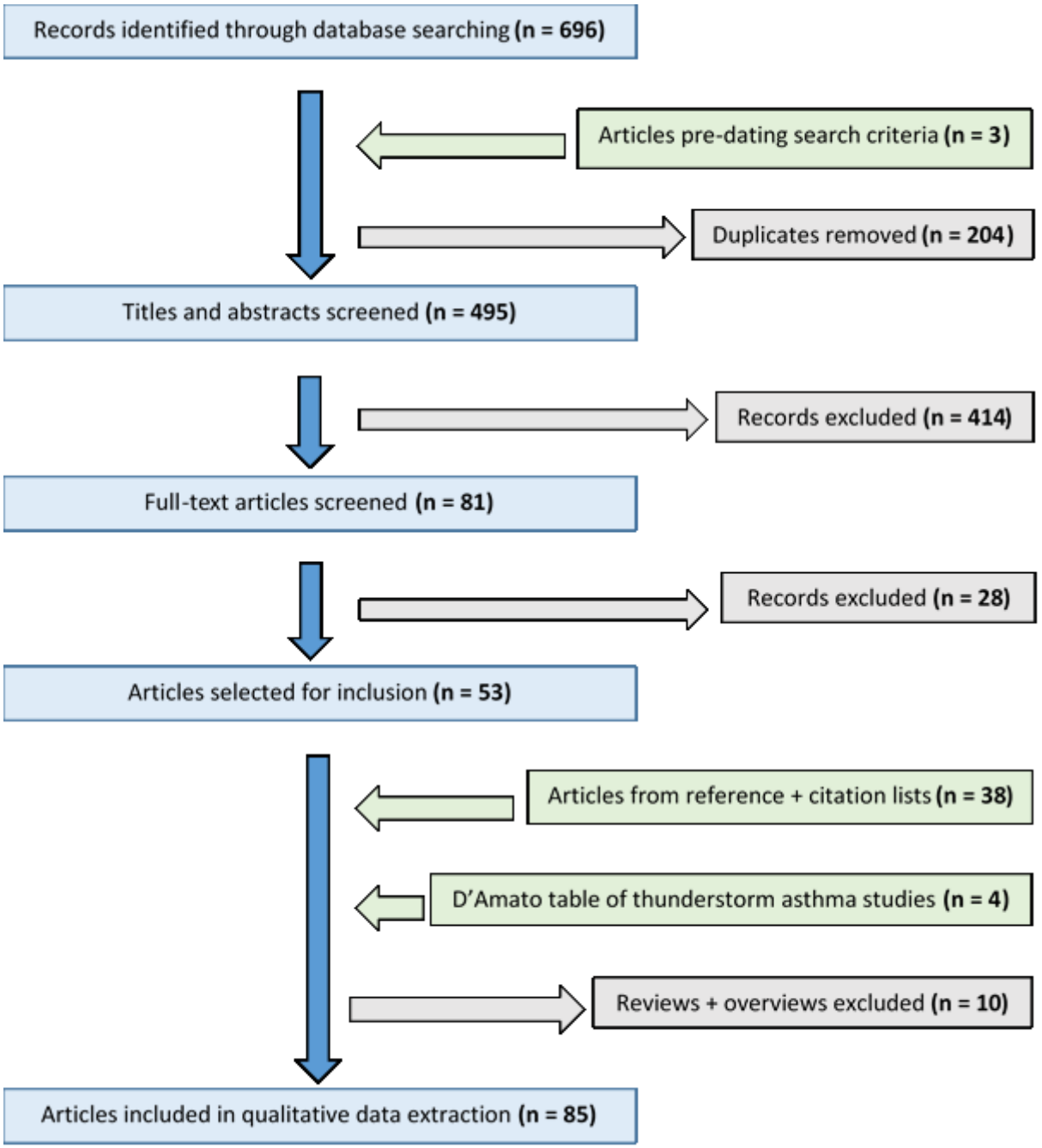


Figure 2-1 Screening process for records identified through database searching.

Table 2-1 Laboratory and clinical methods used in asthma outbreaks associated with soybean, castor bean, thunderstorms or other asthma outbreaks

Laboratory and clinical methods used in asthma outbreaks	Causal agent associated with asthma outbreaks			
	Soybean (n=45)	Thunderstorm (n=31)	Castor bean (n=7)	Other (n=2)
Air sampling	17	16	0	1
SPT	20	6	6	1
Passive transfer of antibodies	0	0	5	0
Microscopy	2	3	0	0
Protein Assay	6	0	0	0
RAST	20	1	0	0
ELISA	9	1	0	0
Western blot	9	0	0	0
CAP	5	2	0	0
Other lab tests	9	3	1	0

2.3.2 Castor bean associated asthma outbreaks

Articles reporting asthma outbreaks associated with castor bean (Table 2-2) were published from 1928 to 1975. These occurred in the US (Ohio), Brazil (Bauru and Ourinhos), an unnamed South African town and France (Marseilles). In all of these reports, an industrial site manufacturing castor bean oil was located in close proximity to the asthma outbreak.

The severity of the outbreaks varied, with 26 individuals affected in Ourinhos (Strauss, 1968), 200 persons affected in the unnamed South African town (Ordman, 1955) and 150 cases as well as 9 deaths reported in the Bauru outbreaks (Mendes and Cintra, 1954). The duration of these castor bean associated asthma outbreaks is unclear. In Ohio, an 'asthma colony' was known to exist for years (Figley and Elrod, 1928). It is likely that outbreaks occurred for as long as the industrial sites were manufacturing castor bean oil. In both Ohio and Ourinhos, the scale of the outbreaks was mostly limited to those living within a mile radius of the oil mill (Figley and Elrod, 1928; Strauss, 1968).

Figley and Elrod, (1928) report the earliest example of an asthma outbreak associated with an unusual peak in airborne allergen. They used a cutaneous scratch method with extracts of castor bean dust, on 30 patients with unexplained asthma attacks that were living within a mile radius of an oil mill. All 30 patients gave positive reactions to the castor bean extract by this method. The scratch method was used in all future reports of asthma outbreaks associated with castor bean.

A 'Passive transfer of antibodies/reagins', also known as the '*Prausnitz-Kustner*' test (described in section 1.1.3) was used in 5 articles reporting on asthma outbreaks associated with castor bean. Briefly, the *Prausnitz-Kustner* test consists of transferring serum from an allergic patient to a non-allergic person, who is then challenged by a SPT with the allergen thought to be the causative agent. When this method was used on asthma-outbreak patients involved in castor-bean related outbreaks occurring in Brazil and France, the test results were in agreement with the scratch method, implicating castor bean as the causative agent.

Rudimentary inhalation challenges were used in 4 articles. The earliest example of this test was in Ohio where 4 asthmatic patients were subject to 'a small amount of castor bean dust…thrown into the air for them to inhale'. All four patients developed 'typical asthmatic

attacks'. In two the attack came on at once, and in the other two it was delayed for a few hours. Similar rudimentary inhalation challenges were used in asthma outbreak studies from Bauru and Marseilles.

There were other less common laboratory and clinical methods used to help explain the cause of asthma outbreaks. In the Ohio outbreak study, microscopic examination (technique not given) showed that many of the castor bean dust particles were 'about the size of ragweed pollen [explaining] the ease with which the wind carries this dust'. Strauss (1968) used passive hemagglutination tests and radioimmunoelectrophoresis to demonstrate asthma-outbreak patients had antibodies specific for castor bean.

In passive hemagglutination testing, erythrocytes were coated with castor bean extract and shown to agglutinate in the presence of patient serum containing skin-sensitising antibodies specific for castor bean allergen. Agglutination of erythrocytes demonstrates a positive test for allergy.

In radioimmunoelectrophoresis, castor bean extract was labelled with radioactive iodine and shown to produce radioactive precipitin lines in the presence of patient serum containing skin-sensitising antibodies specific for the castor bean allergen. The presence of precipitins demonstrates a positive test for serum antibodies produced in the patient serum that are specific for the allergen.

Table 2-2 Data extraction of castor bean associated asthma outbreak articles

CITATION	ARTICLE TYPE + STUDY DESIGN*	EPIDEMIC, DATE + LOCATION	AIR SAMPLING METHODS	SKIN PRICK TESTS	LABORATORY TESTS	CAUSAL AGENT	RESULTS OF LABORATORY + SKIN PRICK TESTS	ALL OTHER RESULTS	CONCLUSIONS
Figley & Elrod, (1928)	Full text Cli, Lab investigation of outbreak EAPs	East Toledo, US, 1927	None	Cutaneous tests by scratch method with castor bean and flaxseed dust extracts.	Microscopic examination of castor bean dust. '…castor bean dust was thrown into the air for them to inhale'.	Castor bean	All patients gave positive reactions to castor bean extract. 4 patients had inhalation tests to castor bean and all developed typical asthmatic attacks.	Workmen suffered toxic effects of castor bean dust if it gained entrance through an abrasion of the skin.	'Thirty cases of asthma are known to have been caused by the inhalation of finely powdered castor bean dust from a linseed and castor oil mill in the neighbourhood'. Castor bean dust could have been transported by wind.
Mendes & Cintra, (1954a)	Full text (translated) Cli, Lab investigation of outbreak EAPs	Bauru, Sao Paulo, Brazil, 1952	None	16 extracts prepared from potentially allergenic materials in the city.	Passive transfer of reagins and experimental reproduction (inhalation challenge).	Castor bean	9 patients reacted positively and intensely to castor bean extract. This was also observed in another 17 patients from Bauru and Trevudos. In transfer of reagins the castor bean extract also gave urticarial reactions in non-allergic patients. All 4 patients tested by experimental reproduction had an asthma attack to castor bean extract.	None.	Castor bean industry cause of asthma. Very similar study group and tests as published in subsequent 1954 article.
Mendes and Cintra, (1954b)	Full text Cli, Lab investigation of outbreak EAPs	Bauru, Sao Paulo, Brazil, 1952	None	'…selection of extracts for cutaneous tests…prepared with coca solution, dialysed [and] filtered'. Intracutaneous tests also conducted.	Local passive transfer of antibodies. Experimental reproduction of symptomology by inhalation of castor-bean pomace.	Castor bean	Strongly positive scratch tests to castor-bean pomace extracts. '…strongly positive Prausnitz Kustner reactions' from local passive transfer of antibodies. In the reproduction of symptomology all 'four patients had serious attacks of bronchial asthma'.	9 deaths and 150 cases registered. 'majority of patients rid themselves of symptoms' after leaving Bauru. 'Symptoms appeared again when the patients returned to Bauru'. 'Symptoms disappeared in all patients allergic solely to castor bean when the crushing mill ceased to work'.	Cases of collective asthma coincided with the changes made in the method of extraction of castor-bean oil'. 'A new outbreak of collective asthma identical with the first one was observed about a month later when the mill resumed work'.

Ordman, (1955)	Full text Cli, Epi investigation of an outbreak	Town in South Africa, 1952	None	Skin tests by 'scratch method' on 131 employees with extracts of castor bean.	None	Castor bean	27 out of 131 skin tests gave positive reactions to castor bean.		Outbreaks of bronchial asthma due to castor bean dust from the castor oil plant. Increased reporting of asthma when solvent extraction processes were in operation.
Panzani, (1957)	Full text Cli, Lab investigation of an outbreak	Marseilles, France, 1951 onwards	None	Scratch method to castor bean and other extracts.	Local passive transfer of antibodies, *Prausnitz-Kustner*. Experimental reproduction of asthma conducted.	Castor bean	102 cases used. Scratch tests always strongly positive and sometimes caused severe systemic reactions. *Prausnitz-Kustner* reaction gave positive results while castor-allergen aerosol never failed to produce experimental fits of asthma.	None	'The disease is often observed among persons in direct contact with castor beans or oil cakes and among people living within close vicinity to the oil-mills. Removal of the patients to a reasonable distance from the sources of castor bean dust…is usually enough to bring about substantial improvement'.
Strauss, (1968)	Full text Cli, Epi, Lab investigation of an outbreak	Ourinhos, Brazil, 1964	None	65 patients submitted to skin tests. Castor bean extract prepared in Coca's solution for scratch testing.	Second castor bean extract prepared to destroy ricin and use for passive transfer and "*in vitro*" testing. Skin sensitising antibodies from patient serum used in 'passive hemagglutination test', 'immuno-diffusion in gel', 'immunoelectrophoresis' and 'radioimmunoelectrophoresis'	Castor bean	Scratch tests with castor bean extract positive in 40% of cases. Passive transfer tests were positive with 18 out of 26 reaginic sera.	60 out of 65 individuals interviewed lived within a 1-mile radius of the castor bean industry. 'Industrial extraction of castor bean oil was done by compression and supplemental extraction of castor bean pomace by solvent, leaving a dry, very dispersible and highly antigenic residue.	Epidemic started in August at the beginning of the industrial castor bean processing season. All patients affected lived near the castor bean mill.

Strauss, (1975)	Full text Long term-follow up investigation of outbreak EAPS	Ourinhos, Brazil, 1964	None	Direct skin tests using extracts: castor bean, cotton, peanuts, coffee, house dust, fungi, pyrethrum and hay.	Passive transfer tests used for titration of castor bean antibody in reaginic serum.	Castor bean	Skin tests with castor bean extract revealed positive reactions in 12 cases, representing a 30% decrease in positivity since 1964. There was also a fall in antibody titre in 8 reaginic serum'.	Clinically, 4.6 years after industrial castor bean processing stopped, 12 individuals [out of 17] were completely free of symptoms. 'Clinical relapses were experienced by 2 individuals after accidental re-exposure to castor bean dust'.	Positive skin reactions to castor bean were still evident in 70% of individuals, 4.6 years later, it may be concluded, that immunological memory persisted in most individuals with castor bean allergy'.

*Abbreviations for study design are as follows, Cl: Clinical, Env: Environmental, Epi: Epidemiological, Lab: Laboratory Des: Descriptive

2.3.3 Soybean associated asthma outbreaks

We identified 45 articles reporting on soybean associated asthma outbreaks. These were categorised into articles reporting on major outbreaks occurring in New Orleans and Barcelona and articles reporting on outbreaks occurring in other Spanish locations.

2.3.3.1 Outbreaks occurring in New Orleans

We identified 10 articles dated between 1962 and 1997 examining soybean associated asthma outbreaks occurring in New Orleans (Table 2-3). In 1962, after an outbreak of asthma in New Orleans (time-frame not given) had affected approximately 100 people and resulted in 3 deaths, a preliminary report by Lewis et al., (1962) was published.

They reported results from microscopic examination of filter extracts used to collect high-volume air samples which had measured different particulate substances, categorised into 10 groups (reddish-brown spheres, coffee and brown grain fragments, starch, good combustion products >1µm, spores, mineral fragments, pollens, finely divided siliceous material, poor combustion products >1 µm and poor combustion products with associated silica). They thought 'a poor combustion particle with associated silica' was the most likely to be associated with the New Orleans asthma outbreaks. On this evidence they localised their air sampling to two city dumps thought to be the likely source of this material and consequently observed large quantities of the same combustion particle.

Weill et al., (1964a) collected high-volume air samples from the emissions of one of these city dumps, made aqueous extracts from the filter paper and used them for scratch and intradermal skin testing in asthma-outbreak patients. They observed positive reactions to the filter paper extracts in 83% of asthma-outbreak patients. Weill et al., (1964b) setup air sampling stations at six strategic locations around the affected area in New Orleans and conducted scratch and intradermal testing from filter extracts in the same way as before (Weill et al., 1964a). They observed positive SPT amongst asthma-outbreak patients to all six sampling locations, with one station producing positive responses in a significantly higher percentage of asthma-outbreak patients. Of note they also reported findings from the preliminary analysis of air samples obtained from a nearby public grain elevator, which they suspected as the source of allergen. This public grain elevator was located closest to the

sampling station that produced the most significant skin test response in asthma-outbreak patients. Using the extract prepared from the filter sampling air closest to the public grain elevator, they observed positive skin test reactions in 86% of 77 asthma-outbreak patients.

Some years later, Salvaggio et al., (1973) used counterimmunoelectrophoresis (CIE) to detect serum precipitins in New Orleans asthma-outbreak patients, against common airborne allergens found in New Orleans. CIE is a laboratory method used to visualise binding of an antibody to its antigen. The antigen and antibody are applied to an electrical field across a diffusion gel and migration towards one another forms a precipitin line which indicates specific binding.

They found serum precipitins to common allergens were present with equal frequency in asthma-outbreak patients as in other asthmatic patients and 'healthy controls'. They concluded that the New Orleans asthma outbreaks resulted from 'sensitisation of the local population by diverse "natural" particulate inhalants in seasonal patterns' and therefore could not be attributed to any local man-made source that could be modified to reduce the outbreaks.

Over a decade later, the New Orleans asthma outbreaks were revisited by Goldstein and Salvaggio, (1984), who observed a decline in the daily average numbers of emergency room visits for asthma since the 1960's in New Orleans. They suggested this to be due to improved socio-economic conditions, rather than any other intervention. There were no laboratory or clinical tests in this article.

In the late 1980's asthma outbreaks with unknown origin were being reported from Barcelona. Analyses in the Barcelona outbreaks identified soybean as the causative agent (as explained in the following section 2.3.3.2). With this in mind, Li et al., (1996) returned to the grain elevator in New Orleans and specifically measured daily airborne soybean allergen. They used automated air samplers and extracted filters which were applied to an inhibition assay. In the inhibition assay, pooled serum from 13 individuals who experienced epidemic asthma in Barcelona (and who were known to have a high antibody titre specific for soybean allergen) was used to measure airborne soybean. They discovered high levels of airborne soybean (similar to those in Barcelona) was present at the grain elevator in New Orleans.

White et al., (1997) used data from 1957-1968 on emergency department visits for asthma in New Orleans to define asthma-epidemic days and determine if there was an association between asthma-epidemic days and those on which soybean was unloaded at the New Orleans harbour. They found that days with 64 or more visits for asthma in New Orleans from 1957-1968 were twice as likely to have occurred when soybean was unloaded at the harbour. It is now generally agreed that the outbreaks of asthma in New Orleans were related to elevated levels of airborne soybean.

Since these results were published, there have been no articles reporting recurring asthma outbreaks in New Orleans, due to soybean or any other airborne allergens. It is worth noting that since these asthma epidemics occurred, new asthma drugs have been available that have probably contributed to the absence of new epidemics.

Around the same time as the New Orleans outbreaks were first being investigated, Cowan et al., (1963) reported an asthma outbreak affecting 13 individuals in Minnesota on 25th January 1962. There was some evidence for an association with air pollutants from a nearby grain industry. Values from dust spot samplers used on the day of the outbreak, showed a build-up of airborne particles around 6am continuing until noon. Asthmatic symptoms began between 8am and noon in eight out of the 13 individuals. Moreover, the authors conducted SPT using a grain mill dust mixture and other allergens on 59 individuals with asthma that had attended an allergy clinic at the University of Minnesota Health Service, between 1961-1962. From this they found an association between those with a positive SPT to the grain mill dust mixture and those coming from farm houses who had already volunteered a clinical history of sensitivity to grain dust or milling odours.

Table 2-3 Data extraction of New Orleans soybean associated asthma outbreak articles

CITATION	ARTICLE TYPE + STUDY DESIGN*	EPIDEMIC, DATE + LOCATION	AIR SAMPLING METHODS	SKIN PRICK TESTS	LABORATORY TESTS	CAUSAL AGENT	RESULTS OF LABORATORY + SKIN PRICK TESTS	ALL OTHER RESULTS	CONCLUSIONS
Lewis et al., (1962)	Full text Cli, Env, Epi. Lab investigation of an outbreak	New Orleans, 1958	High-volume air sampling. Filters extracted for microscopic examination	None.	Microscopic examination to classify 10 particulate substances.	Combustion particle.	None	Asthma outbreaks commonly associated with winds of low speed'. 'Statistically significant relationship between daily asthma admissions and prevalence of a poor combustion particle'.'	'This particle could arise from combustion processes in which siliceous material is present and burning is inefficient'. Outbreaks attributed to dump burning, although microscopy of air pollution also showed hulls of grains and coffee.
Weill et al., (1964a)	Full text Cli, Env, Epi, Lab investigation of an outbreak	New Orleans 1950's/60's	High-volume air sampling from emissions at Agricultural Street dump.	'Aqueous extracts made from the filter paper collections…used for scratch and intradermal skin testing'.	Glass fibre filter extracted and filtered through Seitz filter.	Agriculture street dump emissions.	In outbreak patients, 83% were positive by intradermal tests to one or more of the air sampling extracts. 7.5% of an asthmatic student group and 13% of the outbreak patients were positive to 1 or more scratch tests with air sampling extracts.	None	'The air extracts were probably allergenic' Agriculture street dump now the focus of New Orleans epidemic asthma.
Weill et al., (1964b)	Full text Cli, Env, Epi investigation of an outbreak	New Orleans 1950's/60's	High-volume air sampling stations setup at strategic locations around affected area.	Aqueous extracts prepared from filters as in previous article.	None	Grain elevator suspected.	'Materials from all sampling stations produced positive skin tests' with one station giving positive responses in a significantly higher percentage of patients.	None	'Preliminary evidence indicated an association between developing sensitivity to grain industry pollutants [located near one sampling station] and clinical episodes of asthma'.

Weill et al., (1965)	Full text Cli, Env, Epi investigation of an outbreak	New Orleans 1950's/60's	High-volume air sampling setup on the roof of the suspected public grain elevator.	Aqueous extracts prepared from filters as in previous article.	None	Grain elevator suspected.	85% of asthma-outbreak patients and 74% of other allergic students in New Orleans gave a positive skin test response to the grain elevator extracts.	Large public grain elevator is situated within a mile of a single station whose extracts are causing positive skin reactions. Grain elevator handles wheat, corn and soybeans with some monthly variations in the proportions of each.	'The public grain elevator in the city is a strong suspect…and outbreak patients demonstrate a very high incidence of positive skin reactions to grain dust extracts.'
Kenline, (1966)	Full text Env, Epi investigation of an outbreak	New Orleans 1950's/60's	6 sampling stations using high-volume samplers.	None	None	Grain elevator suspected.	None	No significant relationships found between asthma and any measured air pollution concentrations. Small particles correlated positively with increased asthma.	Small particles had positive correlations with asthma admissions.
Salvaggio and Klein, (1967)	Full text Cli, Epi investigation of an outbreak	New Orleans 1950's/60's	None	Scratch and intradermal tests to 11 common allergens.	None	None	None	Greater eosinophilia seen in non asthma-outbreak patients.	'…host of diverse allergens rather than a point source air pollutant…compatible with the striking immediate skin reactivity to many local inhalant allergens'.
Salvaggio et al., (1973)	Response Cli, Env, Epi Lab investigation of an outbreak	New Orleans 1950's/60's	Spore and pollen aerometric sampling performed. Suspended particulates quantified using high volume air sampling.	None	Counterimmunoelectrophoresis (CIE) used to detect serum precipitins.	Pollen + spores suspected.	Serum precipitins detected with the same frequency in asthma-outbreak patients, non-asthma-outbreak patients and nonatopic individuals.	Epidemic individuals had skin reactivity to many allergens. High summer asthma admission rates associated with high spore and pollen counts. Epidemics associated with climatological variables: humidity, temperature and wind velocity.	Overall findings suggest that New Orleans epidemic asthma results from sensitisation of the local atopic population by diverse "natural" particulate inhalants in seasonal patterns'
Goldstein and Salvaggio, (1984)	Full text Cli, Env, Epi investigation of an outbreak	New Orleans 1950's/60's	None	None	None	Socio economic climate.	None	'Steady decline in daily average numbers of emergency room visits since the mid and late 1960's'.	'Asthma markedly decreased in severity…due to better socio-economic conditions and better out-patient care'.

Li et al., (1996)	Full text Env, Lab investigation of outbreaks	New Orleans May-October 1990	Air samplers placed around grain elevator.	None	Air filters extracted and used in an inhibition assay to measure airborne soybean detected with serum from Barcelona asthma-outbreak patients.	Soybean	Assay results show significant concentrations of soybean airborne allergen around the port of New Orleans.	None	Low molecular mass soybean aeroallergen was present in the air around the grain elevator in New Orleans.
White et al., (1997)	Full text Epi investigation of outbreaks	New Orleans 1950's/60's	None	None	None	Soybean	None	Days with soy exposure were more likely to be identified as epidemic than were days without soy exposure.	Ambient soy dust is very 'asthmogenic'.
Cowan et al., (1963)	Full text Cli, Epi, Env investigation of an outbreak	Minnesota, US. 25th January, 1962	Airborne particles collected using high volume samplers.	SPT with grain mill dust, linseed, wheat, rye, soybeans, corn, fungi + pollens.	None	Grain industry air pollutants suspected.	Definite association in asthmatic patients between clinical history and grain mill dust sensitivity.	Found association with temperature inversion and low velocity winds.	13 patients developed asthma on 25th January 1962 on which the weather and sampling data indicated an increase of air pollutants. Some evidence for asthma outbreak associated with grain industry.

*Abbreviations for study design are as follows, Cl: Clinical, Env: Environmental, Epi: Epidemiological, Lab: Laboratory Des: Descriptive

2.3.3.2 Outbreaks occurring in Barcelona

We identified 25 articles dated between 1983 and 2011 reporting on soybean associated asthma outbreaks in Barcelona (Table 2-4). There were 26 asthma outbreak days that occurred in Barcelona between 1981 and 1987, that affected 687 persons and included 1155 emergency room admissions. During these outbreak days there were sudden increases in acute severe asthma that overwhelmed emergency service providers. Most patients had a quick recovery and were discharged from the emergency room within 3-12 hours of admission (Picado, 1992). Since measures were implemented in 1987 to prevent the dispersion of soybean dust, only one small outbreak due to soybean has been detected in Barcelona (in October 1994).

The earliest reports on asthma outbreaks by Ussetti et al., (1983) and Ussetti et al., (1984), used hospital admission data from asthma-outbreak patients to investigate a potential link with measured pollution levels. They found that 21 patients attended hospital with acute severe asthma, compared with 1.8 patients on control days, and that the number of asthma hospital admissions was correlated with concentrations of nitric oxide and nitrogen dioxide. They concluded that high concentrations of NO and NO_2 may have played a role in the Barcelona asthma outbreaks.

After a total of 8 asthma epidemics had occurred, Antó and Sunyer, (1986) used hospital admission data to investigate time and space clustering of the onset of symptoms in asthma-outbreak patients from the latest Barcelona asthma epidemic. They found on the day of the epidemic there were 43 (adult) and 22 (child) admissions for asthma of which 70% were admitted between the time of noon and 4pm. They also observed geographical clustering for the onset of symptoms, with patients reporting that at the time of onset they had been close to the harbour in Barcelona. In 1986, Anto *et al.* concluded that elevated nitrogen dioxide was unlikely to be the cause of asthma outbreaks in Barcelona, as air pollution monitors showed nitrogen dioxide levels were within the normal ranges during the previous two outbreaks.

In 1989, Antó et al., published in The New England Journal of Medicine evidencing a strong association of asthma-epidemic days with the unloading of soybean at the harbour. They identified all the products unloaded at the Barcelona harbour and the days on which they

were unloaded, between 1985 and 1986. For each product they calculated a risk ratio between 'the probability of an asthma epidemic on the days when the product was being unloaded and the probability of an epidemic when it was not being unloaded'. They identified 13 asthma-epidemic days which all coincided with the unloading of soybean from the harbour, with no epidemic days occurring when soybean unloading did not take place.

The epidemiological results of this article were supplemented with morphological analysis of airborne particles collected in small samplers near the harbour and soybean samples taken from those unloaded in Barcelona during two asthma outbreaks. The analysis was performed with optical microscopy, Lugol's solution to detect starch and Coomassie blue solution to detect protein. In the air filters, spheroidal particles with starch and protein and 'large, trumpet-shaped cells' were detected. The soybean samples collected had aggregates of starch particles and 'large, trumpet-shaped cells, morphologically identical to those in the filters'. With this information the authors thought there could be little doubt that inhalation of soybean dust released from the unloading of soybean at the harbour was causing the outbreaks. In 1989, laboratory methods: paper radioimmunosorbent (RIA) and radioallergosorbent (RAST) were used to measure serum specific-IgE from Barcelona asthma-outbreak patients (Sunyer et al., 1989). They showed that 84.9% of these patients had serum specific-IgE antibodies against soybean dust extract compared to 5.8% of non-epidemic patients.

At the beginning of September 1987, the unloading of soybean in Barcelona was stopped. Three months later, bag filters were installed at the top of the silo (into which soybeans were unloaded), to prevent the dispersal of airborne soybean, and unloading was resumed under controlled conditions. Antó et al., (1989) briefly remark on a preliminary analysis that used data on asthma emergencies for a 16-month period after the installation of filters. At the time of publication, they indicated that since the installation of filters the outbreaks of asthma were not recurring. This analysis was published in further detail by Antó et al., (1993), as described below.

In the following decade, a number of articles attempted to further characterise soybean allergens responsible for causing asthma outbreaks.

Rodrigo et al., (1990) used a protein assay to quantify protein in various extracts of soybean and RIA-inhibition analysis to measure serum specific-IgE in asthma-outbreak patients to hull and dust soybean extracts. They also used electrophoresis, isoelectric focusing and western blot, which showed specific-IgE from asthma-outbreak patients reacted to protein bands from soybean extract with a molecular weight <14.4kDa and isoelectric point <6. Aceves et al., (1991) used RAST inhibition to measure soybean levels in the air on epidemic days. Swanson et al., (1991) identified a glycopeptide less than 14kDa as the major allergen from soybean causing the asthma outbreaks. They used a protein assay to quantify protein in soybean and electrophoresis and western blot. Sunyer et al., (1992) used SPT to assess cutaneous hypersensitivity to common local airborne allergens and RIA to measure total serum IgE in asthma-outbreak patients from Barcelona.

Antó et al., (1993) conducted high-volume air sampling and used RAST inhibition to determine airborne concentrations of soybean in Barcelona, after bag filters to prevent the dispersal of airborne soybean had been fitted. They showed that the installation of filters was very effective in reducing airborne soybean dust (324 units per cubic meter of soybean prior to the intervention, to 25 units per cubic meter after the intervention). The intervention was also effective in preventing outbreaks of asthma, going from 18 asthma epidemic days pre-intervention to zero post-intervention. They also found there was a substantial reduction in admission for asthma to intensive care from 26 per 100 days to 1 per 100 days after the installation of filters.

Pinto (1994) characterised soybean allergens using electrophoresis, isoelectro focusing, western blot and chromatography and identified six allergenic thermostable proteins with molecular weights: 21.4, 19.1, 17.0, 14.4, 10.0 and 6.3kDa in soybean powder extract. Sabrià et al., (1994) measured total serum IgE in asthma-outbreak patients using RAST. Morell et al., (1995) used an ELISA to measure specific-IgE from patient serum to soybean hull and dust extracts, in comparison to SPT diagnosis. They also used a protein assay to quantify protein in soybean extracts.

In October 1994, a small cluster of emergency room visits for asthma occurred in Barcelona, again. It was investigated by Soriano et al., (1995). Using available air filters, RAST inhibition was performed to measure soybean allergen present on this day and on days before and after. They found 1122.5U/m^3 of soybean airborne allergen present during the day of the

asthma cluster, with measures of 8.0 and 4.7U/m^3 on 2 days before and after the asthma cluster. The authors comment that for 'reasons not yet established…soybean unloading released soybean dust and produced acute attacks of asthma in a few sensitised patients'.

Other laboratory methods were used by Codina et al., (1997a). They used an amplified ELISA to quantify other specific immunoglobulins (IgG, IgG_1 IgG_3 IgG_4, IgA and IgM) and electrophoresis and western blot. By western blot they observed three protein bands from soybean, with molecular weights of 8, 7.5 and 7kDa with binding to specific-IgE, IgG and IgG_4. They also quantified protein from soybean extracts using the bicinchoninic acid assay and used RAST to measure specific-IgE.

The 8kDa allergen from soybean was characterised further by Codina et al., (1997b). They dialysed a soybean hull extract to recover proteins specifically between a molecular weight of 10 and 3.5kDa and then fractionated the extract by preparative isoelectrofocusing (IEF). A fraction with a pH range of 5.97 – 6.05 was then studied by electrophoresis and western blot with serum from asthma-outbreak patients. From this, the authors observed the 8kDa allergen, with immune-specificity confirmed by the non-recognition of this protein by pooled control serum from non-allergic individuals. This 8kDa protein band was 'excised and inserted into a blot cartridge and analysed using an automatic sequencer' and the N-terminal sequence (first 20 amino acids) was revealed. They compared the protein sequence with others in the Swiss Protein Sequence Databank and assessed its sequence homology with other proteins. They also performed specific-IgE determination and assessed the allergenicity of the 8kDa allergen by RAST, as well as assessing cross-reactions between the 8kDa allergen and the original unfractionated soybean extract by RAST inhibition.

Genetic polymorphisms were assessed in soybean asthma-outbreak patients by Soriano et al., (1997). They extracted DNA from blood of soybean asthma-outbreak patients and used PCR to amplify human leukocyte antigen (HLA) genes. They found the risk of epidemic asthma was particularly associated with a DRB1*13 gene. They also used a commercial ImmunoCAP method to assess total IgE levels (expressed in kU/L) and found asthma-outbreak patients had a greater serum total IgE level than nonepidemic asthma patients.

Codina & Lockey, (1998) investigated the role of moulds in the Barcelona asthma outbreaks as a secondary allergen source. They cultured soybean hull and identified mould species by

macro and microscopic characteristics and then used RAST to identify specific-IgE in asthma-outbreak patients for the mould species. They found soybean was contaminated by *Aspergillus* and *Penicillium* species and that asthma-outbreak patients had high levels of specific-IgE to these two moulds. The authors conclude by suggesting that moulds may have had a role to play in soybean-induced asthma but no other articles have reported on this.

In 1999, Antó et al., conducted a study on the long-term outcome of soybean epidemic asthma in Barcelona. They took air samples which were assayed for soybean allergen by RAST, measured specific-IgE to soybean and total IgE in asthma-outbreak patients by ImmunoCAP and RAST in addition to SPT. They concluded that levels of soybean were now consistently low and asthma-outbreak patients improved in the following 2 years since the measures to prevent airborne soybean dispersal were implemented.

Codina et al., (1999) used electrophoresis and western blot and observed previously reported allergenic proteins from soybean as well as four additional allergens at higher molecular weights: 25, 27, 34 and 36kDa. An amplified ELISA inhibition assay was used by Rodrigo et al., (2004) to measure post-intervention soybean aeroallergen and compare with pre-intervention soybean allergen levels. As part of longer-term public health monitoring schemes in Barcelona, Villabi *et al.* (2004) and Villalbí et al., (2011) have measured soybean aeroallergen levels in Barcelona using ELISA.

Table 2-4 Data extraction of Barcelona soybean associated asthma outbreak articles

CITATION	ARTICLE TYPE + STUDY DESIGN*	EPIDEMIC, DATE + LOCATION	AIR SAMPLING METHODS	SKIN PRICK TESTS	LABORATORY TESTS	CAUSAL AGENT	RESULTS OF LABORATORY + SKIN PRICK TESTS	ALL OTHER RESULTS	CONCLUSIONS
Ussetti (1983)	Letter Cli, Env, Epi investigation of an outbreak	Barcelona outbreaks - Oct 1981, June, August, Oct 1982, Jan 1983	None	None	None	None	None	21 patients attended with acute severe asthma as opposed to 1.8 patients on control days. 'Time between onset of asthmatic attack and hospital admission was shorter on epidemic days'.	Future research should assume that asthma outbreaks could occur at any season.
Ussetti (1984)	Letter Cli, Env investigation of an outbreak	Barcelona outbreaks - Oct 1983	None	None	None	None	None	Correlation between NO and NO_2 concentration and the number of hospital admissions.	'High concentrations of NO and NO_2 may have played a role in these asthma outbreaks'.
Anto and Sunyer (1986)	Full text Cli, Env investigation of outbreaks	Barcelona outbreaks - 1984	Pollen and spore samplers used.	None	None	None	None	Adult asthma emergency room admissions showed an hour-time cluster. Geographical clustering near the sea for adult asthma admissions. 43 adult cases and 22 children across the city. Air pollution levels and spore counts were both below average.	The industrial area adjacent to the neighbourhood in which the epidemic occurred was a probable source of the outbreak.
Anto et al. (1986)	Letter Cli, Env investigation of outbreaks	Barcelona outbreaks - Jan, May, Sept 1986	None	None	none	None	None	'Most patients did not report a noticeable change in their baseline symptoms' during the air pollution episode. Few reported an increase in respiratory-related medication and none attended an emergency room.	High levels of NO_2 are not a sufficient cause of the outbreaks of asthma in Barcelona. 'Occurrence of asthma outbreaks in Barcelona when NO_2 was within the usual ranges strengthens the evidence against it as a cause'.

Anto et al. (1989) (April)	Full text Env, lab investigation of outbreaks	Barcelona outbreaks 1985/6	Particles were collected in small samplers with cellulose ester filters'	None	Morphological analysis performed by optical microscopy. Lugol's solution for starch staining and Coomassie blue for protein detection. Soybean samples examined by optical microscopy.	Soybean	Spheroidal particles found in air filters were positive for starch and protein. Cells morphologically identical to those in the filters were also found in the episperm of cross-sectioned soybeans.	13 of 23 unusual asthma days were classified as asthma-epidemic days. These days coincided with soybean unloading. 'No epidemic days occurred when soybean unloading did not take place' and 'only 3 of 13 asthma-epidemic days coincided with the unloading of wheat'.	A strong association was found between asthma-epidemic days and unloading of soybean'. Outbreaks were found to be caused by the inhalation of soybean dust released during the unloading of soybean at the city harbour.
Sunyer (1989) (January)	Full text Epi, lab investigation of outbreak EAPs	Barcelona outbreaks	None	None	Total serum IgE, specific-IgE to soybean and other antigens by RAST. Soybean dust extract prepared.	Soybean	74.4% of epidemic cases reactive with commercial soybean. 84.9% reactive with soybean dust extract, compared with 5% of controls.	None	Strong association between specific soybean IgE levels and epidemic asthma.
Ferrer et al. (1990) (April)	Full text Epi investigation of outbreak EAPs	Barcelona outbreaks	None	None	None	Soybean	None	'Epidemic patients were ventilated for fewer hours, admitted fewer days to intensive care and hospitalised fewer days than non-epidemic patients'.	'Differences in patients are consistent with the unusual nature of the aetiological agent, soybean dust'.
Rodrigo et al. (1990) (April)	Full text Lab investigation of outbreak EAPs	Barcelona outbreaks	None	None	Antigen extracts prepared from various forms of soybean. Total protein determined by bicinchoninic acid assay. Soybean specific-IgE measured. Soybean extracts compared by inhibition analysis and applied to electrophoresis, isoelectric focusing and western blot.	Soybean	90% of serum from epidemic patients had high specific-IgE antibodies to soybean hull. Protein bands were observed at 97.4 to <14.4kd and between isoelectric points 6 and 3.5. Western blot showed epidemic patient serum weakly reacted to '2 protein bands ranging from 42 to 21kda and strongly to glycoprotein bands <14.4kda and isoelectric point <6'.	None	'Patients with asthma during an epidemic in Barcelona reacted specifically to an acidic, low molecular weight allergen located in hulls, dust and trash of soybean unloaded in the harbour'.

Aceves et al. 1991 (July)	Full text Env, Lab investigation of outbreaks	Barcelona outbreaks - 1981-1987	Aerosol sampling with high-volume suction pumps and glass microfiber filters.	None	Organic analysis, filter extraction, fractionation to isolate hydrocarbons and alcohols, GC-MS analysis. Allergens from filters assayed by RAST inhibition.	Soybean	Soybean markers identified within aerosol extracts. High values of soybean on epidemic days.	None	Results have 'demonstrated that airborne soybean dust released from the harbour silos was effectively present in the urban atmosphere during [asthma] episodes'.
Swanson et al. 1991 (April)	Full text Lab investigation of outbreak EAPs	Barcelona outbreaks	None	None	Antigen extracts of soybean made. Grain dusts concentrated using a Centricon microconcentrator. RAST inhibition assays. Protein content determined by bicinchoninic method. Electrophoresis and western blot performed.	Soybean	Soybean telae collected in Barcelona was the richest source of allergen reacting with serum specific-IgE. Coomassie staining observed strong bands present at >15kDa with weaker bands below <15kDa from various soybean extracts. Western blot showed intense staining <15kDa with few reactive high molecular weight bands. Most allergens were <14kDa, concentrated in the hull.	None	The major allergen causing the epidemics was a glycopeptide <14kda…abundant in soybean dust'.
Sunyer et al. 1992	Full text Cli, Epi, Lab investigation of outbreak EAPs	Barcelona outbreaks	None	Cutaneous hypersensitivity to common local airborne allergens' was assessed.	Total serum IgE levels assessed by paper radioimmunosorbent test'.	Soybean	No significant difference in positive skin reactions, but more frequent positive reactions in case group. 'Total IgE levels were greater in case than in control subjects'.	As well as soybean exposure, age, atopy and cigarette smoking also had causative roles in Barcelona epidemic asthma.	Results suggest that epidemic asthma was a multifactorial process.

Anto et al. 1993	Full text Cli, Env, Lab long term follow up investigation of outbreak EAPs	Barcelona outbreaks, Jan1987 - Dec 1989	High-volume sampling	Cutaneous hypersensitivity to soybean-was assessed.	Allergens eluted from filters and assayed by RAST inhibition. Specific-IgE antibodies against commercial soybean antigens also detected by RAST.	Soybean	Soybean allergen airborne concentration decreased significantly after filters were installed. Serum soybean specific-IgE antibodies persisted for 2 years after filter installation and cessation of epidemics.	No asthma-epidemic days after filter installation. Decrease In number of days on which there was an unusually large number of emergency room visits for asthma.	Installation of filters prevented the dissemination of allergenic soybean dust from causing outbreaks of asthma.
Pinto 1994	Full text (translated) Cli, Env, Lab investigation of an outbreak	Barcelona, 26 outbreaks since 1981	None	Skin prick tests with 4 extracts	SDS-PAGE, isoelectrofocusing, western blot, chromatography.	Soybean	Soybean powder extract contains 6 allergenic proteins with molecular weights of 21.4, 19.1, 17.0, 14.4, 10.0 and 6.3kD. Allergens were thermostable - no extracts lost allergenic activity after heating.	None	6 allergenic thermostable proteins in soybean powder extract. Approximate fractions at 22,25 and 13-14kD contain most of the allergenicity.
Sabria et al. 1994	Full text Cli, Epi, Lab long term follow up investigation of outbreak EAPs	Barcelona outbreaks	None	Atopy assessed to 16 common allergens.	Total serum IgE concentration assessed using RAST.	Soybean	Patients with epidemic asthma were more atopic and had a higher total IgE than controls.	Patients suffering epidemic asthma reported fewer symptoms during the previous year than non-epidemic subjects, with 20.1% of epidemic patients attending hospital compared to 36.7% non-epidemic subjects.	Results suggest that, 2 years after the elimination of the epidemics, those affected had a favourable prognosis'.
Morell et al. 1995	Full text Cli, Epi, Lab long term follow up investigation of outbreak EAPs	Barcelona outbreaks	None	Skin prick tests	Extracts prepared from hulls and dusts of unloaded soybeans. Protein content determined by bicinchoninic acid assay. Specific-IgE to soybean hull and dust extracts by ELISA.	Soybean	Significant difference of specific-IgE to soybean between epidemic asthmatics and non-epidemic asthmatics.	None	Skin prick tests and ELISAs with soybean hull and dust extracts have proved effective in the diagnosis of soybean asthma'.
Soriano et al. 1995	Full text Env, Lab investigation of outbreak EAPs	Barcelona, October 1994	Air filters obtained for days before and after asthma outbreak day.	None	Air filters assayed for soybean allergen by RAST inhibition.	Soybean	Air filters had significantly less soybean allergen detected on days before 8.0 and after outbreak day 4.7 compared with asthma outbreak day 1122.5 U/m^3	None	Soybean unloading released dust and produced acute attacks of asthma in a few sensitised patients.

Codina et al. 1997a (January)	Full text Lab investigation of outbreak EAPs	Barcelona outbreaks	None	None	Allergen extract prepared from soybean hull unloaded during a Barcelona asthma outbreak. Protein content measured by bicinchonic acid assay. RAST used for specific-IgE determination. An amplified ELISA was performed to determine specific-IgG, IgG1, IgG3, IgG4, IgA and IgM. Cross inhibition study for specific-IgE and IgG4 on the hull extract. Extracts applied to electrophoresis and western blot.	Soybean	Higher serum concentration of specific-IgE and IgG4 in patients with soybean asthma. 3 protein bands observed that bind specific-IgE, IgG and IgG4'. 'Cross-inhibition shows amount of protein to inhibit specific-IgG4 binding is almost 500 times lower than for specific-IgE'.	None	Study suggests that both IgE and IgG4 might be involved in the pathogenesis of soybean asthma'.

Codina et al. 1997b (October)	Full text Lab investigation of outbreak EAPs	Barcelona outbreaks	None	None	Soybean hull allergen extract prepared and dialysed to recover proteins (10-3.5kDa). Extract fractionated by preparative isoelectrofocusing. Specific-IgE determined by RAST with pooled serum from asthmatic-outbreak patients. Cross-reactions of allergens performed by RAST inhibition. Extracts applied to electrophoresis and western blot. 8kDa soybean protein analysed using an automatic sequencer.	Soybean	Specific-IgE to fractionated soybean demonstrated high binding at pH ranges 5.97-6.87. Electrophoresis and western blot demonstrated binding to an 8kDa protein not seen by control pools. RAST inhibition showed extract containing only Gly m 2 allergen is less potent.	None	Study 'demonstrates the existence of a third soybean hull allergen, Gly m 2, which is also responsible for soybean-induced asthma'.
Soriano et al. 1997	Full text Cli, Lab investigation of outbreak EAPs	Barcelona outbreaks	None	Testing with common allergens.	Total IgE levels assessed by ImmunoCAP, DNA extracted from blood taken from soybean asthma patients, underwent PCR and visualised in agarose gels.	Soybean	Asthmatic-outbreak patients were more atopic had a greater total IgE and a 10-fold greater sensitivity to soybean. 'The DRB1*13 gene was associated with epidemic asthma risk, particularly for subjects with the lowest total IgE concentration'.	None	Genetic predisposition could contribute to the response of some asthmatic patients to exposure to soybean dust'.

Codina and Lockey 1998	Full text Lab investigation of outbreak EAPs	Barcelona outbreaks	None	None	Allergen extracts of soybean hull unloaded at Barcelona harbour were prepared. Soybean hull was cultured to isolate and identify mould colonies. RAST was used for specific-IgE determination of patient serum and a cross-inhibition study using pooled serum.	Soybean	Soybean hulls contaminated by *Aspergillus* and *Penicillium* species. Soybean allergen extracts cross-react with *A. flavus* and *A. glaucus*. Serum of asthmatic-outbreak individuals contains higher levels of specific-IgE to many *Aspergillus* and *Penicillium species*.	None	Soybean hulls are contaminated by allergenic mould species… [that] may play a role in soybean-induced asthma'.
Anto et al. 1999	Full text Cli, Epi, Env, Lab long term follow up investigation of outbreak EAPs	Barcelona, 1995-1996	Air samples obtained 1.3 miles from point source.	Atopy assessed by testing to common allergens.	Filters assayed for soybean allergen by RAST inhibition. Total IgE levels assessed by ImmunoCAP. Specific-IgE antibodies to soybean assessed using RAST and CAP method. Testing of skin soybean sensitivity performed using a dry extract from the hull.	Soybean	Levels of soybean allergen in air filters collected during 1995-6 on soybean unloading days were consistently low and not significantly different from levels on non-unloading days. Asthma-outbreak patients were more atopic with a higher total IgE. Skin reactivity to common aeroallergens was similar in cases and controls.	Symptoms of asthma were more likely to improve than to worsen in epidemic and non-epidemic asthma patients'. 'Smaller proportion of epidemic asthmatics showing improvement'.	Initial improvement in soybean epidemic asthma in the 2 years following the intervention with no further improvement in subsequent years'.

Codina et al. 1999	Full text Lab investigation of outbreak EAPs	Barcelona outbreaks	None	None	RAST used for specific-IgE determination to the soybean hull allergen extract. Electrophoresis and western blot performed on soybean extract.	Soybean	83.3% of asthma-outbreak serum contained specific-IgE to soybean hull allergens and gave a positive western blot. Protein bands observed at 8, 7.5 and 7kDa for already reported allergens, but also another allergen at 8.2-8.3kDa and 4 additional allergens with a higher molecular weight.	None	3 main soybean hull allergens detected and additional higher molecular weight allergens, also involved in soybean-induced asthma.
Rodrigo et al. 2004	Full text Cli, Epi, Env, Lab investigation of outbreak	Barcelona 2004	Automated air sampler used	None	Soybean aeroallergen extracted from filters. Soybean concentration determined by an amplified ELISA inhibition assay using a soybean allergic serum pool.	Soybean	Mean soybean aeroallergen levels in the pre-intervention period were significantly higher than in the post-intervention period'.	'Latest protective measures…demonstrated a decrease in mean environmental soybean aeroallergen concentrations on unloading days'. Sensitisation is still present.	Strategies implemented have significantly reduced the concentration of soybean dust in the atmosphere'.
Villalbi et al. 2004	Full text Cli, Env, Lab long term follow up investigation of outbreak	Barcelona outbreaks	Filters from contaminati on control station in the port.	None	Allergen concentration from filters determined by RAST. RAST inhibition gave measure of emissions in terms of allergen units per volume of air.	Soybean	Low levels of allergen with some fluctuations.	A cluster of 5 patients presented with a sudden worsening of their symptoms on a single day.	New filters shown to be effective in the control of soybean dust emissions.
Villalbi et al. 2011	Full text Cli, Epi, Env, Lab investigation of outbreak	Barcelona outbreaks	Allergen measured in monitoring station in Barcelona.	None	Soy allergen extracted and concentrations determined by RAST.	Soybean	4 out of 95 plants studied were above emission reference levels for allergen.	No health effects were detected in the panel of patients nor epidemic asthma days.	System to monitor allergen emissions has shown to be useful in protecting public health.

*Abbreviations for study design are as follows, Cl: Clinical, Env: Environmental, Epi: Epidemiological, Lab: Laboratory Des: Descriptive

Outbreaks occurring in other Spanish locations

We identified 10 articles, dated between 1989 and 2010, on soybean associated asthma outbreaks occurring in Spanish locations other than Barcelona (Table 2-5).

In Valencia, Alvarez-Dardet et al., (1989) showed a statistically significant association between asthma-epidemics and days of soybean unloading. They identified 7 'unusual' asthma days and 3 asthma 'epidemic' days. Five of these days coincided with the unloading of soybean. In 1999, Ballester et al., assayed filters collected in 1995 in Valencia, for soybean allergen by RAST inhibition. They found the maximum concentration of soybean allergen was 35.84U/m^3, and higher concentrations were present on days with soybean unloading than on days without.

Four articles were identified on soybean associated asthma outbreaks in Cartagena (Hernando et al., 1989; González et al., 1991; Navarro et al., 1993 and Zapatero et al. 1994). The local medical services detected three asthma outbreaks from 1987-88 with significant increases in the number of admissions to the emergency room, including 34 individuals affected with acute asthma attacks. In all articles, ELISA and/or RAST were used to measure serum specific-IgE from epidemic-asthmatic patients to soybean extracts. Hernando et al. found 76% of asthma-outbreak patients had specific-IgE for soybean extract, whilst Gonzales et al. found 90% of patients had specific-IgE to soybean shells. Using electrophoresis and western blot methods Gonzalez et al. identified 10 low molecular weight proteins able to bind to specific-IgE. Zapatero et al. found 90% of asthma-outbreak patients gave positive SPT to soybean while 81% had specific-IgE to soybean as measured by ELISA.

Garcia et al. (1997 and 1998) reported two soybean associated asthma outbreaks in Tarragona. SPT, RAST and ImmunoCAP were used as well as electrophoresis and western blot methods. There were 15 patients admitted with 13 and 10 giving positive results to SPT and soybean specific-IgE, respectively. The authors observed specific-IgE binding to a 15.5-17kDa protein in 77% of serum and a 5-6kDa protein in 62% of serum.

In 1994, an outbreak of asthma was detected in L'Hospitalet de Llobregat, a city on the outskirts of Barcelona. Pont et al., (1997) conducted SPT and ImmunoCAP, to measure

soybean specific-IgE in 9 patients that presented with acute asthma attacks. The results were positive for all cases and strongly suggested that soybean exposure caused the outbreak of asthma.

Rovira et al., (2010) investigated the risk of asthma outbreak at the Port of Tarragona where soybean unloading regularly took place. They used an ELISA to measure airborne allergen concentrations at a short distance from the unloading source, which were found to be moderate, with the maximum at 441U/m^3. They did not detect an asthma epidemic during days of soybean unloading, although they expressed concern that allergen dispersion may pose a risk to sensitised patients.

Table 2-5 Data extraction of soybean associated asthma outbreak articles occurring in other Spanish locations

CITATION	ARTICLE TYPE + STUDY DESIGN*	EPIDEMIC, DATE + LOCATION	AIR SAMPLING METHODS	SKIN PRICK TESTS	LABORATORY TESTS	CAUSAL AGENT	RESULTS OF LABORATORY + SKIN PRICK TESTS	ALL OTHER RESULTS	CONCLUSIONS
Alvarez-Dardet (1989) (October)	Editorial Epi investigation of outbreaks	Alicante/ Valencia 1987/1988	None	None	None	Soybean	None	7 unusual days and 3 epidemic days seen in Valencia. 5 of these days coincided with soybean unloading. No unusual or asthma epidemic days found in Alicante.	'Findings strongly support the soybean hypothesis of the Barcelona scientists' for soybean unloading in Valencia.
Hernando et al. (1989) (March)	Letter Cli investigation of outbreak EAPs	Cartagena, Spain, Oct 23, 1987, April 13,14, 1988		SPT to commercial allergens and soybean.	Total serum IgE levels and specific-IgE to commercial soybean were measured by ELISA. Specific-IgE levels for a soybean extract were measured by RAST'.	Soybean	Of emergency room admissions on 'epidemic' days, 69% had high levels of total serum IgE and 63% had specific-IgE to commercial soybean. 'The proportion of cases with specific-IgE to soybean extract was 76%'.	Sulphur dioxide and particle levels normal'.	'High proportion of asthma cases with positive tests, both on skin and in serum, for soybean and absence of other explanations suggest relation between asthma epidemics in Cartagena and unloading of soybean'.
Gonzales et al. (1991) (January)	Full text Lab investigation of outbreak EAPs	Cartagena, Spain, 1987, 1988	None	None	Shells and grains of soybean extracted. Protein concentration determined. Cross inhibition and RAST to measure specific-IgE to soybean components. Electrophoresis and western blot performed.	Soybean	90% of patients had specific-IgE to soybean shells and 13% to soybean grains. Shell component contained mostly low molecular weight proteins with up to 10 able to bind IgE, whereas the grain extract had only 2 weakly stained bands binding serum specific-IgE.	None	Allergenic proteins with molecular weights similar to those reported in the literature were not found in the grain extract, however other proteins were observed in the sample with the ability to bind specific-IgE.
Navarro et al. (1993)	Full text Cli, Lab investigation of outbreak EAPs	Cartagena, Spain, 1987, 1988	None	SPT to commercial soybean, common allergens and soybean extract.	Specific-IgE and total serum IgE measured by ELISA.	Soybean	Serology and SPT were statistically significant for soybean allergens in cases versus their respective controls.	None	'Results support the association between developing an acute asthma attack on an outbreak day and being sensitive to soybean'.

Zapatero et al. (1994)	Full text (translated) Cli, Lab investigation of outbreak EAPs	Cartagena, 23 October 1987, 13 April 1988, 14 April 1988	None	SPT to common allergens and soybean extract	Soybean extracts prepared. Serum total IgE by ELISA. Serum specific-IgE by RAST. Specific-IgE for the soybean extract by ELISA.	Soybean	90% of patients with positive SPT to prepared soybean extract. 75.5% positive to commercial soybean. 81% positive in specific-IgE ELISA to prepared extract and 50% to commercial soybean.	34 patients in total with most having had asthma before the outbreak. Bronchial challenge with soy extract in 3 control asthmatics was negative.	Clinical history and environmental observations strongly suggest asthma outbreaks were caused by the inhalation of soybean dust spread out in the air following soybean transport and industrial activity in the centre of the town.
Pont et al. (1997)	Full text Cli, Env, Lab investigation of an outbreak	L'Hospitalet, Spain, 27 October 1994	Atmospheric particles collected.	SPT to commercial soybean.	Specific-IgE to soybean extract by ImmunoCAP	Soybean	SPT confirmed exposure to soy in all cases.	9 patients with acute asthma attacks. Soy unloaded in harbour on same day.	Preventative measures on soybean unloading need to be reviewed.
Garcia-Ortega (1997)	Letter (translated) Cli, Lab investigation of outbreak EAPs	Tarragona, 15 November 1994, 28 October 1995	None	Performed to soybean seeds.	Determination of specific-IgE to soybean seeds.	Soybean	In first epidemic with 15 patients, 13 and 10 gave positive SPT and specific-IgE to soybean seeds. In the second epidemic one of the 2 new patients was also positive.	15 patients identified in first epidemic and 11 in second epidemic (9 of which were repeaters). Existence of post activity of soybean discharge was confirmed in both epidemics.	Using repeat patients and detection programs can be used for identification and prevention of asthma outbreaks.
Garcia-Ortega (1998)	Full text (translated) Cli, Lab investigation of outbreak EAPs	Tarragona, 15 November 1994,	None	SPT to common allergens and organic products discharged in the silos of the port during epidemic day.	Total serum IgE, specific-IgE with RAST, ImmunoCAP and ELISA. Electrophoresis and western blot performed.	Soybean	In 15 patients, sensitisation to soybean seed powder was verified in 13. Presence of 2 protein bands of 15.5-17kDa in 77% of serum and 5-6kDa in 62% of serum.	All 13 patients sensitised were atopic. The epidemic was particularly severe in over 50's. 4 patients were admitted to intensive care.	There are 2 main allergenic proteins from soybean implicated in the sensitisation of EAPs.
Ballester et al. (1999)	Full text Epi, Env, Lab investigation of possible outbreaks	Valencia and A Coruna, Spain, 1993-1995	Air filters available for short period in 1995, Valencia.	None	Filters assayed for contents of low molecular weight soybean allergen by RAST inhibition.	Soybean	None	Increase in asthma visits was observed during unloading of soya husk and soybeans in A Coruna. The unusual asthma days in this study did not correlate with soybean unloading days.	Lack of overall association between soybean unloading and asthma outbreaks, however the weak associations found should be viewed with concern.

Rovira et al. (2010)	Full text Cli, Env, Lab investigation of an outbreak	Tarragona, Spain, 2007-2008	PM_{10} filters from the port	4 extracts tested	ELISA to measure allergen concentrations. Specific-IgE value determined using ImmunoCAP and western blots performed.	Soybean	Allergen emissions were moderate at 1km from the unloading source. 'Protein patterns of soybean extracts similar to that found in Barcelona.' '92% of patients sensitised to soybean hull extracts.' 'Low molecular weight soybean protein identified as in previous studies, as well as high molecular weight proteins'.	No asthma epidemic was detected during unloading days.	There is allergen dispersion at a short distance from the unloading source, posing a risk to sensitised patients'.

*Abbreviations for study design are as follows, Cl: Clinical, Env: Environmental, Epi: Epidemiological, Lab: Laboratory Des: Descriptive

2.3.4 Thunderstorm associated asthma outbreaks

We identified 31 articles reporting outbreaks of asthma associated with thunderstorm events (Table 2-6). These are dated from 1985 to 2011 and cover 13 isolated outbreaks over 6 countries. A time-series based analysis was used in 8 articles to identify associations of asthma attacks with thunderstorm events. In 15 articles, air sampling methods were used that included volumetric spore counts and pollen traps. Unlike soy or castor bean associated asthma outbreaks, no western blot methods or protein assays were used and the use of RAST and ELISA methods is only reported in one article.

The earliest identified report of a thunderstorm associated asthma outbreak was published by Packe and Ayres in 1985 and referred to events in July 1983 in Birmingham. The average number of daily attendances for asthma ranged between 2 and 20 across 8 local hospitals. However, on the 6 and 7 July there were 36 and 70 attendances, respectively. Packe and Ayres collected measures of pollen and spore counts from a 'volumetric slit sampler' that was in operation during the thunderstorm event and found there was a 'large and sudden increase in numbers of airborne fungal spores, especially *Didymella exitalis* and *Sporobolomyces*, around the time of the outbreak. Morrow-Brown and Jackson, (1985) investigated the same outbreak in Birmingham and also detected large numbers of spores. Using scanning electron microscopy, they observed the spores were accompanied by crystals and identified them as an 'anhydrous form of calcium sulphate', by energy dispersive X-ray microanalysis.

In 1986, Packe & Ayres, conducted SPT on asthma-outbreak patients and found no significant differences in sensitisation, between patients and atopic controls. SPT to mixed grass pollen, house dust, Cladosporium, Sporoblomyces, and *Didymella* was used. They suggested that the SPT results argued against a role for these allergens and that the asthma outbreak was the result of several interacting factors, such as sudden disturbances in weather inducing airway narrowing in allergic patients.

In Nottingham, 20 June 1984, 19 patients in the day and 3 during the night attended A&E reporting acute severe asthma attacks immediately after a thunderstorm. Less than half of these patients gave a past history of hay fever and no obvious cause was identified (Alderman et al., 1986).

In Melbourne, two consecutive thunderstorm associated epidemics of asthma occurred on the 8th and 29th of November 1987 with records of 154 and 277 adults attending hospital presenting with asthma. Bellomo et al., (1992) found that all of the patients had a history of hay fever and using SPT determined they were significantly more likely to test positive to commercial rye grass pollen than non-epidemic asthmatics. In addition, these authors used in their SPT panel 'isolated inhalable components of rye grass pollen …released from rye grass pollen when it is exposed to rain or moisture'. The response was positive to this extract in all storm affected patients (n=12) compared with only 7 of the 13 controls ($P < 0.025$).

Nine articles were identified relating to a single thunderstorm event, on 24th-25th June 1994, across Central and Southern England. The earliest of these (Murray et al., 1994 and Higham et al., 1997) described the sudden increase in asthma attendances. For example, at one hospital 55 people attended for acute severe asthma in contrast to an expected 5-6 cases, representing a 10-fold increase. Campbell-Hewson et al., (1994) was the first to report an association with pollen counts and asthma in this thunderstorm event, in a descriptive and limited analysis. In 1996, daily grass pollen counts for the period of the storm were obtained by Celenza et al., (1996) who showed the thunderstorm was preceded by a steep rise in pollen counts, roughly 9 hours before the thunderstorm. Davidson *et al.* (1996) reported the average pollen count to be exceptionally high (258 grains/ml) 2 days before the epidemic and Hajat et al., (1997) reported that nettle, as well as grass pollen levels were both extremely high on the 22nd of June, 2 days prior to the thunderstorm, with levels on the 22 June the highest recorded in London in 6 years.

Newson et al., (1997) conducted a time series analysis from 1990-1994 on asthma admissions, thunderstorm events and pollen counts. They showed that exceptional thunderstorm activities were associated with a 25% relative excess risk of asthma and high pollen counts 5 days previous to the thunderstorm event were associated with increased asthma admissions.

Laboratory methods were used by Venables et al., (1997) to confirm the association of grass pollen with the thunderstorm asthma outbreak in June 1994. They obtained serum samples from 16 patients attending London A&E departments with respiratory symptoms following the thunderstorm and used ImmunoCAP to measure specific-IgE to grass pollen and mould

mix. They found that 12 patients had high levels of pollen specific-IgE and 3 had low or moderate mould specific-IgE.

An 8-year retrospective study of thunderstorm-associated asthma attacks in Athens was conducted by Ilias, (1998) who did not find an association between thunderstorms and asthma attacks.

Newson et al., (1998) conducted a comprehensive analysis of hospital asthma admissions in English Regional Health Authorities from 1987 to 1994 and showed that over this period there were 56 regionally based 'asthma-epidemics'. To oversimplify, an epidemic was defined statistically as an increase in the number of age specific admissions within a region, that was considerably greater (more than 4-fold increase in coefficient of variation) than would be predicted by a forecasting model. The number of lightning flashes, rainfall and temperature were all higher on epidemic days, and high grass pollen levels increased the probability of an epidemic. However, many of the observed epidemics could not be predicted by either thunder or pollen levels, suggesting that space-time clusters of asthma occur for as yet unexplained reasons.

On 30 October 1997 an epidemic of acute severe asthma following a thunderstorm occurred in Wagga Wagga, Australia. Girgis et al., (2000) performed SPT on asthma-outbreak patients from this event and found 111 cases (96%) were allergic to rye grass pollen compared to 47 (64%) of controls that attended at other times. They also found that 138 (95%) of cases gave a history of hay fever compared to 66 (74%) of controls.

The same thunderstorm associated asthma outbreak in Wagga Wagga was examined by Marks et al., (2001). They collected pollen grains from a volumetric spore trap from the day of the thunderstorm and stained it with a commercial pollen stain. They showed that the concentrations of husks, representing grains which had ruptured and released their contents had increased seven-fold during the thunderstorm compared with the previous hour. Using similar epidemiological methods to that of Newson et al. (1998) described above, Marks et al. (2001) conducted an analysis of emergency asthma admissions in 6 towns located in South-East Australia from 1995 to 1998. They found that over this period 48 'asthma epidemic' days occurred. An epidemic was defined statistically by days on which the observed number of attendances was greater than four standard deviations, in excess of

the expected number. Thunderstorms were detected on 33% of epidemic days, with a stronger association during late spring and summer.

In Calgary, Canada a thunderstorm associated asthma epidemic occurred on the 31 July and 1 August, 2000 with 157 hospital asthma admissions (Wardman et al., 2002). The authors measured a significant increase in the PM_{10} concentration on July 31 in addition to many bioaerosols, that then decreased the day after the storm. Bioaerosols that increased significantly included algae, the families *Amaranthaceae* and *Chenopodiaceae*, the class *Myxomycetes*, the genus *Stemphillium* and *Helicomyces* species.

In the Hunter region of Australia, Wark et al. (2002) investigated asthma cases presenting to the emergency room on 27 October 1998 following a spring thunderstorm. Using SPT they found that the thunderstorm associated cases gave greater allergic reactions than controls, particularly for rye grass pollen. They also used RAST and ELISA methods to measure eosinophil cationic protein and IL-8 from patient sputum.

On July 29 and 30, 2002 there was a sudden increase in asthma admissions in Cambridge, UK associated with a thunderstorm. Pulimood et al., (2007) performed SPT and specific-IgE measurements (methods not given) and showed that 23 of 26 cases were sensitive to *Alternaria* species and 22 cases were positive to grass pollen. They concluded that grass pollen allergy was likely to be an important factor in thunderstorm related asthma.

The first report of thunderstorm-related asthma in the Mediterranean area was in Naples, Italy on 4th July, 2004. D'Amato et al., (2008) found that all 7 patients involved had attacks of severe bronchial asthma and were all sensitised to *Parietaria* species pollen but not sensitised to grasses or other aeroallergens. They also observed that during the thunderstorm, the concentration of airborne *Parietaria* species pollen grains was particularly high, peaking at 144 grains/m^3.

More recently in Italy, a thunderstorm asthma epidemic occurred in Puglia on 27th May 2010 owing to a different pollen species. Losappio et al., (2011) performed SPT on 20 patients affected with sudden and severe asthmatic symptoms after a thunderstorm. They found that all 20 patients were sensitised to olive tree pollen (*Olea europaea*) and observed the mean pollen count was 278 granules/m^3 for olive pollen. This was the first reported thunderstorm associated asthma outbreak owing to olive tree pollen sensitisation.

There has been one other report of an asthma outbreak occurring in Brisbane, Australia, on the night of the 20th April 1959 reported by Morrison (1960). He reported that hundreds of asthmatics awakened with 'violent asthma', between 11.20pm and 12 midnight in a 40-mile radius of Brisbane. However, there were no meteorological conditions that associated the outbreak of asthma with a thunderstorm event. Instead, the authors found the only association was with a temperature inversion that occurred on the eve of the 20th April as well as the following night.

Table 2-6 Data extraction of thunderstorm associated asthma outbreak articles

CITATION	ARTICLE TYPE + STUDY DESIGN*	EPIDEMIC DATE & LOCATION	AIR SAMPLING METHODS	SKIN PRICK TESTS	LABORATORY TESTS	CAUSAL AGENT	RESULTS OF LABORATORY + SKIN PRICK TESTS	ALL OTHER RESULTS	CONCLUSIONS
Packe and Ayres 1985	Full text Cli, Env, Epi investigation of a thunderstorm	Birmingham, 1983	Volumetric slit sampler measuring pollen and spore counts.	None	None	Airborne fungal spores suspected.	None	Air pollution low. Rise in numbers of *Didymella exitialis* ascopores and high concentrations of *Sporobolomyces* basidospores. 36 and 70 attendances on epidemic days.	'Large and sudden increase in airborne fungal spores…around the time of the outbreak, suggests they may have been partly contributory'.
Morrow-Brown and Jackson 1985	Letter Env investigation of a thunderstorm	Birmingham, 1983	Spore trap that sucks 10 litres of air/min through a 0.5mm wide slit.	None	Scanning electron microscopy, analysed crystals by energy dispersive X-ray microanalysis and X-ray diffraction.		'During asthma outbreak in 1983… enormous numbers of spores were seen to be accompanied by many crystals of all shapes and sizes'. '…crystals identified as anhydrous form of calcium sulphate'.	None	'Most likely source may be coal-fired power stations' 'Perhaps crystals could trigger spasm in hyperreactive bronchi, thus contributing to asthma epidemics'.
Alderman 1986	Letter Cli, Env, Epi investigation of a thunderstorm	Nottingham, 20 June 1984	None	None	None	None	None	Patients attended A&E reporting asthma attacks immediately after the thunderstorm. 19 cases in the day and 3 during the night. 9 of these only gave a past history of hay fever and no history of asthma.	Could not identify any obvious cause for the increase in asthma attacks.
Packe and Ayres 1986	Letter Cli investigation of thunderstorm EAPs	Birmingham, 1983	None	SPT to common pollens and spores	None	Multifactorial	No significant difference in SPT between patients and controls. 'Most patients positive to grass pollen, 6 in each group to *Didymella* and over half to *Sporobolomyces'.*	'All patients said their hay fever had been troublesome in the weeks before the thunderstorm, and symptoms had become suddenly worse over the evening and night of the storm'.	Asthma outbreak most likely result of several interacting factors and not singly fungal spores.

Bellomo et al. 1992	Full text Cli, Epi investigation of thunderstorm EAPs	Melbourne, 8th + 29th November 1987	None	SPT to common allergens and rye grass pollen.	None	Rye grass pollen	100% incidence of hay fever in storm affected patients, whom were significantly more likely to test positive to commercial rye grass pollen mixture. Testing against newly isolated inhalable compounds of rye grass pollen released when it is exposed to rain or moisture – found to be positive in all storm affected patients (P<0.025).	'Dramatic rise in the number of patients seeking emergency medical care for acute asthma when compared with average daily attendances'.	'Rye grass pollen may be a major aetiological factor in thunderstorm-associated epidemics of asthma exacerbations'.
Waters 1992	Full text Cli, Env long term follow up of outbreak EAPs	Tamworth, Australia, November 1990	Pollen grain counts collected daily.	None	None	Pollen	None	Grass pollen levels increased dramatically in mid-October 1992', 'diary data indicates that both hay fever and asthma symptoms also tended to increase around this time'.	Epidemic has not recurred. Surveillance has demonstrated that asthma and hay fever symptoms are associated with increased levels of rye and barley grass pollen.
Campbell-Hewson et al. 1994	Letter Des, Env report of attendances during thunderstorm	Cambridge, Peterborough, 24/25th June 1994	None	None	None	Multifactorial	None	Pollen counts considerably increased before rainfall. High proportion of patients with regular seasonal hay fever were affected by the epidemic. Moderate rise in *Didymella,* and *Sporobolomyces* rose only after most patients with asthma attended. Large rise in ascopres of *Phaeospheria nigrans* and Diatrypaceae.	Descriptive, highly limited analysis.
Higham 1994	Letter Des report of GP services during thunderstorm	Luton, 24/25th June 1994	None	None	None	None	None	Most patients complained of hay fever with difficulty in breathing.	Repeat study on acute asthma across the rest of the country would be worthwhile.
Murray et al. 1994	Letter Des report of attendances during thunderstorm	South England, 24/25th June 1994	None	None	None	None	None	None	Epidemic placed great pressure on the emergency services'.

Anderson et al. 1996	Letter Epi investigation of thunderstorm	South England, 24/25th June 1994	None	None	None	None	None	No evidence of rise in attendances for asthma was associated with raised pollutant levels.	Epidemic of asthma not associated with air pollution.
Celenza et al. 1996	Full text Env, Epi investigation of thunderstorm	South England (London), 24/25th June 1994	Daily grass pollen counts obtained.	None	None	Pollen	None	Asthma epidemic significantly associated with temperature drop 6 hours previously and rise in pollen concentration roughly 9 hours before.	Asthma associated with temperature drop and rise in grass pollen. Epidemic asthma patients indicated to be more sensitive to different environmental stimuli than non-epidemic asthmatics.
Davidson et al. 1996	Full text Des, Env investigation of a thunderstorm	South England, 24/25th June 1994	Volumetric spore trap.	None	None	Pollen	None	Exceptionally high daily average pollen count in London (258 grains/ml) 2 days before epidemic. Levels of ascospores also high during and after the storm.	Study supports the view that "thunderstorm associated asthma" is related to aeroallergens'
Hajat et al. 1997	Full text Env, Epi investigation of thunderstorms	South England, 24/25th June 1994	Average aeroallergen levels obtained.	None	None	Pollen	None	Average consultation rate for asthma was 6x higher than average. PM_{10} and ozone were higher on the 24 June. 'Grass and nettle pollen levels were both ...extremely high on the Wednesday before the thunderstorm'. 'Grass pollen levels on 22 June were the highest recorded in London for 6 years'.	The thunderstorm had a substantial effect on the number of people consulting for asthma'.
Higham et al. 1997	Full text Des study of GP services during outbreak	South England, 24/25th June 1994	None	None	None	None	None	During thunderstorm event 29 deputising services across England were involved. 40% of patients complained of hay fever.	Thunderstorms are associated with asthma and can affect many patients.
Newson et al. 1997	Full text Env, Epi investigation of thunderstorm	UK, 1990-1994-time series analysis	Pollen data collected	None	None	None	None	Exceptional thunderstorm activities associated with relative excess risk of asthma - 25%. High pollen counts for previous 5 days, associated with increased risk of asthma admissions.	Typical thunderstorms not associated with asthma epidemics. High sferic densities associated with increased asthma. Large thunderstorm activities associated with moderate rises in hospital admission for acute asthma.

Venables et al. 1997	Full text Cli, Env, Lab investigation of thunderstorm EAPs	South England, 24/25th June 1994	None	None	16 serum samples collected from patients attending London A&E departments with respiratory symptoms following the thunderstorm. Specific-IgE measured by ImmunoCAP to grass pollen and mould mix.	Pollen	12 patients had high levels of IgE to pollen and 3 had a low or moderate IgE level to mould.	Thunderstorm was an unusual and large form of storm with several centres and severe wind gusts. Occurred shortly after a peak grass pollen concentration.	Patients with specific-IgE to grass pollen are at risk of thunderstorm related asthma.
Ilias 1998	Technical Note Epi investigation of thunderstorms	Athens, January 1985-December 1992	None	None	None	None	None	No significant difference of people treated for asthma on thunderstorm days compared to other days.	No clear connection found between thunderstorms and asthma attacks.
Newson et al. 1998	Full text Epi, Env investigation of thunderstorm	England, 1987-1994	Daily pollen counts.	None	None	Pollen	None	On average, days preceding epidemics are warmer by about 1°C than their controls'. 'Rainfall was about twice as high preceding epidemic days as preceding other days'. 'Severe thunderstorms more likely to precede large asthma epidemics than no thunderstorms'. 'Rare combination of severe thunderstorms preceded by high pollen counts is an even stronger risk predictor'.	Association of asthma epidemics found 'whereby thunderstorms mobilise aeroallergens from pollen accumulated over previous days', however this predicts only a small minority of epidemics.
Newson et al. 2000	Full text Epi, Env, Lab investigation of a thunderstorm	Trent, UK, 1987-1994, time series analysis	Spore count data	None	Spores identified by taxonomy and counted microscopically.	None	None	Total mould spore count was not significantly associated with daily admissions for asthma' and 'causal relations were not convincingly found'.	Some evidence that exceptional rates of asthma admission tend to occur on days with high total mould spore counts'. Thunderstorm on 22 August 1987 was associated with exceptionally high counts of Didymella spores.

Girgis et al. 2000	Full text Cli, Epi investigation of a thunderstorm	Wagga Wagga, Australia, 30th October 1997	None	SPT performed against 8 allergens.	None	Pollen	Nearly all cases who attended the hospital at the time of the thunderstorm were allergic to rye grass pollen'. 'Allergy to *Cladosporium* mould was also an independent risk factor'.	Cases who attended during the thunderstorm were four times more likely to have had hay fever in the 12 months prior to their hospital presentation'. 'Asthma tended to be less severe in the thunderstorm cases than in the controls'.	Hay fever sufferers and those who are allergic to rye grass pollen are at risk of experiencing exacerbation of asthma symptoms following thunderstorms in the peak grass pollen season'.
Marks et al. 2001	Full text Epi, Env, Lab investigation of a thunderstorm	Wagga Wagga, Australia, 30th October 1997	Pollen grains collected.	None	Pollen measured and characterised with commercial stain.	Pollen	They observed that the concentrations of husks, representing grains which had ruptured and released their contents had increased seven-fold during the thunderstorm compared with the previous hour.	Excess hospital attendances for asthma during late spring and summer is strongly linked to the occurrence of thunderstorm outflows'. 'Demonstrated that in one [particular] event, the arrival of the thunderstorm outflow was accompanied by a large increase in concentration of pollen grains in ambient air'.	Findings consistent with epidemics of asthma being 'caused by high concentrations of allergenic particles produced by an outflow of colder air, associated with the downdraught from a thunderstorm, sweeping up pollen grains and particles and concentrating them at ground level'.
Wardman et al. 2002	Full text Des, Env investigation of a thunderstorm	Calgary, Canada, 31 July 2000	Particles collected.	None	Microscopy of particles.	Pollen	None	Several pollutants increased on the day of the storm'. There was 'a significant increase in the PM_{10} concentration on July 31st, compared with the preceding and following days. Many bioaerosols increased in concentration on July 31st and then decreased the day after the storm'.	This thunderstorm associated epidemic of asthma affected those with a history of asthma, allergies or hay fever and who were outside before the storm.

Wark et al. 2002	Full text Cli, Epi, Lab investigation of a thunderstorm	Hunter region, Australia, 27 October 1998	None	Sensitisation to 19 common aeroallergens.	Sputum analysis: total cell counts of non-squamous cells and viability performed. Cytokine positive cells assessed. Concentration of eosinophilic cationic protein determined by radioimmunoassay and IL-8 by ELISA.	Pollen	Thunderstorm asthma cases were allergic to each of the grass allergens tested, with greater reactions than controls, particularly for rye grass pollen. Thunderstorm asthma cases had an elevated proportion of eosinophils and a greater proportion of cells staining positive for IL-5 than controls.	All cases had a history of allergic rhinitis and reported their nasal symptoms had worsened along with the asthma'.	Results demonstrate that acute thunderstorm asthma is characterised by an elevation in sputum eosinophils, eosinophil degranulation and a greater proportion of cells positive for IL-5'.
Dales et al., (2003)	Full text Epi, Env investigation of thunderstorm	Ottawa, Canada, 1993-1997	Aeroallergen data collected using rotational implication sampling.	None	None	Fungal spores	None	15% increase in asthma on days with thunderstorms. 'Concentrations of fungal spores almost doubled during thunderstorms'. 'Seasonal differences did not account for the differences between days with thunderstorms and days without'. Air pollutants: ozone and nitrogen dioxide were significantly higher on days with thunderstorms compared to days without. Fungal spores could be shown to influence asthma hospital admissions'.	Both asthma and fungal spores increased during thunderstorms'. 'Mechanism may be through increases in spores that exacerbate asthma'.
Igea & Lázaro, (2003)	Editorial (not peer reviewed) Des study of dry storm	Salamanca, Spain, May - June 2002	Pollen counts	None	None	Pollen	None	No significant correlation between the levels of any pollen and emergency visits, 'only factor showing variation related to dry storms and increase in emergency visits for pollinosis was wind speed'.	Wind affects pollinosis symptoms during a dry storm'.
Villeneuve et al., (2005)	Full text Epi investigation of thunderstorm outbreaks	Ottawa, Canada, 1992-2000	None	None	None	Fog/rain	None	Occurrence of fog or liquid precipitation was associated with an increased number of asthma visits'.	Findings were consistent with previous observations on thunderstorm associated asthma.

Pulimood et al. 2007	Full text Cli, Env, Lab investigation of thunderstorm EAPs	Cambridge, England, 29 - 30 July 2002	Spore traps to monitor pollen.	SPT to common allergens	Specific-IgE serology to number of allergens.	*Alternaria alternata*	Total IgE levels were high in 19 of 23 cases tested. 23 of 26 cases were sensitive to *Alternaria* species, 16 were sensitive to *Cladosporium* species and 22 had positive results to grass pollen'.	Ozone concentrations on July 29th exceeded 180µg/m^3, the high threshold. 'Hourly data for July 28-30th showed that *Alternaria* species values were above the threshold'. '*Cladosporium* species values were above the threshold during July 28-29'. '*Didymella* species values were low on the 28 and 29 but increased dramatically at 1am on July 30. Other ascospores were found in exceedingly high numbers on July 31, together with hyaline basidiospores'. 'Grass pollen was intermittently above threshold values on July 28 and after 4pm on July 29 and 30'.	*Alternaria alternata* sensitivity is a compelling predictor of epidemic asthma in patients with seasonal asthma and grass pollen allergy and is likely to be the important factor in thunderstorm-related asthma'.
D'Amato et al. 2008	Letter Cli, Env investigation of thunderstorm EAPs	Naples, Italy, 4 June 2004	Pollen counts	None	None	*Parietaria* pollen	None	All subjects were outdoors when the thunderstorm struck, all patients were 'sensitised, with allergic respiratory symptoms on exposure, to *Parietaria* species pollen but were not sensitised to grasses or other aeroallergens'. 'During the thunderstorm, the concentration of airborne *Parietaria* species pollen grains was particularly high'.	The same mechanisms in thunderstorm associated asthma might involve different pollen grains in different places.

Grundstein et al., (2008)	Editorial Epi investigation of thunderstorm outbreaks	Atlanta, US, 1993-2004	None	None	None	None	None	Association observed between 'daily counts of asthma emergency department visits and thunderstorm occurrence. 'Asthma visits were 3% higher on days following thunderstorms'. 'Associations with asthma were observed for thunderstorms with rainfall but not for thunderstorms with no recorded rainfall'. 'Associations with asthma were strongest when wind gusts were intermediate and high'.	Findings corroborate previous reports of associating thunderstorm activity with asthma exacerbations.
Howden et al., (2011)	Editorial Epi, Env investigation of a thunderstorm	Melbourne, Australia, 25 November 2010	Pollen counts	None	None	Pollen	None	'Spike in asthma presentations immediately after the storm'. 'Pollen counts were in the extreme range during some of the days before the thunderstorm, but they were only moderate on the day of the storm'.	'Additional warnings of elevated risk of asthma exacerbations in pollen-allergic individuals should be made when springtime and summertime thunderstorms follow several days of high or extreme pollen counts'.
Losappio et al. 2011	Editorial Cli investigation of thunderstorm EAPs	Puglia, Italy, 27 May 2010	None	SPT performed	None	Olive tree pollen	All patients tested were sensitised to olive tree pollen and none to *Alternaria*.	'Mean pollen count was 278 granules/m^3 for olive pollen'. 'All subjects were outdoors when the thunderstorm struck'.	'First report of thunderstorm-related asthma owing to olive tree pollen sensitisation'.
Morrison 1960	Full text Cli, Env investigation of an outbreak	Brisbane, 20th April 1959	None	None	None	Possibly due to a temperature inversion	None	Hundreds of asthmatics were reported to have awakened with violent asthma.	92 patients in the Brisbane area developed asthma when a temperature inversion occurred.

*Abbreviations for study design are as follows, Cl: Clinical, Env: Environmental, Epi: Epidemiological, Lab: Laboratory Des: Descriptive

2.4 Discussion

2.4.1 Overview

In this systematic review 85 articles have been identified that report on outbreaks of asthma thought to be associated with unusual peaks in airborne allergens. The reviewed asthma outbreaks were associated with either sources of castor bean or soybean or associated with increases in pollen or mould during thunderstorm events. The most frequent laboratory and clinical methods used for the investigation in outbreaks of asthma, were air sampling in 34 articles and SPT in 33 articles.

The articles identified were published between 1928 and 2016. Therefore, it was not surprising that the laboratory and clinical methods used during outbreaks of asthma showed significant variation across the articles that were reviewed. For asthma outbreaks associated with castor bean (1928 – 1975), the causative agent was identified using clinical methods available at the time. This included the cutaneous scratch method that was used in all reports, which was effective in demonstrating that the common denominator between asthma-outbreak individuals was allergic sensitisation to castor bean extract. A now updated version of this method, referred to as skin prick testing (SPT) remains the cornerstone for everyday diagnosis of allergy, as it can be rapidly administered, and the allergen responsible for asthmatic symptoms can be identified (Erbruster 1959; Heinzerling et al. 2013).

The ‘Passive transfer of antibodies’ test and basic inhalation challenges were also successfully used to identify castor bean as the causative agent in associated asthma outbreaks. However, the ‘Passive transfer of antibodies’ test has since been abandoned for use in allergy diagnosis, given concerns of disease transmission via the blood (Cohen 2004). Inhalation challenges are now only performed under controlled conditions in a handful of clinical centres across the world and are generally limited to investigation of occupational allergy, rather than for use in the general population (Aasen et al. 2013).

A significant advancement in the laboratory methods used to investigate outbreaks of asthma was shown by Weill et al. (1964a; 1964b). They used high-volume air sampling at strategic locations where asthma-outbreak individuals resided and made extracts from the air samples which they used for scratch testing on asthma-outbreak individuals. From their

results, they were able to identify a point-source (in this case a public grain elevator), that was responsible for the outbreaks of asthma. Anto and Sunyer (1986) were also able to identify a point-source responsible for asthma outbreaks. They investigated asthma outbreaks occurring in Barcelona, but instead of using clinical or laboratory methods, they used epidemiological methods, with geographical data for the onset of symptoms in asthma-outbreak patients. By doing so, they were able to identify a point-source (the local harbour), responsible for the outbreaks of asthma.

Further epidemiological methods were used by Anto et al. (1989) that associated asthma-epidemic days in Barcelona with the unloading of soybean at the local harbour. In the same article, these results were supplemented with optical microscopy laboratory methods. This enabled characterisation of airborne particles collected near the harbour and showed they were morphologically identical to soybean samples taken from those unloaded in Barcelona's harbour.

Other laboratory methods such as RAST, ELISA and western blot were more commonly used to detect airborne soybean allergen as well as to measure serum specific-IgE to soybean in asthma-outbreak patients and to identify individual soybean allergens. Measuring airborne soybean allergen by RAST was especially useful in explaining a small cluster of emergency room visits for asthma occurring in Barcelona in 1994, many years after the cause of asthma-epidemics there was known.

The allergenic protein involved in the soybean associated asthma outbreaks was characterised using electrophoresis, western blot and in one report (Codina et al. 1997b) using N-terminal sequencing. N-terminal sequencing was an early method of partial amino acid sequencing of a protein. It includes comparing the sequence with known sequences held in a database in order to match it with a known protein.

In comparison, the methods mostly used in thunderstorm associated asthma outbreaks were air sampling, pollen monitoring and SPT. The laboratory methods used in soybean epidemics were rarely applied for thunderstorm associated asthma outbreaks and instead, epidemiological methods applied to data from hospital admissions was favoured.

The difference in methods used for the different types of asthma outbreaks is likely due to the difference in the size of the population and geographical area affected. For castor bean

associated asthma outbreaks the individuals affected were mostly within a one-mile radius of an oil mill and for the Barcelona soybean outbreaks the onset of symptoms was clustered to only a small part of the city. The soybean and castor bean associated epidemics were both very characteristic of having a point-source cause. Whereas, in many of the thunderstorm associated asthma outbreaks, hundreds of individuals were affected over a single outbreak across a very large area. Two prime examples of this are the thunderstorm event that occurred on 24-25th June 1994 that stretched across Central and Southern England and the most recent Melbourne thunderstorm asthma outbreak where there were 3500 presentations to emergency departments.

Since the electronic searches were performed for this systematic review in March 2016, one of the world's largest outbreaks of thunderstorm asthma occurred in Melbourne on the 21st of November 2016. There were 3500 presentations to emergency departments, 35 patients requiring intensive care and ten deaths during the thunderstorm associated asthma outbreak (Lee et al., 2017; The Chief Health Officer's Report, April 2017; Thien et al., 2018). SPT and/or serum specific-IgE testing was performed on 81 clinic patients with thunderstorm asthma during this event. Of these, 100% gave positive results for sensitisation to rye grass pollen. The authors concluded that sensitisation, clinical allergic rhinitis and outdoor exposure to ryegrass pollen are the critical risk factor trifecta for thunderstorm asthma (Lee et al. 2017).

It is worth mentioning that a theory has been put forward as to why thunderstorm associated asthma occurs. In a recent review, D'Amato et al., (2016), collated evidence to show that pollen grains can be ruptured in rainwater by osmotic shock. This causes the release of allergenic starch granules from pollen that are of respirable size (Sulphioglu et al. 1998). Furthermore, the cold downdrafts occurring during a thunderstorm can then carry these fragments to ground level, where outflows distribute them further. It is also thought that atmospheric electric charges caused by thunderstorms can enhance the mechanism of pollen rupture (Taylor et al., 2002).

The thunderstorm associated asthma outbreak owing to olive tree pollen sensitisation, shows that the allergen causing thunderstorm outbreaks, may well depend on the predominant pollen species in that area. This is an important consideration when conducting SPT to identify allergens causing thunderstorm asthma. Moreover, it is relevant

for pollen monitoring, to issue public warnings if a high pollen count specific for that location is likely to coincide with a thunderstorm event.

2.4.2 Limitations

In conducting this systematic review, we were limited by the lack of uniform data that could be extracted from the included articles. Articles reviewed were spread over almost a century and the scientific reporting style, statistical testing and laboratory methods varied considerably over time. Some articles used solely laboratory methods, while others used only epidemiological or clinical methods to identify the causative agents responsible for outbreaks of asthma. Moreover, some asthma outbreaks only concerned a small population affected by a point-source, while others (namely thunderstorm associated outbreaks) affected larger populations.

The overall study designs were extremely varied and some lacked sufficient detail to follow the design easily. To maintain some oversight of the steps taken during investigation of these outbreaks we categorised the articles according to their association with castor bean, soybean or thunderstorms to discuss the results of this review. We grouped them broadly into our own study designs (clinical, environmental, epidemiological, laboratory and/or descriptive) during the data extraction, based on discussion between review authors, but present them largely in order of publication.

Colleagues have kindly translated those articles that were published in other languages (Spanish and Portuguese). To the best of our ability we had these articles translated into English by native speakers.

2.4.3 Broader implications

Given that all these outbreaks include sudden life-threatening cases of asthma, it is important that they can be distinguished from other fluctuations in asthma attendances (including seasonal variations). It is possible that these outbreaks may be occurring more frequently and at lower levels than is currently thought, thus making them not easily identifiable. Newson et al. (1998) identified 56 regionally based asthma epidemics in their analysis of hospital admissions in England from 1987-1994 that were not all previously known and Marks et al. (2001) identified 48 asthma epidemic days occurring in six South-Eastern Australian towns, from 1995-1998.

More recently, Burney et al. (2008) found an association between hospital admissions for asthma exacerbations and serum specific-IgE binding to airborne particulate matter (with an aerodynamic size <10μm) collected at the time of asthma exacerbation. From this study, it was not known whether a source of allergen, (such as from an industrial source, like the castor bean oil mill or soybean unloading) was responsible for causing asthma exacerbations.

Therefore, if a method to identify allergen(s) responsible for these outbreaks could be developed, it could be used to prevent future outbreaks of life-threatening asthma. The first step would be to perform air sampling and to identify allergens from filter extracts. To detect allergenic proteins responsible for causing asthma symptoms, serum from asthma-outbreak patients would need to be obtained. Then, using methods such as RAST, ELISA and western blot, allergenic proteins from air filter extracts can be detected by binding of serum specific-IgE. This methodology is shown by articles included in this review.

Developments in protein analysis have seen N-terminal sequencing (used by Codina et al. 1997b) evolve into high throughput and high accuracy amino acid sequencing, now known as modern mass spectrometry (MS). It is now possible to excise an allergenic protein band that has been detected by western blot using serum specific IgE, apply it to MS and identify it by comparing its amino acid sequence with other known protein sequences in freely available protein databases.

Another example in which unknown airborne allergens may be present, is in the occupational setting. Although this review focused on outdoor allergens, the same

laboratory methods, such as air sampling, western blot and SPT can be applied in an occupational setting to identify allergens that may be responsible for causing respiratory symptoms such as occupational asthma or rhinitis.

Inevitably, there are also effects associated with climate change which are likely to impact on these types of asthma outbreaks. The concentrations of different pollen species and other airborne allergens could well increase, and it is not known how the change in weather systems will impact on the frequency and severity of associated thunderstorm asthma events (D'Amato et al. 2015).

2.4.4 Summary and future works

By identifying airborne allergens responsible for causing outbreaks of asthma, we can intervene to prevent further outbreaks. In the case of thunderstorm associated asthma outbreaks, the numerous hospital admissions could possibly be avoided by issuing public warnings directed to those with specific allergic profiles. Given the most recent event in Melbourne in November 2016, the deaths caused, and the demand set on the emergency services, there is a clear need for an effective advance warning system to be in place.

In summary, the laboratory methods that have evolved to identify airborne allergens associated with outbreaks of asthma, mostly depend on demonstrating specific-IgE binding to the allergen. This review shows that air sampling, SPT, RAST and western blot were the most common methods used and almost all of the investigated asthma outbreaks were limited to those associated with castor bean, soybean or thunderstorm events. We believe that the application of MS in combination with specific-IgE detection by western blot, can be used to develop an advanced and efficient technique for the identification of unknown airborne allergens.

In the following chapter are presented the materials and methods that I have used to apply a combined western blot and MS technique, for the identification of airborne proteins responsible for causing allergic asthma.

3 Materials and Methods

3.1 Materials

Table 3-1 Materials used for laboratory work, with manufacturer information

Reagents, kits and equipment	Manufacturer
1-Step™ Ultra TMB-Blotting Solution	ThermoFisherScientific, Leicestershire, UK
2-butanol	Sigma, Hamburg, Germany
5-bromo-4-chloro-3'-indolyphosphate (BCIP)	Sigma, Hamburg, Germany
96-well flat-bottomed microplate	ThermoFisherScientific, Leicestershire, UK
2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS)	Sigma, Irvine, UK
Acetic acid	VWR, UK
Acetonitrile	Sigma, Irvine, UK
Acrylamide/bisulphate	Sigma, Hamburg, Germany
Ammonium carbonate (AHC)	VWR, UK
Ammonium persulphate	Sigma, Hamburg, Germany
Blotting paper	Bio-Rad Laboratories, Germany
Bovine serum albumin	Sigma, Irvine, UK
Bromophenol blue	Sigma, Hamburg, Germany
Citric acid ($C_6H_8O_7$)	VWR, UK
ChemiDoc XRS+ system	Bio-Rad Laboratories, Germany
ChemiDoc™ Imaging System	Bio-Rad Laboratories, Hertfordshire, UK
Coomassie Blue dye (R-250)	Sigma, Irvine, UK
DC™ Protein Assay Kit	Bio-Rad Laboratories, Hertfordshire, UK
dimethylformamide (DMF)	Sigma, Hamburg, Germany
Dithiothretol	Sigma-Aldrich, Gillingham, UK
Dynatech MR5000 plate reader	Dynatech Laboratories, UK
EASY NanoLC system	ThermoFisherScientific, Leicestershire, UK
Ethanol	VWR, UK
Ethylenediamine tetra-acetic acid (EDTA)	VRW, UK
ExtraAvidin-Peroxidase	Sigma-Aldrich, Gillingham, UK
Fluorophore membrane filters, 25mm diameter/1.0µm pore size	ThermoFisherScientific, Leicestershire, UK
Formic acid	VWR, UK
Glass fibre filters, 8" x 10"	Westech, Bedfordshire, UK
Glycerol	Sigma, Hamburg, Germany
Glycine	Sigma, Hamburg, Germany
Goat anti-Human IgE Antibody, HRP conjugate	ThermoFisherScientific, Leicestershire, UK
Hydrogen peroxide (H_2O_2)	VWR, UK
Immuno™ 96 MicroWell™ Maxisorp plate	VWR, UK
Iodoacetamide	Sigma-Aldrich, Gillingham, UK
Potassium chloride (KCl)	Sigma-Aldrich, Gillingham, UK
Monopotassium phosphate (KH_2PO_4)	Sigma-Aldrich, Gillingham, UK
Methanol	VWR, UK
Magnesium chloride (MgCl)	Sigma-Aldrich, Gillingham, UK
Milli-Q grade water	Sigma, Hamburg, Germany
Mouse anti-Human IgE Antibody, AP conjugate	ThermoFisherScientific, Leicestershire, UK
Mouse epithelia skin prick test solution	Allergopharma, Reinbeck
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich, Gillingham, UK
Disodium phosphate (Na_2HPO_4)	Sigma-Aldrich, Gillingham, UK

Sodium chloride (NaCl)	Sigma-Aldrich, Gillingham, UK
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich, Gillingham, UK
Sodium azide (NaN_3)	Sigma-Aldrich, Gillingham, UK
Nitro-blue tetrazolium (NBT)	Sigma, Hamburg, Germany
Nitrocellulose membrane	Watmann, Protran
Nitrocellulose pre-cut blotting membrane (0.2µm pore size) and pre-cut filter paper	ThermoFisherScientific, Leicestershire, UK
Novex® HRP Chromogenic TMB Substrate	ThermoFisherScientific, Leicestershire, UK
NuPAGE™ Bis-Tris Gel, 1.0mm, 12%	ThermoFisherScientific, Leicestershire, UK
NuPAGE™ Bis-Tris Gel, 1.0mm, 4-12%	ThermoFisherScientific, Leicestershire, UK
NuPAGE™ LDS Sample Buffer	ThermoFisherScientific, Leicestershire, UK
NuPAGE™ MES SDS Running Buffer (20X)	ThermoFisherScientific, Leicestershire, UK
NuPAGE™ Transfer Buffer (20X)	ThermoFisherScientific, Leicestershire, UK
Orbital shaker	VWR, UK
Orbitrap Velos Pro	ThermoFisherScientific, Leicestershire, UK
PageRuler™ Plus Prestained Protein Ladder	Bio-Rad Laboratories, Germany
Parafilm®	VWR, UK
Pasteur pipette	VWR, UK
Pierce® Silver Stain Kit	ThermoFisherScientific, Leicestershire, UK
Pierce™ ECL Western Blotting Substrate	ThermoFisherScientific, Leicestershire, UK
Pierce™ Silver Stain for Mass Spectrometry kit	ThermoFisherScientific, Leicestershire, UK
Ponceau Stain	Sigma, Hamburg, Germany
PowerEase® 90W Power supply	ThermoFisherScientific, Leicestershire, UK
PowerPac™ HC High-Current Power supply	Bio-Rad Laboratories, Germany
Polycarbonate membrane filters	SKC Ltd, Dorset, UK
Prestained protein standard	ThermoFisherScientific, Leicestershire, UK
ProteoExtract All-in-One Trypsin Digestion Kit	CalbioChem, Millipore, Darmstadt, Germany
Sodium dodecyl-sulphate (SDS)	Sigma, Hamburg, Germany
Specific Mus m 1 ELISA kit	Indoor Biotechnologies Ltd, Cardiff, UK
Spectrophotometer, Appliskan	ThermoFisherScientific, Leicestershire, UK
Synthetic panel filters	Camfil, Lancashire, UK
Teflon filters, 47mm	Pallflex, Emfab,
Tetraethyleethylenediamine (TEMED)	Sigma, Hamburg, Germany
Thermo Scientific Partisol sampler, 2025	ThermoFisherScientific, Leicestershire, UK
Trans-Blot® SD Semi-Dry Transfer Cell	Bio-Rad Laboratories, Germany
Trifluoroacetic acid (TFA)	Millipore, Darmstadt, Germany
Tris	Sigma, Hamburg, Germany
Tris-HCl	Sigma, Hamburg, Germany
Trypsin	Roche, UK
TUFF 4 Plus I.S. air sample pumps with IOM sampling heads	Casella London Ltd, Bedford, UK
Tween-20	Sigma-Aldrich, Gillingham, UK
Unstained protein standard	ThermoFisherScientific, Leicestershire, UK
XCell II™ Blot Module	ThermoFisherScientific, Leicestershire, UK
XCell *SureLock*™ Mini-Cell	ThermoFisherScientific, Leicestershire, UK
ZipTip®$_{C18}$ pipette tips	Millipore, Darmstadt, Germany
β-mercaptoethanol	Sigma, Hamburg, Germany

3.2 Methods

3.2.1 Sample collection and protein extraction

In the following four chapters (Chapters 4-7), samples were either collected as part of the work described in each chapter or had been previously collected, extracted and stored by the Occupational and Environmental Medicine laboratory within the Population Health and Occupational Disease research group at Imperial College London as follows:

- In Chapters 5 and 6 I used stored allergen samples that were previously extracted overnight in 0.01 mol/l ammonium carbonate at 4°C, dialysed overnight against distilled water (dH_2O), lyophilised and stored at -20°C.
- In Chapters 4 and 7 sample collection and protein extraction were optimised as described in both chapters.

3.2.2 Protein quantification

Protein content in all extracted allergen samples was determined using a *DC*™ Protein Assay Kit, following a protocol based on the Lowry et al., (1951) method for protein measurement using a folin solution as a dye-binding reagent. The kit contained solutions labelled as Reagent S, Reagent A and Reagent B. Following the manufacturer's instructions, I prepared a set of standards using bovine serum albumin (BSA) from 0.1 – 3µg/µl. Standards and extracted samples were added (5µl) in triplicate to a 96-well flat-bottomed microplate. A working reagent was made by mixing 20µl of Reagent S to each millilitre of Reagent A required for a total of 25µl of the working reagent to be added to each well. This was followed by addition of 200µl of Reagent B into each well. The absorbance values for protein were read after 15 minutes at 750nm on an Appliskan spectrophotometer. The absorbance values of the BSA set of standards were plotted against their known protein concentrations to obtain a linear line equation which was used to calculate the protein concentrations of samples using their absorbance values. Only line equations with an R^2 value >0.9 and experimental replicates with a coefficient of variation >0.2 were accepted.

3.2.3 ELISA

A commercial ELISA kit was used to determine the concentration of Mus m 1 present in extracted samples.

The ELISA kit contained 2 vials of polyclonal rabbit anti Mus m 1 antibody, one of which was biotinylated and the other non-biotinylated. The kit also contained a universal allergen standard containing 250ng/ml of Mus m 1 (determined by amino acid analysis). Reagents not provided were made as follows:

- **10X phosphate buffered saline (PBS)** was made by dissolving 80.0g of NaCl, 2.0g of KH_2PO_4, 11.5g of Na_2HPO_4 and 2.0g of KCl in 1 litre of distilled water (dH_2O) and adjusting the pH to 7.4.
- **PBS with 0.05% Tween (PBS-T)** was made by mix pipetting 500µl of Tween-20 in 1 litre of 1X PBS.
- **PBS-T with 1% BSA (PBS-T/BSA)** was made by dissolving 1.00g of BSA in 100ml of PBS-T.
- **50mM carbonate-bicarbonate (coating) buffer** was made by dissolving 1.59g of Na_2CO_3 and 2.93g of $NaHCO_3$ in 1 litre of dH_2O and adjusting the pH to 9.6.
- **70mM citrate-phosphate buffer** was made by first making a 0.1M citric acid solution from 5.25g of $C_6H_8O_7$ dissolved in 250ml of H_2O and then a 0.2M dibasic sodium phosphate solution made from 7.05g of Na_2HPO_4 dissolved in 250ml of H_2O. I then combined 147ml of the citric acid solution with 103ml of dibasic sodium phosphate solution and diluted it with 250ml of dH_2O to make the 70mM citrate-phosphate buffer.
- **Substrate solution (1mM ABTS in 70mM citrate-phosphate buffer)** was made using 500ml of the citrate-phosphate buffer to dissolve 274mg of ABTS. This solution was wrapped in foil and kept at 4°C.

The Mus m 1 ELISA was carried out according to the manufacturer's instructions. In brief, the non-biotinylated polyclonal rabbit anti Mus m 1 antibody was diluted 1:1000 in the 50mM carbonate-bicarbonate buffer and 100µl was used to coat each required well of an Immuno™ 96 MicroWell™ Maxisorp plate, which was incubated for 16 hours at 4°C in a humid box. The plate was washed three times with PBS-T to remove unbound antibody and 100µl of PBS-T/BSA was then added to each required well and the plate was incubated for

30 minutes at room temperature (RT) in a humid box, to prevent non- specific antibody binding. The plate was again washed three times with PBS-T.

Using the universal allergen standard provided in the kit, a control curve was setup using doubling dilutions in PBS-T/BSA to range from 0.5-25 ng/ml of Mus m 1 in a total volume of 100µl per well along with blanks containing 100µl of PBS-T/BSA only, and 100 µl of each sample per well, the plate was then incubated for 1 hour at RT in a humid box. The plate was washed three times with PBS-T to remove any unbound sample. The biotinylated polyclonal rabbit anti Mus m 1 antibody was diluted to 1:1000 in PBS-T/BSA and 100µl was added into each required well and the plate incubated for 1 hour at RT in a humid box. The plate was washed three times with PBS-T to remove any unbound antibody.

ExtraAvidin-Peroxidase was diluted to 1:1000 in PBS-T/BSA and 100µl was added to each required well and the plate was incubated for 30 minutes at RT in a humid box. The plate was washed three times with PBS-T and immediately prior to using the substrate solution, we added 1µl of 30% H_2O_2 per ml of citrate-phosphate buffer solution to the substrate solution. The assay was developed by addition of 100µl of the substrate solution (containing H_2O_2) to each required well and the plate was read when the absorbance values of the highest control reached 2.0 – 2.4. Absorbances were read on a Dynatech MR5000 plate reader. The content of Mus m 1 (ng/ml) in samples was then extrapolated from a sigmoidal dose-response equation derived from the control curve using GraphPad Prism 4 software.

3.2.4 Protein Separation

Extracted protein samples were separated according to protein molecular weight using a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method (Laemmli, 1970). I used a pre-cast gel electrophoresis system for almost all SDS-PAGE and western blot results shown in this thesis. A hand cast gel electrophoresis system was used for some western blot results in Chapters 4 and 5.

3.2.4.1 Pre-cast SDS-PAGE

For pre-cast SDS-PAGE a NuPAGE™ Bis-Tris electrophoresis system was used according to the manufacturer's instructions. Extracted samples were diluted 4X in NuPAGE™ LDS Sample Buffer and heated at 70°C for 10 minutes. Running buffer was made by diluting 20X NuPAGE™ MES SDS Running Buffer in 950ml of dH_2O. Using a Pasteur pipette, diluted running buffer was used to sufficiently wash a NuPAGE™ Bis-Tris 1.0mm thick gel. I used gels with a polyacrylamide percentage of 4-12% with the exception of a 12% pre-cast polyacrylamide gel used in Chapter 5.

Gels were placed in the inner chamber of an XCell *SureLock*™ Mini-Cell and ~400ml of running buffer was used to fill the inner chamber. The sample was loaded using 15-25µl alongside 2µl of either an unstained protein standard or prestained protein standard. Electrophoresis was run at 200 volts constant for 45 minutes using a PowerEase® 90W power supply. Once the run was finished the power supply was stopped, the gel was removed out of the chamber and disassembled from its plastic cassette.

The gel was then either stained (section 3.2.5) or used for western blot analysis (section 3.2.6).

3.2.4.2 Hand-cast SDS-PAGE

For hand-cast SDS-PAGE the following methodology and reagents were provided by the Division of Allergy and Immunology at the University of Salzburg. The separation gel and stacking gel were prepared 'by-hand' between glass plates using reagents listed in Table 3-2 as follows.

Table 3-2 Reagents for separation and stacking gels

Reagent	Separation gel	Stacking gel
Acrylamide/Bisulphate solution	10ml	0.7ml
Lower buffer	5ml	-
Upper buffer	-	1.1ml
dH_2O	5ml	2.6ml
TEMED (Tetramethylethylendiamine)	10µl	5µl
10% Ammonium persulfate (APS)	60µl	40µl

For the separation gel, lower buffer was prepared by dissolving 90.85g of Tris in 400ml of dH_2O and the pH adjusted to 8.8 with hydrochloric acid (HCl) followed by the addition of 20ml of 10% SDS and the final volume was made up to 500ml with dH_2O. We used 5ml of this lower buffer solution and mixed it with 10ml of acrylamide/bisulphate solution, 5ml of dH_2O, 10µl of tetraethyleethylenediamine (TEMED) and 60µl of 10% ammonium persulfate. The mixture was poured between 2 glass slides that were approximately 10x10cm (with the bottom and sides blocked with plastic spacers) until ~1cm from the top. Immediately afterward, this was overlaid with enough 2-butanol to achieve an even surface and then the gel was left to polymerise for 40 minutes. Once set, the remaining 2-butanol was removed, and the gel was washed with dH_2O.

For the stacking gel, the upper buffer was prepared by dissolving 4.43g of Tris in 400ml of dH_2O and the pH adjusted to 6.8 with HCl. To this upper buffer we added 10ml of 10% SDS and the final volume was made up to 500ml with dH_2O. We used 1.1ml of the upper buffer solution and mixed it with 0.7ml of acrylamide/bisulphate solution, 2.6ml of dH_2O, 5µl of TEMED and 40µl of 10% ammonium persulfate. The mixture was then poured over the

separation gel until overflowing the glass slides and a comb was immediately inserted into the stacking gel to form wells. After ensuring no air bubbles were present, the stacking gel was left for 40 minutes to polymerise, the comb was removed, and the wells were washed thoroughly with dH_2O. Gels were wrapped in wet paper towel and stored at 4°C for up to 1 month.

For hand-cast electrophoresis, the extracted samples were diluted 4X in either reducing or non-reducing sample buffer. Both sample buffers were made with the following reagents diluted in dH_2O, with a total volume of 100ml: 3.02g of Tris, 8g of SDS, 40ml of glycerol and 0.004g of bromophenol blue. For the reducing buffer, 20ml of β-mercaptoethanol was also added to the solution. The pH of both solutions was adjusted to 6.75 with HCl prior to use.

The extracted samples diluted in either reducing or non-reducing buffer were then heated at 95°C for 5 minutes. A stock of 10X running buffer was made from 720.6g of 1.92M glycine, 151.3g of 250mM Tris and 50g of 1% SDS, in 5 litres of dH_2O. 50ml of 10X running buffer was diluted in 950ml of dH_2O and ~500ml used to fill the outside of an electrophoresis chamber. The hand-cast gel was unwrapped from paper towel, rinsed in dH_2O and placed into the chamber, ensuring no air bubbles were present. The inner chamber was then filled with the remaining diluted running buffer (~500ml).

The sample was loaded onto the gel alongside 2µl of PageRuler™ Plus prestained protein standard. Electrophoresis was run at 100 volts constant and 20mA using a PowerPac™ HC high-current power supply, until the bromophenol blue in the sample buffer ran out of the gel bottom. Once the run was finished the power supply was stopped and the gel was removed from the chamber and disassembled from its glass slides.

The gel was then either stained with Coomassie Blue (section 3.2.5.3) or used for western blot analysis (section 3.2.6).

3.2.5 Protein Staining

3.2.5.1 Silver stain

A Pierce® Silver Stain Kit was used according to the manufacturer's instructions (Thermo Fisher), following the method by Switzer et al., (1979). The kit included Silver Stain, Silver Stain Sensitizer, Enhancer and Developer. Silver staining was only used on pre-cast gels. After SDS-PAGE, the gel was removed from its plastic cassette and placed in a petri dish. All the following steps were carried out at RT at 60rpm on an orbital shaker using enough solution for the gel to move freely.

- The gel was washed in two changes of dH_2O for 5 minutes each.
- Then fixed in two changes of fixing solution, made from 30% ethanol and 10% acetic acid in dH_2O, for 15 minutes each.
- Then washed in two changes of 10% ethanol and two changes of dH_2O for 5 minutes each.
- Sensitizer working solution was made from 50µl of silver stain sensitizer and 25ml of dH_2O and the gel was incubated in sensitizer working solution for 1 minute, followed by two washes with dH_2O for 1 minute each.
- Stain working solution was made from 0.5ml of Silver Stain Enhancer and 25ml of Silver Stain and the gel was incubated in stain working solution for 30 minutes and washed briefly with two changes of dH_2O.
- Developer working solution was made from 0.5ml of Silver Stain Enhancer and 25ml of Silver Stain Developer and the gel was incubated with developer working solution for up to 3 minutes, or until protein bands appeared on the gel.
- This was replaced with a 5% acetic acid stop solution when bands had sufficiently developed.

The gels were imaged with a ChemiDoc™ Imaging System on a white tray for colorimetric imaging and ImageLab (Version 5.2.1) was used for molecular weight analysis.

3.2.5.2 Silver Stain for Mass spectrometry analysis

When proteins were stained for MS analysis in Chapter 4, a specialist Pierce™ Silver Stain for Mass Spectrometry kit was used to minimise covalent crosslinking of proteins to the gel matrix (as this can affect protein recovery during tryptic digestion). A very similar protocol to the above (3.2.5.1) was used, with some minor changes.

- The gel was washed in two changes of dH_2O for 5 minutes each.
- Then fixed in two changes of fixing solution: made from 30% ethanol and 10% acetic acid in dH_2O, for 15 minutes each.
- Then washed in two changes of 10% ethanol and two changes of dH_2O for 5 minutes each.
- Sensitizer working solution was made from 50µl of silver stain sensitizer and 25ml of dH_2O and the gel was incubated in sensitizer working solution for 1 minute, followed by two washes with dH_2O for 1 minute each.
- Stain working solution was made from 0.25ml of Silver Stain Enhancer and 25ml of Silver Stain and the gel was incubated in stain working solution for 5 minutes and washed briefly with two changes of dH_2O.
- Developer working solution was made from 0.25ml of Silver Stain Enhancer and 25ml of Silver Stain Developer and the gel was incubated with developer working solution for up to 3 minutes, or until protein bands appeared on the gel.
- This was replaced with a 5% acetic acid stop solution when bands developed.

As above the gel was imaged with a ChemiDoc™ Imaging System on a white tray for colorimetric imaging and ImageLab (Version 5.2.1) was used for molecular weight analysis. The relevant specific-IgE binding proteins were excised for in-gel digestion, as described in section 3.2.7.

3.2.5.3 Coomassie Stain

An R-250 Coomassie® blue dye was used for protein staining for both pre-cast and hand-cast gels. After SDS-PAGE the gel was placed in staining solution made from 0.1% Coomassie®R-250, 40% ethanol and 10% acetic acid, for 1 hour. The gel was rinsed briefly with dH_2O and placed in destaining solution made from 10% ethanol and 7.5% acetic acid, overnight. The destaining solution was changed at regular intervals until the background became clear. All steps were carried out at RT at 60rpm on an orbital shaker using enough solution for the gel to move freely. Gels were imaged using either a ChemiDoc™ Imaging System or a ChemiDoc XRS+ system and ImageLab (Version 5.2.1) was used for molecular weight analysis.

3.2.6 Western blot

I used the following western blot method, devised by Towbin et al., (1979), to detect proteins from extracted allergen samples, binding to serum specific-IgE, separated after SDS-PAGE. The following describes protein transfer to nitrocellulose membranes, blocking of the membrane, antibody incubations and detection of serum specific IgE-binding proteins.

3.2.6.1 Membrane transfer

After SDS-PAGE, proteins were transferred to nitrocellulose membranes either by wet or semi-dry transfer.

Wet transfer

For wet transfer, 50ml of 20X NuPAGE™ Transfer Buffer was diluted in 100ml of methanol and 850ml of dH_2O and used to soak blotting pads, nitrocellulose pre-cut blotting membrane (0.2µm pore size) and pre-cut filter paper. Following the NuPAGE™ technical guide (Invitrogen) a gel-membrane sandwich was setup (as shown in Figure 3-1) within the XCell II™ Blot Module, placed in the lower buffer chamber of the XCell *SureLock*™ Mini-Cell and filled with the remaining (~300ml) diluted transfer buffer. The upper buffer chambers were filled with ~500ml of dH_2O and the transfer was run at 30 volts constant for 1 hour.

Semi-dry transfer

For semi-dry transfer, transfer buffer was made with 6.05g of 25mM Tris, 28.8g of 192mM glycine and 400ml of methanol (20% v/v) diluted in 2 litres of dH_2O. After removing the gel from its glass slides, it was equilibrated in 10ml of transfer buffer. A 2µm Watmann nitrocellulose membrane and 2 pieces of blotting paper were all cut to the size of the gel and equilibrated in transfer buffer for 5 minutes. In a Trans-Blot® SD Semi-Dry Transfer Cell a gel-membrane sandwich (a shown in Figure 3-1) was setup. The transfer was run at 15V for 60-90 minutes. To confirm the transfer of the proteins onto the membrane, it was stained for 10 minutes with enough 1% Ponceau Stain to cover the gel.

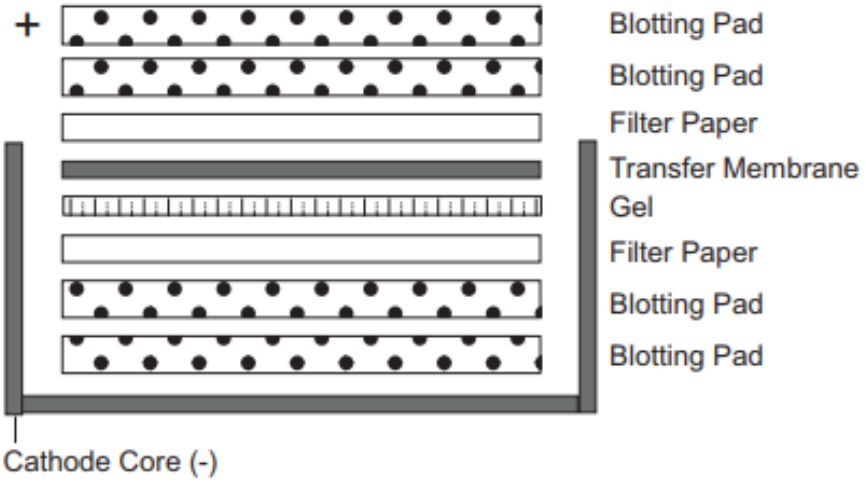


Figure 3-1 Schematic of gel-membrane sandwich. (ThermoFisher, Western blotting handbook, 2015).

3.2.6.2 Membrane blocking

Proteins transferred to nitrocellulose membranes were blocked with either a BSA-phosphate blocking buffer or a BSA-tris blocking buffer, depending on the conjugate of the anti-human IgE binding antibody (see 3.2.6.3). The membrane was removed from the transfer apparatus and using 20ml of either buffer blocked for 1 hours 30 minutes at RT at 60rpm on an orbital shaker.

Phosphate blocking buffer (PBS-T/BSA)

The phosphate blocking buffer (PBS-T/BSA) was made as described in section 3.2.3.

Tris blocking buffer (TBS-T/BSA)

The tris blocking buffer (TBS-T/BSA) was made with the following reagents in 2 litres of dH_2O: 10g of BSA, 17.5g of NaCl, 7.9g of Tris-HCl, 1g of NaN_3 and 8ml of Tween 20.

3.2.6.3 Antibody incubations

I chose stored serum samples from individuals who had elevated levels of specific-IgE to the allergen extract, relevant for each chapter. Levels of serum specific-IgE were previously determined by either the radioallergosorbent test (RAST) or ImmunoCAP. Serum samples were stored at the Occupational and Environmental Medicine Laboratory in the Population Health and Occupational Disease research group at Imperial College London.

To detect specific-IgE binding proteins, serum was either used individually or as a pooled sample. For both approaches the membrane was incubated in serum at 4°C overnight at 60rpm on an orbital shaker. For pooled serum the membrane was removed from blocking buffer and placed in serum prepared as either a 1:10 or 1:5 dilution in blocking buffer in a total volume of 15ml. For individual serum the membrane was cut into 0.5cm strips adjacent to the molecular weight marker. Individual serum was prepared at a 1:10, 1:5 or 1:2 dilution in blocking buffer with a total volume of 500µl or 1500µl. Following incubation with serum, the membranes were washed in either PBS-T or TBS-T (depending on the blocking buffer used), five times for five minutes each.

For membranes blocked in PBS-T/BSA, they were then probed with goat anti-human IgE antibody conjugated with horse radish peroxidase (HRP), diluted 1:1000 in PBS-T/BSA, for 30 minutes at RT. For membranes cut into 0.5cm strips 1500µl of diluted antibody was used per strip and for whole membranes 20ml was used. Membranes were again washed in PBS-T five times for five minutes each, before substrate detection.

For membranes blocked in TBS-T/BSA, they were probed with mouse anti-human IgE conjugated with alkaline phosphatase (AP) diluted 1:10,000 in TBS-T/BSA, for 1 hour at RT. Membranes were washed in TBS-T five times for five minutes each, before substrate detection.

3.2.6.4 Detection

We used both chromogenic HRP and AP substrates and an enhanced chemiluminescent (ECL) HRP substrate for the detection of serum specific-IgE binding proteins.

Chromogenic HRP detection using a TMB substrate

After washing in PBS-T the membrane was incubated with 15ml of either a Novex® HRP Chromogenic TMB Substrate or a 1-StepTM Ultra TMB-Blotting Solution and gently agitated until bands were visible as a coloured precipitate. The reaction was stopped by rinsing the membrane thoroughly with dH_2O.

Chromogenic AP detection using an NBT/BCIP substrate

After washing in TBS-T the membrane was incubated in AP detection buffer made using 12.1g of 100mM Tris-HCl, 5.81g of 100mM NaCl and 1.01g of 5mM MgCl dissolved in 1 litre of dH_2O. Stock solutions for nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolyphosphate (BCIP) were made as follows:

- NBT stock: 200mg of NBT mixed with 1.4ml of dimethylformamide (DMF) and 300µl of dH_2O.
- BCIP stock: 50mg of BCIP mixed with 1ml of DMF.

The substrate solution was made using 10ml of the AP detection buffer, 30µl of NBT stock and 30µl of BCIP stock. The membrane strips were incubated with substrate solution in the dark for up to 30 minutes until bands were visible. The reaction was stopped by rinsing the membrane strips thoroughly with dH_2O. Membrane strips were then imaged with a ChemiDoc XRS+ system and the molecular weight of specific-IgE binding proteins was determined using a prestained protein marker.

Chemiluminescent HRP detection using an enhanced chemiluminescence (ECL) substrate

ECL was used to achieve a more sensitive method of detection for specific-IgE binding proteins. After washing in PBS-T, the membrane was placed on Parafilm® cut ~ 10x15cm (slightly larger than the membrane). The substrate working solution was prepared by mixing 1ml each of Pierce™ ECL Western Blotting Substrate detection reagents 1 and 2. This was pipetted onto the membrane and left at RT for 5 minutes. The membrane was imaged using a ChemiDoc™ Imaging System containing a CCD imaging device, able to capture luminescence emitted from the substrate binding to the conjugate antibody. This was captured in 500 seconds of signal accumulation mode and analysed on ImageLab (Version 5.2.1).

Proteins binding to serum specific-IgE, as detected on the membrane by ECL were matched to proteins detected on either silver or Coomassie stained polyacrylamide gels. These specific-IgE binding regions were selected for protein identification by MS.

3.2.7 Mass spectrometry

The following methods: tryptic digestion, extraction of protein, nano liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) and data processing were all achieved with training and supervision from both Steven Lynham at the Centre of Excellence for Mass Spectrometry at King's College London, Denmark Hill and Professor. Peter Briza from the Division of Allergy and Immunology at the University of Salzburg.

3.2.7.1 In-gel tryptic digestion

Specific-IgE binding regions detected by western blot were excised from either silver or Coomassie stained polyacrylamide gels, for digestion with trypsin using a modification of the Shevchenko et al., (2006) method as described below.

In-gel digestion was performed using either the:

- Reagents and protocol provided by the Centre of Excellence for Mass Spectrometry at King's College London, Denmark Hill
- *or* using a ProteoExtract All-in-One Trypsin Digestion Kit provided by the Division of Allergy and Immunology at the University of Salzburg.

Centre of Excellence for Mass Spectrometry, King's College London, in-gel digestion protocol

For all the steps described, the volume of solution used was enough to cover the gel pieces. All reagents are listed in Table 3-1 on page 116.

Excised regions were cut into 2mm^2 pieces, transferred into Eppendorfs, washed with 100mM ammonium bicarbonate (AHC), for 5 minutes, dehydrated twice with acetonitrile and dried in a SpeedVac for 5 minutes. To reduce the disulphide bonds in the protein, samples were swollen with 10mM Dithiothretol made in 100mM Ammonium hydrogen carbonate (AHC), at 56°C for 30 minutes. This solution was decanted and replaced with 2 changes of acetonitrile and the gel pieces were dried again for 5 minutes. To prevent disulphide bonds from forming, 55mM Iodoacetamide made in 100mM AHC, was added and

gel pieces were incubated at RT for 20 minutes in the dark. The supernatant was discarded, and the gel pieces were washed twice for 5 minutes in 100mM AHC, twice with acetonitrile then dried for 5 minutes.

Gel pieces were then saturated with trypsin (13ng/µl), purchased from Roche as vials of 25µg. Each vial was prepared with addition of 250µl 0.1% trifluoroacetic acid (TFA) and solubilised by vortexing. This solution was split into eight 30µl aliquots and stored at -20°C. Each aliquot was made up to a final trypsin concentration of 13ng/µl by addition of 200µl of 50mM of AHC. Gel pieces were each incubated in enough trypsin to be fully submerged, at 4°C for 20 minutes. Unabsorbed trypsin was removed and a minimal volume of 10-20µl of 50mM AHC (to keep the pieces wet during enzyme cleavage) was used to incubate the gel pieces at 37°C for 2 hours and then RT overnight.

Supernatant from the gel pieces was transferred into new Eppendorf tubes. To obtain the highest peptide yield possible, gel pieces were washed again with 1ml of 50mM of AHC for 5 minutes at 37°C and the residual solution was pooled with the original supernatant. Enough acetonitrile was then added to cover the gel pieces for 10 minutes at 37°C and the solution was also transferred to the pooled supernatant. These steps were repeated twice, and the pooled supernatant was lyophilised in a SpeedVac and stored at -80°C until MS analysis.

ProteoExtract All-in-One Trypsin Digestion Kit, University of Salzburg, for in-gel digestion

A ProteoExtract All-in-One Trypsin Digestion kit was used according to the manufacturer's instructions. The kit included wash buffer solution, digest buffer solution, D-Buffer reagent lyophilizate, reducing agent lyophilizate, blocking agent lyophilizate and trypsin lyophilizate.

The excised bands from Coomassie stained gels were cut into 2mm^2 pieces and transferred into a siliconized microfuge tube. The gel pieces were washed three times for 30 minutes each with 80µl of wash buffer at 37°C and then dried at 90°C for 15 minutes.

Using 0.5ml of digest buffer solution, we dissolved the D-Buffer Reagent lyophilizate. We added 120µl of ultra-pure water (UPW) to a reducing agent lyophilizate and further diluted this 1:10 in UPW. We added 20µl of dissolved digest buffer and 2µl of diluted reducing agent to the gel pieces and incubated them for 10 minutes at 37°C. We added 120µl of UPW to

blocking agent lyophilizate and further diluted this 1:10 in UPW. The gel pieces were removed from the heater, allowed to cool to RT before mixing with 2µl of diluted blocking agent and incubating for 10 minutes at RT.

We dissolved 50µg of trypsin lyophilizate, by addition of 50µl of UPW. We then used 0.5µl of diluted trypsin to the gel piece solution by mix-pipetting. The final concentration of trypsin was 8ng/µl. This was left to incubate for 16 hours at 37°C with gentle agitation, ensuring gel pieces were completely immersed in liquid. The solution was then centrifuged for 10 minutes at 10,000xg and the supernatant containing the tryptic peptides was transferred to a new tube. The sample was then stored at -20°C prior to peptide extraction.

3.2.7.2 In-solution tryptic digestion

The same ProteoExtract All-in-One Trypsin Digestion Kit was also used to digest proteins in-solution, that were not separated by SDS-PAGE. The protocol was followed according to the manufacturer's instructions. Extracted protein samples were dissolved in 25µl of extraction buffer 1, ensuring protein concentration did not exceed 4µg/µl, then centrifuged for 15 minutes at 10,000xg. The supernatant was transferred to a new Eppendorf and to this we added 25µl of digest buffer and 1µl of reducing agent, incubated the solution at 37°C for 20 minutes and allowed it to cool to RT before mixing with 1µl of blocking agent and leaving at RT for 20 minutes. We then added 1µl of trypsin and left the solution at 37°C for a maximum of 16 hours. The sample was then stored at -20°C prior to peptide extraction.

3.2.7.3 Extraction of peptides

A 10µl ZipTip$_{C18}$ pipette tip was used to pick up the digested peptide solution after in-gel or in-solution digestion, to de-salt the solution; producing more concentrated peptides.
To achieve maximum binding to the ZipTip, the peptide solution was checked for acidity using pH paper and addition of 13µl of 0.1% TFA to ensure the solution had a pH <4. The following steps were used to extract peptides:

- Using a maximum volume setting of 10µl, a pipette plunger was depressed to a dead stop and a wetting solution, prepared as 100% acetonitrile, was aspirated into the ZipTip, dispensed to waste, aspirated and dispensed to waste again.
- An equilibration solution, prepared as 0.1% TFA in Milli-Q grade water was aspirated by the same ZipTip, dispensed to waste, aspirated and dispensed to waste again.
- The pipette plunger was again fully depressed to a dead stop and the peptide sample was aspirated and dispensed for 7-10 cycles, by the same ZipTip (without discarding to waste), for maximum binding of peptides to the ZipTip.
- A wash solution, prepared as 0.1% TFA in Milli-Q grade water, was aspirated into the ZipTip and dispensed to waste, twice.
- Using a standard pipette tip, 5µl of elution solution, prepared as 0.1% TFA, 50% acetonitrile in Milli-Q grade water, was placed into a clean Eppendorf. Using the same ZipTip with bound peptides, the elution solution was aspirated and dispensed 3 times in the clean Eppendorf to elute the peptides (without introducing air bubbles).

The sample was dried down in a Speed Vac® for approximately 10 minutes, resuspended in 0.1% TFA and sonicated for 5 minutes, before transfer into a MS vial.

3.2.7.4 Nano liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

The lyophilised pooled supernatant was re-suspended in 10µl of 50mM AHC and analysed with automated nano LC-MS/MS. Peptides were resolved by reverse phase chromatography using an EASY NanoLC system. A 75µm C18 column was used with a 3-step linear gradient of acetonitrile in 0.1% formic acid and peptides were eluted at a flow rate of 300nL/min over 60 minutes.

The eluate was ionised by electrospray ionisation (ESI) using an Orbitrap Velos Pro operated by Xcalibur (version 2.2). The instrument was set up in a data-dependent switching mode, whereby precursor ions were selected after every MS scan based on their intensity. Precursor ions were then sequenced in tandem MS scans using Top20 Collision induced dissociation (CID) fragmentation. The tandem MS analyses were conducted using collision energy profiles, chosen based on the mass-to-charge ratio (*m/z*) and the charge state of the peptide.

The raw MS data was processed by Proteome Discoverer (ThermoScientific version 1.4). This data was searched using the Mascot search algorithm (Matrix Science, London, UK; version 2.2.06) against all taxonomy or to the species in which the specific-IgE binding protein was known to originate, from the UniProt database. Mascot uses an algorithm to generate a peak list and relative abundances from the raw data, that represent peptide fragments for a given mass and charge. The search was made with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 20 ppm. Trypsin was set as the digestion enzyme and oxidation of methionine, acetyl of the n-terminus and carbamidomethyl of cysteine were specified as variable modifications. Charge state deconvolution and deisotoping were not performed.

Database generated files were uploaded into either Scaffold (version Scaffold_4.7.5, Proteome Software Inc., Portland, OR) or PEAKS Studio (Bioinformatics Solutions Inc. Canada), which were used to validate protein identifications. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 unique peptide.

Scaffold and PEAKS Studio software filtered data at the protein and peptide level using different statistical parameters. On Scaffold, protein probability scores (%) were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003) and on PEAKS studio, a score for each protein was calculated based on the quality of each peptide-spectrum match (Bioinformatics Solutions Inc. 2018). Both Scaffold and PEAKS Studio software programs provided results on the unique peptide count, total spectrum count, amino acid sequence coverage (%) and accession number for each identified protein. This information was used to identify the most abundant proteins identified from the excised specific-IgE binding regions.

4 Developing a mass spectrometry (MS)-based method to identify unknown airborne allergens – using known allergens from a mouse urine extract

4.1 Introduction

In the previous chapter, I described the methods for both western blot and mass spectrometry (MS), that are used in the following chapters of this thesis for the identification of airborne allergens. To optimise these methods, I have used, in this chapter, known airborne allergens from mouse urine that are present in animal testing facilities.

It is estimated that 12,000 – 17,000 individuals in the UK conduct experimental work on mice and other small laboratory animals (Draper et al., 2003). The Home Office publishes annual statistics on scientific procedures carried out on living animals, and in 2017 they reported that out of 1.89 million experimental procedures, 58% were conducted on mice. Figure 4-1 is from this report and shows the most common species used for experimental procedures in the UK, in 2017.

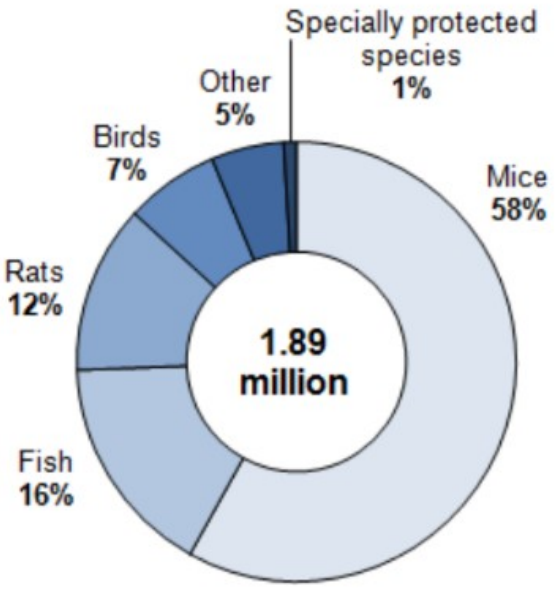


Figure 4-1 Home Office annual statistics of scientific procedures on living animals in 2017. (Annual Home Office review of animal testing, 2018 p. 12)

Workers who are regularly exposed to laboratory animals, such as mice, are at an increased risk of developing symptoms of asthma, rhinitis, urticaria and/or conjunctivitis. These symptoms are caused as an IgE-mediated allergic response from exposure to allergenic proteins derived from laboratory animals. When associated with exposure to small laboratory animals, this symptomology is often referred to as laboratory animal allergy (LAA) (Agrup et al. 1986).

Prevalence of LAA

There is a wide variation in the prevalence of LAA, which has been demonstrated in the literature for over 40 years (Lincoln et al., 1974; Platts-Mills et al., 1987; Cullinan et al., 1994; Heederik et al., 1999; Hewitt et al., 2008). Reported rates of LAA vary from 10 to 50%, amongst animal facilities from different countries and those reported in different studies. In Table 4-1 (on page 141) is a summary of 28 cross-sectional studies that have estimated the prevalence of LAA (adapted from Jeal et al. 2010: p.39-41).

In 1981, a detailed clinical survey by Slovak and Hill, of workers within a UK based pharmaceutical company reported that 30% of the workers had LAA. Then, in 1988 Venables et al., showed that 44% of workers in another UK based pharmaceutical company had LAA. A few years later, a large cross-sectional survey in Japan of 5641 workers across 137 animal facilities showed 23.1% had LAA (Aoyama et al., 1992).

More recently, Feary et al. (2017a) conducted a cross-sectional survey of 663 laboratory animal workers exposed to mice, across six UK research institutions. They observed a sensitisation prevalence to mouse proteins of 6.1%, which varied from 0 to 9.1% across the six institutions. Moreover, they showed that the sensitisation prevalence was 2.6% for those who had only ever worked in facilities using individually ventilated cages (IVC), compared with 8.4% for those who worked in facilities using a mix of IVC's and open cages. IVC's have been increasingly replacing conventional open cages used in animal research institutions and early evidence by Feary et al. (2017b) shows the change is associated with lower mouse aeroallergen levels, which may affect the prevalence of LAA.

However, the prevalence of LAA may often be underestimated in cross-sectional studies. This is due to the 'healthy worker effect', which is a type of survivor bias. Workers that develop LAA are more likely to seek other employment, thus leaving behind a population of 'healthy workers'. This effect was suggested by Venables et al., (1988) who showed a negative relationship between the duration of employment and the development of LAA chest symptoms in their cross-sectional study of UK pharmaceutical company workers.

Domestic allergy to mice

Allergic symptoms caused by exposure to mouse allergens may also occur in other settings besides the workplace - potentially in deprived areas and environments with poor housing. In inner-city homes in Baltimore, levels of airborne mouse allergen were shown to be similar to those found in animal facilities and were considered to be high enough to cause symptoms in already sensitised individuals (Matsui et al., 2005). Moreover, the prevalence of mouse sensitisation in inner-city children in the US was significantly higher when mouse allergen levels in the home were above average (Phipatanakul et al., 2000). Therefore, mouse allergen may be an important allergen in the home environment for aggravating asthma, in particular for vulnerable individuals such as children and those living in poor housing environments.

Table 4-1 Prevalence of LAA from cross-sectional studies (adapted from Jeal et al. 2010: p.39-41)

Citation	Country	Facility	Species	N (% response)	Exposure duration	Prevalence of allergic symptoms (%)	Prevalence of sensitisation SPT or IgE (%)	
							Mouse	Other
Lincoln et al., 1974	US	Research	M, R, Rb, Gp, H	238 (NA)	NA	11%	NA	10% (SPT)
Lutsky 1975	US	Mixed	M, R, Rb, Gp, C	1293 (NA)	NA	15%	NA	NA
Taylor 1976	UK	Research, Pharma	LA	474 (NA)	NA	23%	NA	NA
Gross 1980	US	Research	M, R, Rb, Gp	399 (100%)	NA	15%	NA	NA
Cockroft 1981	UK	Research	M, R, Gp, Rb, S	179 (61%)	NA	27%	NA	16% (SPT)
Davies 1981	UK	Research, Pharma	LA	585 (NA)	NA	20%	NA	NA
Schumacher 1981	Australia	Research	M	121 (82%)	3 years	32%	33%	NA
Slovak 1981	UK	Pharma	LA	146 (NA)	NA	30%	NA	15% (SPT)
Agrup 1986	Sweden	Research	M, R, Rb, Gp, H, C	101 (100%)	NA	30%	18%	19% (SPT + IgE)
Bland 1986	US	Research	R, Rb, Gp, C	549 (93%)	NA	24%	NA	NA
Lutsky 1986	Israel	Research	R, M, Rb, Gp	90 (NA)	9 years	7%	NA	NA
Platts-Mills 1987	UK	Research	R	213 (NA)	NA	17%	NA	NA
Venables 1988	UK	Pharma	R, M, Rb, Gp	138 (87%)	9 years	44%	NA	26%
Venables 1988	UK	Mixed	R, M, Rb, Gp	296 (NA)	8 years	47%	NA	17% (SPT)
Aoyama 1992	Japan	Research, breeding	R, M, Gp, b, H, C, D	5641 (64%)	NA	23%	NA	NA
Cullinan 1994	UK	Research	R	323 (88%)	21 months	31%	NA	NA
Bryant 1995	Australia	Research	LA	130 (NA)	NA	56%	NA	NA
Hollander 1996	Netherlands	Research, pharma	R	458 (77%)	11 years	19%	NA	NA
Hollander 1996	Netherlands	Research, pharma	M	377 (77%)	10 years	10%	10% (SPT)	NA
Hollander 1997	Netherlands	Research, pharma	R	398 (77%)	NA	20%	NA	NA
Heederik 1999	Sweden	Research, pharma	R	74 (82%)	<4 years	NA	NA	NA
Heederik 1999	Netherlands	Research, pharma, training	R	219 (77%)	<4 years	NA	NA	NA
Heederik 1999	UK	Research, pharma	R	357 (88%)	<4 years	NA	NA	NA
Lieutier-Colas 2002	France	Research	R	113 (100%)	NA	39%	NA	NA
Jeal 2006	UK	Research	R	689 (96%)	8 years	22%	NA	NA
Krakowiak 2007	Poland	Vets	R, M, H, Gp	200	NA	NA	16% (SPT)	H: 6%, Gp: 13%
Hewitt 2008	New Zealand	Research	LA	50 (NA)	11 years	22%	4%	Gp: 2%
Feary 2017	UK	Research	M	663 (88%)	<5 years	13%	6.1%	NA

M, mouse; R, rat; Rb, rabbit; Gp, guinea pig; H, hamster; D, dog; C, cat; S, sheep; LA, laboratory animals unspecified. NA, not available.

Mouse urine proteins

In both the occupational and domestic setting, the main source of allergenic proteins from mice is derived from their urine. Allergens in mouse urine belong to a family of proteins, known as the mouse urinary protein (MUP) complex (Finlayson et al., 1965). These proteins are synthesised in the liver, circulated in the plasma and excreted via the kidneys under hormonal control. Mice excrete very high levels of urinary protein compared to other mammalian species, due to having a state of 'permanent proteinuria' (Virtanen et al., 1999). Mouse urine can therefore have very high concentrations of allergenic proteins.

There are fifteen different MUPs, coded by thirty-five genes that have been isolated so far from mouse urine. Expression of these MUPs varies between different strains and sexes of mice and across their lifespan (Lücke et al., 1999). For example, male mice excrete 5-10mg of MUPs per day, which is four times more than female mice. This is thought to be because of the role MUPs play in mating behaviour, where they selectively bind male pheromones and concentrate them in the urine (Bacchini et al., 1992). This hypothesis is supported by Hilger et al., (2012) who showed that MUPs elicit different behaviours in male and female mice, and that females were attracted, but male mice repelled by higher levels of MUPs. Moreover, Gordon et al. (1993) showed that as male rats get older they excrete higher levels of urinary proteins.

The major mouse allergen – Mus m 1

The major mouse allergen, Mus m 1 is a 19kDa prealbumin derived from the MUP complex and is the most well-characterised mouse allergen. In 1977, Taylor et al., demonstrated that a major mouse urinary prealbumin of this molecular weight was capable of eliciting allergic reactions by specific inhalation and SPT in five laboratory workers.

The amino acid sequence for Mus m 1 was determined in 1984 and is now known to consist of two allergenic isoforms: Mus m 1.0101 (also known as major urinary protein 6 (MUP6)) and Mus m 1.0102 (known as MUP2), which differ by only two amino acids (Clark et al., 1984; Hilger et al. 2012). These Mus m 1 variants are the only allergens from mouse that are approved and officially recognised by the WHO/IUIS Allergen Nomenclature Sub-committee.

In Table 4-2 are listed all the MUPs recognised as allergenic variants of Mus m 1, by either the WHO/IUIS Allergen Nomenclature or the Allergome platform.

Table 4-2 Mouse urinary proteins (MUPs) identified as variants of Mus m 1 by either the WHO/IUIS Allergen Nomenclature or the Allergome platform. Accession numbers given according to the UniProt online protein database.

Protein	Allergen	Registered database	Accession no.
MUP6	Mus m 1.0101	WHO/IUIS Allergen Nomenclature	P02762 / A2CGB6
MUP2	Mus m 1.0102	WHO/IUIS Allergen Nomenclature	P11589 / A2AKN9
MUP1	Mus m 1	Allergome platform	P11588 / A2CEK9
MUP3	Mus m 1	Allergome platform	P04939
MUP4	Mus m 1	Allergome platform	P11590 / AZANT5
MUP5	Mus m 1	Allergome platform	P11591
MUP7	Mus m 1	Allergome platform	Q58EV3 / L7MUC7
MUP8	Mus m 1	Allergome platform	A2AKN8
MUP9	Mus m 1	Allergome platform	A2BIN0 / A9C496
MUP10	Mus m 1	Allergome platform	A2BIN1
MUP11	Mus m 1	Allergome platform	P04938
MUP13	Mus m 1	Allergome platform	A2CEK6 / L7N222
MUP14	Mus m 1	Allergome platform	A2CEK7 / B8JI96
MUP15	Mus m 1	Allergome platform	A9R9W0
MUP17	Mus m 1	Allergome platform	B5X0G2
MUP18	Mus m 1	Allergome platform	A2BIM8
MUP19	Mus m 1	Allergome platform	A9C497
MUP20	Mus m 1	Allergome platform	Q5FW60
MUP21	Mus m 1	Allergome platform	A9R9V7

Mus m 1 belongs to the lipocalin family of proteins. There are many animal allergens that belong to the lipocalin family such as Rat n 1, a urinary protein from rat, Fel d 7 from cat, Can f 4 and Can f 6 from dog and Cav p 2 and Cav p 3 from guinea pig (Virtanen et al. 1999; Virtanen, 2001). Lipocalins can be characterised by their common tertiary structure. They have three highly conserved sequence motifs that lie close to one another on the molecular surface and form a common cell surface receptor binding site (Wood, 2001). However, primary amino acid sequences amongst the lipocalin family can be divergent, with sequence identity comparisons as low as 15%. Figure 4-3 (on page 145) compares the sequence identities of all known lipocalin allergens (Hilger et al. 2012).

Although it is not common, laboratory animal workers can be sensitised to both Rat n 1 and Mus m 1 lipocalin allergens, despite only being exposed to one of these species (Jeal et al., 2009). This dual sensitivity is almost certainly explained by cross-reactivity between these allergenic proteins which share 64% amino acid sequence similarity. Jeal et al. (2009) conducted a cross-sectional study of 776 laboratory animal workers and used detailed RAST and western blot inhibition experiments to show IgE cross-reactivity between Rat n 1 and Mus m 1. Over half of the individuals sensitised to rat, were also sensitised to mouse (62%) and almost all of the individuals sensitised to mouse were sensitised to rat (91%). Sequence alignment of these two allergens shown in Figure 4-4, on page 145 (Jeal et al. 2009 p.859) revealed short stretches of amino acids where high sequence similarity was observed.

The three-dimensional structures of Mus m 1 and Rat n 1 were determined by Böcskei et al., (1992) using X-ray crystallography. They observed the 3-D structure of these proteins to be consistent with other lipocalin allergens, both containing an eight-stranded β-barrel and an α-helix with loops between the β-strands. The three-dimensional structure of Mus m 1 is shown below (Figure 4-2).

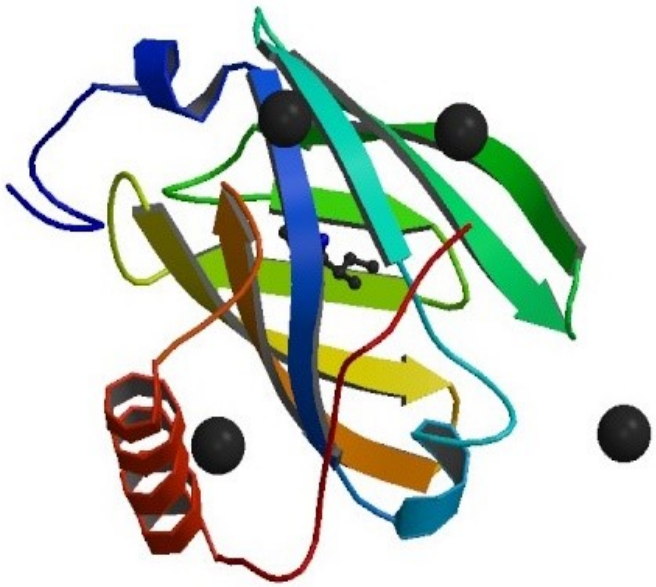


Figure 4-2 The 3-D structure of mouse major urinary protein (at 2.4 A resolution) using X-ray diffraction. Image from the RCSB PDB (www.rcsb.org) of PDB ID 1MUP (Bocskei et al. 1992).

Fel d 7	19											
Can f 1	24	62										
Can f 2	25	22	23									
Can f 4	25	21	19	25								
Can f 6	67	24	25	24	26							
Equ c 1	67	25	25	24	28	57						
Bos d 2	30	16	23	20	31	27	31					
Bos d 5	25	27	26	20	16	23	20	20				
Cav p 2	31	22	22	14	33	27	33	39	19			
Cav p 3	28	20	19	23	32	27	28	39	18	43		
Rat n 1	55	24	21	28	28	52	47	26	23	30	25	
Mus m 1	49	21	19	26	26	47	46	26	18	27	24	64
	Fel d 4	**Fel d 7**	**Can f 1**	**Can f 2**	**Can f 4**	**Can f 6**	**Equ c 1**	**Bos d 2**	**Bos d 5**	**Cav p 2**	**Cav p 3**	**Rat n 1**

Figure 4-3 Amino acid sequence identities (%) between mammalian lipocalins (Hilger 2012: p. 444)

```
RAT n 1    EEASSTRGNLDVAKLNGDWFSIVVASNKREKIEENGSMRVFMQHIDVLENSLGFKFRIKE  60
Mus m 1    EEASSTGRNFNVEKINGEWHTIILASDKREKIEDNGNFRLFLEQIHVLENSLVLKFHTVR  60
           ******  *  * **** *  *  ** ******  *    *   * ******  **

RAT n 1    NGECRELYLVAYKTPEDGEYFVEYDGGNTFTILKTDYDRYVMFHLINFKNGETFQLMVLY 1120
Mus m 1    DEECSELSMVADKTEKAGEYSVTYDGFNTFTIPKTDYDNFLMAHLINEKDGETFQLMGLY 1120
             ** **  ** **   *** * *** ***** *****   * **** * ******* **

RAT n 1    GRTKDLSSDIKEKFAKLCEAHGITRDNIIDLTKTDRCLQAR 161
Mus m 1    GREPDLSSDIKERFAQLCEEHGILRENIIDLSNANRCLQAR 161
           **  ******** ** *** *** * *****    ******
```

Figure 4-4 Sequence alignment of Rat n 1 and Mus m 1. Asterisks denote identical amino acid residues between molecules. Letters highlighted in bold indicate identical sequences >5 amino acids in length. Boxed regions denote structurally conserved domains of the lipocalins. (Jeal et al., 2008 p.859)

Other mouse allergens

Other mouse allergens coming from non-urinary sources, have been less well characterised. A 17kDa glycoprotein has been identified as the mouse allergen Mus m 2. The biological function of this protein is not well-understood, but it is known to originate from the hair follicles of mice and as such has been associated with mouse pelt and mouse epithelium (Price and Longbottom, 1987).

Serum albumin, also known as Mus m 4 is a 68kDa protein found in large quantities in mouse serum. Up to 30% of mouse-allergic patients will develop specific-IgE to mouse serum albumin (Price & Longbottom, 1990). It is the most abundant plasma protein amongst mammalian species and is essential for maintaining oncotic pressure to distribute bodily fluids.

Serum albumin has been shown to participate in IgE-mediated cross-reactions (Díaz-Perales et al., 2013). It's cross reactivity spreads across species of cat, dog, rodents, cow, horse and chickens. It is thought that its presence in animal dander is its major origin of exposure that causes allergic respiratory symptoms in humans (Spitzauer et al., 1995; Chruszcz et al., 2013).

Both Mus m 2 and Mus m 4 are not currently registered as allergens by the WHO/IUIS Allergen Nomenclature Sub-committee. However, they are widely cited as allergenic proteins derived from mice and are listed as allergens within the Allergome platform. Allergome provides an exhaustive repository of allergen data and gives allergenicity scores based on specific-IgE binding, skin tests and epidemiological results in the available literature. This is compared with the WHO/IUIS Allergen Nomenclature which is a far more limited allergen repository and has very strict inclusion criteria as discussed in Chapter 1. A list of the most well-characterised allergens from mouse is shown in Table 4-3 on page 147.

There is one report by Hollander et al., (1997) of an allergic reaction to a mouse urine protein with a molecular weight of 40kDa. The authors obtained serum samples from 47 mouse-sensitised workers across 7 laboratory animal facilities in the Netherlands and demonstrated using western blot, that over half had serum specific-IgE binding to a 40kDa protein. However, no further characterisation of this 40kDa protein has been attempted.

Table 4-3 Allergens from mouse (Mus musculus). (Hilger, 2012)**.**

Allergen	Approximate molecular weight (kDa)	Source	Biological function	Registered database
Mus m 1	19	Hair, dander, urine	Transport protein	WHO/IUIS Allergen Nomenclature and Allergome platform
Mus m 2	16	Hair, dander	Unknown	Allergome platform
Mus m 4 (serum albumin)	68	Serum	Regulates oncotic pressure	Allergome platform

Airborne mouse allergens

When mice spray urine onto their surroundings, the urinary proteins can become aerosolized, allowing them to be inhaled by laboratory animal workers and cause symptoms of respiratory allergy. Mouse urinary proteins may remain airborne for prolonged periods of time and can be carried on a wide range of particle sizes that range 0.4 - 10µm (Ohman et al. 1994).

In 1980, Price and Longbottom used immunohistochemical staining to show that antigens present in mouse hair follicles were also present in dust extracts that were collected from animal testing facilities. In 1982, Twiggs et al., showed airborne allergens from mouse urine and pelt present within an animal testing facility could be detected and quantified. They were able to measure airborne allergen originating from either mouse pelt or mouse urine and recorded levels that ranged from 1.8 to 825ng/m^3. They also observed that the levels varied depending on the number of mice, type of work activity taking place within the animal facility and air circulation in the room where sampling took place.

Although an increased number of mice per room is associated with higher allergen levels, there are other factors that affect a worker's allergen exposure. Workers that handle contaminated bedding are exposed to high levels of mouse allergen, this was observed by Gordon et al., (1992). More recently, ventilated cage-changing wagons have been introduced to remove contaminated bedding. This change has been shown to reduce mouse allergen exposure for laboratory animal workers, from 77 to 17 ng/m^3 (Thulin et al., 2002).

Currently, exposure to airborne mouse allergen is determined by measuring the major mouse allergen, Mus m 1. A commercial kit for measuring Mus m 1 has been available for over a decade and is based on a sandwich ELISA technique, as described in section 1.4.4.1, produced by Indoor Biotechnologies. Because this kit is standardised it allows for comparisons to be made between different laboratories, unlike mouse allergens levels published previously. Its use in animal testing facilities in the UK has been demonstrated by Canizales et al., (2016) who measured both static and personal exposures to Mus m 1 within animal testing facilities.

Diagnosis of LAA

To diagnose LAA, a full clinical picture is ascertained, which usually includes SPT and/or blood testing to assess the presence of allergen specific-IgE. Intradermal, nasal and bronchial challenge testing also serve as diagnostic tools; however, they are not commonly used to diagnose LAA. Currently, the SPT used is a commercial mouse epithelia extract (as no commercial mouse urine extract is available) and is a rapid means of determining sensitisation by the presence of specific-IgE. However, concentrations of Mus m 1 in SPT extracts are known to differ substantially according to their manufacturing company, which can lead to variability in test results (Sharma et al., 2008). Moreover, mouse epithelia SPT and other SPT used in occupational allergy clinics are becoming increasingly difficult to source from commercial outlets (personal communications from Bernadette Fitzgerald and Julie Cannon healthcare professionals working for the Royal Brompton Hospital occupational asthma clinic).

For blood testing, a serum sample is taken from the patient and tested for specific-IgE to either mouse urine or mouse epithelium using the ImmunoCAP method (as described in section 1.4.2.3). This is based on a sandwich assay and is a diagnostic test trademarked by Thermo Scientific. Figure 4-5 (on page 149) shows the principle of ImmunoCAP used to test for specific-IgE. This is a standardised assay, where results are comparable between laboratories.

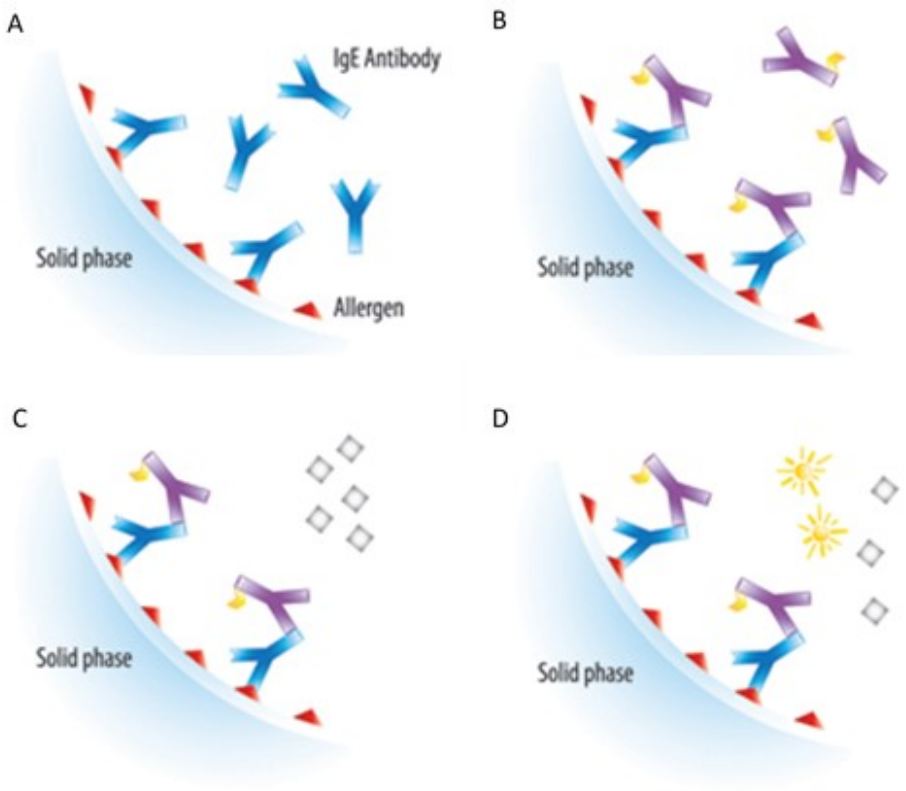


Figure 4-5 Principle of ImmunoCAP specific IgE testing. The test is designed as a sandwich immunoassay**.** The allergen component is covalently coupled to the solid phase which reacts with specific-IgE in patient serum (A). Enzyme-labelled antibodies against specific-IgE are then added which bind to specific-IgE (B). The bound complex is then incubated with a developing agent (C) and fluorescence of the complex is measured (D). The higher the fluorescence, the more specific-IgE is present in the sample (adapted from the Phadia website, accessed June 2018).

Many workers with LAA will test positive to mouse urine as well as mouse epithelium. However, some workers with LAA will test positive to mouse epithelium and negative to mouse urine. Canizales et al., (2015) investigated how many laboratory animal workers may be positive to mouse epithelium and negative to mouse urine. Of 321 laboratory animal workers that they recruited, 35 and 31 tested positive to mouse epithelium and mouse urine ImmunoCAP, respectively. Of the 35 positive to epithelium, nine were negative to mouse urine ImmunoCAP. This shows that if mouse urine is solely used to diagnose LAA, some individuals will be misdiagnosed.

Moreover, the 321 participants in the same study all underwent SPT to mouse epithelia. Of 11 positive SPT results, 1 was negative to mouse urine ImmunoCAP, and of 310 negative SPT results, 24 were positive to mouse urine ImmunoCAP. This demonstrates discordance between the two diagnostic tests - ImmunoCAP and SPT, for 8% (n=25) of the laboratory animal workers included in this study. Neither of these tests were sensitive enough to identify all individuals with LAA.

It is important to identify all the separate mouse allergen components from within mouse urine and mouse epithelium, that may be responsible for causing allergic sensitisation. Therefore, it is necessary to develop a diagnostic test that is sensitive enough to detect all laboratory animal workers with LAA.

In this chapter we sought to identify which allergens were present in extracts of mouse urine and mouse epithelium. We also sought to identify which allergens would be detected in airborne samples collected from an animal testing facility housing mice.

As an integral part of this thesis, the purpose of this chapter was also to optimise the MS-based methodology required to identify unknown airborne allergens.

Objective

To identify mouse allergens, present in mouse urine, mouse epithelium and airborne samples collected within an animal testing facility.

Aims

1. Optimise western blot and MS methods to identify known allergen Mus m 1 from both mouse urine and airborne samples.
2. Identify allergens from mouse epithelium extract, using the optimised MS-based method.
3. Compare individual sensitisation to mouse epithelium and mouse urine in laboratory animal workers.

4.2 Methods

4.2.1 Collection of crude allergen samples

Mouse urine extract

I used a stored extract of crude mouse urine. Urine was previously collected from male adult mice and extracted by dialysis against four changes of dH_2O, over 48 hours at RT, then lyophilised and stored at -20°C.

Mouse epithelium

I collected adult mice after necropsy (from Imperial College animal research facilities) and prepared mouse epithelium by removing the skin from each dead mouse. This was stored at -20°C until extraction.

SPT of mouse epithelia

A mouse epithelia SPT solution was purchased from Allergopharma, Reinbeck (labelled as 10,000 BU/ml).

Mouse hair

Hair was shaved as close as possible to the skin from adult mice after necropsy, using an electrical shaver. This was stored at -20°C until extraction.

4.2.2 Collection of airborne samples

Air samples

Air samples were collected during cage-cleaning (a known high exposure task), from an animal facility housing mice. I collected air samples using TUFF 4 Plus I.S. air sample pumps with IOM sampling heads and 25mm diameter, 1.0µm pore size fluorophore membrane filters. Filters were placed inside sampling heads, attached to the pump, placed next to the cage-cleaning area and run for approximately 7 hours at a flow rate of 2 litres per minute. Filters were stored in individual petri dishes at 4°C until extraction.

Synthetic panel filters

I collected synthetic panel filters used to ventilate stacks of cages housing mice from an Imperial College animal facility and stored them in individual plastic bags at -20°C until extraction.

Ventilation filters for a cage-changing station

Filters used to ventilate a fume cupboard, used as a mice cage-changing station were collected from an animal facility at the University of Salzburg, Austria and were extracted immediately.

4.2.3 Protein extraction

The extraction of all samples (except for mouse urine) was optimised (Table 4-4). In brief, I used three different buffers and extracted protein for either 3 or 16 hours, at RT on a rock 'n' roller. PBS-T was prepared as in section 3.2.3.

Table 4-4 Optimisation of protein extraction at RT for all samples collected for mouse allergen detection.

Samples	Sample size	Buffer		
		Distilled water (dH_2O)	*PBS 0.05% Tween-20 (PBS-T)*	*0.1M Ammonium hydrogen carbonate (AHC)**
		3 hours		16 hours
Mouse epithelium	From 1 mouse	20ml	20ml	20ml
Mouse hair	From 1 mouse	20ml	20ml	20ml
Air samples	From 1 membrane filter	1ml	1ml	5ml
	From 16 membrane filters (pooled)	-	1ml	-
Synthetic panel filter	6x2cm piece	20ml	20ml	20ml
Ventilation filters	6x2cm piece	20ml	20ml	20ml

*Samples extracted in AHC were dialysed against two changes of dH_2O over 24 hours at RT.

After extraction, half the volume (10ml) of each sample was stored at 4°C and the other half was freeze dried to avoid storage degradation. Samples were freeze-dried by freezing the solutions at -20°C, covering containers with parafilm (ensuring air could escape) and placing them into a freezer dryer (Scanvac Coolsafe) with a vacuum pump attached at -54°C. The solutions were left overnight or until no ice crystals were present. These were then stored at -20°C.

4.2.4 ELISA

An ELISA was used to determine the concentration of Mus m 1 present in the extracted samples. An ELISA kit was used according to the manufacturer's instructions, as described in Chapter 3, section 3.2.3.

Briefly, a microwell plate was coated with anti-Mus m 1 polyclonal antibody, washed with PBS-T, blocked with PBS-T/BSA and washed with PBS-T again. A universal allergen standard provided in the kit was used to setup a control curve using doubling dilutions, and 100µl of extracted allergen sample was used per well. A biotinylated anti-Mus m 1 polyclonal antibody was added and ExtraAvidin-Peroxidase and an ABTS substrate solution were used to detect Mus m 1 levels, by calculating from absorbance values detected by a Dynatech MR5000 plate reader.

4.2.5 Protein quantification, separation and staining

Methods for protein quantification, separation and staining, were followed as described in Chapter 3, sections 3.2.2-3.2.5.

Briefly, protein content in the extracted samples was quantified using a *DC*™ Protein Assay kit. Proteins were separated using the NuPAGE™ Bis -Tris electrophoresis system according to the Laemmli method and their molecular weight calculated according to either a prestained or unstained protein standard. Proteins were stained using either using a Pierce® Silver Stain Kit, a Pierce® Silver Stain Kit for Mass spectrometry analysis or a 0.1% R-250 Coomassie dye.

4.2.6 Protein separation under reducing conditions

The crude mouse urine extract was subject to reducing and non-reducing SDS-PAGE conditions to determine if mouse urinary proteins undergo conformational changes or split into monomer units under reducing conditions. SDS-PAGE was performed using a hand cast electrophoresis system on a 12% gel. This system is described in Chapter 3, section 3.2.4.2.

4.2.7 Western blot

4.2.7.1 Serum samples

I chose stored serum samples from laboratory animal workers who had attended an occupational allergy clinic at the Royal Brompton Hospital in London and whom were found to have elevated levels of serum specific-IgE to mouse urine, as determined by ImmunoCAP (Table 4-5). A pool of serum was also made using 4ml of each of the five laboratory animal workers.

Table 4-5 ImmunoCAP score of specific IgE antibody (kU/L) to mouse urine and mouse epithelium in serum from five laboratory animal workers and in a pooled sample of the same workers.

Serum Sample Type/No.	ImmunoCAP Score (kU/L)	
	Mouse Urine	Mouse Epithelium
Individual	18.9	14.6
Individual	26.0	4.4
Individual	36.9	>100.00
Individual	51.9	13.0
Individual	>100	6.1
Pool of all above	56.2	69.2

4.2.7.2 Chromogenic dot blot

We initially used a dot blot method as a quick way to establish whether we could detect specific-IgE binding proteins from mouse urine. The buffers and reagents used are described under western blot methods in Chapter 3, section 3.2.6. I applied either 2 or 5µl of mouse urine (containing 1.54 or 3.85µg of protein respectively) onto a pre-cut 0.2µm nitrocellulose membrane which was dried at RT for 15 minutes. The membrane was blocked with 20ml of

phosphate blocking buffer (PBS-T/BSA) for 1 hour and 30 minutes at RT and probed with 20ml of pooled serum from Table 4-5 (diluted 1:10 with PBS-T/BSA) with gentle agitation. The membrane was washed five times with 20ml of PBS-T and then probed with 20ml of goat anti-human IgE antibody conjugated with HRP at RT. The dilutions and incubation times of serum and anti-human IgE antibody were optimised over three dot blot experiments as shown in Table 4-6.

Table 4-6 Optimisation of dilutions and incubations times of serum and anti-human IgE antibody. Serum and anti-human IgE antibody were diluted in PBS-T/BSA to a total volume of 20ml.

Experiment number	Serum		Anti-human IgE antibody	
	Dilution	Incubation time	Dilution	Incubation time
1	1:10	1 hour	1:1000	1 hour 30 mins
2	1:10	16 hours	1:1000	1 hour 30 mins
3	1:10	16 hours	1:1000, 1:5000	30 mins

Following a further five washes with 20ml of PBS-T, the membrane was developed with 15ml of either Novex® HRP Chromogenic TMB Substrate or 1-Step™ Ultra TMB-Blotting Solution and gently agitated until bands were visible as a coloured precipitate. The reaction was stopped by rinsing the membrane thoroughly with dH_2O.

4.2.7.3 Enhanced chemiluminescence (ECL) western blot

Methods for an ECL western blot, were followed as described in Chapter 3 sections 3.2.6. Briefly, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane, incubated with phosphate blocking buffer (PBS-T/BSA), then probed with pooled or individual serum samples from Table 4-5 for 16 hours at 4°C, and diluted goat anti-human IgE antibody for 30 minutes at RT. Specific-IgE binding proteins were detected with an ECL western blotting substrate and a ChemiDoc™ Imaging System.

4.2.8 Mass spectrometry

The following methods: tryptic digestion, peptide extraction, nano LC-MS/MS and data processing were all achieved with training and supervision from both Steven Lynham at the Centre of Excellence for Mass Spectrometry at King's College London, Denmark Hill and Prof. Peter Briza from the Division of Allergy and Immunology at the University of Salzburg.

4.2.8.1 Tryptic digestion of protein

Specific-IgE binding regions from the mouse urine extract, synthetic panel filter extract and mouse epithelium extract detected by ECL western blot were excised from silver stained polyacrylamide gels for in-gel tryptic digestion. The whole mouse urine extract, synthetic panel filter extract and ventilation filter extract were also analysed by MS after in-solution tryptic digestion.

In-gel tryptic digestion

Methods for in-gel tryptic digestion were followed as described in Chapter 3, section 3.2.7.1, following the Centre of Excellence for Mass Spectrometry, in-gel digestion protocol. Briefly, specific-IgE binding regions were excised from silver stained polyacrylamide gels (after SDS-PAGE), then washed, dehydrated and dried. In-gel digestion was performed using either a single excised gel piece or pooled gel pieces of the same molecular weight from the same sample. To digest proteins into peptides, the enzyme trypsin was used. After digestion, the sample was stored at -20°C prior to peptide extraction.

In-solution tryptic digestion

A ProteoExtract All-in-One Trypsin Digestion Kit was used to digest proteins in-solution, that were not separated by SDS-PAGE, as described in Chapter 3, section 3.2.7.2. The protocol was followed according to the manufacturer's instructions. Briefly, extracted protein samples were dissolved in an extraction buffer, centrifuged, then incubated with kit reagents including digest buffer, reducing agent, blocking agent and trypsin. After digestion with trypsin, the sample was stored at -20°C prior to peptide extraction.

4.2.8.2 Extraction of peptides

Methods for peptide extraction were followed as described in Chapter 3, section 3.2.7.3. Briefly, a 10µl ZipTip$_{C18}$ tip was used to pick up the peptide solution after in-gel or in-solution digestion, to de-salt the solution; producing concentrated peptides. The peptide solution was checked for acidity using pH paper and addition of 13µl of TFA. The sample was dried down, resuspended in 0.1% TFA and sonicated before transfer into a MS vial.

4.2.8.3 Nano liquid chromatography coupled to tanem mass spectrometry (LC-MS/MS)

Methods for nano LC-MS/MS were followed as described in Chapter 3, section 3.2.7.4. Briefly, MS vials were placed into an automated EASY nano liquid chromatograph and peptides were resolved by reverse phase chromatography. This was coupled to an Orbitrap Velos Pro by ESI in which peptides were ionised by charged hydrogen ions and then separated based on their mass-to-charge ratio (*m/z*). Raw data was processed by Proteome Discoverer and searched for peptide matches within the UniProt database. The search was limited to mouse taxonomy and employed a 95% protein identification threshold. Based on the probability score, number of unique peptides and sequence coverage the most abundant proteins identified in each MS digest have been listed.

4.3 Results

4.3.1 Optimising western blot methods to identify Mus m 1 from mouse urine

4.3.1.1 Chromogenic dot blots of mouse urine extract

We were unable to detect allergens from the mouse urine extract with sufficient sensitivity using a chromogenic blot. This was despite optimising dilutions and incubation periods of both serum and anti-human IgE antibody. The dot blot results are shown in the following three experiments.

In the first dot blot experiment, it was difficult to detect any specific-IgE binding to protein from the mouse urine extract. As shown in Figure 4-6, 1.54µg of protein from the mouse urine extract was applied to three separate boxes in a 2x3 grid format, incubated with pooled serum (mouse urine specific-IgE level of 56.2kU/l) for 1 hour at a 1:10 dilution and incubated with anti-human IgE for 1 hour 30 minutes at a 1:1000 dilution.

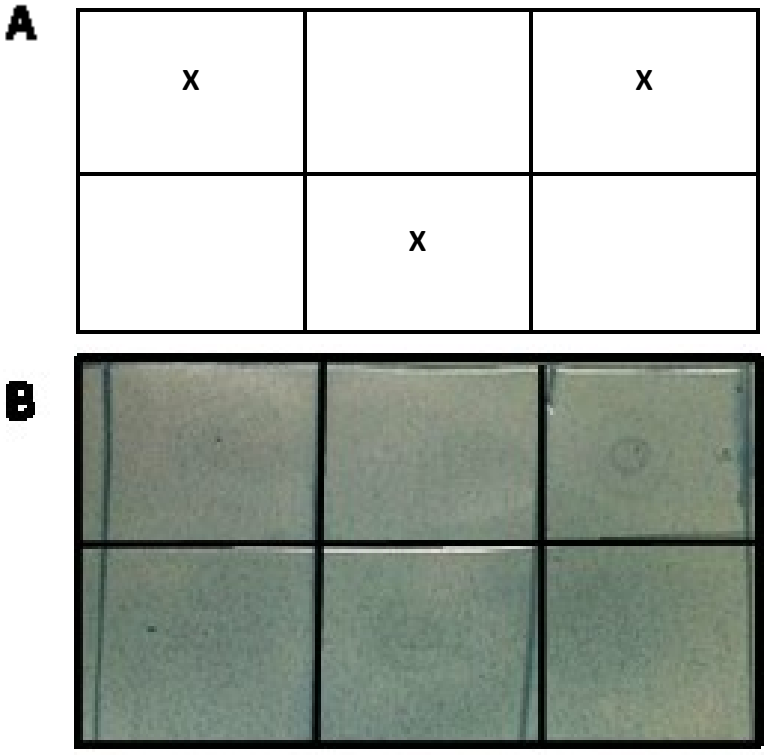


Figure 4-6 Chromogenic dot blot of mouse urine extract (experiment one). Specific-IgE binding present as blue precipitate (B) in boxes marked as shown (A). 1.54µg of mouse urine protein in 2µl was applied to 3 separate boxes in a 2x3 grid format. The membrane was blocked for 1 hour 30 minutes, incubated in serum for 1 hour at a 1:10 dilution, washed with PBS-T, incubated with anti-human IgE for 1 hour 30 minutes at a 1:1000 dilution and detection was using 20ml of TMB substrate solution. All steps were at RT.

In the second dot blot experiment (Figure 4-7) we ran the blot under the same conditions as before, except for extending the serum incubation time to 16 hours. Briefly, 1.54µg (2µl) and 3.85µg (5µl) of protein from the mouse urine extract was applied in three separate boxes each in a 2x3 grid format, incubated with pooled serum for 16 hours at a 1:10 dilution and incubated with anti-human IgE for 1 hour 30 minutes at a 1:1000 dilution.

We were unable to detect specific-IgE binding given the very low signal with very high background staining.

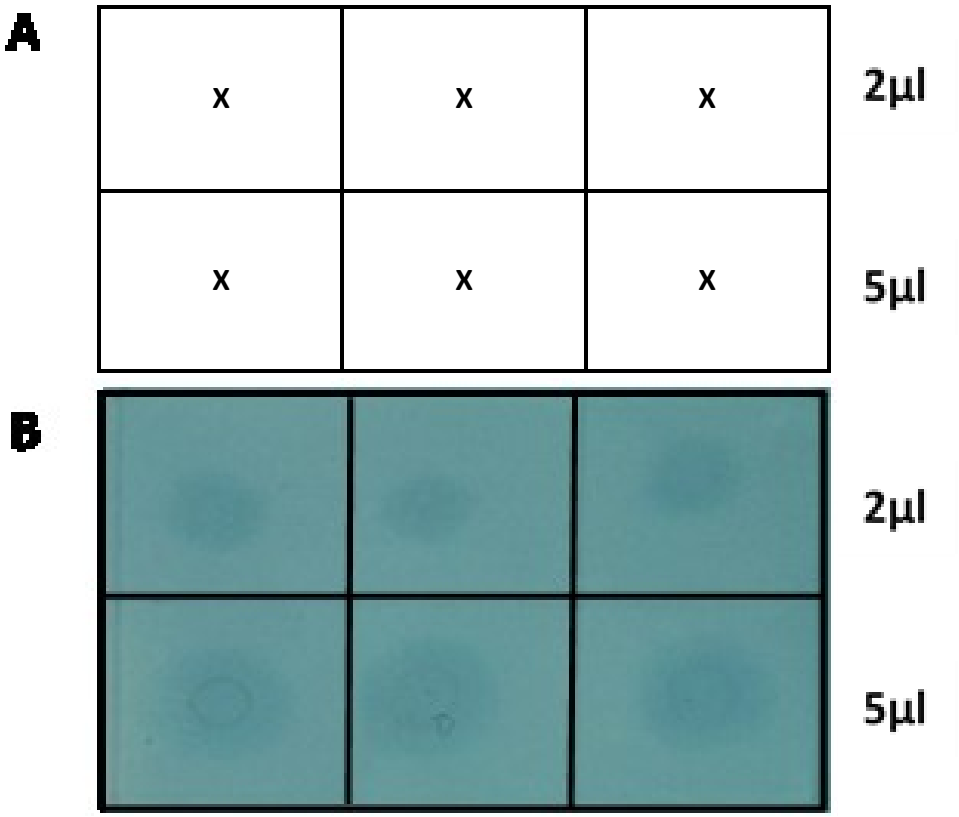


Figure 4-7 Chromogenic dot blot of mouse urine extract (experiment two). Specific-IgE binding present as blue precipitate (B) in boxes marked as shown (A). 1.54µg in 2µl (top row) and 3.85µg in 5µl (bottom row) of mouse urine protein was applied to 3 separate boxes each in a 2x3 grid format. The membrane was blocked for 1 hour 30 minutes, incubated in serum for 16 hours at a 1:10 dilution, washed with PBS-T, incubated with anti-human IgE for 1 hour 30 minutes at a 1:1000 dilution and detection was using 20ml of TMB substrate solution. All steps were at RT.

In the third dot blot experiment (Figure 4-8), the blot was run under the same conditions, except for reducing the anti-human IgE incubation time to thirty minutes (according to protocol recommendations for reducing background), and using two dilutions of anti-human IgE (1:1000 and 1:5000). We also used an Ultra-TMB substrate for higher detection sensitivity. Briefly, 1.54µg (2µl) of protein from the mouse urine extract was applied in six separate boxes in a 4x3 grid format, incubated with pooled serum for 16 hours at a 1:10 dilution, incubated with anti-human IgE antibody for 30 minutes at either a 1:1000 or 1:5000 dilution and then incubated with an Ultra-TMB detection substrate.

The incubation with a 1:5000 dilution of anti-human IgE antibody gave a reduction in background staining, compared with the 1:1000 dilution. Given the low sensitivity of chromogenic detection seen in these dot blot experiments, we sought to optimise a more appropriate detection method for future western blot experiments.

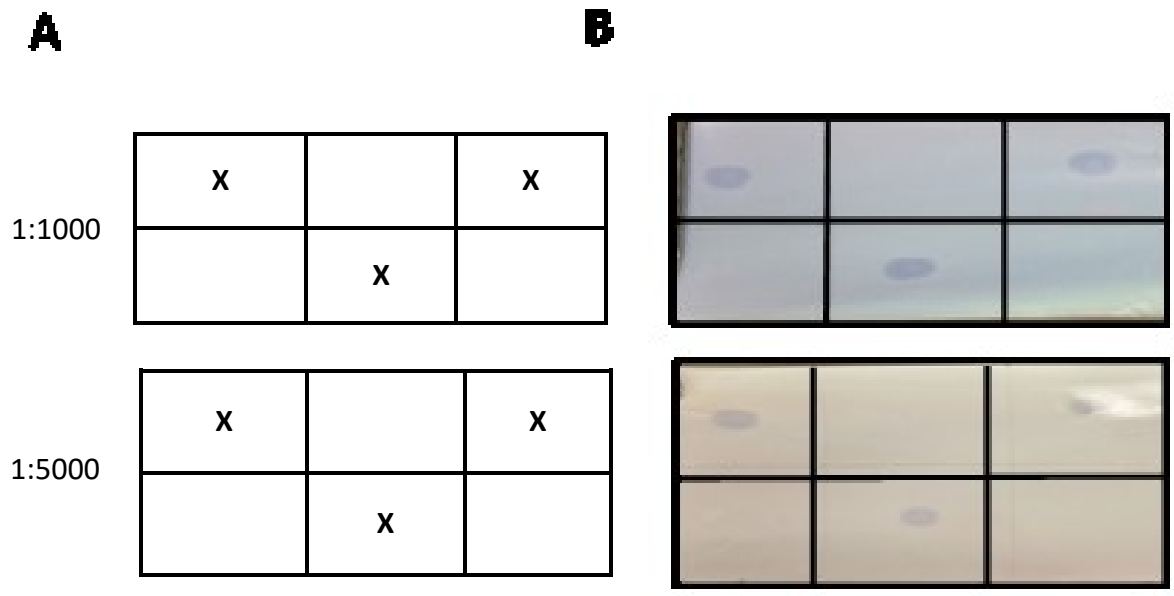


Figure 4-8 Chromogenic dot blot of mouse urine extract (experiment three). Specific-IgE binding present as blue precipitate (B) in boxes marked as shown in (A). 1.54µg in 2µl of mouse urine protein was applied to 6 separate boxes in a 4x3 grid format. The membrane was blocked for 1 hour 30 minutes, incubated in serum for 16 hours at a 1:10 dilution, washed with PBS-T, incubated with anti-human IgE for 30 minutes at a 1:1000 dilution and 1:5000 dilution and detection was using 20ml of Ultra-TMB substrate solution. All steps were at RT.

4.3.1.2 Silver stain protein separation of mouse urine extract

I separated 10, 5 and 2.5µg of mouse urine protein (lanes 2, 3 and 4, respectively) by SDS-PAGE as shown in Figure 4-9. Bands were observed by silver staining at approximately 14, 16, 19, 35 and >50kDa. The strongest bands were between 14 and 20kDa. According to their molecular weight, the bands observed from mouse urine extract, most likely corresponded to Mus m 1, Mus m 2 and Mus m 4, as labelled below.

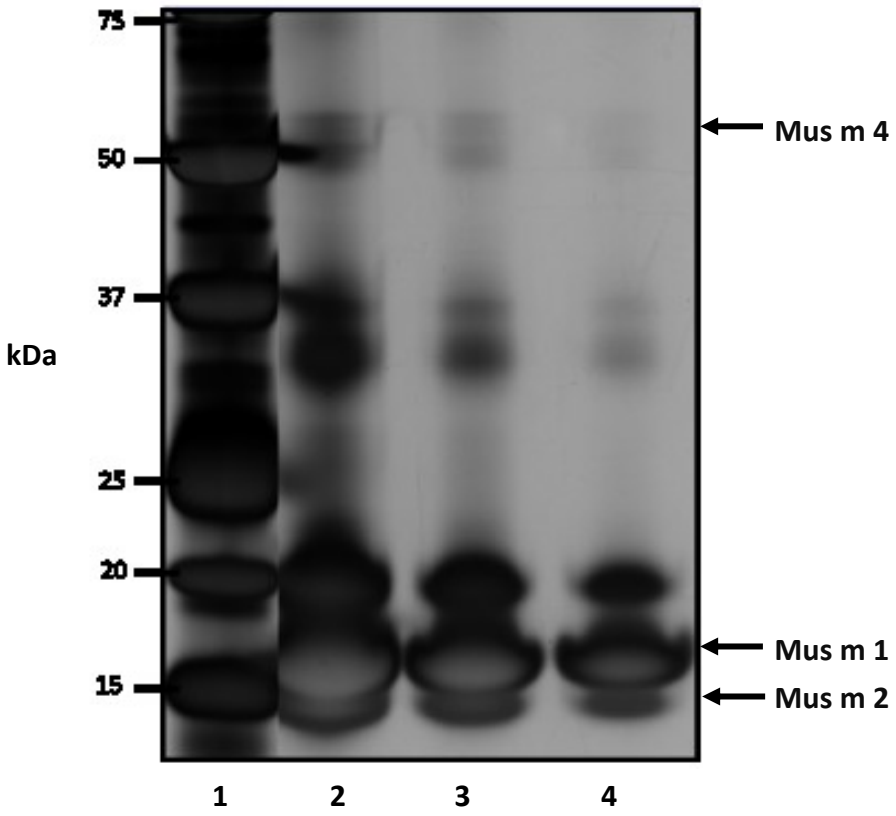


Figure 4-9 Silver stained SDS-PAGE of mouse urine extract. A serial dilution of 10µg (lane 2), 5µg (lane 3) and 2.5µg (lane 4) of mouse urine protein was applied to SDS-PAGE, each in a total volume of 25µl. The molecular weight was determined with a pre-stained protein standard (lane 1). Proteins of interest are labelled on the right.

4.3.1.3 ECL western blot of mouse urine extract

I applied an ECL western blot method to 10, 5 and 2.5µg of mouse urine protein (lanes 2, 3 and 4 respectively), separated by SDS-PAGE. Protein was incubated with pooled serum for 16 hours at 4°C at a 1:10 dilution and with anti-human IgE for 30 minutes at RT at a 1:5000 dilution. Specific-IgE binding was observed at three molecular weight regions; 15-21kDa, 35kDa and 60kDa, as shown in Figure 4-10. This included Mus m 1, Mus m 2, Mus m 4 and an unknown specific-IgE binding protein at approximately 35kDa. When compared with the previous silver stained SDS-PAGE of mouse urine extract (Figure 4-9), proteins were detected at the same molecular weight regions. This ECL detection method was used in all subsequent western blot experiments.

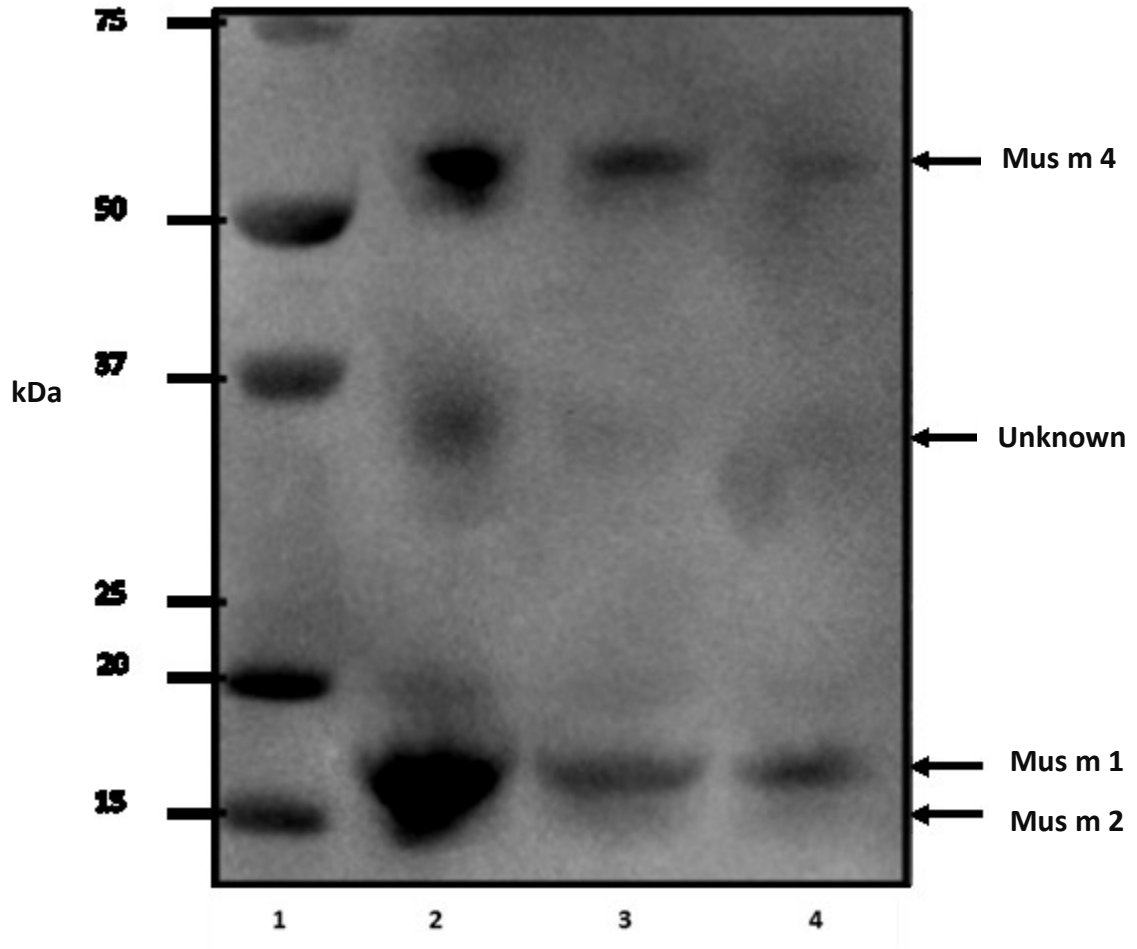


Figure 4-10 ECL western blot of mouse urine extract. A serial dilution of 10µg (lane 2), 5µg (lane 3) and 2.5µg (lane 4) of mouse urine protein was applied to SDS-PAGE each in a total volume of 25µl. Protein was transferred to a nitrocellulose membrane under normal conditions, then blocked for 1 hour 30 minutes, incubated in pooled serum (1:10 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution. The molecular weight was determined with a prestained protein standard (lane 1). Specific-IgE binding proteins of interest are labelled on the right.

4.3.1.4 Determining the limit of detection for mouse urine proteins by silver stained SDS-PAGE and mouse urine specific-IgE binding proteins by ECL western blot

In Figure 4-11 I performed silver stained SDS-PAGE (A) and an ECL western blot (B) using a serial dilution of protein from mouse urine extract and pooled sensitised serum, to determine the limit of detection for mouse urine proteins and mouse urine specific-IgE binding regions, respectively. The protein serial dilution ranged 10 to 0.0781µg (lanes 2-9) in a volume of 25µl for both silver stained SDS-PAGE and the ECL western blot.

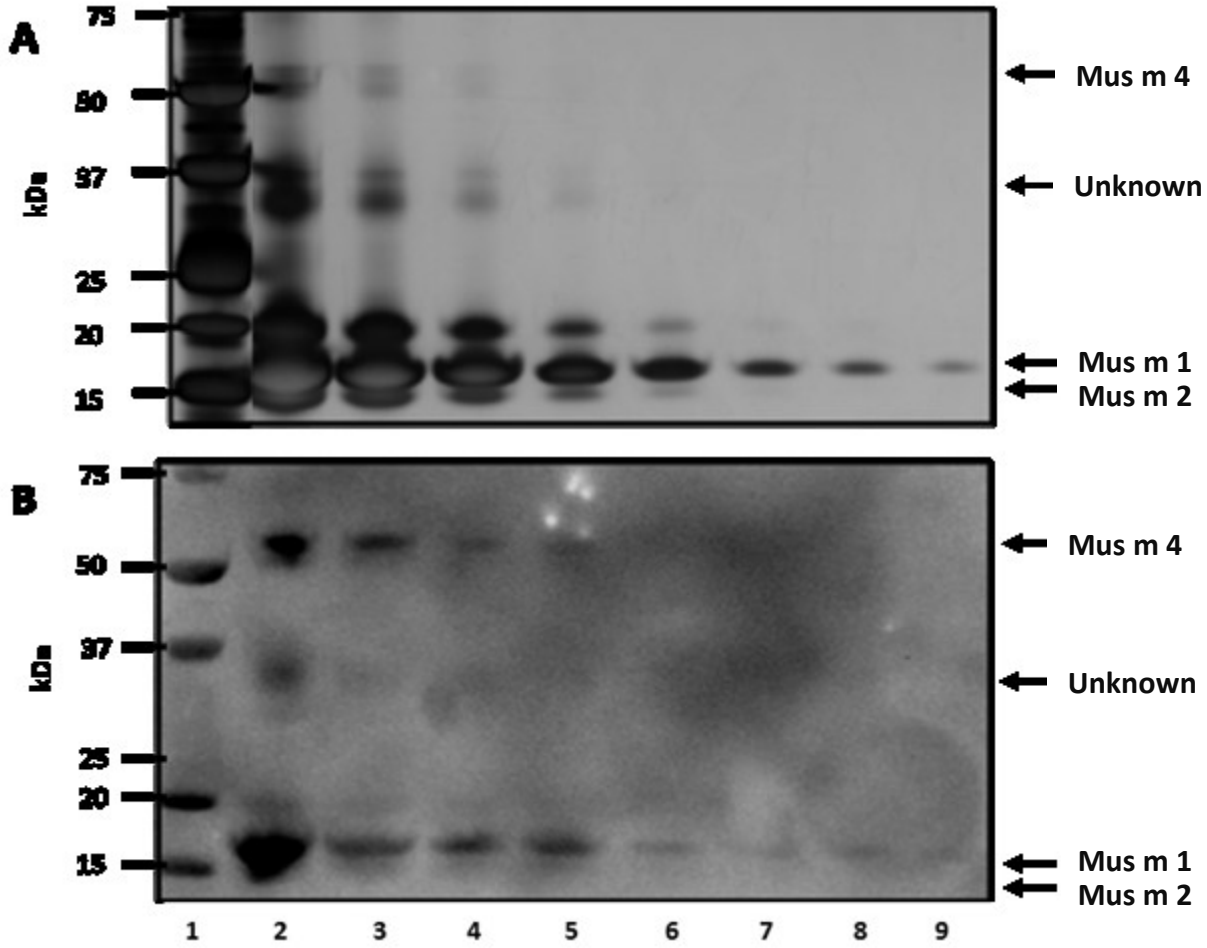


Figure 4-11 Silver stained SDS-PAGE (A) and ECL western blot (B) of mouse urine extract. A serial dilution of 10 (lane 2), 5 (lane 3), 2.5 (lane 4), 1.25 (lane 5), 0.625 (lane 6), 0.3125 (lane 7), 0.1625 (lane 8) and 0.078µg (lane 9) of mouse urine protein was applied to SDS-PAGE, each in a total volume of 25µl and then was either detected with silver stain or transferred to a membrane under normal conditions, blocked for 1 hour 30 minutes, incubated in pooled serum (1:10 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detected using 2ml of ECL substrate solution. The molecular weight was determined with a prestained protein standard (lane 1). Proteins and specific-IgE binding regions of interest are labelled on the right.

The limit of detection in *μg* for mouse urine proteins by silver stained SDS-PAGE and mouse urine specific IgE-binding proteins by ECL western blot is shown in Table 4-7. Mus m 1 was detected in up to 0.078μg for both silver stained SDS-PAGE and ECL western blot. The unknown specific IgE-binding protein at approximately 35kDa was only detected in >1.25μg by silver stained SDS-PAGE and >5μg by ECL western blot.

Table 4-7 Limit of detection for mouse urine proteins by silver stained SDS-PAGE and mouse urine specific-IgE binding proteins by ECL western blot.

Allergen	Approximate molecular weight (kDa)	Limit of detection for proteins (μg) observed by silver stained SDS-PAGE (lane no.)	Limit of detection for specific-IgE binding proteins (μg) observed by ECL western blot (lane no.)
Mus m 1	19-21	0.078 (9)	0.078 (9)
Mus m 2	15-19	0.625 (6)	1.25 (5)
Unknown	~35	1.25 (5)	5 (3)
Mus m 4	>50	2.5 (4)	1.25 (5)

4.3.1.5 Effect of SDS-PAGE reducing conditions on mouse urine proteins

The same proteins were observed in mouse urine extract under both reducing (lane 3) and non-reducing conditions (lane 2), as shown in Figure 4-12. Reduced mouse urine proteins migrated at a higher molecular weight than non-reduced proteins and no separate monomer units were formed from the reduction of mouse urinary proteins. Protein names that are underlined refer to those separated under non-reducing conditions in lane 2.

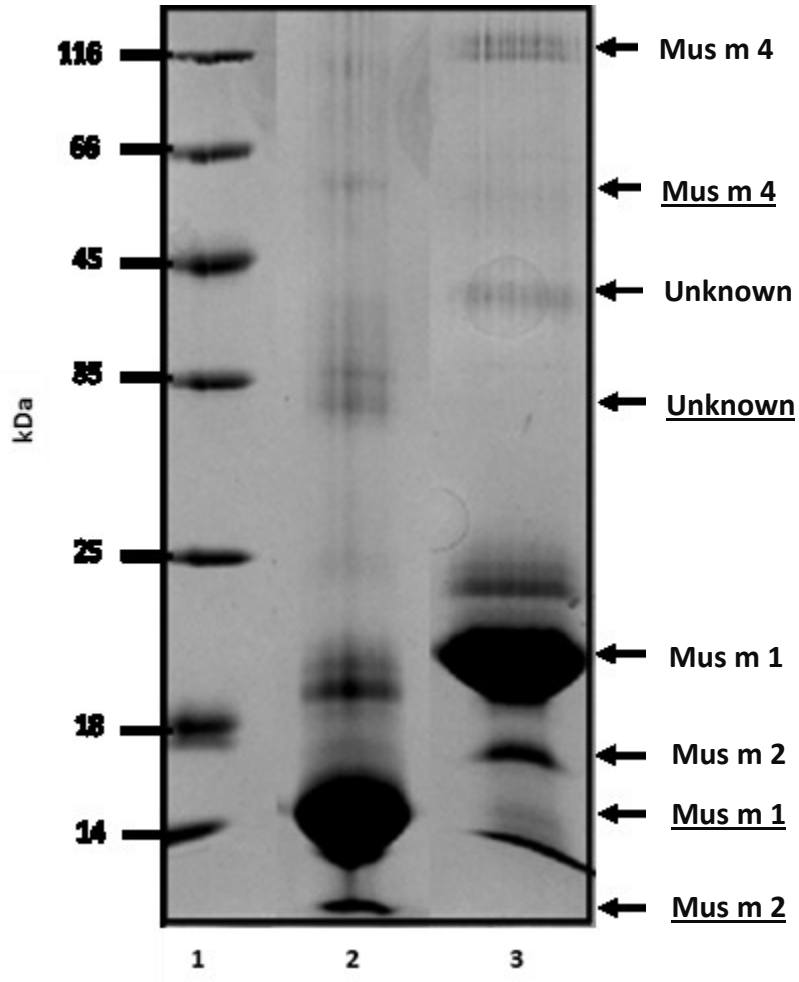


Figure 4-12 Coomassie stained SDS-PAGE of mouse urine extract separated under reducing and non-reducing conditions. 40µg of mouse urine protein was separated under non-reducing (lane 2) and reducing conditions (lane 3) on a 12% hand-cast gel at 200 volts for 35 minutes and was stained with Coomassie. The molecular weight was determined with an unstained protein standard (lane 1). Proteins of interest are labelled on the right, those underlined refer to non-reduced proteins.

4.3.2 Optimising MS methods to identify Mus m 1 from mouse urine

The following specific-IgE binding regions at approximately 15-21kDa, 35kDa and 60kDa from mouse urine extract were excised from silver-stained gels and digested with trypsin for MS analysis as listed in Table 4-8 and shown in Figure 4-13.

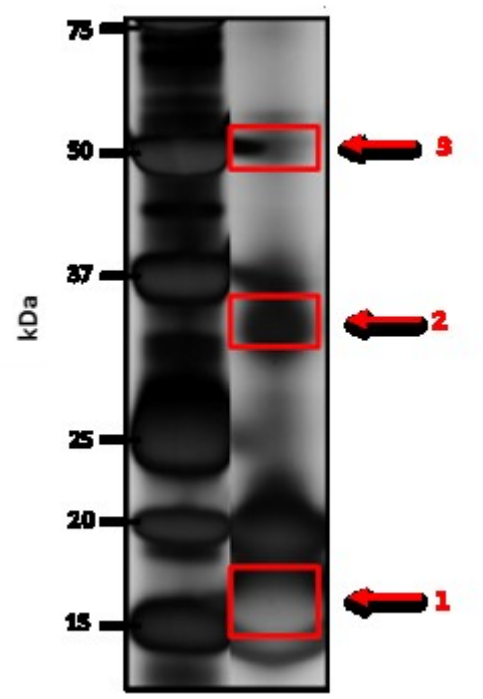


Figure 4-13 In-gel excision of specific-IgE binding regions from mouse urine extract. 10µg of mouse urine protein was applied to SDS-PAGE in a total volume of 25µl and detected using an MS-compatible silver stain. The molecular weight was determined with a prestained protein standard.

Table 4-8 Molecular weight (kDa) and allergen name of specific-IgE binding regions from mouse urine extract excised for MS analysis.

Number	Approximate molecular weight (kDa)	Allergen name
1	15-21	Mus m 1 Mus m 2
2	~35	Unknown
3	60	Mus m 4

Protein identifications from an in-gel 15-21kDa mouse urine digest

We identified 23 proteins from the 15-21kDa digest, with the most abundant being major urinary protein 2 (Prot. Acc: P11589), also known as the mouse urine allergen variant Mus m 1.0102. Sequence coverage was 79.40% and 21 unique peptides were identified, as depicted in Figure 4-14. Major urinary protein 6 (Prot. Acc: P02762), known as the allergen variant Mus m 1.0101, was also identified in this digest.

The 10 most abundant proteins identified in this peptide analysis are shown in Table 4-9 with those registered on either the WHO/IUIS Allergen Nomenclature or Allergome platform indicated. The following Mus m 1 variants, according to Allergome were also detected: MUP1 (Prot. Acc: P11588), MUP17 (Prot. Acc: B5X0G2), MUP20 (Prot. Acc: Q5FW60) and MUP3 (Prot. Acc: P04939).

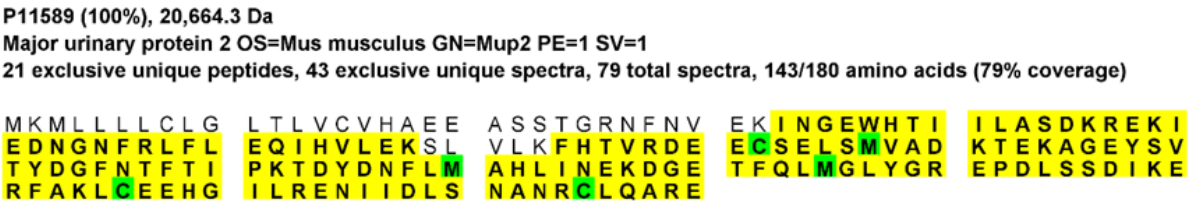


Figure 4-14 Sequence coverage of major urinary protein 2 (Mus m 1.0102), identified by nano LC-MS/MS from an in-gel 15-21kDa mouse urine digest. The mouse urine extract was resolved by SDS-PAGE and visualised with an MS-compatible stain. The specific-IgE binding region at 15-21kDa, was excised and digested with trypsin before injection into HPLC coupled to a nano ESI source into MS/MS. Raw MS data was processed by Proteome Discoverer, searched against mouse taxonomy and uploaded to Scaffold software from which sequence coverage was documented.

Table 4-9 Proteins identified by nano LC-MS/MS from an in-gel 15-21kDa mouse urine digest.

Protein name	Accession Number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)	WHO/IUIS Allergen Nomenclature	Allergome platform
Major urinary protein 2 (Mus m 1.0102)	P11589	100%	21	79%	21	✓	✓
Major urinary protein 1 (Mus m 1)	P11588	100%	4	73%	21		✓
Major urinary protein 6 (Mus m 1.0101)	P02762	100%	2	73%	21	✓	✓
Major urinary protein 17 (Mus m 1)	B5X0G2	100%	2	68%	21		✓
Major urinary protein 20 (Mus m 1)	Q5FW60	100%	7	29%	21		✓
Major urinary protein 3 (Mus m 1)	P04939	100%	2	13%	21		✓
Pigment epithelium-derived factor	P97298	95%	3	8%	46		
Ral GTPase-activating protein subunit alpha-2	A3KGS3	95%	3	4%	210		
Kinesin-like protein KIF26B	Q7TNC6	96%	2	3%	226		
Pericentrin	P48725	98%	4	3%	329		

Protein identifications from in-gel 35kDa and 60kDa mouse urine digests

Initially, MS analysis did not give any significant protein identifications from the 35kDa and 60kDa digests, possibly due to insufficient protein concentrations. To overcome this, we pooled nine bands of each of the 35kDa (2) and 60kDa (3) proteins as shown in Figure 4-5. Each pooled sample was then digested with trypsin as before, for MS analysis.

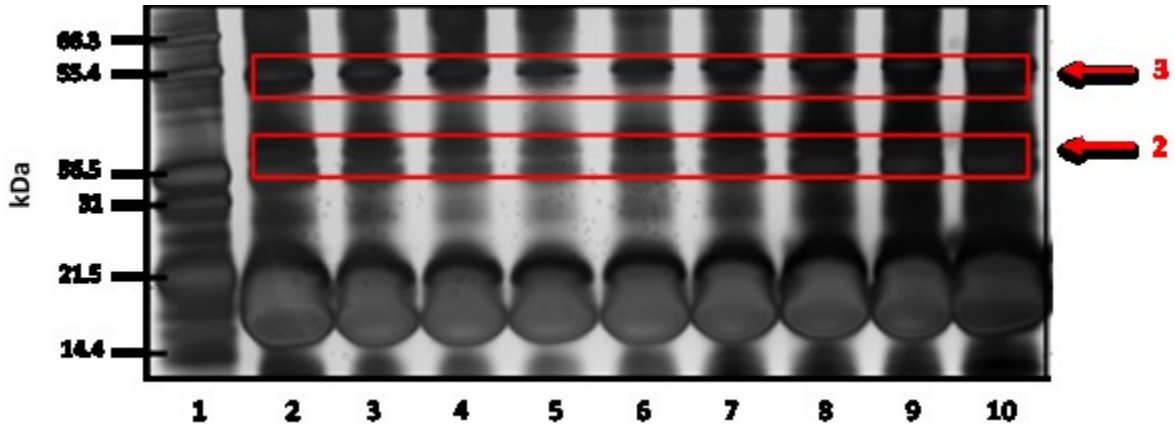


Figure 4-15 In-gel excision of pooled specific-IgE binding regions at 35kDa (2) and 60kDa (3) from mouse urine extract. 10µg of mouse urine protein was applied to SDS-PAGE in a total volume of 25µl in 9 lanes and was detected using an MS-compatible silver stain (lane 2-10). The molecular weight was determined with an unstained protein standard (lane 1).

Pooled 35kDa in-gel mouse urine digest

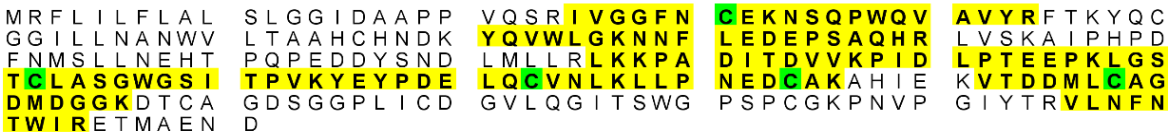
We identified 51 proteins from the 35kDa digest, with the most abundant being major urinary protein 6 also known as the allergen variant Mus m 1.0101. Sequence coverage was 86% and 5 unique peptides were identified. Many of the most abundant proteins identified were major urinary proteins, (as already identified from the 15-21kDa digest, likely to have aggregated through the SDS gel). Therefore, we removed protein identifications with a molecular weight <25kDa from these results and removed those with two or less unique peptides to increase the identification of a likely match. The 10 most abundant proteins identified in this peptide analysis are shown in Table 4-10 and the most abundant protein identified was kallikrein (Prot. Acc: P15947). Sequence coverage was 48% and 11 unique peptides from kallikrein were identified as depicted in Figure 4-16.

Pooled 60kDa in-gel mouse urine digest

We identified 76 proteins from the 54kDa digest with the most abundant being serum albumin (Prot Acc: P07724), registered on Allergome as the mouse allergen Mus m 4. Sequence coverage was 75% and 56 unique peptides were identified as depicted in Figure 4-16. The 10 most abundant proteins identified in this peptide analysis with a molecular weight >55kDa and three or more unique peptides are shown in Table 4-11.

Kallikrein from the 35kDa digest

P15947 (100%), 28,775.3 Da
Kallikrein-1 OS=Mus musculus GN=Klk1 PE=1 SV=3
11 exclusive unique peptides, 15 exclusive unique spectra, 15 total spectra, 125/261 amino acids (48% coverage)



Serum albumin (Mus m 4) from the 60kDa digest

P07724 (100%), 68,692.9 Da
Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
56 exclusive unique peptides, 108 exclusive unique spectra, 337 total spectra, 456/608 amino acids (75% coverage)

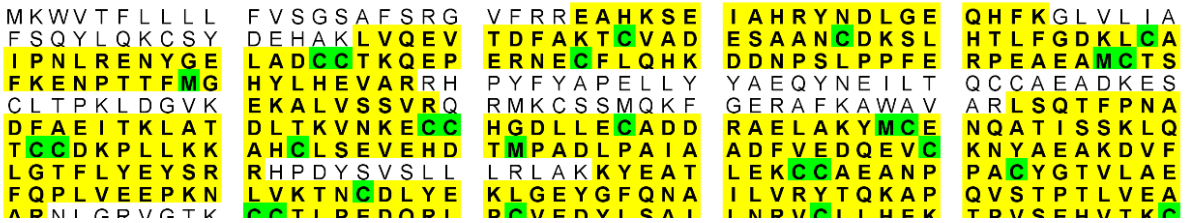


Figure 4-16 Sequence coverage of kallikrein and serum albumin (Mus m 4) identified by nano LC-MS/MS from in-gel mouse urine digests. The mouse urine extract was resolved by SDS-PAGE and visualised with an MS-compatible stain. Specific-IgE binding regions at 35kDa and 60kDa were excised and digested with trypsin before injection into HPLC coupled to a nano ESI source into MS/MS. Raw MS data was processed by Proteome Discoverer, searched against mouse taxonomy and uploaded to Scaffold software from which sequence coverage was documented.

Table 4-10 Proteins identified by nano LC-MS/MS from an in-gel 35kDa mouse urine digest

Protein name	Accession Number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)
Kallikrein-1	P15947	100%	11	48%	29
Complement factor D	P03953	100%	9	46%	28
Carcinoembryonic antigen-related cell adhesion molecule 10	Q61400	100%	8	31%	29
Protein AMBP	Q07456	100%	10	29%	39
Pro-epidermal growth factor	P01132	100%	22	24%	133
Deoxyribonuclease-1	P49183	100%	6	22%	32
Intelectin-1b	Q80ZA0	100%	4	17%	35
Insulin-like growth factor-binding protein 7	Q61581	100%	3	17%	29
Protein-glutamine gamma-glutamyltransferase 4	Q8BZH1	100%	6	9%	76
Complement factor B	P04186	100%	3	6%	85

Table 4-11 Proteins identified by nano LC-MS/MS from an in-gel 60kDa mouse urine digest.

Protein name	Accession number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)
Serum albumin	P07724	100%	56	75%	69
Protein-glutamine gamma-glutamyltransferase 4	Q8BZH1	100%	29	46%	76
Pancreatic alpha-amylase	P00688	100%	18	39%	57
Pro-epidermal growth factor	P01132	100%	29	26%	133
Beta-hexosaminidase subunit beta	P20060	100%	12	25%	61
Dipeptidyl peptidase 2	Q9ET22	100%	9	20%	56
Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	P45700	100%	5	9%	73
Cadherin-1	P09803	100%	3	8%	98
Gamma-glutamyltranspeptidase 1	Q60928	100%	3	7%	62
Complement C1r-B subcomponent	Q8CFG9	100%	3	6%	80

Protein identifications from an in-solution digest of mouse urine extract

We also performed an in-solution digest of the entire mouse urine extract and identified 166 proteins, with the most abundant being mouse urinary protein 2 (Mus m 1.0102) with a sequence coverage of 85.56%. Mouse urinary protein 6 (Mus m 1.0101) was identified as well as 14 other mouse urinary proteins recognised as Mus m 1 variants on Allergome. Mus m 4 and kallikrein were also identified from the in-solution mouse urine digest.

4.3.3 Optimising ECL western blot methods to identify Mus m 1 from airborne samples collected within an animal testing facility

Air sampling

Air sampling filters were extracted using three different buffers as described earlier in this chapter in section 4.2.3 (p.153). Protein was not detected from any of these filter extracts by the *DC*™ Protein Assay or silver stained SDS-PAGE. Using a specific Mus m 1 ELISA kit, I was able to detect 5.95ng/ml and 6.48ng/ml of Mus m 1 from filters that were extracted in dH_2O and PBS-T, respectively.

Filters were then pooled (n=16) and extracted using PBS-T buffer (PBS-T was shown to yield the highest protein content from mouse epithelium, shown later in section 4.3.5.1 (on page 180). However, protein was not detected from these pooled filters by the *DC*™ Protein Assay or silver stained SDS-PAGE.

Synthetic panel filters

Protein was detected from synthetic panel filters, extracted using dH_2O, PBS-T and AHC buffers by the *DC*™ Protein Assay and Mus m 1 was detected using a specific Mus m 1 ELISA kit. Total protein (µg/µl) and Mus m 1 (ng/ml) detected in synthetic panel filters for each extraction buffer is shown in Table 4-12. Extraction of the filter with PBS-T, yielded the highest protein and Mus m 1 content.

Table 4-12 Total protein (µg/µl) and Mus m 1 (ng/ml) detected in synthetic panel filter extracts. Synthetic panel filters collected from animal facilities were extracted in 20ml of either dH_20, PBS-T or AHC overnight. Samples extracted in AHC were dialysed at RT against 2 changes of dH_2O. Total protein was measured by a *DC*™ Protein Assay with absorbances read at 750nm and Mus m 1 was detected by a specific Mus m 1 ELISA kit.

Extraction Buffer	Total protein (µg/µl)	Mus m 1 (ng/ml)
dH_2O	0.49	5.33
PBS-T	0.51	6.76
AHC	0.082	6.17

4.3.3.1 Silver stain protein separation and ECL western blot of the synthetic panel filter extracts

The same three synthetic panel filter extracts tested by *DC™* Protein assay, were applied to silver stained SDS-PAGE and the PBS-T extract was also applied to an ECL western blot (Figure 4-17). Protein was detected at 16-19kDa in the dH_2O extract (lane 3) and at 15-21kDa and ~62kDa in the PBS-T extract (lane 4). No protein was observed from the AHC extract (lane 2).

Specific-IgE binding in the PBS-T extract by ECL western blot, was observed at three molecular weight regions; 15-21kDa, 35kDa and 60kDa (lane 5). This included Mus m 1, Mus m 4 and an unknown specific-IgE binding protein at ~35kDa, as labelled below.

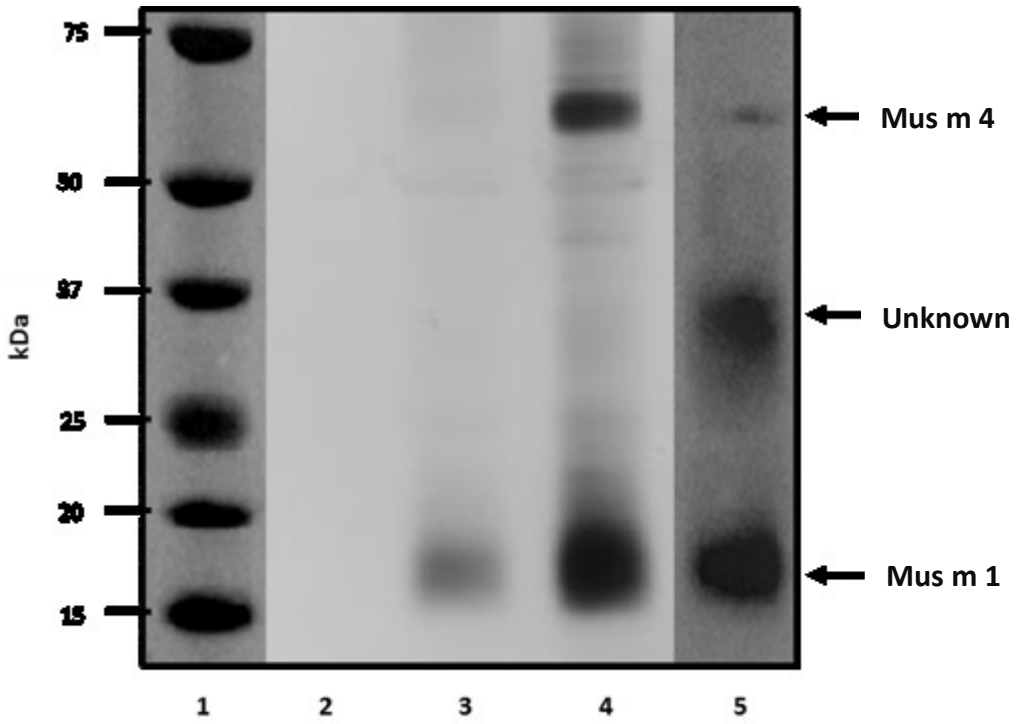


Figure 4-17 Silver stained SDS-PAGE (lanes 2-4) and ECL western blot (lane 5) of synthetic panel filter extracts. I applied 2.5µg of protein from three synthetic panel filter extracts to SDS-PAGE, extracted in AHC (lane 2), dH_2O (lane 3) and PBS-T (lane 4). The PBS-T extract was also transferred to a nitrocellulose membrane under normal conditions, blocked for 1 hour 30 minutes, incubated in pooled serum (1:5 dilution) for 16 hours, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution (lane 5). The molecular weight was determined with a prestained protein standard (lane 1). Specific-IgE binding proteins of interest are labelled on the right.

4.3.4 Optimising MS methods to identify Mus m 1 from airborne samples collected on a synthetic panel filter within an animal testing facility

The following specific-IgE binding regions at approximately 15-21kDa, 35kDa and 62kDa listed in Table 4-13 detected from a synthetic panel filter extracted in PBS-T, were excised from silver-stained gels, each pooled (n=9) and digested with trypsin for MS analysis as shown in Figure 4-18.

Table 4-13 Molecular weight (kDa) and allergen name of specific-IgE binding regions from a synthetic panel filter extract excised for MS analysis.

Number	Approximate molecular weight (kDa)	Allergen name
1	15-21	Mus m 1 Mus m 2
2	~35	Unknown
3	62	Mus m 4

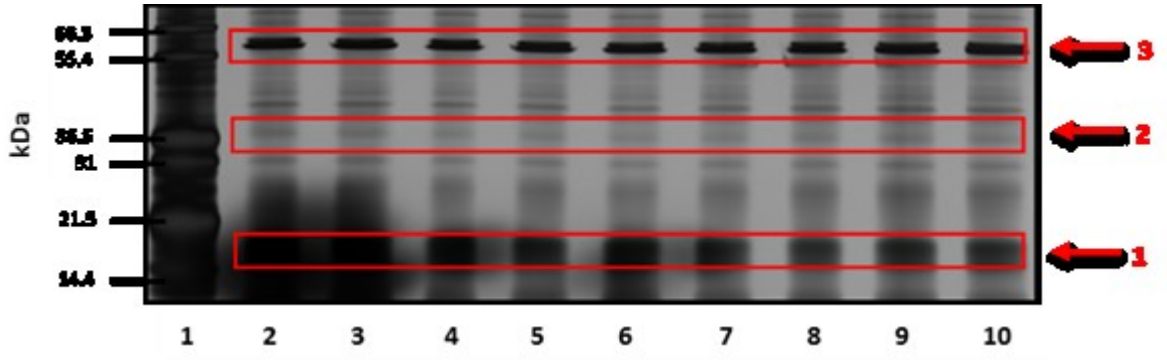


Figure 4-18 In-gel excision of pooled specific-IgE binding regions at 15-21kDa (1), ~35kDa (2) and 62kDa (3) from a synthetic panel filter extract. 10µg of protein from a synthetic panel filter (extracted in PBS-T) was applied to SDS-PAGE in a total volume of 25µl in 9 lanes and was detected using an MS-compatible silver stain (lane 2-10). The molecular weight was determined with an unstained protein standard (lane 1).

Protein identifications from three in-gel digests from a synthetic panel filter extract

We identified proteins from all three digests excised from the synthetic panel filter extract. The results are summarised in Table 4-14. From the 15-21kDa digest the most abundant protein identified was major urinary protein 6, known as the allergen variant Mus m 1.0101 and from the 62kDa digest the most abundant protein identified was serum albumin, known as the mouse allergen Mus m 4. Kallikrein was not identified in the ~35kDa digest.

Protein identifications from an in-solution digest of a ventilation filter extract

As mentioned in section 4.2.2 (on page 152), I also collected filters used to ventilate a fume cupboard used as a mice cage-changing station, at an animal facility in Salzburg, Austria. We performed an in-solution digest on one of these ventilation filters.

We identified 71 proteins, of which 13 were Mus m 1 variants (according to Allergome). The most abundant protein identified was odorant-binding protein 7 (Prot. Acc: A0A0D3) with a sequence coverage of 80% and 18 unique peptides. Serum albumin (Mus m 4) and kallikrein were identified from this digest.

Table 4-14 Proteins identified by nano LC-MS/MS from three in-gel digests of specific-IgE binding regions observed from a synthetic panel filter extract.

Specific-IgE binding regions (kDa) from the synthetic panel filter extract excised for MS analysis	Total proteins identified	Most abundant protein at the expected molecular weight	Accession Number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)
15-21	49	Major urinary protein 6	P11589	100	4	22	21
35	53	stAR-related lipid transfer protein 9	Q80TF6	96	3	0.72	500
60	28	Serum albumin	P07724	100	2	4.4	69

4.3.5 Identification of allergens from mouse epithelium by optimised ECL western blot methods

4.3.5.1 Protein content in mouse hair and mouse epithelium

We attempted to detect protein from mouse hair and mouse epithelium using three extraction buffers. Protein was not detected from mouse hair by the *DC*™ Protein Assay using any of the three buffers. Protein was detected from mouse epithelium by the *DC*™ Protein Assay, that was extracted using any of the three extraction buffers (dH_2O, PBS-T and AHC). Using a Mus m 1 specific ELISA, Mus m 1 was detected from mouse hair and mouse epithelium that was extracted using any of the three extraction buffers. These results are shown in Table 4-15.

Extraction of mouse hair with PBS-T and mouse epithelium with dH_2O, yielded the highest Mus m 1 content for each sample. Extraction of mouse epithelium with PBS-T yielded the highest total protein content and was used for subsequent experiments. I also found that subsequently to freeze-drying the total protein content in mouse epithelium extracted in PBS-T, was significantly reduced from 1.425 to 0.089 µg/µl.

Table 4-15 Total protein (µg/µl) and Mus m 1 (ng/ml) in mouse hair and epithelium extracts.
Mouse hair and epithelium was extracted in 20ml of either dH_20, PBS-T or AHC overnight. Samples extracted in AHC were dialysed at RT against 2 changes of dH_2O. Total protein was measured by a DC™ Protein Assay with absorbances read at 750nm and Mus m 1 was measured by a specific Mus m 1 ELISA kit.

Extraction Buffer	Mouse hair		Mouse epithelium		Freeze-dried mouse epithelium
	Total protein (µg/µl)	Mus m 1 (ng/ml)	Total protein (µg/µl)	Mus m 1 (ng/ml)	Total protein (µg/µl)
dH_2O	-	5.71	0.699	6.69	-
PBS-T	-	6.85	1.425	6.45	0.089
AHC	-	6.62	1.164	6.46	-

4.3.5.2 Silver stain protein separation and ECL western blot of mouse epithelium, with a comparison of before and after freeze-drying

Prior to freeze-drying (Figure 4-19: A), protein was detected between 10 and 150kDa using silver stained SDS-PAGE (lane 2) and specific-IgE binding was detected between 24 and 150kDa using an ECL western blot (lane 3). More intense specific-IgE-binding was seen in lane 3 for bands between 50 and 75kDa.

In the freeze-dried extract of mouse epithelium (Figure 4-19: B), protein was detected between 10 and 150kDa using silver stained SDS-PAGE (lane 2) and specific-IgE binding was detected between 24 and 150kDa using an ECL western blot (lane 3).

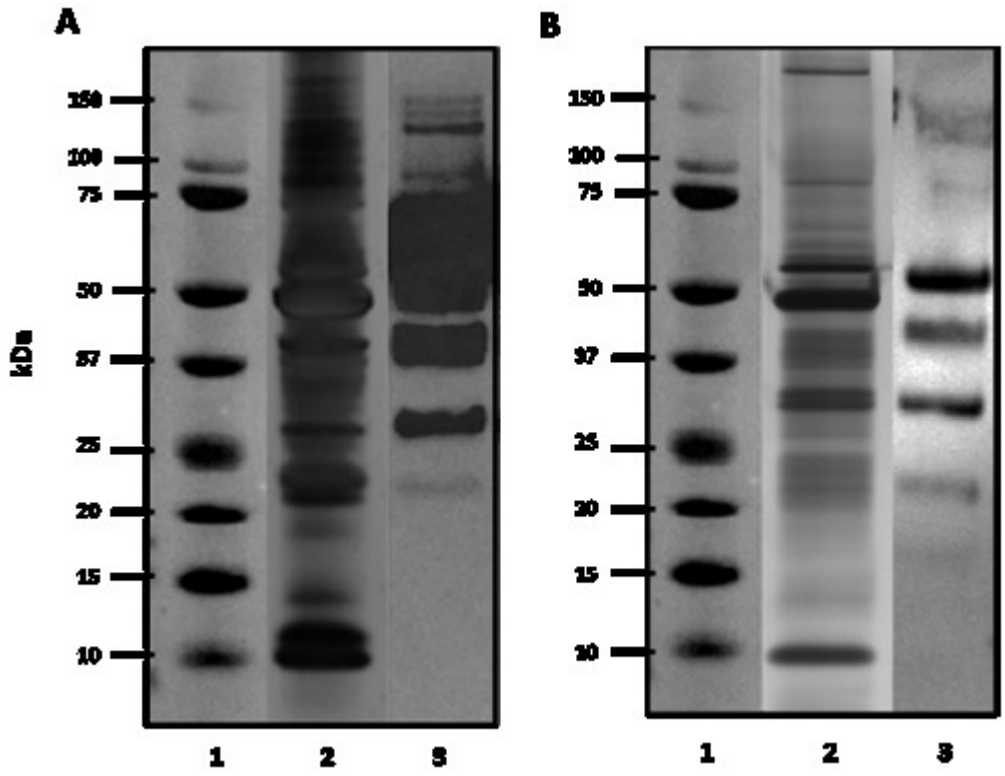


Figure 4-19 Silver stained SDS-PAGE and ECL western blot of mouse epithelium prior to (A) and after (B) freeze-drying. 20µg of protein prior to freeze-drying (A) and 4µg of protein after freeze-drying (B) from mouse epithelium was applied to silver stained SDS-PAGE (lane 2). Protein was also transferred to a nitrocellulose membrane under normal conditions, blocked for 1 hour 30 minutes, incubated in pooled serum (1:5 dilution) for 16 hours, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution (lane 3). The molecular weight was determined with a prestained protein standard (lane 1).

4.3.6 Identification of allergens from mouse epithelium by optimised MS methods

The following specific-IgE binding regions between approximately 24-150kDa in mouse epithelium were excised from silver-stained gels, each pooled (n=9) and digested with trypsin for MS analysis, labelled 1-6 in Figure 4-20 below.

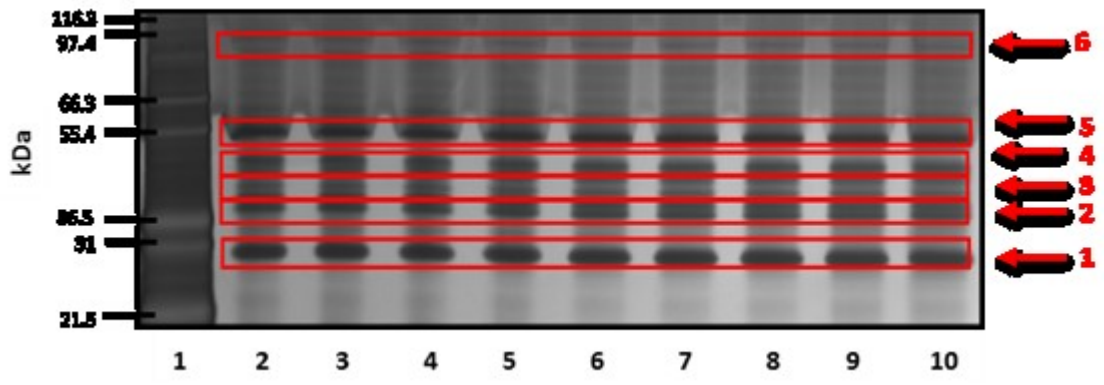


Figure 4-20 In-gel excision of pooled specific-IgE binding regions between 24 and 150kDa (1-6) from mouse epithelium extract. 10µg of mouse epithelium protein was applied to SDS-PAGE in a total volume of 25µl in 9 lanes and was detected using an MS-compatible silver stain (lanes 2-10). The molecular weight was determined with an unstained protein standard (lane 1).

We identified 25, 18, 20, 20, 45 and 103 proteins in digests 1 to 6 respectively. In digest 1 to 5 the most abundant protein identified was serum albumin (Mus m 4) and in digest 6 it was titin (Prot. Acc. A2ASS6). Sequence coverage of titin was 0.2% and 3 unique peptides were identified. After serum albumin, titin was the most abundant protein identification in digest 5, with a sequence coverage 0.31% of and 6 unique peptides.

In addition to serum albumin, 5 unique peptides of a Serotransferrin mouse protein (Prot Acc: Q921I1) were identified in digest 2, and in both digests 3 and 4 there were 7 unique peptides of an alpha-1-antitrypsin mouse protein (Prot. Acc: P22599) identified.

4.3.7 Comparison of protein in mouse epithelium extract and skin prick test (SPT) epithelia solution by optimised ECL western blot methods

I wanted to compare the specific-IgE-binding regions present in SPT epithelia solution with those in the mouse epithelium extract. In the mouse epithelium extract (Figure 4-21: A, lane 2), protein was detected using silver stained SDS-PAGE, between 12 and 116kDa, compared with between 6 and 116kDa in the mouse epithelia SPT solution (lane 3). Proteins were detected between 6 and 20kDa in the SPT that were not observed in the mouse epithelium extract.

Specific-IgE binding regions in the SPT were detected at >50kDa and >100kDa (Figure 4-21: B, lane 3). These areas of specific-IgE binding were seen in the crude mouse epithelium extract (Figure 4-21: B lane 2), in addition to three other regions of specific-IgE-binding <50kDa.

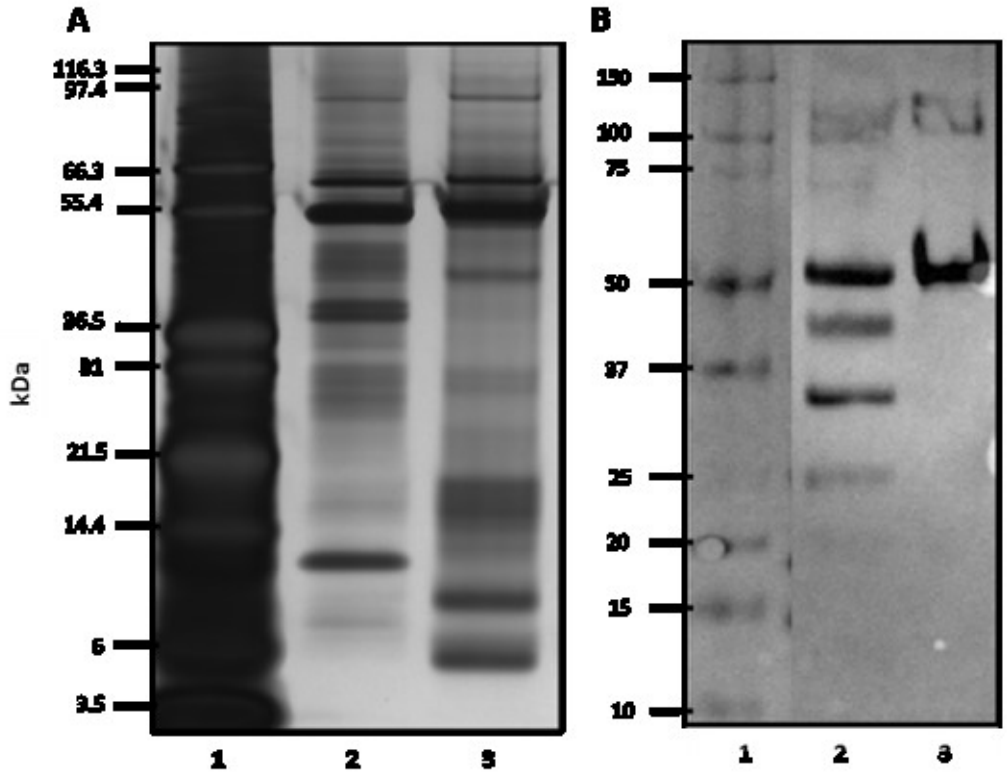


Figure 4-21 Silver stained SDS-PAGE (A) and ECL western blot (B) of mouse epithelium (lane 2) and mouse epithelia SPT (lane 3). 4µg of freeze-dried mouse epithelium protein (lane 2) and 80µg of mouse epithelia SPT protein (lane 3) was applied to silver stained SDS-PAGE (A). Protein was also transferred to a nitrocellulose membrane under normal conditions, blocked for 1 hour 30 minutes, incubated in pooled serum (1:5 dilution) for 16 hours, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution (B). The molecular weight was determined with an unstained standard for SDS-PAGE (A; lane 1) and a prestained protein standard for the ECL western blot (B; lane 1).

4.3.8 Comparing individual sensitisation to mouse epithelium and mouse urine amongst laboratory animal workers

I applied the ECL western blot method to 10µg of protein from mouse urine extract and 4µg of protein from mouse epithelium extract and incubated with individual serum from 5 laboratory animal workers for 16 hours at a 1:5 dilution, as shown in Figure 4-22:A and B, lanes 2-6. A pooled sample of the same serum was used in lane 7 in both A and B.

For mouse urine (Figure 4-22: A), all five individuals had specific-IgE binding within the 15-21kDa region, two individuals had specific-IgE binding to the 35kDa region and one individual had specific-IgE binding to the 62kDa region.

For mouse epithelium (Figure 4-22: B), two individuals had specific-IgE binding at approximately 30, 38, 48 and 60kDa. One individual had specific-IgE binding at approximately 22kDa and >100kDa.

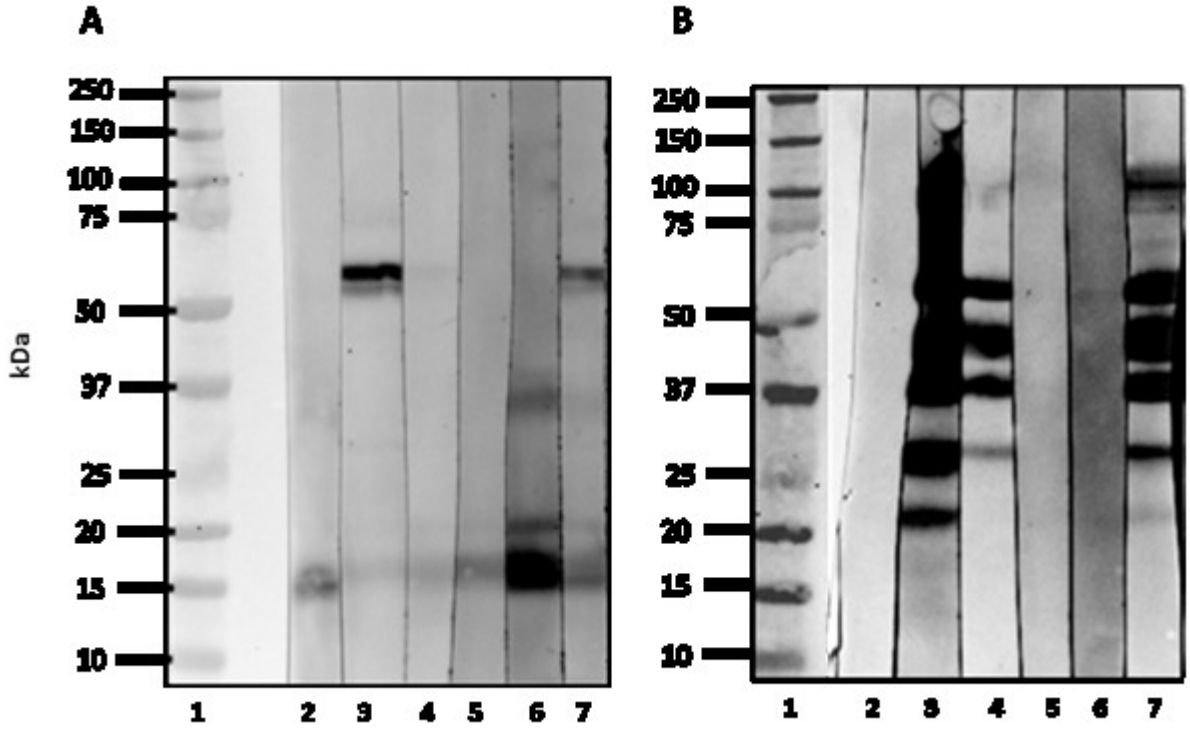


Figure 4-22 ECL western blot of individual specific-IgE binding to mouse urine (A) and mouse epithelium (B). 60µg of mouse urine protein and 24ug of mouse epithelium protein was applied to SDS-PAGE each in a total volume of 225µl. Protein was transferred to a nitrocellulose membrane under normal conditions, then blocked for 1 hour 30 minutes. The membrane was then cut using a clean scalpel into 7 strips and incubated in individual serum (1:5 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution. Molecular weight was determined with a prestained protein standard (lane 1). Individual serum was used in lanes 2-6 and a pooled sample of the same serum was used in lane 7.

Table 4-16 shows the ImmunoCAP scores to mouse urine and mouse epithelium for the 5 laboratory animal workers alongside their pattern of specific-IgE binding to both mouse urine extract and mouse epithelium extract.

Table 4-16 ImmunoCAP scores and specific-IgE binding present to mouse urine and mouse epithelium from five laboratory animal workers.

	ImmunoCAP score		Specific- IgE binding present (kDa)					
			Mouse urine extract			Mouse epithelium extract		
	Mouse Urine	Mouse Epithelium	15-21	35	60	<60	60	>100
Lane 2	51.9	13.0	✓	✓				
Lane 3	36.9	>100.0	✓		✓	✓	✓	✓
Lane 4	18.9	14.6	✓			✓	✓	
Lane 5	26.0	4.4	✓					
Lane 6	>100	6.1	✓	✓				

4.4 Discussion

4.4.1 Overview

In this chapter I have optimised the identification of specific-IgE binding proteins from mouse urine, mouse epithelium and airborne samples collected from animal testing facilities. Using the optimised MS-based method we were able to detect the major mouse allergen Mus m 1, as well as the serum albumin allergen, Mus m 4. In addition, we were able to identify other potential 'new' mouse allergens, (e.g. an uncharacterised specific-IgE binding protein at approx. 35kDa). This was an important finding as it suggested that Mus m 1 may not be the only relevant allergen in occupational asthma due to exposure to mouse proteins. Using this method and broad approach I have shown how allergens from mouse urine and mouse epithelium can be identified.

From mouse urine and airborne samples from the synthetic panel filter extract, I identified Mus m 1, Mus m 4 and an uncharacterised ~35kDa specific-IgE binding protein. From the mouse epithelium extract, I identified Mus m 4 and another uncharacterised specific-IgE binding protein at ~150kDa.

Mus m 1 is a urinary protein with an approximate molecular weight of 19kDa and is a well-cited major airborne allergen from mouse that is known to be present in animal testing facilities (Twiggs et al. 1982; Newman Taylor et al. 1997; Jeal et al. 2009; Canizales et al. 2016). Mus m 4 is a serum albumin protein with an approximate molecular weight of 68kDa and is a less well-cited mouse allergen that is derived from mouse serum. It is thought that it's most relevant exposure that causes allergic symptoms is its presence in animal dander. Moreover, serum albumin has been identified as an allergen from many other mammalian species, such as cat, dog and horse (Spitzauer et al. 1995; Chruszcz et al. 2013).

The ~35kDa and ~150kDa proteins were putatively identified as potential novel mouse allergens - kallikrein and titin, respectively. These are not known as mouse allergens but have been identified as allergenic proteins from other species.

Kallikrein has previously been identified as a major allergen from dog urine (Mattsson et al., 2009) and was recently named Can f 5 by the WHO/IUIS Allergen Nomenclature sub-committee. In their study of 37 serum from subjects with dog allergy, 26 (70%) had specific-IgE antibodies to a prostatic kallikrein protein present in dog urine. In 2016, Uriarte and

Sastre, showed 33% of 159 patients with dog allergy were sensitised to Can f 5 and that there was a strong association with Can f 5 sensitisation and symptoms of rhino-conjunctivitis.

As mentioned in the introductory part of this chapter, there is only one report by Hollander et al. (1997) of an allergic reaction to a mouse urine protein with a molecular weight close to 35kDa. They used a chromogenic western blot method to observe serum specific-IgE binding to mouse urine and detected a 40kDa binding region that they did not detect when staining the extract with Coomassie blue after SDS-PAGE. Out of 47 serum samples from mouse-sensitised workers, 50% showed specific-IgE binding to the 40kDa region, however the authors did not attempt any further characterisation of the specific-IgE binding region. In the mouse epithelium extract there was also specific-IgE binding at ~35kDa, however MS analysis did not identify a mouse kallikrein protein was present in MS digests from mouse epithelium extract. Kallikrein functions as a serine protease enzyme, is expressed at high levels in the salivary gland and prostate and is regulated by hormonal control. In humans, prostatic kallikrein has been used as a valuable biomarker in prostate cancer for over 20 years (Lawrence et al., 2010).

The ~150kDa allergen, putatively identified from mouse epithelium as titin, is a muscle protein. Titin has been identified as an allergen from black tiger prawn by Kamath et al., (2014). They observed 11 (68%) out of 16 patients with a clinical history of allergic reactivity to shellfish react to this protein by specific-IgE binding observed by western blot. However, titin is not a registered allergen on the WHO/IUIS Allergen Nomenclature. A serotransferrin protein was also identified in a MS digest of a mouse epithelium specific-IgE-binding protein. Transferrin proteins have been previously implicated in cow's milk allergy (Hochwallner et al., 2014), but are not currently registered on the WHO/IUIS Allergen Nomenclature.

In further experiments, I was able to demonstrate that mouse urine proteins, under reducing conditions, migrate at a higher molecular weight compared with non-reducing conditions. Under reducing conditions, the protein disulphide bridges are broken and the protein being applied to SDS-PAGE becomes less compact. As well as affecting protein migration, this can cause the protein to split into monomer units to produce two distinct proteins (Thermo Fisher Scientific Handbook, 2015). Although reducing conditions affected the protein migration of mouse urine and therefore it's molecular weight, the protein did

not split into monomer units. This could explain the difference in the observed and expected molecular weight of Mus m 4.

I optimised the detection of specific-IgE binding, by using an ECL method of detection in replacement of a chromogenic method. Chromogenic detection of specific-IgE binding to proteins from mouse urine had limited sensitivity. This was despite extending the incubation period with pooled allergic serum as well as using a more sensitive substrate (Ultra-TMB). Increasing the dilution of anti-human IgE (from 1:1000 to 1:5000) however, did show an increase in the signal-to-noise ratio on the chromogenic blot.

Although chromogenic detection is the simplest and most cost-effective method, chemiluminescence is currently the most popular detection method for western blot analysis. ECL substrates detect proteins down to the low-femtogram level with high signal-to-noise ratios (Jain and Magrath 1991). I established the limit of detection for mouse urine allergens using the ECL western blot method. This ranged from 0.078µg of protein to detect Mus m 1 and 2.5µg of protein to detect Mus m 4. It was interesting to note that while the detection limit was the same for Mus m 1 for both silver staining and ECL western blot, it was lower for Mus m 4, by ECL western blot (1.25µg) than by silver staining (2.5µg). The detection of specific-IgE binding proteins by ECL western blot depends on both the protein concentration and the strength of serum specific-IgE binding.

The allergens Mus m 1, Mus m 4 and kallikrein were identified from synthetic panel filters using the optimised ECL western blot and MS method. We expected to detect Mus m 1 as an airborne allergen, however the presence of Mus m 4 as an airborne allergen is less well-documented in the literature. Albumin animal allergens are minor allergens, known to extensively cross-react, but there is no definite consensus on whether they are spread through animal dander or another source (Chruszcz et al. 2013; Konradsen et al., 2015). Moreover, the identification of kallikrein from synthetic panel filters, was a novel finding.

The mouse epithelium collected was extracted in three different buffers and a portion was freeze-dried to avoid storage degradation. We measured the protein content of all these extracts and found that while the PBS-T buffer extracted the highest protein content (1.425µg/µl), this was significantly reduced after freeze-drying (0.089µg/µl). This was unexpected as lyophilisation by freeze-drying is a common method to remove water and

other solvents from an extract so that it is more suitable for long-term storage. The process of lyophilisation can cause biological stresses however, such as low temperature, pH changes and structural changes which may be irreversible depending on the protein (Wang, 2000). This may explain the apparent loss of mouse epithelium protein after freeze-drying.

I also used the optimised ECL western blot method to compare the specific-IgE binding proteins present in the mouse epithelium extract with the epithelia solution used for SPT diagnosis of laboratory animal allergy. Mus m 4 and the uncharacterised specific-IgE binding protein, putatively identified as titin were present in both solutions, however my epithelium extract had three additional uncharacterised specific-IgE binding proteins between 25 and 50kDa. The content of SPT extracts is known to differ depending on the manufacturing company they are produced from (Sharma et al. 2008), so it is important to identify which allergens they contain. The significance of titin as a mouse allergen and its potential contribution to LAA should be investigated further.

I also looked at individual specific-IgE binding sensitisation patterns to the mouse urine and mouse epithelium extracts amongst five laboratory animal workers. There was variation in specific-IgE binding present, however the small number of individuals made it difficult to make a valuable interpretation on the potential sensitisation prevalence to the putatively identified allergenic proteins.

4.4.2 Limitations

We did expect to detect mouse allergens from air samples collected using TUFF Plus pumps and fluorophore membrane filters. This air sampling method has been previously used to quantify levels of airborne Mus m 1 by Canizales et al. (2016). However, in their study they used a specific Mus m 1 ELISA kit, which has a sensitivity of 0.05ng/ml and detection limit of 0.2ng/ml. Although this method has high sensitivity, it does not allow for the identification of other allergenic proteins besides Mus m 1.

Although we had interesting findings from the synthetic panel filters, they are not a wholly accurate representation of inhalable particles that laboratory animal workers may be exposed to. Rather, these were filters used to ventilate stacks of cages housing mice. In order to detect particles that are truly airborne using these optimised methods, it is likely that a higher concentration of allergen is needed, which could possibly be achieved with higher flow rates and longer sampling times.

Moreover, the extracted samples were obtained and used in a crude format and were not subject to any purification experiments. It is not uncommon to purify allergenic proteins using such methods as fractionation and affinity chromatography (Rigaut et al., 1999). However, implementing these methods was beyond the scope of optimising our MS-based method for allergen identification. Some of the results were also limited because of the small number of allergic laboratory animal workers that were used for both individual and pooled serum ECL western blot experiments. Using a larger panel of individuals in a multicentre study would have given a more representative depiction of all the allergens associated with LAA and may have altered the prevalence of sensitisation to mice that was possibly induced by domestic exposure.

4.4.3 Summary and future works

In summary, we have identified both known and previously unknown allergenic proteins from both crude mouse urine and mouse epithelium extracts, as well as from airborne samples collected within an animal testing facility, that are capable of binding to serum specific-IgE obtained from patients diagnosed with LAA.

Future works investigating the importance of putatively identified proteins – kallikrein and titin, could be important in detecting laboratory animal workers suffering with LAA, whom may be currently misdiagnosed because they do not have specific-IgE to the well-known mouse allergens that are currently tested for. In collaboration with pharmaceutical companies that manufacture reagents for diagnosing allergy, there is also an opportunity to create purified or recombinant kallikrein and titin allergens that could be used in SPT's.

In this chapter we have successfully developed and optimised a MS-based method to identify allergenic proteins. It is now our intention to apply this method in the following chapters of this thesis to identify novel uncharacterised allergenic proteins that may be responsible for causing symptoms of respiratory allergy.

5 Application of the mass spectrometry (MS)-based method to identify novel occupational allergens associated with *Drosophila melanogaster*

5.1 Introduction

In the previous chapter I reported experiments related to laboratory animal allergy (LAA), caused by exposure to mice in laboratory animal testing facilities. However, exposure to insect species has also been associated with the onset of allergic symptoms in laboratory animal workers. Exposure to insects such as bees, moths, beetles, locusts and flies (Feinberg et al., 1956; Spieksma et al., 1986; Lierl et al., 1994; Kausar et al., 2007; Cantillo et al., 2017) have all been implicated as causes of allergic respiratory symptoms. In the UK, during the years 1999 and 2000 the annual incidence of occupational asthma and rhinitis for those carrying out research with insects, was estimated at 0.24% and 2.1%, respectively (Draper et al., 2003).

Insects as inhalant allergens

For over half a century, a variety of insects have been studied as potential sources of inhalant allergen (Feinberg et al., 1956). Early examples of isolated asthma and rhinitis cases caused by occupational insect exposure, include beekeepers (Ellis and Ahrens 1932; Ostrom et al., 1986) and mushroom growers exposed to the mushroom fly, *Aphiochaeta agarici* (Kern 1938). Perlman (1958), who was prompted by a series of isolated case histories of insect allergy, collected insects by 'light trap catches', in order to prepare insect allergens for scratch and intradermal allergy testing. He also commented on the protein chemistry of the insect cuticle, with regards to the cuticle acting as the source of allergen.

In 1987, a study in Kyoto, Japan, aimed to demonstrate the importance of inhalant insect allergens, in patients with bronchial asthma (Kino et al., 1987). Researchers prepared insect extracts from silkworms, caddis fly and chironomid, and assessed their allergenicity using *in vivo* and *in vitro* testing, in a group of 56 bronchial asthmatic patients. The patients were

selected from an outpatients' asthma clinic and had no occupational exposure to insects. Positive intracutaneous reactions to insect extracts were demonstrated in 50% of the patients and of those, 80% had specific-IgE antibodies to insect extracts, as observed by RAST. Intracutaneous skin tests to common inhalant allergens were also performed on the 56 patients with bronchial asthma, and the percent of positive results for these, as well as the insect extracts is shown in Figure 5-1.

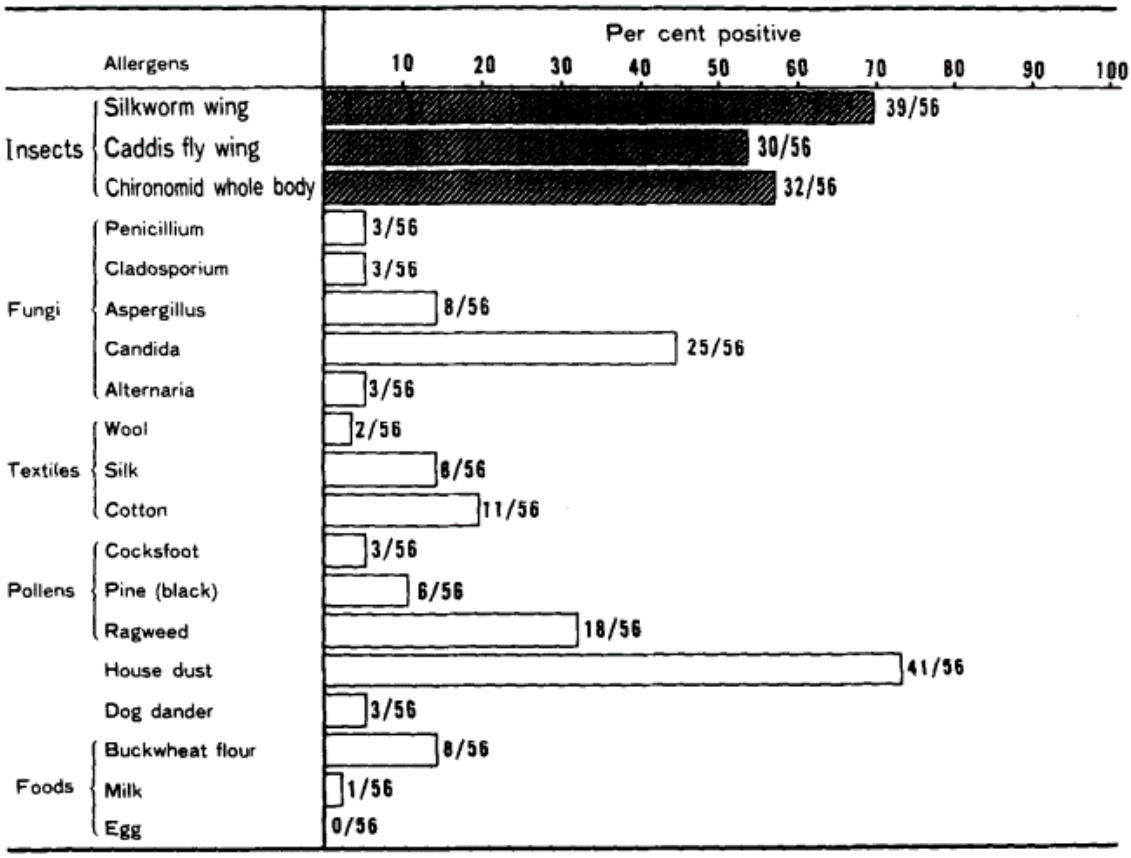


Figure 5-1 Intracutaneous skin reactions against common inhalant allergens and insect extracts in patients with bronchial asthma randomly selected from an outpatient's clinic in Kyoto, Japan, with no occupational insect exposure. (Kino et al. 1987, p.862).

The percent of positive reactions to insect allergens was almost as high as reactions to house dust which was considered as the most common sensitizer in Japan at the time of the study. The authors do not address the potential cross-reactivity of allergens derived from house dust mite, that may likely be present in the samples of house dust. They did use RAST-inhibition experiments to show the presence of insect-related aeroallergens in dust,

measuring <10µm in diameter, collected using a high-volume air sampler. The authors concluded that the presence of insect-related allergens in dust, may explain the high frequency of positive reactions to insect extracts that were observed (Kino et al., 1987).

Baldo and Panzani (1988), prepared insect extracts in order to detect serum specific-IgE antibodies by RAST in 41 people aged 5 to 66 who had been diagnosed with inhalant insect allergy (based on case histories and/or SPT). About one-third gave positive reactions by RAST to all seven species of house fly, blowfly, the common clothes moth, warehouse moth, cockroach, carpet beetle and silverfish. Using western blot methods with patient serum they also observed 15 specific-IgE binding proteins from 11 different insect species. They found a common specific-IgE binding protein with a molecular weight of 37kDa, present in each of the fly extracts. The presence of specific-IgE binding at the same molecular weight across these insect species, likely indicates a highly cross-reactive insect allergen.

In Ohio, 1994, Lierl et al., studied children with allergic asthma to assess the prevalence of serum specific-IgE binding to insect allergens. Extracts from ant, cricket, grasshopper, housefly and moth were used for detection of specific-IgE, by coupling to paper discs for RAST analysis. For cricket, grasshopper, housefly and moth the prevalence of specific-IgE was significantly higher in serum from children with allergic asthma than serum from a nonallergic panel. Cross-reactivity was investigated using RAST-inhibition experiments, but was not observed, suggesting individuals had species specific-IgE and insect allergens were not cross-reactive.

Workers based within an entomological research centre were tested by Kaufman et al., and Baldo et al., in 1989, for allergic sensitisation to the blowfly, *Lucilia cuprina*. Fifteen of 53 workers had clinical manifestations of allergy including rhinitis and lower respiratory symptoms. Using RAST analysis, 10 of the workers with allergic symptoms were shown to have serum specific-IgE to an extract of *L. cuprina* blowfly (Kaufman et al., 1989). Baldo et al., (1989) used the same blowfly extract and performed SDS-PAGE and western blot, to detect specific-IgE binding regions. Serum of 30 exposed workers, all of whom showed a positive RAST result to the blowfly extract was used and a total of 21 specific-IgE binding regions from blowfly were detected. The specific-IgE binding region from blowfly that reacted with the highest number of serum samples used (70%) was at 67kDa. Despite these research efforts there are still no allergens from blowfly officially registered on the

WHO/IUIS Allergen Nomenclature, and to my knowledge a protein from this 67kDa binding region has not been identified.

Characterisation of insect allergens

There is a comprehensive list of insect allergens currently registered on the WHO/IUIS Allergen Nomenclature. This includes species of mosquito, bee, tick, cockroach, midge, flea, hornet, fly, beetle, silverfish, louse, ant and moth. Of these allergens, 31 are classified under the *Diptera* order of the *Insecta* class, which includes species of mosquito, midge and fly. These allergens are listed in Table 5-1.

The yellow fever mosquito, *Aedes aegypti* listed in Table 5-1 is known to induce allergic respiratory symptoms. Specific-IgE binding to *A. aegypti* was demonstrated by Kausar et al., (2007) in sensitised individuals suffering with asthma and/or rhinitis. It has been suggested that mosquitoes induce respiratory symptoms via the inhalation of suspended allergens that are derived from the insect body, referred to as emanations and insect detritus (Cantillo et al., 2014).

In a more recent report, Cantillo et al., (2017) used modern proteomic techniques to characterise the allergenic proteins from *A. aegypti*. Total protein was extracted from mosquito bodies and applied to two-dimensional electrophoresis (which combines isoelectrofocusing and SDS-PAGE). Specific-IgE binding proteins were detected using serum by western blot, from 15 individuals with asthma and/or rhinitis who were sensitised to mosquito. Specific-IgE binding spots from two-dimensional electrophoresis were then selected for protein identification by MS. They identified 10 different proteins corresponding to 25 specific-IgE reactive spots and went on to show that sensitisation to any one of three specified identified allergens was enough to detect 80% of individuals sensitised to at least one of the *A. aegypti* allergens.

Table 5-1 Registered allergens from the WHO/IUIS Allergen Nomenclature under the *Diptera* order of the *Insecta* class.

Allergen	Biochemical name	Molecular weight	Route of exposure
***Aedes aegypti* (Yellow fever mosquito)**			
Aed a 1	Apyrase	68	Unknown
Aed a 2	Salivary D7 protein	37	Injection
Aed a 3	Undefined 30kDa salivary protein	30	Unknown
Aed a 4	Glucosidase	67	Unknown
Aed a 5	Sarcoplasic Ca+ binding protein	28.5	Unknown
Aed a 6	Porin 3		Unknown
Aed a 7	Undefined protein		Unknown
Aed a 8	Heat shock cognate protein-70		Unknown
Aed a 10	Tropomyosin	32	Unknown
Aed a 11	Lysosomal aspartic protease		Unknown
***Aedes albopictus* (Asian Tiger Mosquito)**			
Aed al 2	D7 like salivary odorant binding protein	33	Injection
Aed al 3	30kDa Salivary protein	30	Injection
***Anopheles dirus B* (Malaria mosquito SE Asia)**			
Ano d 2	Odorant binding protein	15	Injection
***Chironomus kiiensis* (Midge)**			
Chi k 10	Tropomyosin		Unknown
***Chironomus thummi* (Midge)**			
Chi t 1	Hemoglobin, component III/IV	16	Unknown
Chi t 2	Hemoglobin, component I/IA	16	Unknown
Chi t 3	Hemoglobin, components II-beta, VI, VII, VIII, IX	16	Unknown
Chi t 4	Hemoglobin, component IIIA	16	Unknown
Chi t 5	Renamed to Chi t 3.0201		Unknown
Chi t 6	Renamed to Chi t 3.03 and 3.04		Unknown
Chi t 7	Renamed to Chi t 3.05 – 3.08		Unknown
Chi t 8	Renamed to Chi t 3.09		Unknown
Chi t 9	Hemoglobin, component X	16	Unknown
***Culex quinquifasciatus* (Southern House Mosquito)**			
Cul q 2	Salivary odorant binding protein	33	Injection
Cul q 3	Salivary odorant binding protein 2	35	Injection
***Forcipomyia taiwana* (Biting midge)**			
For t 1	Serine, threonine-protein kinase	14	Unknown
For t 2	Eukaryotic translation initiation factor 3 subunit	36	Unknown
***Glossina morsitans* (Savannah Tsetse Fly)**			
Glo m 5	Tsetse antigen 5	27	Unknown
***Tabanus yao* (Horsefly)**			
Tab y 1	Apyrase	70	Unknown
Tab y 2	Hyaluronidase	35	Unknown
Tab y 5	Antigen 5-related protein	26	Unknown

Inhalant allergens from fruit fly

The Mediterranean fruit fly (*Ceratitis capitate*), also known as the medfly has been implicated in two cases of occupational asthma in Spain (de las Marinas et al., 2014). In both cases, patients gave positive SPT results to extracts of medfly, and their serum was collected for western blot analysis. Specific-IgE binding was observed at 27.5, 55, 60, 66 and 80kDa for the first case and 13 and 16kDa for the second. The authors comment that this difference may be explained by patterns of work exposure to medfly. Both individuals worked for the same company producing medflies, but the first individual attributed their symptoms got worse when working 'in the room where adult flies were housed', and the second reported an onset of wheezing symptoms when working in 'rooms with a higher density of flies'. The authors gave a simple description of the case-studies and did not further characterise or identify the allergenic proteins involved.

The common fruit fly, *Drosophila melanogaster*, is an insect species frequently used in animal testing laboratories, particularly in the field of genetics. Exposure to *D. melanogaster* was first implicated in respiratory allergy in the Netherlands in 1986, by Spieksma et al., In a study within a single scientific laboratory, six of 22 workers who complained of respiratory symptoms demonstrated immediate-type skin reactions and a positive RAST result to an extract of *D. melanogaster*. Using RAST-inhibition experiments, the authors confirmed the presence of fruit fly-allergen in the dust of the laboratory room, suggesting inhalational exposure to allergens from *D. melanogaster*.

In a recent 'clinical communication', Colomb et al., (2017), sent a questionnaire to 59 individuals working with *D. melanogaster* in a research institute based in Montpellier. They performed SPT and collected serum to quantify specific-IgE using extracts from adult fruit fly. They found negative SPT results from all controls (those who were not exposed and those exposed but symptom free) and positive SPT results for 7 out of 11 (64%) of symptomatic workers, whom also had serum specific-IgE to fruit fly, as determined by RAST.

In the same study (Colomb et al., 2017) western blot results showed that 6 of the 7 sensitised workers had specific-IgE binding to proteins from the fruit fly extract, and all 6 had specific-IgE binding to an 80kDa protein. The authors used polyclonal antibodies, specific for larval serum proteins in *D. melanogaster*, on adult fly extracts, based on recent

evidence of similar hexamerin ~80kDa allergens identified from German and American cockroach (Khurana et al., 2014). These polyclonal antibodies were able to detect a *D. melanogaster* specific larval serum protein the same as the 80kDa band that was detected with the serum of allergic workers. Hexamerin proteins have also been identified as allergens from species of locust and cricket (Srinroch et al., 2015).

Other specific-IgE binding proteins shown using western blot by Colomb et al., (2017) corresponded to predicted *D. melanogaster* allergens registered on the Allergome online platform, including a 33kDa tropomyosin protein (Dro m 7), a 40kDa arginine kinase protein (Dro m 9) and a 25kDa superoxide dismutase protein (Dro m MnSOD).

A far larger cross-sectional survey of workers exposed to *D. melanogaster* was conducted in 2017 by Jones et al., In a large UK academic institute, 286 workers were recruited to complete a questionnaire. SPT was performed and serum was obtained from 95% and 86%, respectively. Serum specific-IgE to whole fruit fly extract was determined by RAST and the overall sensitisation prevalence amongst the exposed workers was 6%.

Moreover, the workers recruited by Jones et al., (2017) were categorised into one of four groups according to their estimated exposure to *D. melanogaster* ('High + frequent', 'high + infrequent', 'low + frequent' and 'low and infrequent'). From this, authors found a dose-response relationship, between the presence of specific-IgE and estimated fruit fly exposure. Table 5-2 shows the number (and %) of workers that had specific-IgE to *D. melanogaster*, according to each of the four exposure groups.

Table 5-2 Specific-IgE *D. melanogaster* sensitisation amongst four exposure groups (Jones et al., 2017). The percent of workers with *D. melanogaster* specific-IgE is expressed as a percent of the total number of workers in each exposure group.

	Exposure group			
	High + frequent	**High + infrequent**	**Low + frequent**	**Low + infrequent**
Total number of workers tested; n (%)	72 (25)	19 (7)	48 (17)	147 (51)
Workers with *D. melanogaster* specific-IgE; n (%)	10 (15)	1 (6)	2 (5)	2 (2)

The specific-IgE binding proteins from *D. melanogaster* causing occupational asthma within the population studied by Jones et al., (2016) were not characterised. Furthermore, despite the immunological characterisation described by Colomb et al., (2017) their experiments were conducted for only 6 workers, and to date, allergens from *D. melanogaster* have not been registered in the WHO/IUIS Allergen Nomenclature.

We hypothesised that using the MS-based method optimised in the previous chapter, allergenic proteins from the fruit fly, *Drosophila melanogaster*, causing occupational asthma, could be identified.

Objective

To identify allergenic proteins, present in the fruit fly, *Drosophila melanogaster*.

Aims

1. Detect specific-IgE binding proteins from *D. melanogaster* using optimised western blot methods.
2. Identify specific-IgE binding proteins from *D. melanogaster* using optimised MS-based methods.

The experimental work for the first aim was conducted in two locations. Firstly, within the Division of Allergy and Immunology at the University of Salzburg in Austria (during a 3-month Short term research training fellowship funded by the European Respiratory Society) and then within my own department; Population Health and Occupational Disease, at Imperial College London. The fellowship was an opportunity to gain experience in laboratory methods that are used in allergen proteomics from experts in the research field.

Because of this, there were two approaches for western blot methods used in this chapter:

- At the University of Salzburg, I followed a routinely used protocol setup by the laboratory research group for the chromogenic detection of serum specific-IgE binding proteins. In addition, specific-IgE binding proteins were excised from Coomassie stained gels rather than silver stained gels, as the preferred method for MS analysis within this research group. For this work I was supervised by Prof. Peter Briza.

- At Imperial College London, I followed the methodology I optimised in Chapter 4, using ECL detection of serum specific-IgE binding proteins.

5.2 Methods

5.2.1 Sample collection

Fruit fly (*D. melanogaster*) were collected and extracted as described in Jones et al., (2017). Briefly, fruit flies were collected from a large academic institute within the UK and extracted overnight in 0.01mol/L ammonium carbonate at 4°C, then centrifuged at *500 g* at 4°C for 10 minutes. The supernatant was then dialysed against dH_2O, lyophilised in a freeze dryer and stored at -20°C.

5.2.2 Protein quantification

Methods for protein quantification were followed as described in the Chapter 3, section 3.2.2. Briefly, protein content in the fruit fly extract was quantified using a *DC*™ Protein Assay kit.

5.2.3 Serum samples

I selected 11 stored serum samples from laboratory animal workers, exposed to fruit fly who had attended the occupational allergy clinic at the Royal Brompton Hospital. Serum was chosen based on having a high level of specific IgE to fruit fly extract, as determined by RAST, with specific IgE ≥2% binding considered positive. Serum was used for either chromogenic (n=3) or ECL (n=8) western blot methods (Table 5-3).

Table 5-3 Level of specific IgE antibody (% binding) to fruit fly extract as determined by RAST in eleven laboratory animal workers.

Specific-IgE antibody (% binding) to fruit fly extract as determined by RAST used for either chromogenic or ECL western blot methods	
Chromogenic	**ECL**
4	10.4
3.2	9.6
2.2	2.5
	2.6
	3.1
	3.2
	3.8
	3.5

5.2.4 SDS-PAGE and western blot

For SDS-PAGE and western blot of the fruit fly extract I used two approaches:

The first was conducted using the reagents, equipment and expertise from the Division of Allergy and Immunology at the University of Salzburg and followed a routinely used protocol for the chromogenic detection of serum specific-IgE binding proteins. These methods are described in full in Chapter 3, sections 3.2.4-3.2.6. Briefly, proteins in the fruit fly extract were applied to a hand-cast gel electrophoresis system and separated proteins underwent semi-dry transfer to a nitrocellulose membrane, which was blocked using a tris-blocking buffer. The membrane was incubated with serum from workers with elevated levels of specific-IgE to fruit fly and following washing was probed with a mouse anti-human IgE antibody conjugated with alkaline phosphatase, before detection with an NBT/BCIP chromogenic substrate. The protein molecular weight was calculated according to a prestained protein standard and was imaged using a ChemiDoc XRS+ system.

The second approach was conducted using the methodology I optimised in Chapter 4, for protein separation and ECL detection of serum specific-IgE binding proteins. These methods are described in full in Chapter 3, sections 3.2.4-3.2.6. Briefly, proteins in the fruit fly extract were separated using the NuPAGE™ Bis-Tris electrophoresis system and proteins were stained either using a Pierce® Silver Stain Kit or a 0.1% R-250 Coomassie dye. The protein molecular weight was calculated according to an unstained protein standard. Protein from the fruit fly extract was transferred to nitrocellulose membranes by wet transfer and blocked using a phosphate-blocking buffer. The membrane was incubated with serum from workers with elevated levels of specific-IgE to fruit fly and following washing was probed with a goat anti-human IgE antibody conjugated with horseradish peroxidase, before detection with an ECL substrate. The protein molecular weight was calculated according to a prestained protein standard and imaged using a ChemiDoc™ System.

For both approaches, after blocking, the membrane was cut using a disposable scalpel into either six or eleven membrane strips, depending on the number of individual sera used and one membrane strip was incubated with pooled serum.

5.2.5 Mass spectrometry

The following methods: tryptic digestion and extraction of protein, nano LC-MS/MS and data processing were all achieved with training and supervision from Prof. Peter Briza at the Division of Allergy and Immunology at the University of Salzburg.

5.2.5.1 In-gel tryptic digestion

Methods for in-gel tryptic digestion were followed using a ProteoExtract All-in-One Trypsin Digestion Kit, as described in Chapter 3, section 3.2.7.1. Briefly, specific-IgE binding proteins were excised from Coomassie stained polyacrylamide gels and washed, dehydrated and dried. Trypsin was used to digest the proteins into peptides and after digestion, the sample was stored at -20°C prior to peptide extraction.

5.2.5.2 Extraction of peptides

Methods for peptide extraction were followed as described in Chapter 3, section 3.2.7.3. Briefly, a 10µl ZipTip$_{C18}$ tip was used to pick up the peptide solution after digestion and de-salt the solution; producing concentrated peptides. The peptide solution was checked for acidity using pH paper and addition of 13µl of TFA. The sample was dried down, resuspended in 0.1% TFA and sonicated before transfer into a MS vial.

5.2.5.3 Nano liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Methods for nano LC-MS/MS were followed as described in Chapter 3, section 3.2.7.4. Briefly, MS vials were placed into an automated EASY nano liquid chromatograph and peptides were resolved by reverse phase chromatography. This was coupled to an Orbitrap Velos Pro by ESI in which peptides were ionised by charged hydrogen ions and then separated based on their mass-to-charge ratio (*m/z*). Raw data was processed by PEAKS Studio. Peptide matches were sought within the UniProt database against the fruit fly taxonomy and the search was limited to a 95% protein identification threshold.

5.3 Results

5.3.1 Chromogenic western blot of *D. melanogaster*

In a first attempt I applied 120µg of fruit fly protein to hand-cast SDS-PAGE, using individual and pooled serum and a chromogenic substrate to detect specific-IgE binding proteins. I identified two regions of specific-IgE binding to fruit fly extract with molecular weights of approximately 76 and 107kDa, from serum of three sensitised workers (Figure 5-2: lanes 2-5). Lane 5 was a pooled serum sample from the same three sensitised workers. One individual had an additional four regions of specific-IgE binding, with molecular weights of approximately 12, 28, 54 and 183kDa (lane 2). In a control blot with serum replaced by tris-blocking buffer, no non-specific binding to fruit fly extract was observed (lane 6).

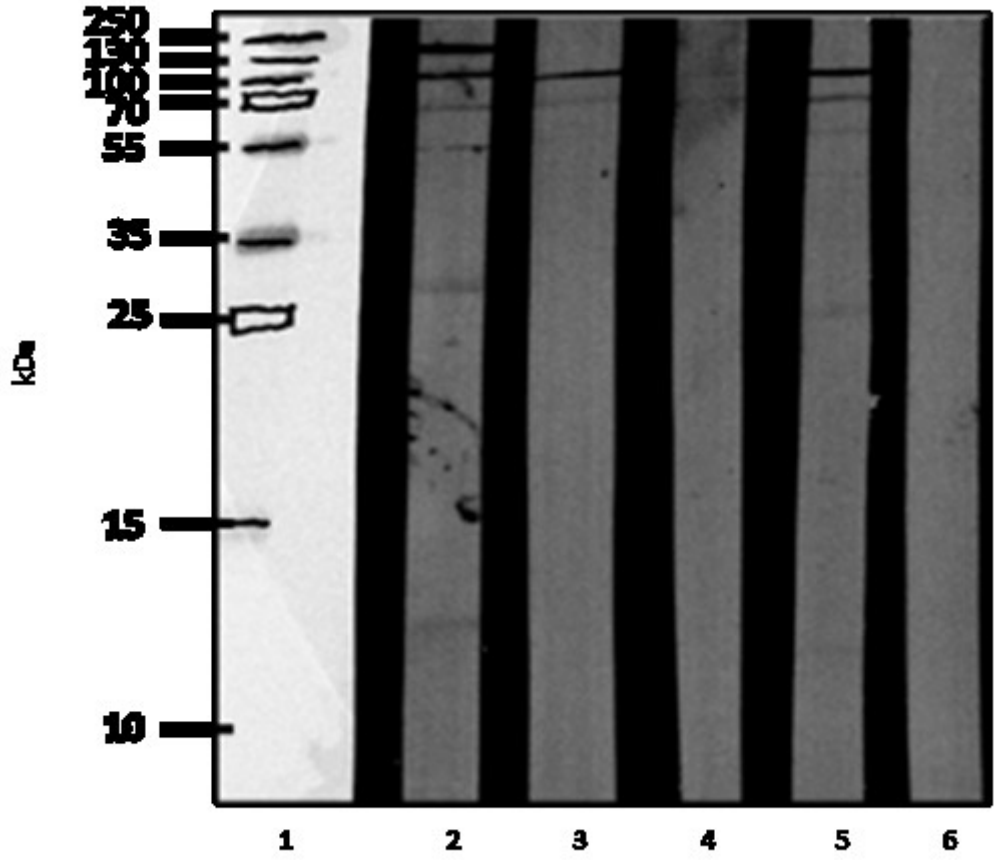


Figure 5-2 Chromogenic western blot of fruit fly extract. I applied 120µg of fruit fly protein to hand-cast SDS-PAGE in a total volume of 200µl. Protein was transferred to a nitrocellulose membrane using semi-dry transfer and blocked in tris-blocking buffer for 1 hour 30 minutes. The membrane was divided into strips and incubated with individual and pooled serum (1:5 dilution) for 16 hours at 4°C. Membrane strips were washed in PBS-T, incubated in anti-human IgE, with an AP conjugate (1:5000 dilution) for 30 minutes and specific-IgE binding regions were detected using 2ml of a NBT/BCIP substrate solution prepared in AP detection buffer. The molecular weight was determined with a prestained protein standard (lane 1). Lanes 2-4: individual fruit fly-sensitised serum, lane 5: pool of serum used in 2-4, Lane 6: control without serum.

5.3.2 ECL western blot of *D. melanogaster*

Using an ECL western blot with serum from nine sensitised workers, I identified three regions of specific-IgE binding to fruit fly extract, with molecular weights of approximately 10-13kDa, 100-110kDa and 230-250kDa (Figure 5-3: lanes 2-11). Lane 11 was a pooled serum sample from the same nine sensitised workers. Serum dilutions were 1:10 for individual blots (lanes 2-10) and 1:5 for the pooled blot (lane 11), as there was a limited volume of serum available. Using individual serum, specific-IgE binding was only observed below ~30kDa, most predominantly between 10 and 22kDa (lanes 2-10). Using pooled serum, specific-IgE binding was observed at ~10-13, 100-110 and 230-250kDa (lane 11). Negative (white) staining was observed in lane 11, especially from 15-30kDa.

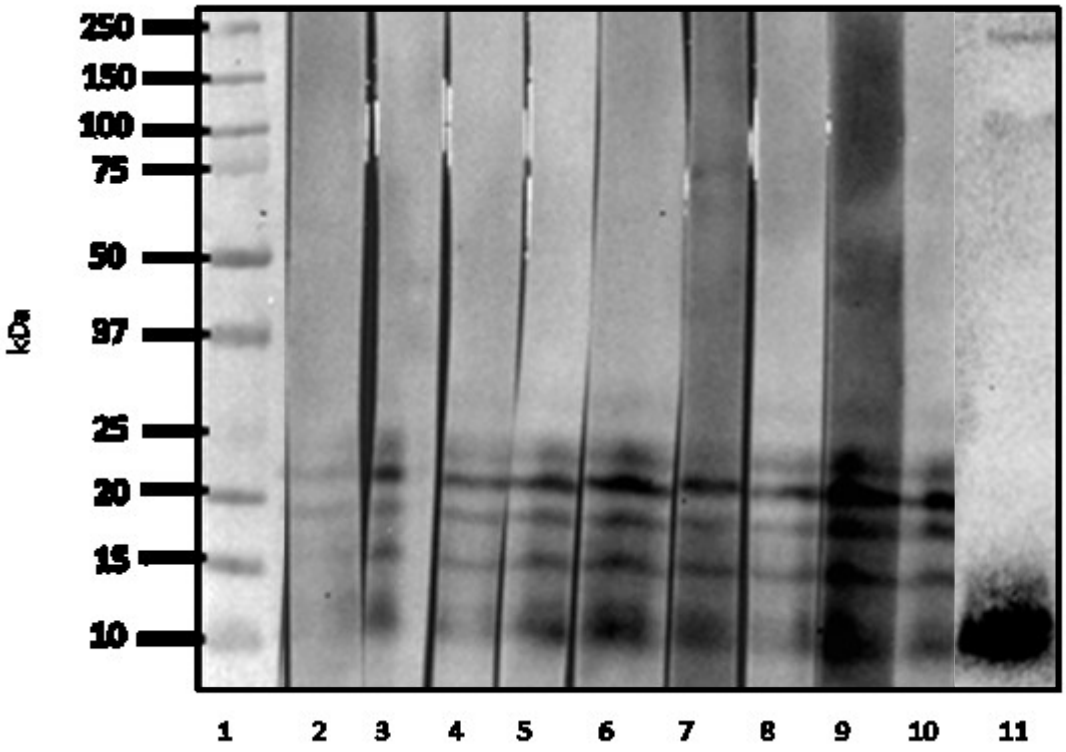


Figure 5-3 ECL western blot of fruit fly extract. I applied 150µg of fruit fly protein to a precast NuPAGE gel in a total volume of 250µl. Protein was transferred to a nitrocellulose membrane using wet transfer and blocked in phosphate-blocking buffer for 1 hour 30 minutes. The membrane was divided into strips and incubated with individual serum (1:10 dilution) and pooled serum (1:5 dilution) for 16 hours at 4°C. Membrane strips were washed in PBS-T, incubated in anti-human IgE, with HRP conjugate (1:5000 dilution) for 30 minutes and specific-IgE binding regions were detected using 2ml of ECL substrate solution. The molecular weight was determined with a prestained protein standard (lane 1). Lanes 2-10: individual fruit-fly sensitised serum, lane 11: pool of serum used in 2-10.

5.3.3 Determining the limit of detection for *D. melanogaster* proteins by Coomassie and silver stained SDS-PAGE

To determine the limit of detection of fruit fly extract run on SDS-PAGE and stained with either Coomassie or silver I performed a serial dilution of the extract using 1.25-20µg of fruit fly protein as measured by the *DC™* protein assay (Figure 5-4: A+B: lanes 2-6). This was necessary to determine the protein mass needed for in-gel digestion and MS analysis.

By Coomassie staining, proteins were detected from 6-25kDa (Figure 5-4:A) and by silver staining proteins were detected between 6 - >200kDa (Figure 5-4:B). Strong bands were observed at approximately 6, 10, 15 and 25kDa in 2.5µg of protein by silver staining (lane 5), whereas strong bands could only be detected in 10µg of protein by Coomassie staining (lane 3). Bands at approximately 70, 130 and >200kDa were also detected in 10µg of protein by silver staining (lane 3).

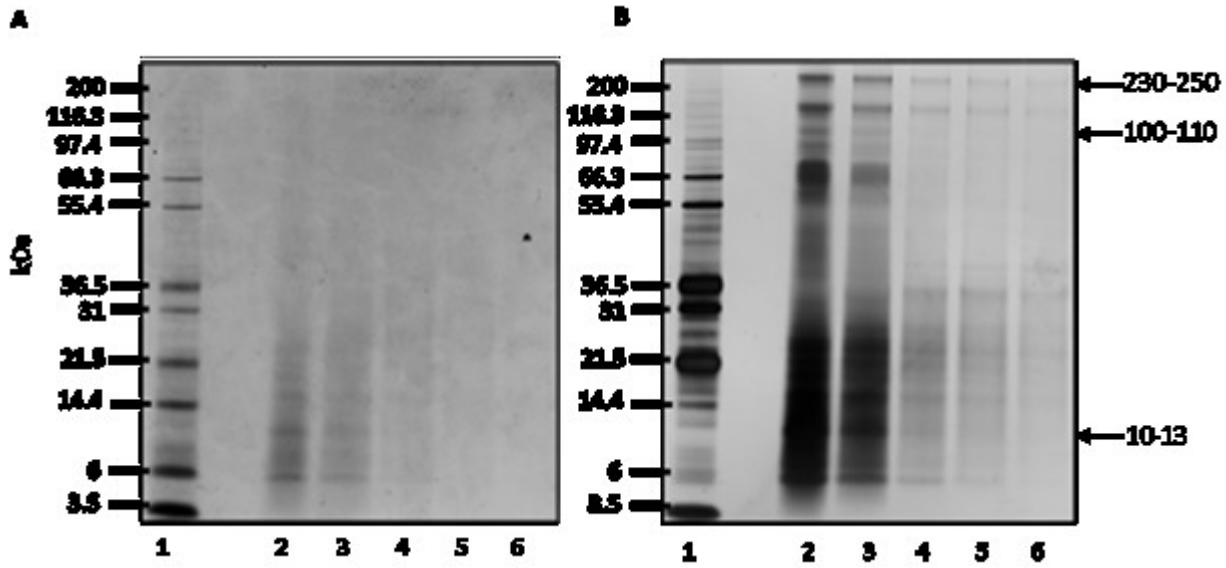


Figure 5-4 Coomassie (A) and silver stained (B) SDS-PAGE of fruit fly extract. A serial dilution of 20, 10, 5, 2.5 and 1.25µg of fruit fly protein (lanes 2-6) was applied to a precast NuPAGE gel, each in a total volume of 25µl. The molecular weight was determined with a prestained protein standard (lane 1). Specific-IgE binding proteins detected in the previous figure are labelled on the right.

5.3.4 Identification of allergens from *D. melanogaster* by MS

To excise the specific-IgE binding proteins from a Coomassie stained gel that were detected by ECL western blot, I increased the protein content applied to SDS-PAGE from 20µg to 90µg.

The following specific-IgE binding regions at approximately 10-13kDa, 100-110kDa and 230-250kDa shown in Figure 5-5 were excised from a Coomassie stained gel and digested with trypsin for MS analysis.

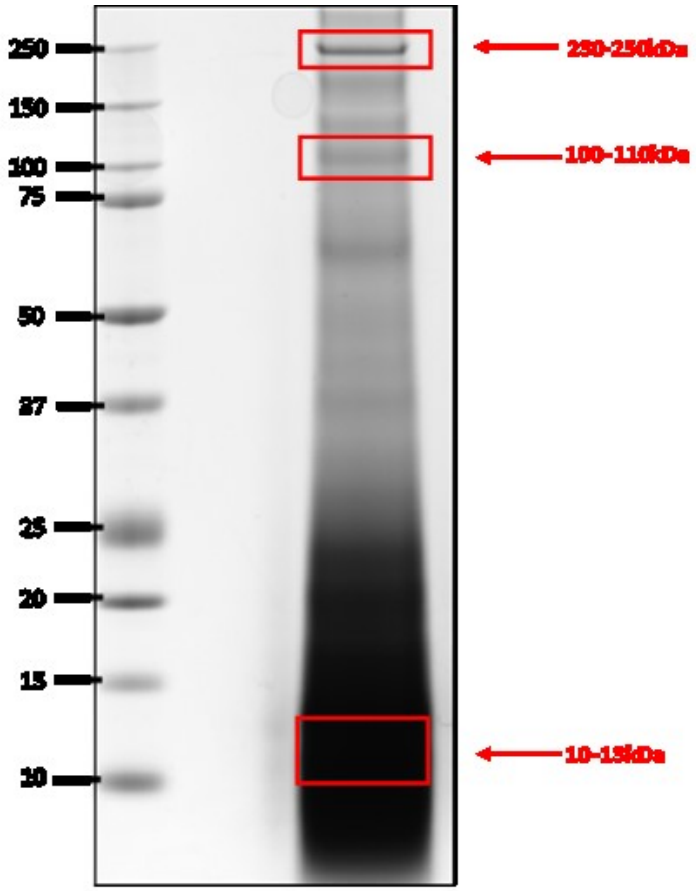


Figure 5-5 In-gel excision of specific-IgE binding regions from fruit fly extract. 90µg of fruit fly protein was applied to SDS-PAGE in a total volume of 25µl and was detected using Coomassie stain. The molecular weight was determined with a prestained protein standard.

Protein identifications from an in-gel 10-13kDa fruit fly digest

We identified 273 proteins from the 10-13kDa digest, with the most abundant being fructose-bisphosphate aldolase (Prot Acc: C8VV14). Sequence coverage was 56% and 32 unique peptides were identified (Figure 5-6:A). In Table 5-4 are the 10 most abundant proteins identified from this digest, after excluding those with less than 3 unique peptides.

Of note, larval serum protein (Prot Acc: X2JCV2) was identified from this digest with a sequence coverage of 28% and 37 unique peptides.

Protein identifications from an in-gel 100-110kDa fruit fly digest

We identified 67 proteins from the 100-110kDa digest, with the most abundant being alpha mannosidase (Prot Acc: Q9VKV1). Sequence coverage was 34% and 41 unique peptides were identified (Figure 5-6:B). In

Table ***5-5*** are the 10 most abundant proteins identified from this digest, after excluding those with less than 3 unique peptides.

Of note, superoxide dismutase (Prot Acc: P6185) was identified from this digest with a sequence coverage of 63% and 10 unique peptides.

Proteins identifications from an in-gel 230-250kDa fruit fly digest

We did not identify proteins from the 230-250kDa digest. This was thought to be due to instrument error with reasons discussed later in this chapter.

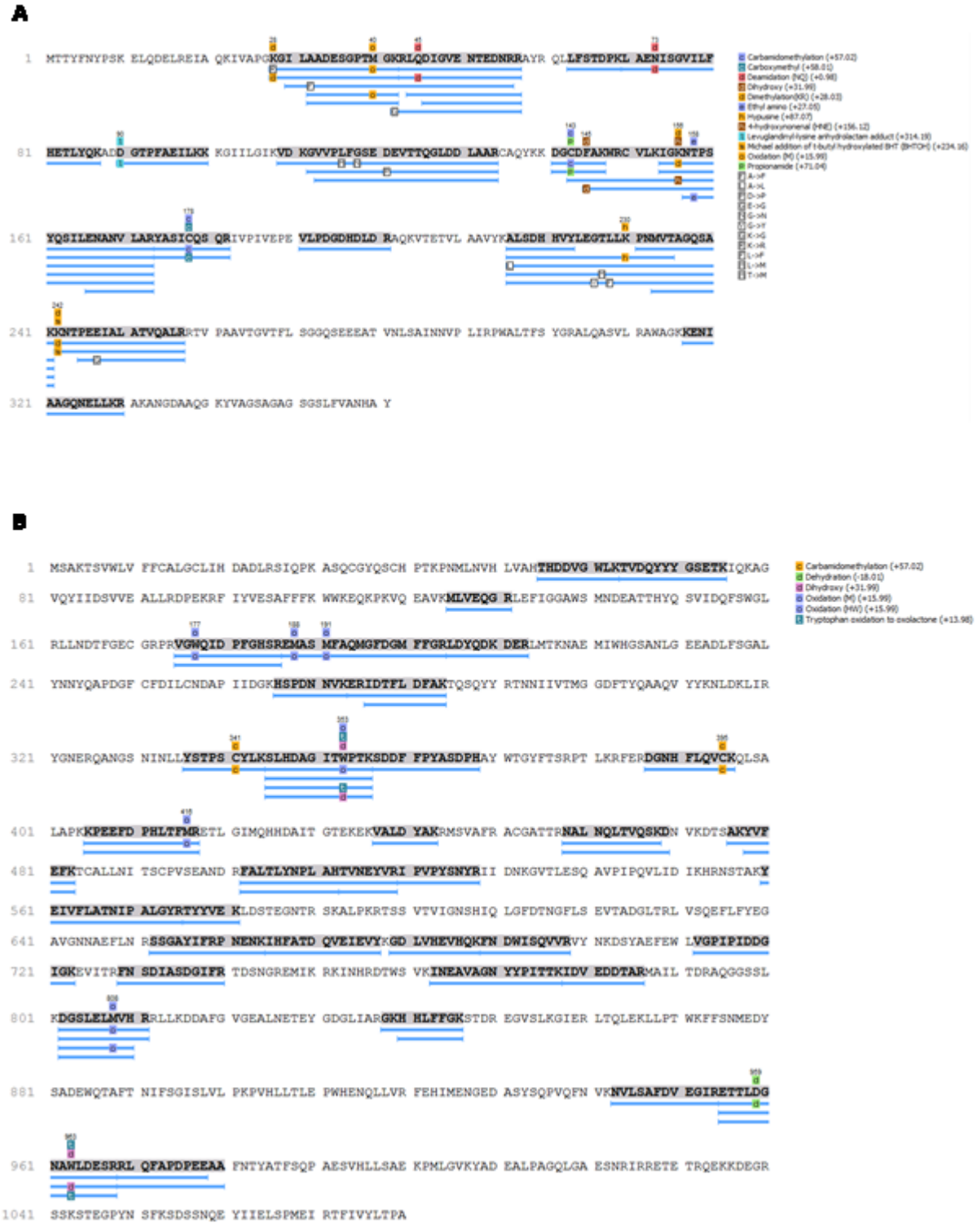


Figure 5-6 Sequence coverage of fructose-bisphosphate aldolase (A) and alpha-mannosidase (B) identified by nano LC-MS/MS from in-gel 10-13kDa and 100-110kDa fruit fly digests. The fruit fly extract was resolved by SDS-PAGE and visualised with Coomassie stain. Specific-IgE binding regions at 10-13kDa (A) and 100-110kDa (B) were excised and digested with trypsin before injection into HPLC coupled to a nano ESI source into MS/MS. Raw MS data was processed by PEAKS Studio and searched against fruit fly taxonomy.

Table 5-4 Proteins identified by nano LC-MS/MS from an in-gel 10-13kDa fruit fly digest.

Protein name	Accession Number	Score	No. of Unique Peptides	Coverage (%)	MW (kDa)
Projectin (Fragment)	O76281	208.5	3	7	743
Larval serum protein 2, isoform B	X2JCV2	203.98	37	28	83
Sallimus, isoform U	M9PEA5	194.05	35	2	1810
Fructose-bisphosphate aldolase	C8VV14	189.41	32	56	39
Alpha actinin, isoform F	F0JAG6	180.23	23	21	103
Apolipophorins	Q9V496	162.3	22	6	372
Dihydrolipoyl dehydrogenase	Q9VVL7	151.69	11	26	53
GH15296p	Q8MSI2	147.32	14	50	21
Obscurin	A8DYP0	146.84	14	4	475
Transferrin	Q9VWV6	137.26	12	19	71

Table 5-5 Proteins identified by nano LC-MS/MS from an in-gel 100-110kDa fruit fly digest.

Protein name	Accession Number	Score	No. of Unique Peptides	Coverage (%)	MW (kDa)
Alpha-mannosidase	Q9VKV1	217.43	41	34	122
Thioredoxin peroxidase 1, isoform C	X2JF59	176.31	10	56	21
Beta galactosidase, isoform A	Q9VMJ5	172.44	14	26	75
Dipeptidase B, isoform A	Q9VFQ9	168.57	15	33	55
Trehalase	Q9W2M2	159.14	14	25	67
Superoxide dismutase [Cu-Zn]	P6185	155.35	10	63	15
Aminopeptidase	Q8SWX4	153.84	12	12	108
Putative ferric-chelate reductase 1 homolog	Q8MSU3	149.4	8	14	70
Alpha-amylase	A0A0B4	142.71	8	19	53
Iron regulatory protein 1B	Q9VGZ3	139.35	10	14	98

5.3.5 Protein separation and ECL western blot of *D. melanogaster* at a higher polyacrylamide percentage

As a 'post-hoc' experiment, we attempted to further separate the specific-IgE binding region between 10-13kDa, using an SDS gel with a higher polyacrylamide percentage. I applied 70µg of fruit fly protein to a precast gel with a polyacrylamide concentration of 12% (instead of 4-12% as previously used) and stained with Coomassie (Figure 5-7:A). In addition, I applied 70µg of fruit fly protein to an ECL western blot using a precast 12% polyacrylamide gel, with pooled sensitised workers serum (as used previously) in Figure 5-7:B. Strong protein staining was still shown between 10 and 13kDa and specific-IgE binding showed more discrete bands at approximately 11, 16 and 19kDa. Due to time and financial restrictions I was unable to excise regions from this separation for MS analysis.

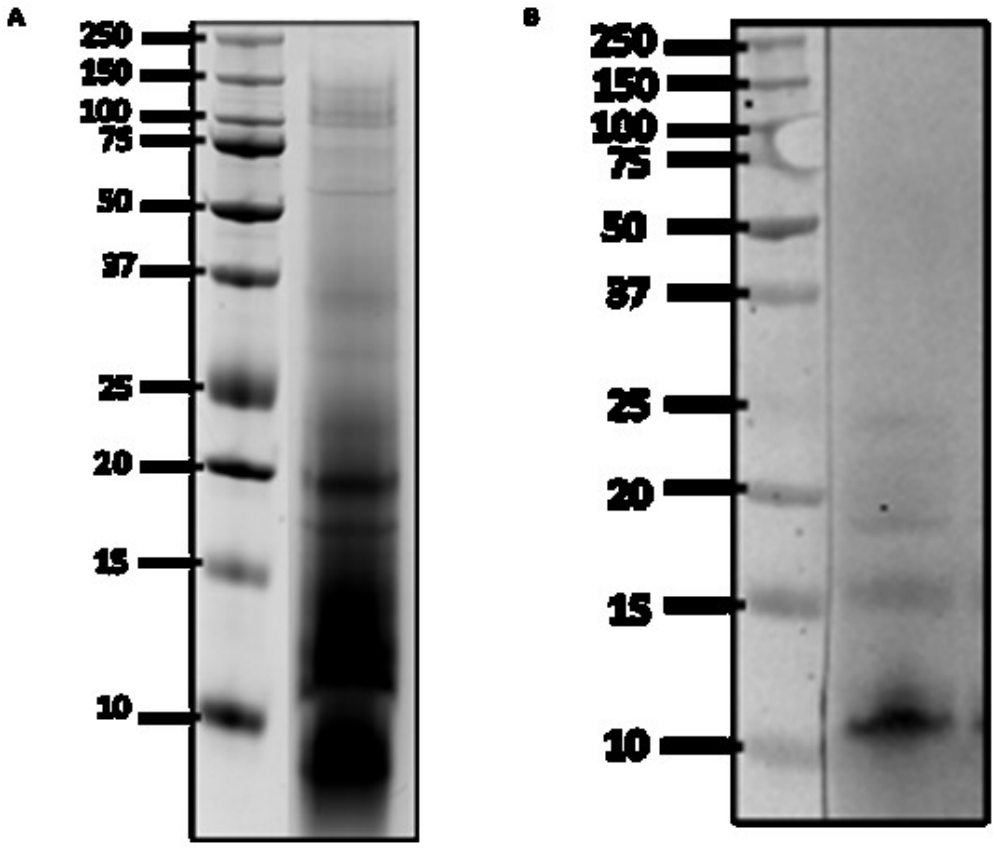


Figure 5-7 Coomassie SDS-PAGE (A) and ECL western blot (B) of fruit fly extract on a 12% polyacrylamide concentrated pre-cast gel. I applied 70µg of fruit fly protein in a total volume of 25µl to 2 x SDS-PAGE running in parallel. One gel was stained with Coomassie for 1 hour (A) and the other was transferred to a membrane under normal conditions and blocked for 1 hour 30 minutes. The membrane was incubated in pooled serum (1:10 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution (B). The molecular weight was determined with a prestained protein standard.

5.4 Discussion

5.4.1 Overview

In this chapter I have successfully applied an optimised MS-based method and have identified potential novel allergenic proteins from the common fruit fly, *Drosophila melanogaster*. Specific-IgE binding proteins from the fruit fly extract were detected using both chromogenic and ECL western blot methods, and the modern proteomic tool MS allowed for identification of these specific-IgE binding proteins. Using this novel approach, allergens were putatively identified from a species that is not currently registered in the WHO/IUIS Allergen Nomenclature.

The number of workers exposed to the fruit fly, *D. melanogaster* is not currently known as a Home Office licence is not required for animal testing on insects. However, for the several hundred individuals in the UK that carry out research with insects, the annual incidence of occupational asthma and rhinitis was estimated at 0.24% and 2.1%, respectively (Draper et al., 2003).

To the best of my knowledge, there are only three published reports on occupational allergy associated with exposure to *D. melanogaster*. Spieksma et al., (1986) used RAST methods to show specific-IgE binding to fruit fly extract from six workers exposed in a scientific laboratory in the Netherlands. Colomb et al., (2017) used western blot methods to show specific-IgE binding to fruit fly in six laboratory workers from Montpellier, and Jones et al., (2017) used RAST methods to report a prevalence of serum specific-IgE binding to fruit fly extract of 6% amongst 247 workers in a UK academic institute.

In my work, I have also used western blot methods to detect specific-IgE binding proteins to fruit fly, with serum from eleven laboratory workers who attended occupational allergy clinics in London. More importantly, I have extended the current literature on *D. melanogaster* allergy, through using MS to identify the specific-IgE binding proteins that may be causing allergic sensitisation in workers exposed to fruit fly in animal testing facilities.

In my ECL western blot, serum specific-IgE binding was observed at approximately 10, 110 and 240kDa, using pooled serum from eight sensitised workers. ECL is a more sensitive

western blot detection method than chromogenic and so these three regions of specific-IgE binding were excised for in-gel digestion and MS analysis.

In the ~10kDa in-gel MS digest, the most abundant protein identified was fructose-bisphosphate aldolase (also known as just, aldolase). Aldolase has been identified as an allergen from other species and it is a registered allergen on the WHO/IUIS Allergen Nomenclature. Kuehn et al., (2013) identified aldolase allergens from cod, salmon and tuna which have since been termed Gad m 3.0101, Sal s 3.0101 and Thu a 3.0101, respectively in the WHO/IUIS Allergen Nomenclature. Aldolase is an enzyme involved in the glycolytic cycle and is often found in muscle tissue and while cross-reactivity among allergens from different fish species is widely accepted, cross-reactivity of aldolase between fish and chicken meat has also been recently recognised (Kuehn et al., 2016).

Ishiguro et al., (1992) identified antigens binding to serum specific-IgE from the yeast *Candida albicans*. Using N-terminal sequencing they showed the partial sequence of a 37kDa excised protein from *C. albicans* shared significant levels of homology with aldolase from another yeast species that had already been sequenced. Aldolase has also been identified as an allergenic protein present in venom from the Asian wasp, *Vespa affinis*, responsible for sting-derived clinical manifestations, such as IgE mediated-anaphylaxis (Sookrung et al., 2014). Similar western blot and MS methods as I have shown here, were used by Sookrung et al., to report that eight out of 15 wasp allergic patients reacted to aldolase, in addition to other major and minor allergens from *V. affinis*.

I also identified larval serum protein as one of the most abundant proteins from the ~10kDa digest. In the latest publication on *D. melanogaster* allergens to date, Colomb et al., (2017) proposed that an 80kDa protein to which 6 sensitised individuals showed specific-IgE binding, corresponded to a hexamerin larval serum protein. This was evidenced through polyclonal antibodies raised against larval serum protein (anti-LSP) used in a western blot of fruit fly extract, to show binding of anti-LSP polyclonal antibodies matched the binding of serum from sensitised workers to the 80kDa protein. Moreover, an 80kDa specific-IgE binding protein has also been implicated in a Spanish case of occupational asthma to medfly, as observed by western blot experiments from de las Marinas et al., (2014).

In the ~110kDa in-gel MS digest, the most abundant protein identified was alpha mannosidase. To my knowledge this protein is not registered on any of the primary allergen databases (WHO/IUIS Allergen Nomenclature, Allergome etc.).

We did however, identify a 25kDa protein called superoxide dismutase (SOD) in the ~110kDa digest. SOD is listed as an *D. melanogaster* allergen on the Allergome platform and is termed Dro m MnSOD. Evidence for this allergen entry to Allergome is given by Flückiger et al., (2002) and consists of two parts. Firstly, the high sequence similarity (71.5%) observed between SOD from *D. melanogaster* and the allergen SOD from *A. fumigatus*, and secondly the specific-IgE binding from patients sensitised to *A. fumigatus*, to a recombinant form of *D. melanogaster* SOD. Although this allergen is registered on Allergome, this evidence is insufficient for Dro m MnSOD to be listed on the WHO/IUIS Allergen Nomenclature.

On the Allergome platform there are two other allergens from *D. melanogaster* listed which I did not identify in my results: Dro m 7, a 33kDa tropomyosin protein and Dro m 9 a 40kDa arginine kinase protein. Tropomyosin is a major shrimp allergen, that has high cross-reactivity with other crustacean and mollusc species. Leung et al., (1996) were able to demonstrate that tropomyosin from fruit fly (as well as grasshopper and cockroach) had a high cross-reactivity with the shrimp tropomyosin major allergen. This was achieved through western blot and inhibition experiments of the fruit fly extract with serum from patients with shrimp allergy, showing specific-IgE binding to the tropomyosin protein from fruit fly.

The 40kDa fruit fly allergen, arginine kinase (according to Allergome) has demonstrated allergen cross-reactivity from species of moth, cockroach and other insect groups (Binder et al., 2001; Sookrung et al., 2006). However, serum specific-IgE binding to arginine kinase from fruit fly has never been demonstrated. The Allergome evidence for arginine kinase as a fruit fly allergen relies on its high sequence homology of 87%, with the arginine kinase allergen from the cat flea, *C. felis* (Werr et al., 2009).

Although in the previous chapter ECL was discussed as a more sensitive method for detection of specific-IgE binding proteins (when compared to chromogenic detection), I included a chromogenic western blot in this chapter. This was because some of the work was conducted using the equipment from another academic institute that had expertise specifically in allergen proteomics (Division of Allergy and Immunology at the University of

Salzburg). A routinely used protocol for chromogenic detection of serum specific-IgE binding proteins was followed and using serum from three sensitised fruit fly workers, specific-IgE binding was observed at approximately 12, 28, 54, 76, 107 and 183kDa. However, not all these specific-IgE binding regions were observed using the ECL western blot method. This difference between the chromogenic and ECL methods could be possibly explained by different polyacrylamide percentage gels that were used. Hand-cast 12% gels were used for the chromogenic blot, while pre-cast 4-12% gels were used for the ECL blot; this may have well caused differences in separation of the proteins within the fruit fly extract.

Although we were unable to perform MS for all specific-IgE binding regions, it could be suggested that the 28 and 76kDa regions observed by the chromogenic blot may correspond to the SOD and hexamerin larval serum *D. melanogaster* allergens, already discussed.

I established the limit of detection for *D. melanogaster* proteins by SDS-PAGE, stained with either Coomassie or silver. As expected, silver stained SDS-PAGE had a far lower detection limit, with *D. melanogaster* proteins observed in 1.25µg of total protein, compared to Coomassie, where proteins were not observed below 2.5µg of total protein. Silver staining also detected a far greater range of molecular weight proteins (6 to >200kDa), compared with Coomassie staining (6 to 25kDa). Silver staining methods are known to be considerably more sensitive than Coomassie staining methods and are commonly used to detect smaller amounts of protein, whilst Coomassie is used as a rapid and inexpensive means of staining (Sasse & Gallagher, 2009).

For the in-gel 10-13kDa digest, a significant number of proteins were identified. This is not surprising, given the strong staining shown by western blot at this molecular weight region. As a post-hoc experiment we used an SDS gel with a higher polyacrylamide percentage, to further separate the proteins between 10-13kDa. As I was using a precast NuPAGE gel electrophoresis system, I was limited to the precast gel manufactured products available to detect protein at a lower molecular weight range. Using a 12% polyacrylamide gel, more discrete specific-IgE binding regions at 11, 16 and 19kDa were observed by ECL western blot. However, due to the cost of MS analysis, we were unable to carry out digests for protein identifications on these individual binding regions.

5.4.2 Limitations

Limitations concerning SDS-PAGE and western blot methods in this chapter include the appearance of negative staining and the difference in observed and expected molecular weights for some of the specific-IgE binding proteins. I detected specific-IgE binding proteins from *D. melanogaster* using a 1:5 dilution of pooled serum from nine sensitised workers, but also observed negative (white) staining on the membrane, especially in the region of 15-30kDa. Negative staining can occur from too much protein, too much serum or too much of the secondary antibody (anti-human IgE) (Mahmood and Yang, 2012).

For some of the MS identified proteins, the theoretical molecular weight did not match the molecular weight of the in-gel digest. For example, the 80kDa larval serum protein identified from the ~10kDa digest, and the 25kDa superoxide dismutase protein identified from the ~100kDa digest. This is not uncommon in MS-based proteomics, as it is expected that larger proteins may have degradation products that appear with a lower molecular weight by SDS-PAGE. The protein can still be identified as it exists prior to degradation from a lower molecular weight digest, because the partial amino acid sequence detected still remains a highly accurate match. The existence of protein degradation products in the fruit fly extract, could also explain the high number of proteins and their divergent molecular weights that were identified from the ~10kDa MS database search. Furthermore, lower molecular weight proteins can aggregate higher in an SDS gel and appear at a higher molecular weight than would be expected. This can occur because of protein overloading in the gel (Weber et al., 1972).

We were also limited by MS instrument error that resulted in proteins not being identified from the specific-IgE binding region at 230-250kDa. The cause of this was not known, however was thought to be due to a fault in the calibration settings of the instrument.

The volume and number of serum samples available from fruit fly sensitized individuals was limiting. We had chosen stored serum samples from those who had attended an occupational allergy clinic and had elevated levels of specific-IgE as determined by RAST, but soon ran out of suitable serum which meant we could not conduct repeat experiments.

5.4.3 Summary and future works

Future work would include MS identification of the specific-IgE binding proteins that were either unsuccessful or not attempted (i.e. the 230-250kDa digest and those from the chromogenic blot). To overcome negative staining, the protein amount applied to western blot would need to be optimized further, while still being a sufficient quantity for in-gel excision to MS. Hand-cast gels with a higher polyacrylamide percentage could also be designed to better separate the specific-IgE binding proteins at 10-13kDa. The allergenicity and clinical relevance of fructose-bisphosphate and alpha mannosidase as *D. melanogaster* proteins, as well as those registered on the Allergome platform should be investigated as part of future works. This would include tests of allergenicity such as basophil activation and histamine release which should be used on purified or recombinant versions of these proteins.

Investigation into fruit fly sensitization is warranted by the large number of workers in animal testing facilities that have been and will continue to be frequently exposed to *D. melanogaster*. I have identified specific-IgE binding proteins, detected from a small subset of allergic individuals, where previous studies have used no more than 6 individuals. Larger scale studies, collecting both clinical (e.g. SPT, RAST and western blot) and potentially epidemiological data (e.g. sensitization prevalence and exposure type) should be established across academic and industry research sectors to assess the extent of the occupational allergy burden that is caused by exposure to *D. melanogaster*. These workplaces could then consider methods to limit fruit fly exposure using approaches similar to those used for limiting exposure to airborne allergens from mice, responsible for LAA.

In summary, we have identified potential novel allergenic proteins, capable of binding serum specific-IgE, derived from the allergen source *D. melanogaster*, that are not currently recognized by the leading allergen database, WHO/IUIS Allergen Nomenclature. I applied western blot and MS-based methods and successfully detected serum specific-IgE binding proteins. Future works should seek to purify these proteins and assess their allergenicity, in addition to larger scale efforts to determine the prevalence of allergic sensitisation to *D. melanogaster* amongst exposed laboratory animal workers.

6 Application of the mass spectrometry (MS)-based method to identify novel occupational allergens associated with 'improver' enzymes used in supermarket bakeries

6.1 Introduction

In the previous chapter I have shown how, using an optimised MS-based method, I was able to identify potential novel allergenic proteins from fruit fly. In this chapter I have investigated occupational allergens associated with the baking industry. The prevalence of asthma in bakers has been estimated between 15-30% (Armentia et al., 1990) and exposure to flour proteins and the fungal α-amylase enzyme are well-known to elicit symptoms of occupational asthma. Currently, the panel of 'improver' enzymes used to enhance the baking process is increasing and the extent to which they are responsible for causing occupational asthma within bakeries is unknown.

Baker's asthma

Baker's asthma is one of the oldest recognised occupational diseases, first described by Bernardo Ramazzini in the 1700's, as respiratory symptoms among bakers caused by exposure to flour dust. It is still regarded as one of the most frequently occurring forms of occupational asthma in Europe, and in the UK during 1992-1997 the annual incidence was estimated as 951 per million bakery workers (McDonald et al., 2000).

The main causes of baker's asthma have been identified as airborne allergens derived from wheat (*Triticum aestivum*), rye (*Secale cereale*) and barley flour (*Hordeum vulgare*) (Houba et al., 1998). There are now 27 allergens from *Triticum aestivum* listed on the WHO/IUIS Allergen Nomenclature, including those characterised as food allergens (Raulf, 2018). Since the 1970's, the enzyme fungal α-amylase, derived from the fungus *Aspergillus oryzae*; has been added to flour to enhance the baking process and has been shown to cause baker's asthma (Baur et al., 1986; Quirce et al., 1992). This fungal α-amylase enzyme is listed in the WHO/IUIS Allergen Nomenclature and has been given the allergen term Asp o 21. In Table

6-1 (on page 222) are all the allergens that have been implicated in baker's asthma which are registered within the WHO/IUIS Allergen Nomenclature.

Fungal α-amylase

Fungal α-amylase stimulates the growth and fermentation process of yeast which facilitates the rising of the dough and improves quality of the bread. The first description of allergy to enzymes used in the baking industry was published by Baur et al., in 1986, who identified the enzyme fungal α-amylase as the protein responsible for causing allergic symptoms. Using *in vivo* and *in vitro* clinical tests, they studied 118 workers from bakeries and confectioners, 35 of whom had bronchial asthma, rhinitis and/or conjunctivitis after contact with flour. They found out that the enzyme α-amylase from *A. oryzae* was being added to flour, and thus decided to test all 118 serum samples for specific-IgE antibodies to commercially available α-amylase from *A.oryzae*, using RAST. They found that 34% (n=12) of the symptomatic workers and 2% of all those exposed, had elevated levels of serum specific-IgE to α-amylase. SPT confirmed immediate-type hypersensitivity to α-amylase in workers with positive RAST results. Furthermore, 4 symptomatic bakers had a positive inhalation challenge to α-amylase with an immediate onset of rhinitis or an asthmatic response.

Baur et al., (1986) used western blot methods with serum from sensitised bakers to identify specific-IgE binding regions from the fungal α-amylase enzyme (Figure 6-1, on page 223). A specific-IgE binding region at 52kDa was detected after incubation with serum that was positive for specific-IgE to α-amylase according to RAST (n=5). This was compared with no binding observed using serum from a healthy control.

Quirce et al., (1992) investigated further the role of α-amylase in bakers' asthma, using SPT, enzyme immunoassays for specific-IgE determination, histamine release testing, western blot and bronchial provocation tests. They identified five bakery workers with positive SPT and specific-IgE to α-amylase, and in four out of five, histamine release testing was also positive. By western blot, using pooled serum from the bakers, they observed a specific-IgE binding region with a molecular weight of 52kDa from a 6mg/ml extract of α-amylase (as determined by the Lowry method for protein concentration).

Table 6-1 Registered allergens from the WHO/IUIS Allergen Nomenclature implicated in baker's asthma.

Allergen source	Allergen	Biochemical name	Allergenicity reference	MW (kDa)	Food allergen	Remarks
Triticum aestivum (Wheat)	Tri a 12	Profilin	Raulf 2016	14	Yes	Relevant for cross-reactivity with grass pollen
	Tri a 14	Non-specific lipid transfer protein 1	Sander et al., 2011	9	Yes	One subject with baker's asthma out of 40 had IgE binding
	Tri a 15	Monomeric alpha-amylase inhibitor	Sander et al., 2015		No	Relevant in patients with baker's allergy
	Tri a 21	Alpha-beta-gliadin	Sander et al., 2011		No	Four subjects with baker's asthma out of 40 had IgE binding
	Tri a 25	Thioredoxin	Weichel et al., 2006		Yes	Not exclusively recognised by specific IgE from bakers
	Tri a 27	Thiol reductase homologie	Sander et al., 2011	27	No	Six subjects with baker's asthma out of 38 had IgE binding
	Tri a 28	Dimeric alpha-amylase inhibitor	Sander et al., 2011	13	No	Ten subjects with baker's asthma out of 40 had IgE binding
	Tri a 29	Tetrameric alpha-amylase inhibitor	Sander et al., 2015	13	No	Not exclusively recognised by specific IgE from bakers
	Tri a 30	Tetrameric alpha-amylase inhibitor	Sander et al., 2015	16	No	Relevant in patients with baker's allergy
	Tri a 31	Triosephosphate-isomerase	Sander et al., 2015		No	Not exclusively recognised by specific IgE from bakers
	Tri a 32	1-cys-peroxiredoxin	Sander et al., 2015		No	Relevant in patients with baker's allergy
	Tri a 33	Serpin	Sander et al., 2015		No	Recognised by 8% of the patients with baker's allergy
	Tri a 34	Glyceraldehyde-3-phosphate-deydrogenase	Sander et al., 2011		No	Two subjects with baker's asthma out of 40 had IgE binding
	Tri a 35	Dehydrin	Sander et al., 2011		No	Two subjects with baker's asthma out of 38 had IgE binding
	Tri a 39	Serine protease inhibitor-like protein	Sander et al., 2015		No	18 of 101 European bakers (Spain, Netherlands and Germany) had IgE binding
	Tri a 40	Alpha amylase inhibitor	Sander et al., 2016	15.96	No	Eight subjects with baker's asthma out of 94 had IgE binding
Secale cereal (Rye)	Sec c 38	Dimeric alpha-amylase/trypsin inhibitor	García-Casado et al., 1995	13.5	No	Fifteen subjects with baker's asthma out of 21 had +ve SPT.
Hordeum vulgare (Barley)	Hor v 15	Alpha-amylase inhibitor	Barber et al., 1989	14.5	Yes	Identified as a major IgE-binding component of serum from baker's asthma patients.
Triticum turgidum (Durum wheat)	Tri tu 14	nsLipid transfer protein 1	Poster at 7th ISMA conference	9.2	Yes	23/32 subjects with baker's asthma or wheat food allergy had +ve IgE to recombinant protein.
Aspergillus oryzae (Rice mold)	Asp o 21	TAKA-amylase A (α-amylase)	Baur et al., 1994	53	No	35% and 32% of symptomatic bakers (with asthma and rhinitis), were +ve by SPT and specific IgE.
Aspergillus niger (Black mold)	Asp n 14	Beta-xylosidase	Sander et al., 1998	105	No	11% of 171 bakers with workplace-related symptoms had specific IgE to xylanase by western blot.

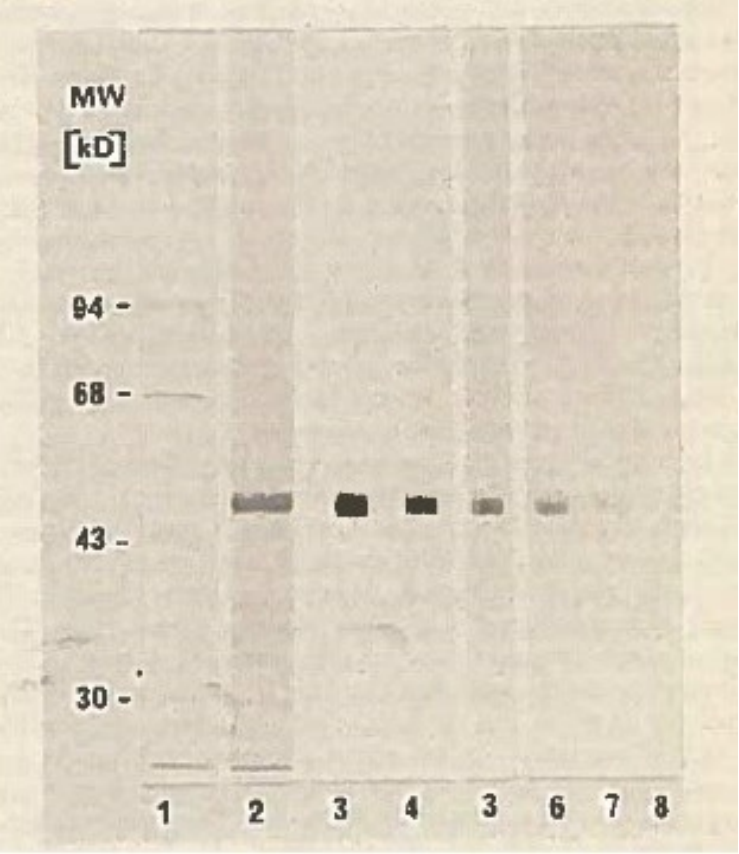


Figure 6-1 Detection of specific-IgE binding regions of *A. oryzae* amylase by western blot. An 8% polyacrylamide gel containing SDS was used to separate protein, samples were transferred to nitrocellulose sheets with a Bio-Rad electroblot apparatus and incubated with 100µl of patients serum followed by anti-human IgE and autoradiographic detection. Molecular weight markers stained with Coomassie brilliant-blue (lane 1). Proteins stained with Coomassie brilliant-blue (lane 2). Autoradiograms of nitrocellulose sheets after incubation with serum of individual sensitised patients (lanes 3-7), and serum of a healthy control (lane 8).

Other enzymes in the baking industry

In the last 20 years, other enzymes in addition to α-amylase have been commonly used in the baking industry, and these have been implicated as causes of allergic respiratory symptoms in bakery workers.

The allergen Asp n 14, which is a β-xylosidase protein from *Aspergillus niger* (black mould) was initially identified by Sander et al., (1998). Using serum from 171 German bakers with workplace-related symptoms, 11% were shown to have specific-IgE to xylanase, determined by enzyme allergosorbent testing, and 7 of the 8 who were sensitised also showed specific-IgE binding to a 105kDa region determined by chromogenic western blot methods. This region was excised from the gel, destained and digested with trypsin, for protein

identification by MS. A sequence coverage of 39% was identified for the *A. niger* derived protein, β-xylosidase. This allergenic protein has since been registered in the WHO/IUIS Allergen Nomenclature.

In the same year, Baur et al., (1998) published a case study of a 26-year-old baker with symptoms of rhino-conjunctivitis, cough and shortness of breath. The baker underwent an inhalation challenge to aerosolised xylanase and a significant asthmatic reaction occurred after application of ~0.5μg of the xylanase enzyme.

Glucoamylase is another enzyme derived from *A. niger* that is used in the baking industry and is implicated in bakers' asthma. In the study by Sander et al., (1998), 7.6% of the 171 German bakers had specific-IgE to glucoamylase as shown by enzyme allergosorbent testing. In 2002, Quirce et al., described four male bakery employees with work-related allergic respiratory symptoms, who all gave positive SPT to glucoamylase and fungal α-amylase. Specific inhalation challenges to glucoamylase elicited early asthmatic responses in three of the employees and a chemiluminescence western blot using their serum showed specific-IgE binding regions to commercial glucoamylase from *A. niger* (purchased from Sigma) between 30 and 100kDa. The most frequent specific-IgE binding regions were at 33.5, 47 and 54kDa.

In 2003, 135 serum samples were collected from workers across 18 small bakeries in Scotland and tested by RAST against a mixed solution of the *A. niger* derived enzymes: cellulase, hemicellulose and xylanase as well as fungal α-amylase (Elms et al., 2003). They found that 6% (n=8) were sensitised to the enzyme mixed solution, of whom 2 were negative to fungal α-amylase. This and the previous case report demonstrate the importance of not only measuring sensitisation to fungal α-amylase but also to the full range of enzymes used in different bakery facilities.

More recently, a case report was published (Lipińska-Ojrzanowska et al., 2016) in which routine clinical tests alone (specific-IgE determination for flour and α-amylase allergens) would have resulted in a false-negative diagnosis for baker's asthma. A 38-year-old baker was hospitalised due to a suspicion of occupational asthma and SPT gave positive results on flours and α-amylase. Specific inhalation challenges with the same agents were negative. The presence of specific-IgE to xylanase and cellulase from *A. niger* was demonstrated by coupling them to an ImmunoCAP and testing with the baker's serum. Specific inhalation

challenges using these enzymes triggered dry cough and wheezing symptoms in the baker, thus demonstrating an allergic response to improver enzymes used in the baking industry, other than fungal α-amylase.

Between the years 2000 and 2010 there has been a change in exposure and sensitisation rates to enzymes used in the baking industry. Simonis et al., (2014) studied 433 bakers across Germany who experienced allergic rhinitis and/or allergic bronchial asthma or were at real risk of being affected by occupational disease. Of these, 299 were tested between 1999 and 2001 (2000) and 134 between 2009 and 2011 (2010), for serum specific-IgE to baking enzymes. The results suggested a 50% reduction from 2000 to 2010 for sensitisation to α-amylase, (26% to 13%). Sensitisation rates for glucoamylase however, were significantly higher in the 2010 group (28%) and sensitisation of cellulase (16%) was higher than for α-amylase (13%). The authors concluded that the high sensitisation rate to glucoamylase, warrants better monitoring of exposure to enzymes other than fungal α-amylase that are used in the baking industry.

Other industries

Occupational asthma caused by exposure to airborne enzymes, is not unique to the baking industry. In several other trades, such as food processing, laundry detergent production, paper manufacture and the brewing industry, allergic symptoms caused by enzyme exposure has been observed (Raulf et al., 2018). Table 6-2 (on page 226) is taken from Budnik et al., (2017) and shows the large variety of industrial enzymes now used.

When enzymes were first introduced into biological washing powders in the 1960's, it led to allergic sensitisation and asthmatic symptoms in both exposed workers (Flindt, 1969) and consumers (Belin et al., 1970). Afterward, enzymes were used in an encapsulated form which appeared to reduce the occurrence of occupational asthma (Juniper et al., 1977). Since then the number of encapsulated enzymes has increased and now includes amylases, cellulases, proteases and lipase amongst many others.

Table 6-2 Examples of known industrial enzyme applications.

Application	Enzymes used
Food processing	Amylases, proteases, aspartases, cellulases
Baby foods	Trypsin
Brewing industry	Amylases, glucanases, proteases, β-glucanases, arabino xylanases
Fruit juices	Cellulases, pectinases
Dairy industry	Rennin, lipases, lactases, microbial-produced enzymes
Meat tenderisers	Papain
Starch industry	Amylases, amyl glucosidase, glucoamylases, glucose isomerase
Paper industry	Amylases, xylanases, cellulases, liginases
Biofuel industry	Cellulases, liginases
Contact lens cleaners	Proteases
Rubber industry	Catalases
Photographic industry	Proteas (ficin)
Molecular biology	Restriction enzymes, DNA ligase, polymerases
Fragrance/flavours industry	Lipolytic enzymes, proteases, alcohol dehydrogenase, amine oxidase
Pharmaceutical industry	Papain, bromelain, trypsin, pancreatinin, lipase, amylases
Detergent industry	Amylases, lipases, cellulases (stainzyme, savinases), proteases

In 2000, Cullinan et al., reported an outbreak of asthma in a detergent factory that was using encapsulated enzymes. They conducted SPT and serum specific-IgE measurements by RAST to amylase, protease and cellulase from 342 employees working in the factory, recording whether individuals had upper and/or lower respiratory work-related symptoms. They showed that 26% (n=90) of the employees had a positive SPT to one or more enzymes, with positive tests to amylase being the most common at 23% (n=80). Overall, they estimated there were 50 cases of occupational asthma in the workforce and that sensitisation was associated with levels of airborne enzyme exposure which was related to specific job roles.

More recently, Budnik et al., (2016) reported a cross-sectional study of allergic sensitisation amongst workers from various industries that were exposed to modified enzymes. They obtained serum samples from 813 workers based in the food, chemical, detergent and pharmaceutical industries, whom were all occupationally exposed to enzymes. They measured serum specific-IgE using a fluorescence enzyme-labelled immunoassay, showing

that 23% of all exposed workers had specific-IgE antibodies against their workplace enzymes, with the highest sensitisation frequency observed for α-amylase (44%), followed by stainzyme (41%) and pancreatinin (35%). In a smaller subset of workers (n=134) from the same study, a higher prevalence of sensitisation to enzymes was shown in those clinically diagnosed with work-related symptoms, compared with asymptomatic exposed workers.

Airborne

The few research papers that have measured airborne levels of enzymes used in the baking industry have mainly measured α-amylase exposure, with little or no data on other enzymes (Baur et al., 1988; Merget et al., 2001; Quirce et al., 2002). Vanhanen et al., (1996) was able to use a dot blot technique with specific commercial antibodies to measure levels of airborne xylanase in four Finnish bakeries. High volume sampling at a flow rate of 25m^3/h and glass fibre filters were used. They found that concentrations of airborne xylanase varied widely from 1-200ng/m^3.

In a larger study, sensitive assays were developed to measure airborne enzymes in 195 air samples taken across 55 British craft baking establishments (Elms et al., 2006). They collected personal 'long-term' samples in the workers' breathing zone, using IOM sampling heads (patented by the Institute of Occupational Medicine) with glass fibre filters at a flow rate of 2 litres per minute. By conjugating peroxidase labels to polyclonal antibodies, they were able to detect amyl-glucosidase (9%), fungal α-amylase (6%) and bacterial amylase (7%) in air samples (n = 195), taken from 55 craft baking establishments. The only enzyme not detected in air samples was glucose oxidase.

Sensitisation to a panel of eight improver enzymes used in two UK supermarket bakeries

In a recent study, Jones et al., (2016) assessed the prevalence of allergic sensitisation in bakers (n = 269) from two large UK supermarket scratch bakeries to a panel of eight improver enzymes, between 2008 and 2012. Scratch bakeries use raw ingredients to make bread, rather than purchasing ready-made dough as some other supermarket bakeries do. Serum specific-IgE was measured to the enzymes using RAST. They found that 25% of bakers

were sensitised to one or more enzyme. Of those not sensitised to flour or fungal α-amylase (n=187), 5% were sensitised to one or more other enzymes. Table 6-3 shows the sensitisation rates to each of the improver enzymes (Jones et al., 2016). Current diagnostic testing for baker's asthma relies on determining sensitisation to either flour or α-amylase. This study shows that sensitisation can occur to eight other enzymes currently used in the baking industry.

Table 6-3 Sensitisation to improver enzymes with species of origin and enzyme function, stratified by sensitisation to flour and/or α-amylase. Taken and adapted from Jones et al., (2016).

Improver enzyme	Sensitisation to improver enzyme (%)			Species of origin	Function
	All bakers	Bakers co-sensitised to flour and/or fungal α-amylase	Bakers not sensitised to flour or α-amylase		
Maltogenic amylase	13/268 (5)	11/81 (14)	2/187 (1)	*Bacillus subtilis*	Breaks down starch, produces maltose sugar.
Cellulase	12/122 (10)	9/27 (33)	3/95 (3)	*Trichoderma reesei*	Improves crumb structure and colour.
Fungal xylanase	41/268 (15)	37/82 (45)	4/186 (2)	Not given	Decreases the rate of crumb firming during storage.
Phospholipase	8/121 (7)	7/26 (27)	1/95 (1)	*Aspergillus oryzae*	Used in cheese production to reduce the loss of fat and milk solids.
Lipase	17/147 (12)	17/55 (31)	0/92 (0)	Not given	Improves dough stability and gives finer crumb structure.
Bacterial xylanase	36/269 (13)	36/86 (42)	0/183 (0)	*Bacillus subtilis*	Increase the specific volume of bread.
Glucose oxidase	30/268 (11)	28/85 (33)	2/183 (1)	*Aspergillus niger*	Oxidises ascorbic acid, modifies gluten.
Bacterial α-amylase	16/269 (6)	15/86 (17)	1/183 (0.5)	*Bacillus subtilis*	Breaks down starch.
Any of the above	66/269 (25)	57/86 (66)	9/187 (5)	-	-

The panel of improver enzymes used in the baking industry is constantly changing and often the supermarket is unaware which enzymes are contained within the improver mix. Most occupational asthma clinics measure serum specific-IgE to flour and α-amylase only, which leaves the possibility that bakers who are not sensitised to flour and α -amylase but sensitised to other improver enzymes may remain undiagnosed. Unfortunately, due to commercial secrecy, the enzymes within the 'improver' mix are not identified, so there is a need for a method to be able to detect and identify enzymes used within the improver mix, to aid accurate determination of potential allergic sensitisation and cases of occupational asthma (personal communication with Dr M. Jones).

In this chapter we sought to use the optimised MS-based method to identify these genetically modified enzymes. This would be as a 'proof of principle' application to determine whether this novel MS-based approach has the potential to identify unknown allergenic enzymes found in bakeries and various other workplaces.

Objective

To identify the allergenic proteins, present in eight improver enzymes, used in two large UK supermarket scratch bakeries.

Aims

1. Detect specific-IgE binding proteins from the eight improver enzymes, using an optimised western blot method.
2. Identify specific-IgE binding proteins from the eight improver enzymes, using optimised MS-based methods.

6.2 Methods

6.2.1 Sample collection

Samples of eight 'improver' enzymes used in two large UK supermarket scratch bakeries were obtained and extracted as described in Jones et al., (2016). Each enzyme was extracted overnight in 0.01 mol/l ammonium carbonate at 4°C, dialysed overnight against distilled water, lyophilised and stored at -20°C. The eight improver enzymes were maltogenic amylase, cellulase, fungal xylanase, phospholipase, lipase, bacterial xylanase, glucose oxidase and bacterial alpha amylase, as listed previously in Table 6-3 (on page 228).

6.2.2 Protein quantification, SDS-PAGE and staining

Methods for protein quantification, SDS-PAGE and staining were followed as described in Chapter 3, sections 3.2.2-3.2.5.

Briefly, protein content was quantified in a 5mg/ml solution of each of the eight lyophilised enzyme extracts dissolved in dH_2O, using a *DC*™ Protein Assay kit. Proteins were separated using the NuPAGE™ Bis-Tris electrophoresis system according to the Laemmli method and protein molecular weight was calculated according to a prestained protein standard. The gel was stained for proteins using a 0.1% R-250 Coomassie dye.

6.2.3 Serum samples

I used 28 stored serum samples from UK supermarket scratch bakers that were collected for the Jones et al., (2016) study. Serum samples were collected between 2008-2012 from 269 bakers working in two UK supermarkets that were using the improver enzymes. Serum was chosen based on having a high level of specific-IgE to one or more improver enzyme, as previously determined by RAST (Table 6-4, on page 233). Specific-IgE ≥2% binding was considered positive. Levels of specific-IgE are given for serum used in both individual and pooled western blot experiments. The previously determined levels of specific-IgE binding to flour and alpha α-amylase, as determined by ImmunoCAP (flour and α-amylase) and RAST (just α-amylase), are given for all serum that were used in this study. Serum from either

exposed non-sensitised bakers or non-exposed mouse sensitised individuals was used as the control.

6.2.4 ECL western blot

For detection of specific-IgE binding proteins I used optimised ECL western blot methods, described in full in Chapter 3, section 4.2.7.3. Briefly, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane by wet transfer and incubated with phosphate-blocking buffer. Serum incubation was with either pooled or individual samples:

- When using pooled serum (as indicated with an asterisk in Table 6-4) the entire membrane was incubated with serum for 16 hours at 4°C.
- When using individual serum, the membrane was cut using a disposable scalpel into 7, 8, 9 or 10 strips, depending on the number of individual sera used. In addition, one membrane strip was incubated with pooled serum, and one membrane strip was incubated with control serum. All membrane strips were incubated in serum for 16 hours at 4°C.

The membrane was washed and probed with goat anti-human IgE conjugated with horseradish peroxidase, before detection with an ECL substrate on a ChemiDoc™ imaging device and the protein molecular weight was calculated according to a prestained protein standard.

Table 6-4 Level of specific-IgE antibody (% binding) to eight improver enzyme extracts as determined by RAST amongst 28 scratch bakers from two UK supermarkets.
Specific-IgE levels given in shaded boxes were used for individual western blots.

Serum	**Improver enzymes** (% specific IgE binding, as determined by RAST)								**Flour** (kU/L of specific IgE, as determined by ImmunoCAP)	**Alpha amylase** (kU/L of specific IgE, as determined by ImmunoCAP and (RAST % binding))
	Maltogenic amylase	Cellulase	Fungal xylanase	Phospholipase	Lipase	Bacterial xylanase	Glucose oxidase	Bacterial alpha amylase		
1	5.26*	4.34*		4.41*					0.4	<0.35 (1.6)
2	4.83*	4.09*		7.54*					9.4	<0.35 (11.1)
3	3.23*	4.17*		2.73					0.4	<0.35 (0.5)
4	2.97*	3.53		3.94					10.7	0.4 (0.6)
5	2.84*								9.2	<0.35 (1.8)
6	2.74								2.7	<0.35 (0.5)
7	2								1.3	<0.35 (1.3)
8		26.4*		35*					0.5	<0.35 (19.6)
9		2.18		3.98*					10.3	<0.35 (0.8)
10			12.11*		15.45*	13.44*			44.3	<0.35 (0.6)
11			6.27*		4.99	5.4	5.32*	2.13*	4.9	<0.35 (18.5)
12			5.3*						1.4	<0.35 (1.9)
13			6.5		9.69*	13.6*			19.9	0.4 (7.7)
14			3.26						0.5	<0.35 (0.5)
15			3.41*		7.54*				1.6	<0.35 (16.5)
16					3.32*				0.6	<0.35 (1.5)
17					2.53	8*	6.66*		55.4	<0.35 (1.8)
18					2.44	3.87*			4.8	<0.35 (3.9)
19						3.19		2.55*	2.3	<0.35 (7.2)
20						4.08*			6	<0.35 (1.2)
21					4.08*	3.42			0.6	<0.35 (1.2)
22			3.5*				4.44*		2.7	<0.35 (1.9)
23							2.54*		11.2	<0.35 (1.8)
24							2.43*		0.7	<0.35 (8.8)
25								3.79*	9.8	5.5 (16)
26								3.6*	0.8	<0.35 (18.3)
27		4*							1.4	<0.35 (0.6)
28				4.5					1.2	1.3 (5.8)

*serum samples use for pooled western blots

6.2.5 Heat map analysis

Heat map analysis was used to display the frequency of proteins with specific-IgE binding to sensitised individuals. Row clusters were used to represent the allergic profile of each individual baker to an enzyme extract, and column clusters to represent the number of bakers sensitised to a specific-IgE binding region. Results showing specific-IgE binding regions with the highest reactivity amongst individuals were chosen for MS analysis.

6.2.6 Mass spectrometry

The following methods: tryptic digestion and extraction of protein, nano LC-MS/MS and data processing were all achieved with training and supervision from Prof. Peter Briza at the Division of Allergy and Immunology at the University of Salzburg.

6.2.6.1 In-gel tryptic digestion

Methods for in-gel tryptic digestion were followed using a ProteoExtract All-in-One Trypsin Digestion Kit, as described in Chapter 3, section 3.2.7.1. Briefly, specific-IgE binding proteins were excised from Coomassie stained polyacrylamide gels and washed, dehydrated and dried. Trypsin was used to digest the proteins into peptides and after digestion, the sample was stored at -20°C prior to peptide extraction.

6.2.6.2 Extraction of peptides

Methods for peptide extraction were followed as described in Chapter 3, section 3.2.7.3. Briefly, a 10µl ZipTip$_{C18}$ tip was used to pick up the peptide solution after digestion and de-salt the solution; producing concentrated peptides. The peptide solution was checked for acidity using pH paper and addition of 13µl of TFA. The sample was dried down, resuspended in 0.1% TFA and sonicated before transfer into a MS vial.

6.2.6.3 Nano liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Methods for nano LC-MS/MS were followed as described in Chapter 3, section 3.2.7.4. Briefly, MS vials were placed into an automated EASY nano liquid chromatograph and peptides were resolved by reverse phase chromatography. This was coupled to an Orbitrap Velos Pro by ESI in which peptides were ionised by charged hydrogen ions and then separated based on their mass-to-charge ratio (*m/z*). Raw data was processed by PEAKS Studio. Peptide matches were sought within the UniProt database. The search was limited to a 95% protein identification threshold and we removed proteins derived from mammal or bird species or with no unique peptides.

6.3 Results

6.3.1 Protein content in 'improver' enzyme extracts

The protein content in the 5mg/ml solutions of eight improver enzyme extracts varied from a minimum of 0.97µg/µl in maltogenic amylase to a maximum of 3.38µg/µl in bacterial α-amylase, as shown in Table 6-5.

Table 6-5 Protein content in improver enzyme extracts.

Improver enzyme extract	Protein (µg/µl)
Maltogenic amylase	0.97
Cellulase	1.54
Fungal xylanase	2.48
Phospholipase	1.89
Lipase lipopan	1.20
Bacterial xylanase	1.69
Glucose oxidase	1.78
Bacterial α-amylase	3.38

6.3.2 Protein separation of the eight improver enzyme extracts

I applied 10µg, 15µg and 20µg from each of the eight enzyme extracts (as determined by protein quantification) to Coomassie stained SDS-PAGE (Figure 6-2:A,B and C respectively). Using 10µg, protein was observed for five out of eight enzyme extracts (there is some staining in lane 8 of Figure 6-2:A, which is likely to be an artefact, given that staining is not observed at this molecular weight in Figure 6-2:B or C). Using 15 and 20µg, protein was observed for all eight enzyme extracts. The molecular weight of all proteins varied from ~12 – 200kDa amongst the extracts. The molecular weight of the most intense bands for each extract is listed in Table 6-6 (on page 238). Different molecular weight bands were identified for all of the improver enzymes. There were other less intense bands visible for all improver enzymes when 15 and 20µg of protein was applied to SDS-PAGE.

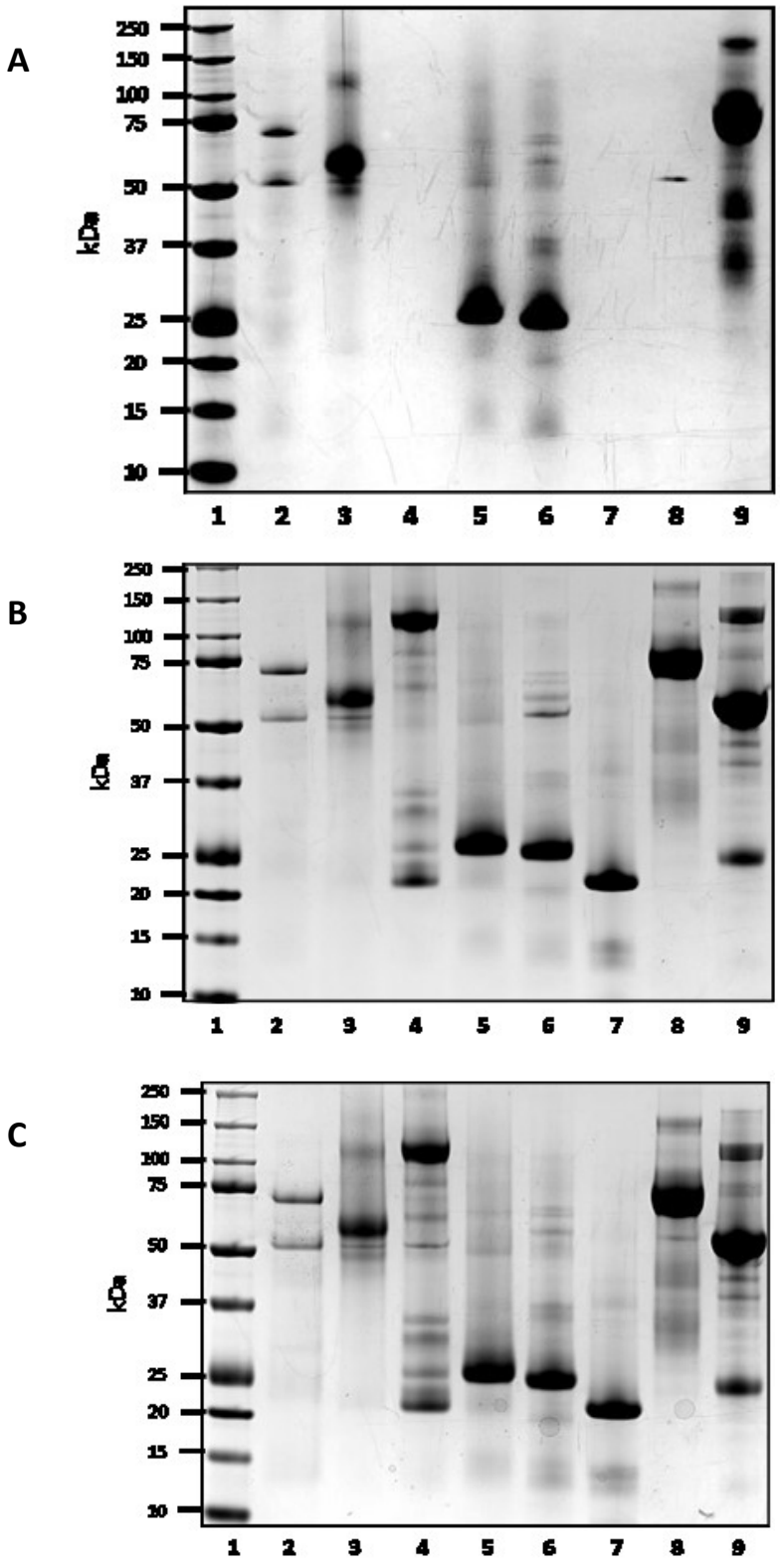


Figure 6-2 Coomassie stained SDS-PAGE of 10µg (A), 15µg (B) and 20µg (C) of eight improver enzyme extracts. Protein was applied in a total volume of 25µl. Lane 1: molecular weight marker, lane 2: maltogenic amylase, lane 3: cellulase, lane 4: fungal xylanase, lane 5: phospholipase, lane 6: lipase, lane 7: bacterial xylanase, lane 8: glucose oxidase, lane 9: bacterial alpha amylase.

Table 6-6 Approximate molecular weight of the most intense bands (kDa) observed from eight improver enzyme extracts by Coomassie stained SDS-PAGE.

Improver enzyme extract	Approximate molecular weight of intense bands (kDa)
Maltogenic amylase	53, 71
Cellulase	60
Fungal xylanase	22, 121
Phospholipase	27
Lipase lipopan	26, 54
Bacterial xylanase	22
Glucose oxidase	74
Bacterial α-amylase	25, 56, 128

6.3.3 ECL western blot of eight improver enzyme extracts using pooled sensitised serum

I applied 20µg of protein from each of the eight enzyme extracts to an ECL western blot using pooled serum from sensitised workers. In Figure 6-3 below specific-IgE binding was observed for all enzymes (lanes 2-9), except for glucose oxidase (lane 8). The approximate molecular weight of specific-IgE binding regions varied from 19-250kDa. These are listed in Table 6-7 (on page 240). The most intense specific-IgE binding was observed to phospholipase and lipase lipopan enzyme extracts (lanes 5 and 6).

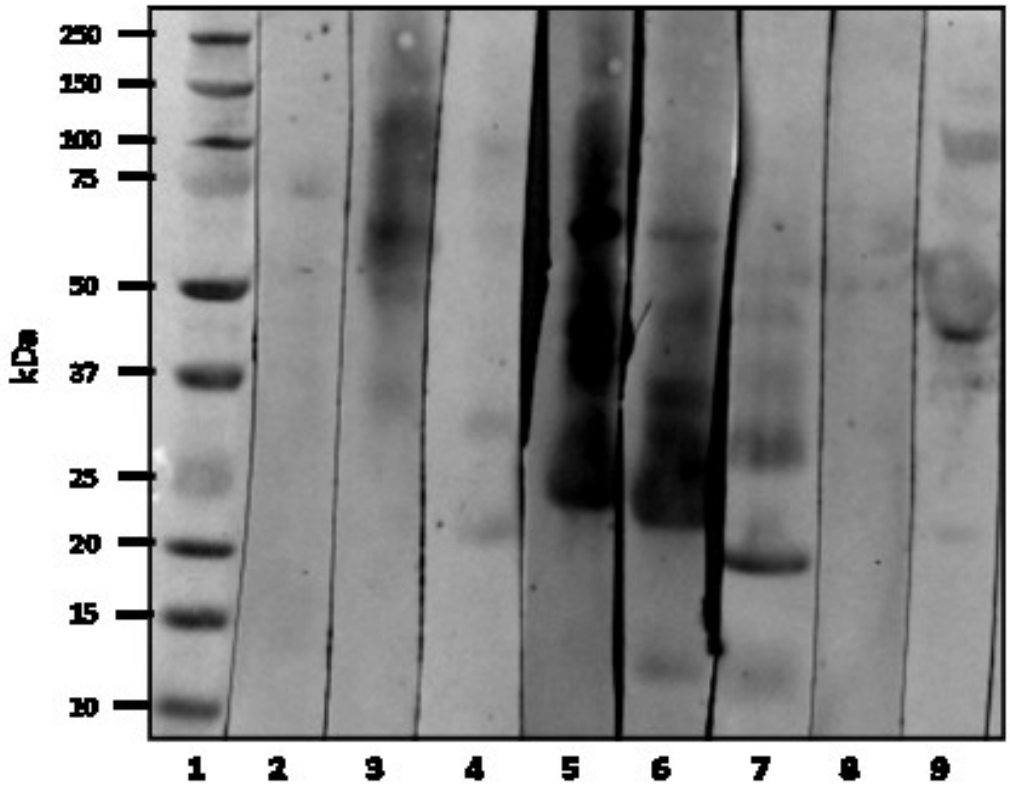


Figure 6-3 ECL western blot of eight improver enzyme extracts. 20µg of protein from each extract was applied to SDS-PAGE, each in a total volume of 25µl. Protein was transferred to a nitrocellulose membrane under normal conditions, then blocked for 1 hour 30 minutes, incubated in pooled serum (1:5 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in goat anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution. Molecular weight was determined with a prestained protein standard. Lane 1: molecular weight marker, lane 2: maltogenic amylase, lane 3: cellulase, lane 4: fungal xylanase, lane 5: phospholipase, lane 6: lipase, lane 7: bacterial xylanase, lane 8: glucose oxidase, lane 9: bacterial alpha amylase.

Table 6-7 Approximate molecular weight of the most intense bands (kDa) observed by Coomassie stained SDS-PAGE, compared with specific-IgE binding regions (kDa) observed using pooled sensitised serum in an ECL western blot, from eight improver enzyme extracts.

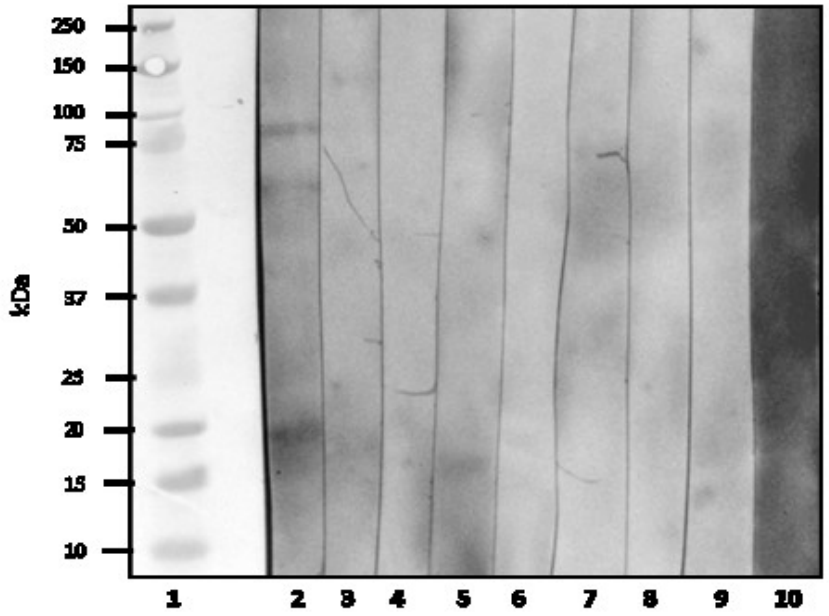
Improver enzyme extract	**Approximate molecular weight of intense bands by Coomassie staining (kDa)** see Figure 6-2:C	**Approximate molecular weight of specific IgE binding regions by ECL western blot (kDa)** see Figure 6-3
Maltogenic amylase	53, 71	74
Cellulase	60	35, 50, 62, 115
Fungal xylanase	22, 121	21, 31
Phospholipase	27	24, 44, 64
Lipase lipopan	26, 54	24, 34, 62
Bacterial xylanase	22	19, 28
Glucose oxidase	74	-
Bacterial α-amylase	25, 56, 128	43, 97

6.3.4 ECL western blots of eight improver enzyme extracts using individual sensitised serum

I applied an ECL western blot to each of the eight enzyme extracts using a minimum of 20µg to detect protein and using individual serum from sensitised workers. Specific-IgE binding proteins were observed to every improver enzyme extract as shown in the following 8 figures (Figure 6-4 to Figure 6-11). Each western blot figure is accompanied by a heat map analysis of the enzyme extract.

6.3.4.1 Maltogenic amylase

A



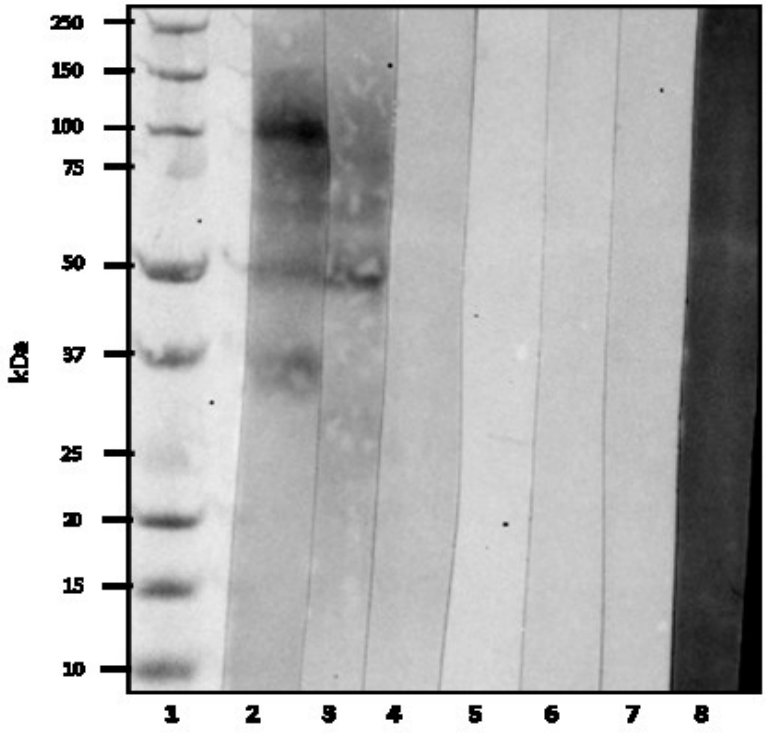
B

Level of specific IgE antibody from individual baker serum, as determined by RAST (Lane no.)	~Molecular weight of specific-IgE binding region			Total number of specific- IgE binding regions present per individual baker
	16-18	60	80	
5.26 (2)	■	■	■	3
4.83 (3)	■			1
3.23 (4)				0
2.97 (5)	■			1
2.84 (6)				0
2.74 (7)				0
2 (8)				0
	3	1	1	
	Number of bakers sensitised to a specific-IgE binding region			

Figure 6-4 (A) ECL western blot and (B) heat map analysis of maltogenic amylase extract using individual serum. A minimum of 20µg of protein (per strip) was applied to SDS-PAGE, in a total volume of >150µl. Protein was transferred to a nitrocellulose membrane under normal conditions, then blocked for 1 hour 30 minutes. The membrane was then split into 10 strips and each strip was incubated in serum (1:5 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution. The molecular weight was determined with a prestained protein standard in lane 1. Lanes 2-8: individual sensitised baker serum, lane 9: pooled sensitised baker serum, lane 10: individual non-exposed serum.

6.3.4.2 Cellulase

A



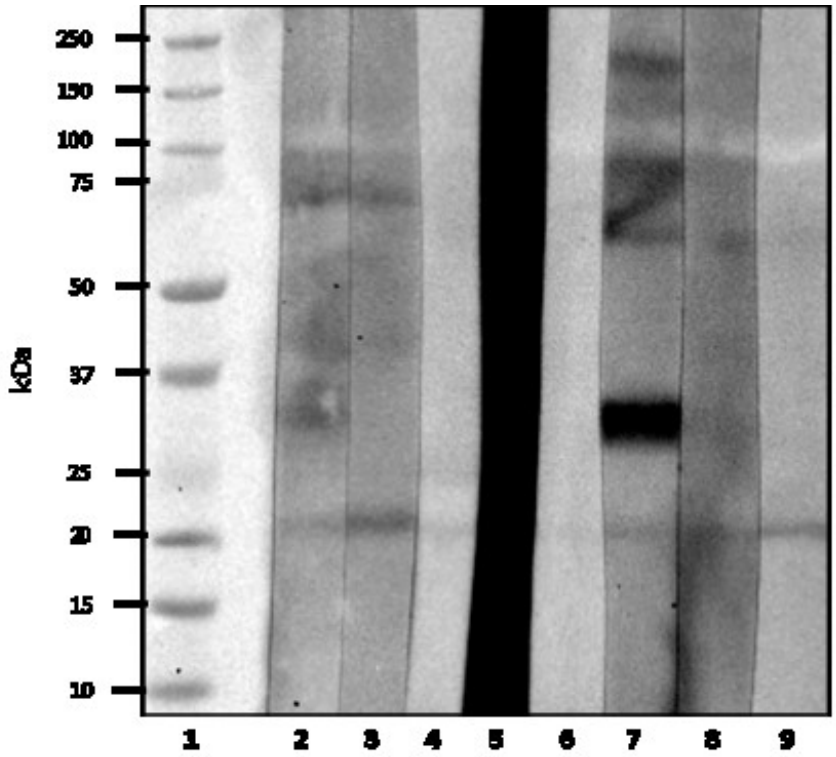
B

Level of specific IgE antibody from individual baker serum, as determined by RAST (Lane no.)	~Molecular weight of specific-IgE binding region (kDa)						Total number of specific- IgE binding regions present per individual baker
	37	49	65	87	100	150	
26.4 (2)	■	■	■	■	■	■	6
4.34 (3)		■	■	■	■		4
4.09 (4)							0
3.53 (5)							0
2.18 (6)							0
	1	2	2	2	2	1	
	Number of bakers sensitised to a specific-IgE binding region						

Figure 6-5 (A) ECL western blot and (B) heat map analysis of cellulase extract using individual serum. A minimum of 20µg of protein (per strip) was applied to SDS-PAGE, in a total volume of >150µl. Protein was transferred to a nitrocellulose membrane under normal conditions, then blocked for 1 hour 30 minutes. The membrane was then split into 8 strips and each strip was incubated in serum (1:5 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution. The molecular weight was determined with a prestained protein standard in lane 1. Lanes 2-6: individual sensitised baker serum, lane 7: pooled sensitised baker serum, lane 8: individual non-exposed serum.

6.3.4.3 Fungal xylanase

A



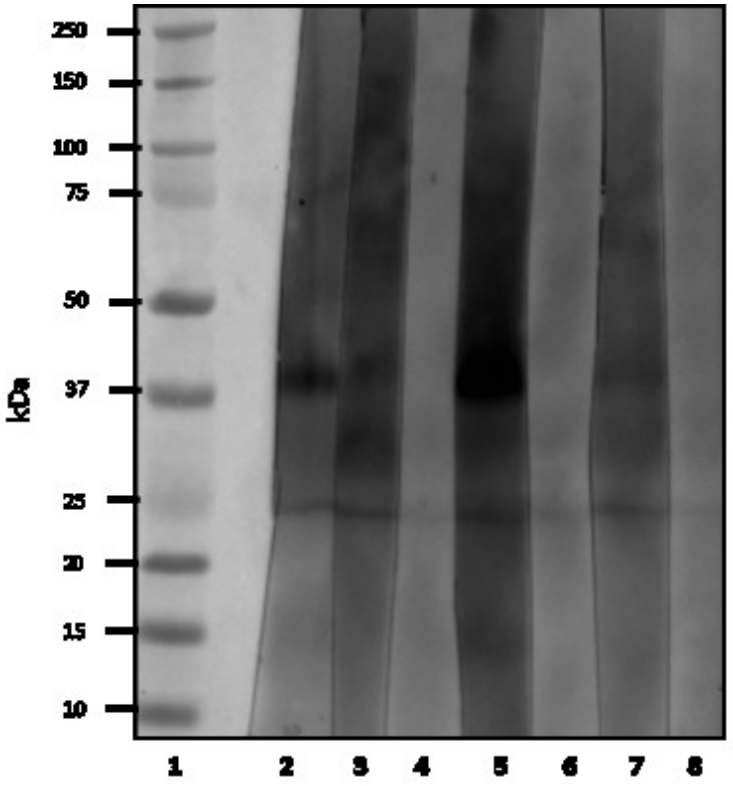
B

Level of specific IgE antibody from individual baker serum, as determined by RAST (Lane no.)	~Molecular weight of specific-IgE binding region (kDa)							Total number of specific- IgE binding regions present per individual baker
	35	42	63	72	87	145	203	
12.11 (2)	■	■		■				3
6.27 (3)	■			■				2
5.3 (3)								0
6.5 (4)								0
3.26 (5)								0
3.41 (6)	■		■		■	■	■	5
	3	1	1	2	1	1	1	
	Number of bakers sensitised to a specific-IgE binding region							

Figure 6-6 (A) ECL western blot and (B) heat map analysis of fungal xylanase extract using individual serum. A minimum of 20µg of protein (per strip) was applied to SDS-PAGE, in a total volume of >150µl. Protein was transferred to a nitrocellulose membrane under normal conditions, then blocked for 1 hour 30 minutes. The membrane was then split into 9 strips and each strip was incubated in serum (1:5 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution. The molecular weight was determined with a prestained protein standard in lane 1. Lanes 2-7: individual sensitised baker serum, lane 8: pooled sensitised baker serum, lane 9: individual exposed non-sensitised baker serum.

6.3.4.4 Phospholipase

A



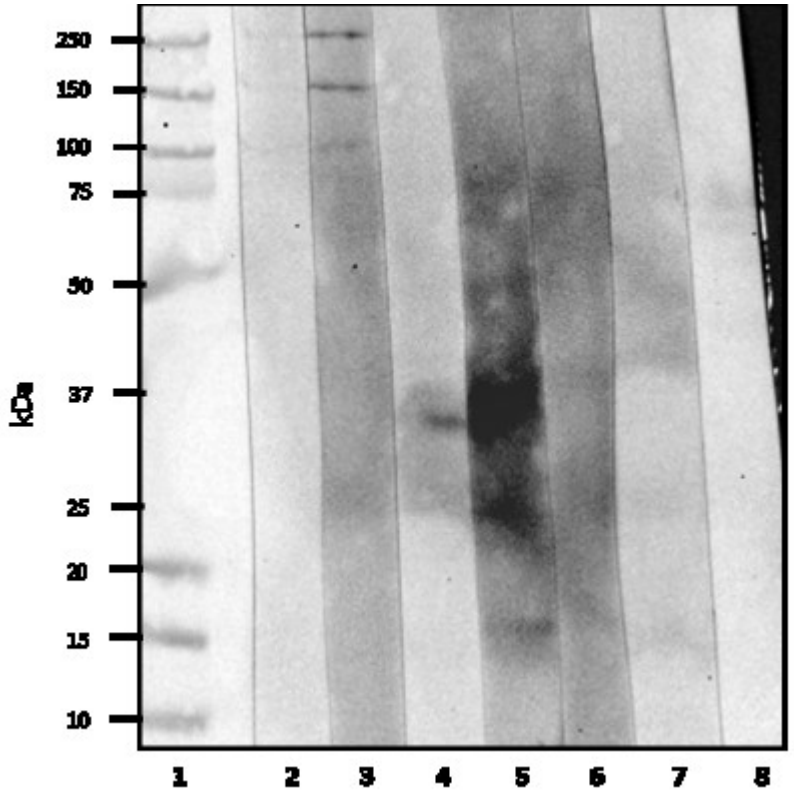
B

Level of specific IgE antibody from individual baker serum, as determined by RAST (Lane no.)	~Molecular weight of specific-IgE binding region (kDa)			Total number of specific- IgE binding regions present per individual baker
	14	30	40	
7.54 (2)			■	1
4.41 (3)		■	■	2
3.98 (4)				0
3.94 (5)	■		■	2
2.73 (6)				0
	1	1	3	
	Number of bakers sensitised to a specific-IgE binding region			

Figure 6-7 (A) ECL western blot and (B) heat map analysis of phospholipase extract using individual serum. A minimum of 20µg of protein (per strip) was applied to SDS-PAGE, in a total volume of >150µl. Protein was transferred to a nitrocellulose membrane under normal conditions, then blocked for 1 hour 30 minutes. The membrane was then split into 8 strips and each strip was incubated in serum (1:5 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution. The molecular weight was determined with a prestained protein standard in lane 1. Lanes 2-6: individual sensitised baker serum, lane 7: pooled sensitised baker serum, lane 8: individual exposed non-sensitised baker serum.

6.3.4.5 Lipase

A



B

Level of specific IgE antibody from individual baker serum, as determined by RAST (Lane no.)	~Molecular weight of specific-IgE binding region (kDa)						Total number of specific- IgE binding regions present per individual baker
	16	25	29	35	38	49	
4.99 (2)							0
3.32 (3)		■					1
2.53 (4)		■	■	■			3
9.69 (5)	■	■	■	■	■	■	6
2.44 (6)	■	■			■	■	4
	2	4	2	2	2	2	

Number of bakers sensitised to a specific-IgE binding region

Figure 6-8 (A) ECL western blot and (B) heat map analysis of lipase extract using individual serum. A minimum of 20µg of protein (per strip) was applied to SDS-PAGE, in a total volume of >150µl. Protein was transferred to a nitrocellulose membrane under normal conditions, then blocked for 1 hour 30 minutes. The membrane was then split into 8 strips and each strip was incubated in serum (1:5 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution. The molecular weight was determined with a prestained protein standard in lane 1. Lanes 2-6: individual sensitised baker serum, lane 7: pooled sensitised baker serum, lane 8: individual exposed non-sensitised baker serum.

6.3.4.6 Bacterial xylanase

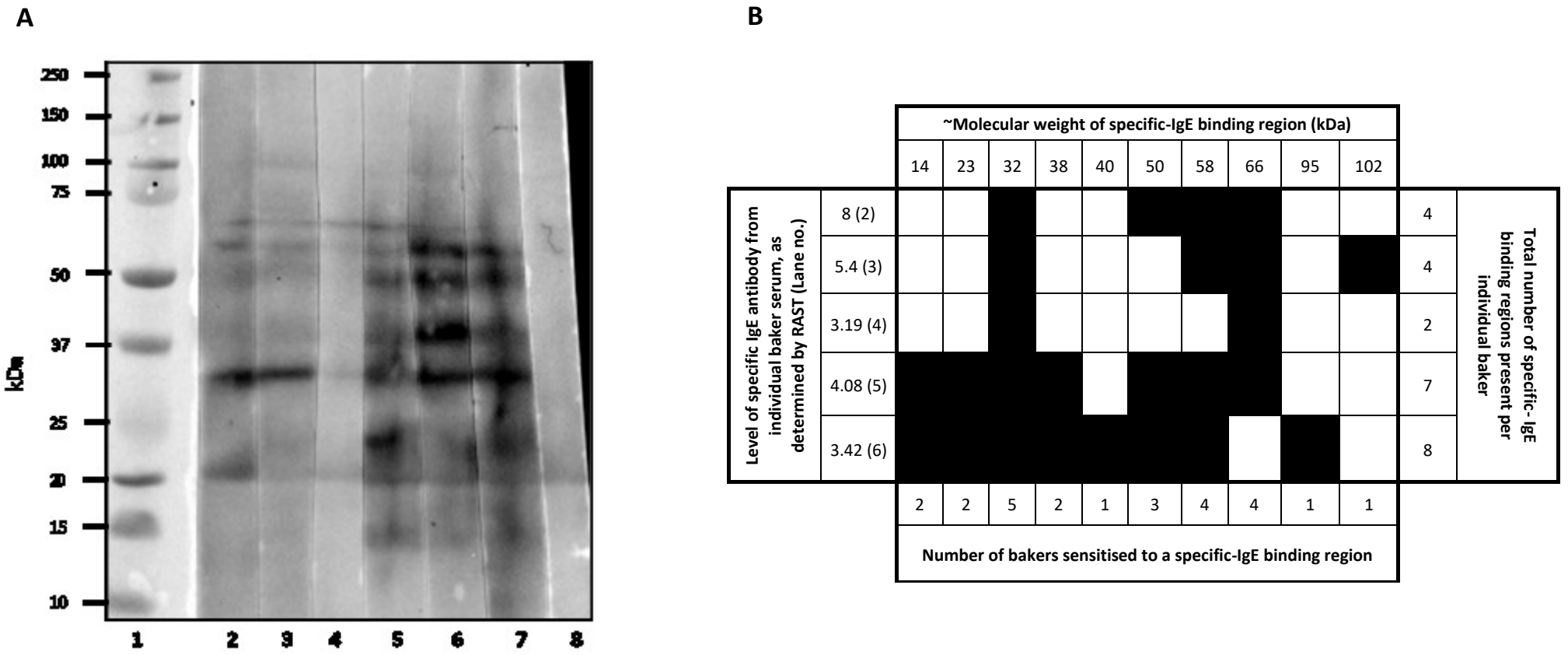
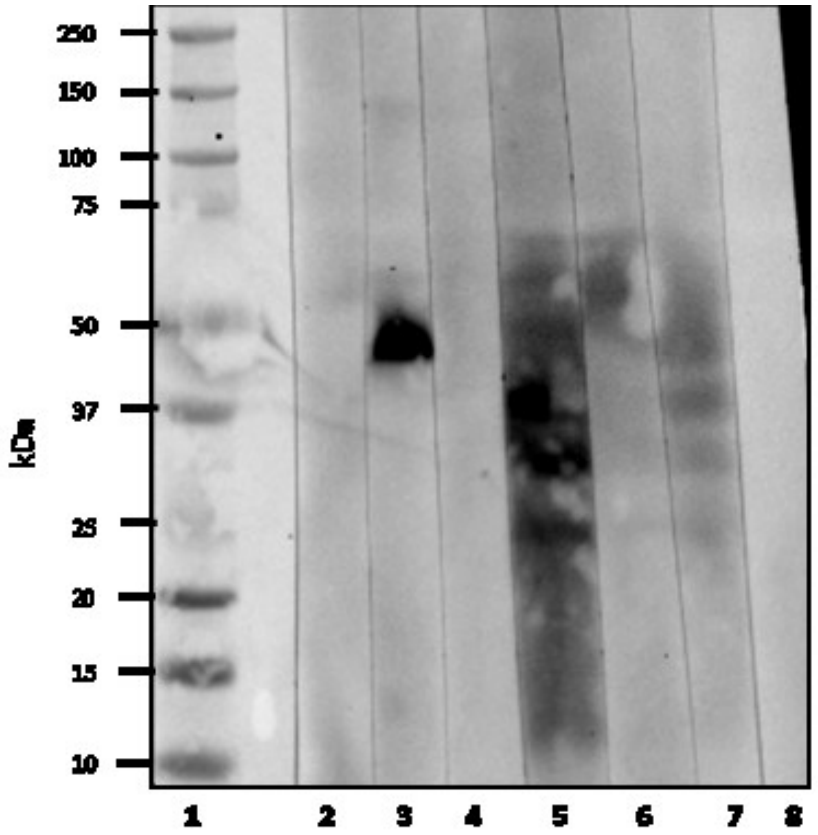


Figure 6-9 (A) ECL western blot and (B) heat map analysis of bacterial xylanase extract using individual serum. A minimum of 20µg of protein (per strip) was applied to SDS-PAGE, in a total volume of >150µl. Protein was transferred to a nitrocellulose membrane under normal conditions, then blocked for 1 hour 30 minutes. The membrane was then split into 8 strips and each strip was incubated in serum (1:5 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution. The molecular weight was determined with a prestained protein standard in lane 1. Lanes 2-6: individual sensitised baker serum, lane 7: pooled sensitised baker serum, lane 8: individual exposed non-sensitised baker serum.

6.3.4.7 Glucose oxidase

A



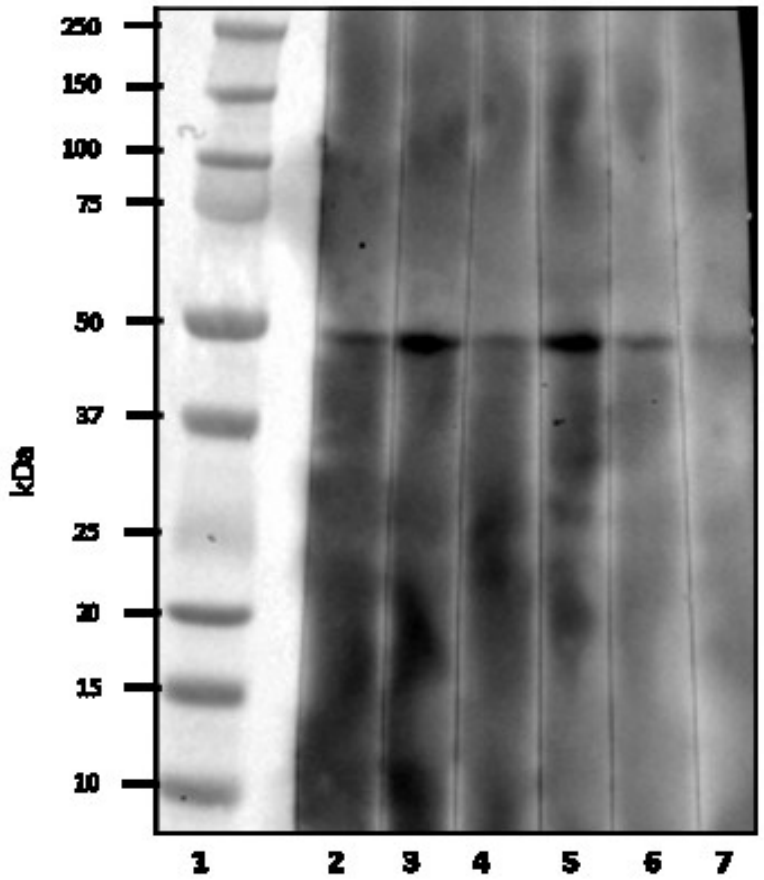
B

Level of specific IgE antibody from individual baker serum, as determined by RAST (Lane no.)	~Molecular weight of specific-IgE binding region (kDa)								Total number of specific-IgE binding regions present per individual baker
	25	31	38	45	47	56	66	138	
5.32 (2)									0
4.44 (3)				■	■	■		■	4
6.66 (4)									0
2.54 (5)	■	■	■		■	■	■		6
2.43 (6)	■	■				■	■		4
Number of bakers sensitised to a specific-IgE binding region	2	2	1	1	2	3	2	1	

Figure 6-10 (A) ECL western blot and (B) heat map analysis of glucose oxidase extract using individual serum. A minimum of 20µg of protein (per strip) was applied to SDS-PAGE, in a total volume of >150µl. Protein was transferred to a nitrocellulose membrane under normal conditions, then blocked for 1 hour 30 minutes. The membrane was then split into 8 strips and each strip was incubated in serum (1:5 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution. The molecular weight was determined with a prestained protein standard in lane 1. Lanes 2-6: individual sensitised baker serum, lane 7: pooled sensitised baker serum, lane 8: individual exposed non-sensitised baker serum.

6.3.4.8 Bacterial alpha amylase

A



B

Level of specific IgE antibody from individual baker serum, as determined by RAST (Lane no.)	~Molecular weight of specific-IgE binding region (kDa)					Total number of specific IgE binding regions present per individual baker
	21	23	26	27	140	
3.79 (2)	■			■		2
3.6 (3)	■			■		2
2.55 (4)		■	■	■	■	4
2.13 (5)	■		■	■	■	4
	3	1	2	4	2	
	Number of bakers sensitised to a specific-IgE binding region					

Figure 6-11 (A) ECL western blot and (B) heat map analysis of bacterial alpha amylase extract using individual serum. A minimum of 20µg of protein (per strip) was applied to SDS-PAGE, in a total volume of >150µl. Protein was transferred to a nitrocellulose membrane under normal conditions, then blocked for 1 hour 30 minutes. The membrane was then split into 7 strips and each strip was incubated in serum (1:5 dilution) for 16 hours at 4°C, washed in PBS-T, incubated in anti-human IgE (1:5000 dilution) for 30 minutes and detection was using 2ml of ECL substrate solution. The molecular weight was determined with a prestained protein standard in lane 1. Lanes 2-5: individual sensitised baker serum, lane 6: pooled sensitised baker serum, lane 7: individual exposed non-sensitised baker serum.

The following in Table 6-8 summarises the number of specific-IgE binding regions that were detected in each enzyme extract, the number of sensitised bakers (as determined by RAST) that showed specific-IgE binding to each extract and the binding region from each enzyme that reacted with the highest number of sensitised bakers.

Table 6-8 Summary of the number of specific-IgE binding regions, number of sensitised bakers showing specific-IgE binding and binding regions (kDa) that reacted with the highest number of sensitised bakers, from eight improver enzyme extracts, observed by ECL western blot.

Improver enzyme extract	Number of specific-IgE binding regions	Number of sensitised bakers showing specific-IgE (n=27)	Most reactive specific-IgE binding region (kDa)
Maltogenic amylase	3	3	16-18
Cellulase	6	2	100*
Fungal xylanase	7	3	35
Phospholipase	3	3	40
Lipase lipopan	6	4	25
Bacterial xylanase	10	5	32
Glucose oxidase	8	3	56
Bacterial α-amylase	5	4	27

*There were four different specific-IgE binding regions in cellulase that were all observed to have binding from two sensitised bakers, but the 100kDa region showed the most intense binding.

The improver enzyme extract, bacterial xylanase gave the highest number of specific-IgE binding regions (n=10) and had the highest number of sensitised bakers showing specific-IgE binding (n=5). Glucose oxidase had the next highest number of specific-IgE binding regions (n=8). From these results I decided to excise specific-IgE binding proteins from the enzymes, bacterial xylanase and glucose oxidase for MS analysis. Limited funding meant not all specific-IgE binding regions from all improver enzyme extracts could be excised for MS analysis in this proof of principle study.

6.3.5 Identification of allergens from bacterial xylanase and glucose oxidase improver enzyme extracts by MS

The following specific-IgE binding proteins from extracts of bacterial xylanase (grown in *Bacillus subtilis*) and glucose oxidase (grown in *Aspergillus niger*) were excised from a Coomassie stained gel and digested for MS analysis (Figure 6-12:A + B). These specific-IgE binding regions were chosen based on which showed binding with the highest number of sensitised bakers.

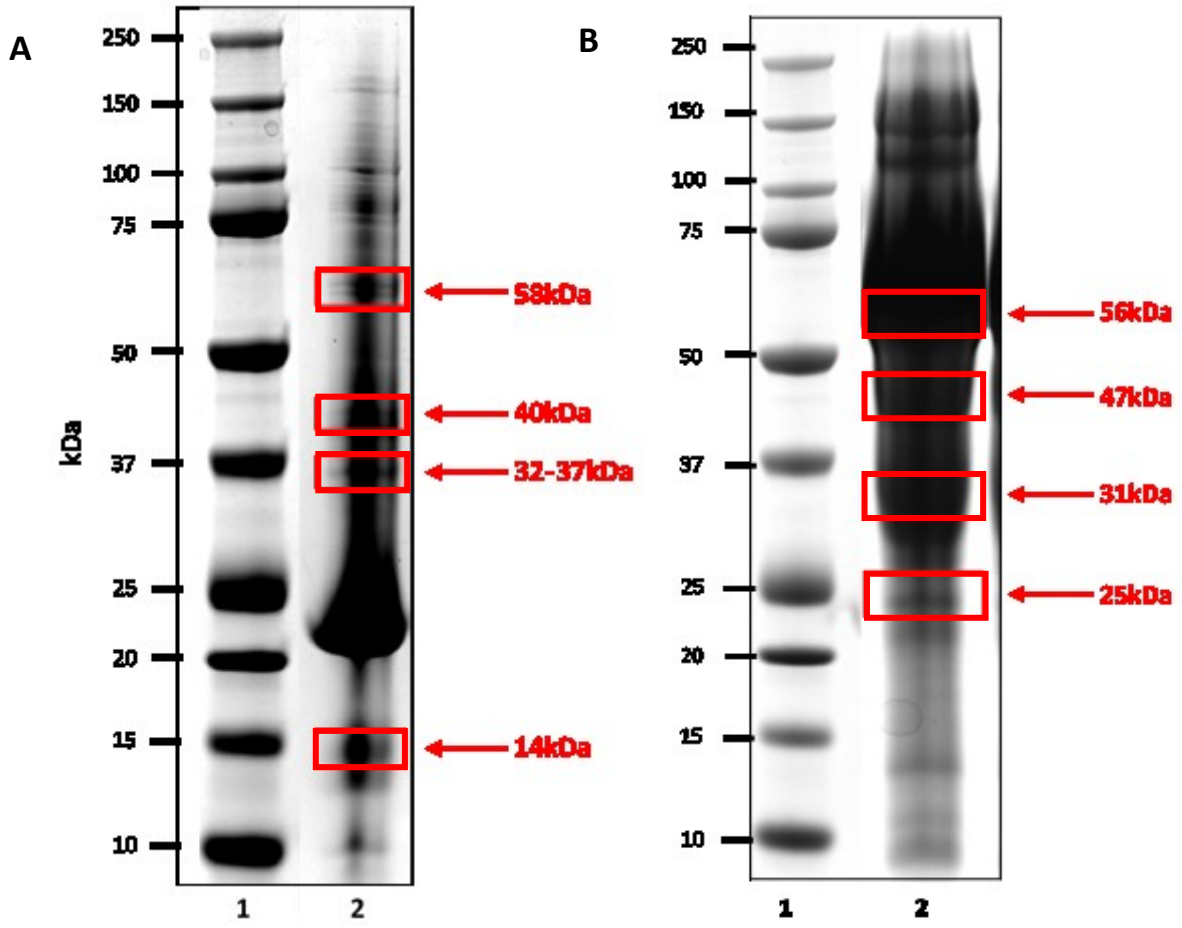


Figure 6-12 In-gel excision of specific-IgE binding proteins from (A) bacterial xylanase and (B) glucose oxidase. 40µg of protein from the bacterial xylanase extract and 140µg of the glucose oxidase extract was applied to SDS-PAGE (lane 2) in a total volume of 25µl and detected using Coomassie. The molecular weight was determined with a prestained protein standard (lane 1).

6.3.5.1 Proteins identified from the bacterial xylanase improver enzyme extract

We identified 1854, 2336, 2868 and 1922 proteins in digest samples excised at 14, 32-37, 40 and 58kDa respectively, from the bacterial xylanase extract.

Protein identifications from an in-gel 14kDa bacterial xylanase digest

The most abundant protein identified from this digest was glucose oxidase from *Aspergillus niger* (Prot Acc: C5J0H1), with a molecular weight of 65kDa, sequence coverage of 33% and 14 unique peptides identified. In Table 6-9 are the top 10 proteins identified from this digest.

Of note:

- An endo-1, 4-beta-xylanase protein (also known as bacterial xylanase) from *Bacillus subtilis* was identified (Prot Acc: Q45VU1), with a molecular weight of 23kDa, sequence coverage of 46% and 2 unique peptides.
- A cell wall-binding protein (Prot Acc: P54484) from *Bacillus subtilis* was identified with a molecular weight of 15kDa, sequence coverage of 68% and 12 unique peptides.
- *and* an alpha-amylase/trypsin inhibitor (Prot Acc: P17314) from *Triticum aestivum* was identified with a sequence coverage of 76% and 1 unique peptide. This identified protein is a known allergen registered as Tri a 30 on the WHO/IUIS nomenclature.

Protein identifications from an in-gel 32-37kDa bacterial xylanase digest

The most abundant protein identified from this digest was porin (Prot Acc: Q8THN2) from an *Acidovorax* species, with a molecular weight of 36kDa, sequence coverage of 61% and 21 unique peptides. In Table 6-10 are the top 10 proteins identified from this digest.

Of note:

- Malate dehydrogenase from *Bacillus subtilis* was identified (Prot Acc: A0A164WTU7) with a molecular weight of 33kDa, sequence coverage of 71% and 6 unique peptides.

- Phosphate binding proteins were also identified from *Variovorax*, *Pantoea* and *Acidovorax*, all with a molecular weight of 36kDa.

Protein identifications from an in-gel 40kDa bacterial xylanase digest

The most abundant protein identified from this digest was porin (Prot Acc: P8ZNQ8) from *Variovorax* species, with a molecular weight of 44kDa, sequence coverage of 67% and 21 unique peptides. In Table 6-11 are the top 10 proteins identified from this digest.

Of note:

- From the *Bacillus subtilis* species, an uncharacterised protein with a molecular weight of 43kDa (Prot Acc: A0A1L9DEB7) and a cell surface protein with a molecular weight of 44kDa (Prot Acc: A0A164Z453) were identified.
- Endo-1, 4-beta-xylanase (bacterial xylanase) (Prot Acc: A0A1J5X4R7) from *Bacillus subtilis* (23kDa) was also identified.

Protein identifications from an in-gel 58kDa bacterial xylanase digest

The most abundant protein identified from this digest was chaperonin (Prot Acc: P9C073) from *Variovorax* species, with a molecular weight of 57kDa, sequence coverage of 71% and 1 unique peptide. In

Table ***6-12*** are the top 10 proteins identified from this digest.

Of note:

- Proteins were identified from the *Triticum aestivum* species.
- Glucose oxidase (Prot Acc: P13006) from *Aspergillus niger* was also identified.

Table 6-9 Proteins identified by nano LC-MS/MS from an in-gel 14kDa bacterial xylanase digest.

Protein name	Species	Accession Number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)
Glucose oxidase	*Aspergillus niger*	C5J0H1	214.86	14	33	65
Glucose oxidase	*Aspergillus niger*	M4VR58	214.86	14	33	65
Uncharacterized protein	*Aspergillus tubingenesis*	A0A1L9NE01	214.86	14	33	65
Alpha-amylase/trypsin inhibitor CM3	*Triticum aestivum*	P17314	187.28	1	76	18
Alpha amylase inhibitor protein	*Triticum aestivum*	Q53YX8	187.28	1	76	18
Endo-1 4-beta-xylanase	*Bacillus subtilis*	Q45VU1	176.7	2	46	23
Alpha-amylase/trypsin inhibitor CM3	*Aegilops tauschii*	M8BV45	176.54	4	52	24
Cell wall-binding protein	*Bacillus subtilis*	P54484	164.58	12	68	15
Uncharacterized protein	*Triticum aestivum*	A0A1D5XLP1	149.67	1	49	17
Endo-1 4-beta-xylanase	*Bacillus subtilis*	Q3HLJ4	148.9	1	34	23

Table 6-10 Proteins identified by nano LC-MS/MS from an in-gel 32-37kDa bacterial xylanase digest.

Protein name	Species	Accession Number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)
Porin	*Acidovorax*	A0A0Q8THN2	170.8	21	61	36
Phosphate-binding protein PstS	*Variovorax paradoxus*	A0A0N8P9K0	163.05	2	74	36
Glyceraldehyde-3-phosphate dehydrogenase	*Pantoea agglomerans*	A0A0L7DK62	158.37	1	70	35
Malate dehydrogenase	*Bacillus subtilis*	A0A164WTU7	153.43	6	71	33
Phosphate-binding protein PstS	*Pantoea agglomerans*	A0A059IAS1	151.73	10	60	36
Phosphate-binding protein PstS	*Variovorax paradoxus*	E6UZH6	151.24	1	59	36
Phosphate-binding protein PstS	*Acidovorax*	A0A0Q8S8A2	148.95	5	56	36
Glucose oxidase	*Aspergillus niger*	M4VR58	148.42	15	34	65
Uncharacterized protein	*Aspergillus tubingenesis*	A0A1L9NE01	148.42	15	34	65
Uncharacterized protein	*Triticum aestivum*	A0A1D5XS09	128.07	1	23	68

Table 6-11 Proteins identified by nano LC-MS/MS from an in-gel 40kDa bacterial xylanase digest.

Protein name	Species	Accession Number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)
Porin	*Variovorax paradoxus*	A0A0P8ZNQ8	201.34	21	67	44
Uncharacterized protein	*Bacillus subtilis*	A0A1L9DEB7	164.58	16	44	43
Cell surface protein	*Bacillus subtilis*	A0A164Z453	164.58	16	44	44
Endo-1 4-beta-xylanase	*Bacillus subtilis*	A0A1J5X4R7	156.67	1	57	23
Ketol-acid reductoisomerase (NADP(+))	*Bacillus subtilis*	A0A125UKR0	156.13	13	56	37
Porin	*Acidovorax*	A0A0Q8T5D6	154.57	2	53	40
Sugar ABC transporter substrate-binding protein	*Pantoea agglomerans*	A0A059I8Z5	153.44	18	54	42
Porin	*Acidovorax*	A0A0Q8TB96	145.85	1	47	40
Aminopeptidase YpdF (MP-MA-MS-AP-NP-specific)	*Bacillus subtilis*	A0A164X802	144.65	5	50	38
Outer membrane phosphoporin protein E	*Pantoea agglomerans*	A0A059IEL9	142.16	3	38	41

Table 6-12 Proteins identified by nano LC-MS/MS from an in-gel 58kDa bacterial xylanase digest.

Protein name	Species	Accession Number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)
60 kDa chaperonin	*Variovorax paradoxus*	A0A0P9C073	184.6	1	71	57
60 kDa chaperonin	*Acidovorax*	J0U9H1	174.07	2	60	57
Beta-amylase	*Triticum aestivum*	M8B5G5	149.4	3	39	58
Beta-amylase	*Triticum aestivum*	A0A1D5XGF3	147.77	1	37	58
Beta-amylase	*Aegilops tauschii*	M8AQX5	143.82	1	35	59
Beta-amylase	*Triticum aestivum*	A0A1D5XXC8	143.82	1	35	59
Beta-amylase	*Triticum aestivum*	W5EKI0	143.82	1	34	61
60 kDa chaperonin	*Brevundimonas*	A0A0Q5LSB4	139	2	44	57
Glucose oxidase	*Aspergillus niger*	P13006	131.47	8	19	65
60 kDa chaperonin	*Mucilanginibacter*	H1Y4X3	126.46	1	31	57

6.3.5.2 Proteins identified from the glucose oxidase improver enzyme extract

We identified 1211, 1182, 736 and 499 proteins in digest samples excised at 25, 31, 47 and 56kDa respectively, from the glucose oxidase extract.

Protein identifications from an in-gel 25kDa glucose oxidase digest

The most abundant protein identified from this digest was YkgB (Prot Acc: A0A100IT92) from *Aspergillus niger*, with a molecular weight of 42kDa, sequence coverage of 90% and 12 unique peptides. In Table 6-13 are the top 10 proteins identified from this digest.

Of note:

- Glucose oxidase (65kDa) from *Aspergillus niger* was identified with a sequence coverage of 50% and 17 unique peptides.
- The known allergen registered as Tri a 30 on the WHO/IUIS nomenclature was identified with a sequence coverage of 39% and 6 unique peptide (not listed in Table 6-13).

Protein identifications from an in-gel 31kDa glucose oxidase digest

The most abundant protein identified from this digest was YkgB from *Aspergillus niger* (as in the 25kDa digest), with a sequence coverage of 86% and 32 unique peptides. In Table 6-14 are the top 10 proteins identified from this digest.

Of note:

- Glucose oxidase (65kDa) from *Aspergillus niger* was identified with a sequence coverage of 62% and 8 unique peptides.

Protein identifications from an in-gel 47kDa glucose oxidase digest

The most abundant protein identified from this digest was an uncharacterised protein from *Aspergillus tubingensis* (Prot Acc: A0A1L9NE01) with a molecular weight of 65kDa, sequence coverage of 64% and 1 unique peptide. In Table 6-15 are the top 10 proteins identified from this digest.

Of note:

- Sulphydryl oxidase Sox (A0A117E1C5) and Sulphydryl oxidase (Q68CM8) were identified from *Aspergillus niger*, both with molecular weights of 43kDa, sequence coverages of 66% and 53% and having 5 and 3 unique peptides, respectively.

Protein identifications from an in-gel 56kDa glucose oxidase digest

The most abundant protein identified from this digest was GMC oxidoreductase from *Aspergillus luchensis* (Prot Acc: A0A1M3T1N5) with a molecular weight of 65kDa, sequence coverage of 75% and 2 unique peptides. In Table 6-16 are the top 10 proteins identified from this digest.

Of note:

- Glucose oxidase (65kDa) from *Aspergillus niger* was identified with a sequence coverage of 55% and 1 unique peptide.

Table 6-13 Proteins identified by nano LC-MS/MS from an in-gel glucose oxidase 25kDa digest.

Protein name	Species	Accession Number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)
YkgB	*Aspergillus niger*	A0A100IT92	322.57	12	90	42
Uncharacterized protein	*Aspergillus tubingensis*	A0A1L9NDZ7	322.57	12	90	42
YkgB	*Aspergillus kawachii*	G7XS21	296.76	2	81	42
Uncharacterized protein	*Aspergillus luchuensis*	A0A1M3T1Q2	296.76	2	81	42
Glucose oxidase	*Aspergillus niger*	M4VR58	257.99	17	50	65
Glucose oxidase	*Aspergillus niger*	C5J0H1	257.99	17	50	65
Uncharacterized protein	*Aspergillus tubingensis*	A0A1L9NE01	257.99	17	50	65
Aspergillus niger contig An01c0470 genomic contig	*Aspergillus niger*	A2QBC2	232.4	1	52	41
Glucose oxidase	*Aspergillus niger*	Q9P8M8	221.51	2	40	65
Glucose oxidase (Fragment)	*Aspergillus niger*	Q0PGS3	216.21	1	43	62

Table 6-14 Proteins identified by nano LC-MS/MS from an in-gel 31kDa glucose oxidase digest.

Protein name	Species	Accession Number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)
YkgB	*Aspergillus niger*	A0A100IT92	361.91	32	86	42
Uncharacterized protein	*Aspergillus tubingensis*	A0A1L9NDZ7	361.91	32	86	42
YkgB	*Aspergillus kawachii*	G7XS21	328.46	7	86	42
Uncharacterized protein	*Aspergillus luchuensis*	A0A1M3T1Q2	328.46	7	86	42
Glucose oxidase	*Aspergillus niger*	M4VR58	292.69	8	62	65
Aspergillus niger contig An01c0470 genomic contig	*Aspergillus niger*	A2QBC2	281.6	7	59	41
Glucose oxidase (Fragment)	*Aspergillus niger*	Q0PGS3	278.57	2	59	62
Uncharacterized protein	*Aspergillus brasiliensis*	A0A1L9UQW5	257.48	12	68	42
Glucose oxidase	*Aspergillus niger*	Q9P8M8	251.33	1	45	65
Catalase	*Aspergillus kawachii*	G7XXW2	239.68	2	46	80

Table 6-15 Proteins identified by nano LC-MS/MS from an in-gel 47kDa glucose oxidase digest.

Protein name	Species	Accession Number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)
Uncharacterized protein	*Aspergillus tubingensis*	A0A1L9NE01	280.91	1	64	65
Sulphydryl oxidase Sox	*Aspergillus niger*	A0A117E1C5	262.22	5	66	43
Sulphydryl oxidase Sox	*Aspergillus kawachii*	G7XN45	257.08	3	63	43
Sulphydryl oxidase Sox	*Aspergillus luchuensis*	A0A146FPT4	256.68	3	63	43
Sulphydryl oxidase	*Aspergillus niger*	Q68CM8	254.45	3	53	43
Uncharacterized protein	*Aspergillus brasiliensis*	A0A1L9UR98	228.68	1	38	65
YkgB	*Aspergillus niger*	A0A100IT92	224.22	26	66	42
Catalase	*Aspergillus tubingensis*	A0A1L9NDA5	223.64	2	51	80
Uncharacterized protein	*Aspergillus niger*	A0A100I535	221.91	5	57	41
Catalase	*Aspergillus niger*	A2Q7T0	214.79	2	41	80

Table 6-16 Proteins identified by nano LC-MS/MS from an in-gel 56kDa glucose oxidase digest.

Protein name	Species	Accession Number	Probability (%)	No. of Unique Peptides	Coverage (%)	MW (kDa)
GMC oxidoreductase	*Aspergillus luchensis*	A0A1M3T1N5	392.5	2	75	65
Glucose oxidase	*Aspergillus luchensis*	A0A146F2H4	391.89	3	76	65
Glucose oxidase	*Aspergillus niger*	Q9P8M8	370.14	1	55	65
Uncharacterized protein	*Aspergillus brasiliensis*	A0A1L9UR98	334	13	51	65
Glucose oxidase	*Aspergillus niger*	Q9HFQ1	292.69	3	36	65
Catalase	*Aserpgillus tubingensis*	A0A1L9NDA5	244.55	1	45	80
Catalase	*Aspergillus niger*	A2Q7T0	240.97	1	36	80
Catalase	*Aspergillus brasiliensis*	A0A1L9UU23	234.86	1	39	80
YkgB	*Aspergillus niger*	A0A100IT92	224.7	16	55	42
Uncharacterized protein	*Aspergillus tubingensis*	A0A1L9NDZ7	224.7	16	55	42

6.4 Discussion

6.4.1 Overview

In this chapter I have shown that serum specific-IgE binding proteins are present in up to eight different enzymes that are used to improve the dough making process in UK supermarket scratch bakeries. Using the optimised MS-based method, a very high number of proteins were identified from specific-IgE binding regions in two enzyme extracts, bacterial xylanase and glucose oxidase. The proteins were most commonly derived from species of *Aspergillus*, *Bacillus* and *Triticum*. Both *Aspergillus* and *Triticum* are listed on allergen databases, such as the WHO/IUIS Allergen Nomenclature and the Allergome platform.

The most monitored enzyme in the baking industry, that is known to elicit occupational asthma is fungal α-amylase. This enzyme is derived from *Aspergillus oryzae*, has a molecular weight of 53kDa and has been given the allergen name Asp o 21. Currently, the clinical diagnosis of baker's asthma relies on detecting specific-IgE sensitisation to allergens derived from wheat, rye and other flours, as well as to the fungal α-amylase enzyme. Clinical testing does not consider other enzymes used in the baking process, which may cause allergic respiratory symptoms. Jones et al., (2016) has shown that 5% of 269 bakers, who were not sensitised to flour or fungal α-amylase, were sensitised to one or more of eight other enzymes used in supermarket bakeries. Moreover, clinical tests such as inhalation challenges, RAST and SPT have all been used to demonstrate sensitisation to enzymes other than fungal α-amylase in bakers with work-related respiratory symptoms (Sander et al., 1998; Baur et al., 1998; Quirce et al., 2002; Merget et al., 2001; Elms et al., 2003; Lipińska-Ojrzanowska et al., 2016).

In this work, I have shown specific-IgE binding proteins from maltogenic amylase, cellulase, fungal xylanase, phospholipase, lipase, bacterial xylanase, glucose oxidase and bacterial α-amylase, that were detected using serum from sensitised bakers, by an optimised ECL western blot method. Based on having the highest number of specific-IgE binding regions and the most sensitised bakers showing specific-IgE binding, the specific-IgE binding regions from bacterial xylanase and glucose oxidase were chosen for MS protein identification. Via

personal communication with Dr M. Jones, it was known that bacterial xylanase and glucose oxidase were grown in the species; *Bacillus subtilis* and *Aspergillus niger*, respectively.

In the 14kDa and 40kDa bacterial xylanase digests, endo-1 4-beta-xylanase (also known as bacterial xylanase), derived from *Bacillus subtilis*, was identified amongst the top 10 most abundant proteins. It has been previously shown that enzyme preparations made from the species *Bacillus subtilis*, were responsible for causing occupational allergic respiratory diseases in workers manufacturing laundry powders, by both SPT and inhalation tests (Pepys et al., 1969; Pepys 1992), however individual allergenic proteins were not identified. Currently, no allergens from *Bacillus subtilis* are listed on the WHO/IUIS Allergen Nomenclature. In the bacterial xylanase MS digests, many proteins were identified from not only *Bacillus,* but also *Variovorax, Acidovorax* and *Pantoea*. These identifications are not surprising, given that these are all species of bacteria and therefore likely to have high sequence homology with *Bacillus subtilis*.

For the specific-IgE binding regions digested from the glucose oxidase extract, most of the proteins identified by MS were from *A. niger, A. tubingensis* and *A. luchensis*. In the 25, 31 and 56kDa glucose oxidase digests; the protein, glucose oxidase derived from *Aspergillus niger*, was identified amongst the top 10 most abundant proteins. Glucose oxidase derived from *Aspergillus niger* is not a registered allergen on the WHO/IUIS Allergen Nomenclature or on the Allergome platform. However, another protein derived from *Aspergillus niger*, β-xylosidase has been previously identified as an allergen by Sander et al., (1998) and is registered as Asp n 14 on the WHO/IUIS Allergen Nomenclature.

In the MS results from both bacterial xylanase and glucose oxidase extracts, there were proteins identified from the *Triticum aestivum* (bread wheat) species. Of note, the 16kDa wheat flour allergen Tri a 30 (an alpha-amylase/trypsin inhibitor), was identified from both the 14kDa bacterial xylanase digest and the 25kDa glucose oxidase digest. This contamination in both enzyme extracts with wheat flour proteins is most likely explained by the experimental conditions of the previous study (Jones et al., 2016). They measured serum specific-IgE levels by RAST to an in-house preparation of wheat flour as well as to the same improver enzyme extracts used in the methods shown here. This demonstrates how highly sensitive and susceptible to contamination MS is, as a method for protein identification (Schuchardt and Sickmann 2007).

When comparing the eight improver enzyme extracts there was a large variation in their protein content. It varied from 0.97µg/µl to 3.38µg/µl amongst the extracts. None of the enzymes were encapsulated and they were all extracted into a lyophilised form using ammonium carbonate, overnight H_2O dialysis and freeze-drying as reported in Jones et al., (2016). On visual inspection of the enzymes, prior and subsequent to extraction, they differed in many physical qualities (i.e. colour and texture (sticky or powder-like)). We suggest that the most likely reason for the variation in protein content is due to these physical differences. It is also reasonable to suggest that there may have been protein degradation in these samples as lyophilisation can cause structural changes which may be irreversible depending on the protein (Wang 2000).

All proteins and specific-IgE binding proteins from the enzyme extracts were compared by Coomassie stained SDS-PAGE and ECL western blot using pooled serum. Some specific-IgE binding proteins were detected that were not observed in the Coomassie stain. Whether a protein is detected by western blot, not only depends on the amount of protein in the sample but also on the strength of serum specific-IgE binding. This can be particularly challenging when in-gel digestion for MS analysis requires that the specific-IgE binding protein detected is also sufficiently detected in the SDS-PAGE stained gel. This can usually be overcome by using a more sensitive staining method, such as silver stain, or by increasing the protein concentration.

Heat map analysis was a useful visual tool for displaying the number of bakers with serum specific-IgE binding to each molecular weight region, as well as displaying the allergic profile of each baker. This analysis also determined which improver enzymes would be analysed by MS. Given that MS is still governed by modern and costly equipment, the expenses available for this study limited our analysis to identify proteins from only eight specific-IgE binding regions detected by ECL western blot in two enzyme extracts.

Heat map analysis has a proven success in assessing specific-IgE binding proteins and their allergenicity against a panel of allergic individuals. Saha et al., (2015), Ghosal et al., (2016) and Dey et al., (2016) who identified pollen and fungal allergens from coconut, *Lantana camara* and *Curvularia pallescens*, respectively, all portrayed specific-IgE binding to different components of an extract by heat map analysis. Dey et al., (2016) used a heat map constructed in MS-Excel to show which allergens the sensitised population were most

frequently allergic to and therefore, was able to represent major allergens from the *Curvularia pallescens* species. This was the same approach I used, however Saha et al., (2015) and Ghosal et al., (2016) used heat maps with more advanced hierarchical clustering to further divide specific-IgE binding regions into groups based on their reactivity and to divide patients into groups based on the number of binding regions they reacted with.

To the best of our knowledge, this is the most comprehensive analysis of the proteins present within enzymes that are used in the UK supermarket scratch baking industry. The strengths of our work compared to the available literature, include the number of sensitised serum samples made available and prior knowledge of the enzyme ingredients, both of which were obtained from Jones et al., (2016). Furthermore, in this work, I have shown the first western blot and MS-based results given together for such a large panel of enzymes used in the baking industry.

6.4.2 Limitations

The optimised MS-based method was used in this chapter as a 'proof of principle' application to determine whether unknown enzymes with allergenic potential, used in the baking industry can be identified. Although the correct protein and species of the improver enzymes were identified by MS, the results gave a high number of other protein identifications for every digest that was analysed. This is a significant limitation of the MS-based method for application in this field of identifying unknown allergens. The high sensitivity of MS means that the results output will often be a list of highly likely protein candidates, rather than a conclusive single protein identification. Moreover, where the species of the allergenic protein is not known, proteins from other species sharing significant homology may also be listed. Despite this, MS is still an invaluable proteomics tool and if used in conjunction with tests to measure allergenicity of identified protein candidates, it could still be crucial in the identification of unknown allergens in the baking industry.

Not all sensitised individuals with a RAST score ≥2% showed specific-IgE binding by ECL western blot. However, with the exception of glucose oxidase, the serum with the highest RAST scores for each enzyme always showed specific-IgE binding.

Although serum was collected from a large population of bakers, the volume and number of serum samples previously collected was limiting. This meant we were unable to conduct any repeat western blot experiments. In addition, a comparison of allergic profiles from a larger number of sensitised serum samples would have given a more appropriate recommendation for the major and minor allergens present within these enzyme extracts.

6.4.3 Summary and future works

More investigation into the contribution these improver enzymes have on the burden of occupational asthma in bakeries is necessary, given that there are workers still exposed to them on a daily basis. Larger scale studies and cooperation with companies manufacturing the enzymes will likely be required to determine the prevalence of allergic sensitisation as well as to further characterise the allergenic enzymes. As mentioned in previous chapters, there are other laboratory experiments which can offer more in-depth characterisation of the allergenicity and immunogenicity of these proteins. This includes purifying and/or making recombinant versions of the relevant proteins as well as conducting basophil activation and histamine release tests.

In summary, specific-IgE binding proteins have been detected from eight different improver enzyme extracts using ECL western blot methods, and a high number of proteins have been identified using MS, most of which are derived from either *Aspergillus*, *Triticum* or species of bacteria. Currently, diagnosis of baker's asthma relies on determining sensitisation to fungal α-amylase or allergens from flour. In order to test appropriately for occupational asthma in bakers, a wide panel of tests beyond flour proteins and fungal α-amylase should be used.

7 Application of the mass spectrometry (MS)-based method to identify outdoor airborne allergens

In the previous three chapters, I have optimised and applied a MS-based method to identify airborne allergens present in occupational environments that are responsible for symptoms of respiratory allergy. In this chapter, I have applied similar methods to identify outdoor airborne allergens, which may be responsible for unexplained respiratory symptoms, occurring in the general population.

7.1 Introduction

The systematic review presented in Chapter 2 showed that outbreaks of asthma have been linked with increased levels of airborne allergens in the outdoor environment. These allergens may be well known (for example rye grass pollen after thunderstorm events) or have to be identified by epidemiological, clinical and laboratory methods (for example soybean in the Barcelona outbreaks). Within the systematic review there were 2 papers (Newson et al., 1998; Marks et al., 2001) suggesting that outbreaks of asthma were occurring on a smaller scale throughout the UK – providing some support for the hypothesis that allergens in outdoor air may be responsible for more asthma morbidity than is currently recognised.

A comprehensive literature of scientific evidence now exists that associates outdoor air pollution with adverse respiratory health effects (Sweileh et al., 2018). In 2016, the World Health Organisation (WHO) estimated that 3 million deaths worldwide were caused by outdoor air pollution (World Health Organisation, 2016). Outdoor particulate matter (PM), specifically with an aerodynamic diameter below 10μm (PM_{10}) is commonly measured in urban settings, where there are increased levels of traffic-related air pollution. Elevated levels have been linked to asthma (Künzli et al., 2009; Peacock et al., 2011; Kelly & Fussell, 2011; Samoli et al., 2016; Atkinson et al., 2016). Airborne PM_{10} is especially relevant in respiratory disease due to its small aerodynamic size, which allows it to penetrate into the

respiratory tract. This is particularly the case for particles with an aerodynamic diameter below 2.5µm.

One study has examined whether asthma exacerbations in patients attending a London GP practice (n=297) were associated with serum specific-IgE binding to airborne PM_{10}, collected at the time of asthma exacerbation (Burney et al., 2008). Using a high-volume sampler with a flow rate of $1.2m^3$ per second, located on the roof of a London GP practice, PM_{10} was collected daily onto glass fibre hydrophilic filters. Strips were cut from the filters, sized 2 x 18cm and extracted in 1ml of 0.1M ammonium bicarbonate buffer on a rock 'n' roller for 2 hours. The filter extracts were fixed directly to ELISA plates, which were then blocked with BSA-phosphate buffer and incubated with serum from patients who experienced asthma exacerbations on the same day PM_{10} was collected. Serum specific-IgE binding to filter extracts was then detected with a labelled anti-human IgE antibody.

From these experiments, Burney et al., (2008) detected a 25% increase in specific-IgE binding to airborne PM_{10} that was collected at the time of asthma exacerbation, compared with PM_{10} collected two weeks before or after the asthma exacerbation. These findings suggest that an outdoor protein present in airborne PM_{10} was able to bind with specific-IgE and may have been responsible for causing asthma exacerbations.

The study by Burney et al., (2008) did not however identify any allergenic proteins associated with asthma exacerbations. The authors argued that pollen was unlikely to explain the specific-IgE binding to PM_{10}, given that; a) pollen allergic subjects did not show greater risk; b) the effect of pollen allergens had been largely controlled for by the matching of event and control times by season and c) the effect was not confined to the pollen season. Furthermore, specific-IgE binding was not higher in patients who reacted to allergens used in SPT and were considered 'atopic', which included mould allergic patients. The authors did not exclude possible industrial or commercial sources of allergen that may have been the cause of unexplained asthma exacerbations.

A similar study was set up by the same authors to identify the allergen(s) responsible for causing an increase in specific-IgE binding to PM_{10} at the time of asthma exacerbation. This study was called ALLOHA (ALLergen and Oxidative particles and Hospitalisation with Airway

disease). As before, a high-volume sampler with a flow rate of 1.2m^3/s was used to collect airborne PM_{10} on the roof of a London hospital onto glass fibre hydrophilic filters and serum was collected from patients with asthma exacerbations attending the same hospital. The filters were extracted with ammonium bicarbonate on a rock 'n' roller for 2 hours, fixed to ELISA plates and analysed using the same method as before. However, in this study there was no association of asthma exacerbations with specific-IgE binding to PM_{10} (unpublished).

The results from the ALLOHA study were surprising in view of the previous associations observed by Burney et al., (2008), that airborne PM_{10} was binding to specific-IgE and thus was associated with causing asthma exacerbations.

As part of this PhD I wanted to determine whether specific-IgE binding proteins could be detected on filters from particulate monitoring equipment by using different laboratory methods. Possible explanations for the negative findings in the ALLOHA study could have been that the extraction buffer and/or method may not have been suitable to extract protein from these filters or a larger portion of the filter for extraction may have been required to detect protein. It is also possible, of course, that there was no protein on the filters collected during the ALLOHA study. Moreover, to the best of our knowledge, there is currently no published methodology to identify unknown outdoor airborne proteins or allergens.

Therefore, I undertook a series of experiments to determine whether it was possible to detect protein from filters used for particulate monitoring in a range of different scenarios – i.e. the stored ALLOHA filters, fresh filters from a roadside pollution monitoring site in London and fresh filters used to measure particulates near a composting site.

Objective

To identify outdoor airborne specific-IgE binding proteins from filters collecting particulate matter (PM).

Aims

1. Detect protein from stored PM_{10} glass fibre filters collected during the ALLOHA study, using recognised extraction methods.
2. Detect protein from fresh PM_{10} Teflon filters collected from a London air pollution monitoring site, using recognised extraction methods and newly reported methods.
3. Compare the quantity of protein extracted from spiked Teflon filters, using recognised and newly reported methods.
4. Detect protein from fresh polycarbonate filters collecting PM at an outdoor composting facility, using recognised and newly reported methods.

7.2 Detecting protein from stored PM_{10} glass fibre filters, using recognised extraction methods

The first aim was to determine if protein could be detected from PM_{10} glass fibre filters collected during the ALLOHA study, which had since been stored for ~8 years.

7.2.1 Methods

7.2.1.1 PM_{10} collection

In the ALLOHA study, PM_{10} particles were collected on 8" x 10" glass fibre filters using a high-volume sampler (purchased from Westech, Bedfordshire) which was set at a flow rate of 1.2 m^3/s. The sampler was located on the roof of Chelsea and Westminster hospital (see Figure 7-1) and filters were changed every 3 days, with each start and end time of sampling recorded. The sampling period during the ALLOHA study was between July 2008 and August 2010. After sampling, these filters were stored at RT, out of direct sunlight, in individually labelled A4 plastic envelopes, in an office cupboard.

Figure 7-1 Image of PM_{10} high volume sampler located on the roof of Chelsea and Westminster hospital

7.2.1.2 Filter selection

Filters collecting outdoor PM_{10} during the last 2 weeks of May and all 4 weeks of June and July were selected, reasoning that at the very least, proteins from grass pollen should be present. Sampling did not occur on every day of the study period, due to holidays, equipment malfunction and the high-volume sampler requiring repair from March 2009 to June 2009. Based on this, the following four filters in Table 7-1 were selected for the detection of protein.

Table 7-1 Start and end sampling dates of PM_{10} filters used for detection of protein

Filter number	Start date	End date
1	23/07/2008	25/07/2008
2	03/07/2009	06/07/2009
3	14/06/2010	16/06/2010
4	19/07/2010	21/07/2010

Stored serum samples from patients admitted with asthma on the same days as air sampling took place were identified from the stored ALLOHA study serum bank.

7.2.1.3 Filter extraction

To identify the best method for extracting protein from the PM_{10} glass fibre filters, I tested different sized strips cut from the filter and three different recognised extraction buffers.

From each filter I cut either:

- nine 3x3mm filter pieces that were placed into 0.5ml of buffer or,
- one 85x80mm filter piece that was placed into 10ml of buffer.

From each cut filter I extracted in each of the following recognised extraction buffers:

1. Distilled water (dH_2O)
2. 0.1M Ammonium hydrogen carbonate (AHC)
3. 'Extraction buffer' taken from an All-in-One Trypsin Digestion Kit (see Chapter 3, section 3.2.7.1).

For extraction, filters were placed into Eppendorf's and left overnight at RT on a rock 'n' roller. The remaining solution from each filter extract was transferred into a new Eppendorf and dried down in a SpeedVac. The dried pellet was stored at -20°C prior to MS analysis.

7.2.1.4 In-solution tryptic digestion, peptide extraction and nano LC-MS/MS

The following methods were conducted under the supervision of Prof. Peter Briza at the Division of Allergy and Immunology at the University of Salzburg.

To determine whether any protein had been extracted from these filters we performed an in-solution tryptic digestion of the filter extracts for MS analysis.

A ProteoExtract All-in-One Trypsin Digestion Kit was used to digest the filter extracts as described in the Chapter 3, section 3.2.7.2. Briefly, extracted samples (except for those already extracted in 'extraction buffer from this kit), were dissolved in 25µl of extraction buffer 1, ensuring the protein concentration did not exceed 4µg/µl and this was centrifuged for 15 minutes at 10,000xg. The supernatant was transferred to a new Eppendorf and 25µl of digest buffer and 1µl of reducing agent were added. This solution was incubated at 37°C for 20 minutes and then to cooled to RT before mixing with 1µl of blocking agent and leaving at RT for 20 minutes. We then added 1µl of trypsin and left the solution at 37°C for a maximum of 16 hours.

Next, a ZipTip protocol was used to extract peptides from the solution as described in the Chapter 3, section 3.2.7.3. A 10µl ZipTip$_{C18}$ tip was used to pick up and de-salt the solution, to create a more peptide concentrated solution. This was checked for acidity using pH paper and addition of 13µl of TFA. The sample was dried down, resuspended in 0.1% TFA and sonicated before transfer into a MS vial.

Methods for nano LC-MS/MS were also followed as described in Chapter 3, section 3.2.7.4. Briefly, MS vials containing concentrated peptide solutions were placed into an automated EASY nano liquid chromatograph, whereby peptides were resolved by reverse phase chromatography. This was coupled by ESI to an Orbitrap Velos Pro where peptides were ionised by charged hydrogen ions and then separated based on their mass-to-charge ratio (*m/z*). Raw data was processed by Proteome Discoverer and experimental peptide spectra generated were searched against theoretical peptide spectra to identify matches within the UniProt database. The generated files were uploaded into PEAKS studio to interpret peptide and protein identifications.

7.2.2 Results and Interpretation

Using these extraction methods and MS analysis, I was unable to detect **any** grass pollen-derived or other proteins from the stored PM_{10} glass fibre filters. From this, we were forced to conclude that the stored filters from the ALLOHA study contained no protein, for which there were two main possible explanations:

1. *Either*, there had never been any protein on them, even at the time of collection
2. *or* there was a loss/degradation of protein over the storage period (~8 years) which meant protein was no longer present and could not be detected.

To investigate whether proteins are present on filters that routinely quantify particulate matter in urban air, I decided to examine fresh filters from equipment collecting outdoor PM_{10} as part of the Air Quality Network monitoring program in London. The methods and findings of this are described in the following section.

7.3 Detecting protein from <u>fresh</u> PM_{10} Teflon filters, using recognised and newly reported extraction methods

7.3.1 Methods

7.3.1.1 PM_{10} collection

A Thermo Scientific Partisol sampler (2025) was used to collect outdoor PM_{10} onto a 47mm Teflon filter for a 48-hour continuous sampling period. The sampler was located on a standalone cabin at a pollution monitoring site approximately 1 metre from a frequently congested location on Marylebone Road, London, UK.

Air flow through the filter was provided by a vacuum pump, and mass flow controllers maintained a constant volumetric flow rate of 2.7×10^{-4} m^3/s. The filter compartment was ventilated continuously to maintain filter temperature within 5°C of the ambient temperature. No more than four days after the start of the sampling period, the filter cassette was removed from the storage magazine and the sample was kept in foil at RT overnight and extracted for protein the following day.

Air sampling was conducted by Anja Tremper, Anna Font and Dr. David Green from the MRC-PHE Centre for Environment and Health at King's College London.

Filters collecting airborne PM_{10} were obtained in December 2017, January 2018 and April 2018. As another research project demanded the use of the sampling equipment, I was unable to obtain filters from the 24th January to 26th April.

7.3.1.2 Filter extraction

To establish the optimal method for extracting protein from fresh PM_{10} Teflon filters, I used the following three recognised extraction buffers and two more recently reported methods*. For each buffer, duplicate filters were tested.

1. Distilled water (dH_2O)
2. 0.1M Ammonium hydrogen carbonate (AHC)
3. Phosphate buffered saline, 0.05% Tween-20 (PBS-T)
4. SDS-based*
5. Methanol*

For extraction using dH_2O and AHC the filter was placed in 2ml of buffer and left overnight at RT on a rock 'n' roller. For extraction using PBS-T the filter was placed in 2ml of buffer, sonicated for 30 minutes (based on protocol recommendations) and left overnight at RT on a rock 'n' roller. PBS-T was used here as it extracted the highest level of protein from mouse epithelium samples in Chapter 4.

The following more recently reported methods*, had been previously used by Chen and Hilderman (2009) and Roper et al., (2015), specifically to extract protein from filters containing PM and were as follows.

For extraction using the SDS-based buffer, the filter was cut into ~1cm^2 pieces, placed in 1ml of buffer, incubated at 60°C for 15 minutes and left overnight at RT on a rock 'n' roller. The SDS-based buffer was prepared using: 2% sodium dodecyl sulphate (SDS), 0.4mM ethylenediamine tetra-acetic acid (EDTA) and 10mM Tris-HCl.

For extraction using the methanol buffer, the whole filter was placed in 2ml of buffer, sonicated for 30 minutes and left overnight at RT on a rock 'n' roller. The methanol buffer was prepared by mixing methanol and dH_2O at a ratio of 9:1.

7.3.1.3 Protein quantification

To determine whether any protein had been extracted from these filters I performed a quick and relatively inexpensive protein quantification assay as described in Chapter 3, section 3.2.2.

Briefly, protein content in the filter extract was quantified using a *DC*™ Protein Assay kit following the manufacturer's instructions. Using the protein concentration as determined by the assay, the mass of protein (mg) in the filter extract was calculated as the:

Concentration of protein (mg/ml) × volume of extraction buffer (ml).

Each filter extract solution was tested in triplicate and values are given as an average.

7.3.2 Results

Using the recognised methods for extraction and the *DC*™ Protein Assay I was unable to detect protein from fresh PM_{10} Teflon filters. I was also unable to detect protein using a methanol buffer.

I was however, able to detect protein from two fresh PM_{10} Teflon filters by the *DC*™ Protein Assay, using the SDS-based buffer. The quantity of protein (mg) detected in these two filters is shown below in Table 7-2.

Table 7-2 Mass of protein (mg) detected from two fresh PM_{10} Teflon filters extracted in SDS-based buffer. Each filter extract was tested in triplicate by DC™ Protein Assay with values given as an average.

Filter number	Mass of protein in each filter extract (mg)
1	0.12
2	0.17

As a 'post-hoc' experiment, the filters extracted with SDS-based buffer were then applied to protein separation by silver stained SDS-PAGE, to separate the proteins within the extract according to their molecular weight. I applied 2.5µg of protein from both filter number 1 (volume of 20µl) and filter number 2 (volume of 14µl). However, no protein was observed by silver stained SDS-PAGE from either filter extract.

7.3.3 Interpretation

Using low volume sampling techniques that are widely adopted for PM_{10} monitoring within the London Air Quality Network, I successfully applied a newly reported extraction method that used an SDS-based buffer and detected protein from Teflon filters. However, I was unable to detect protein from Teflon filters when using other more widely used extraction buffers: dH_2O, AHC and PBS-T. This was unexpected, as other authors, such as Burney et al., (2008) have used buffers such as these to successfully detect protein from glass hydrophilic fibre filters collecting PM_{10} by high volume sampling.

It should be noted that we were unable to apply our extraction methods to filters that had been collected at the same time, as we were unable to use the equipment continuously (other ongoing projects requiring use of the pollution monitoring equipment). The extraction buffers: dH_2O, AHC, PBS-T and methanol were all applied to filters collected in December 2017 and January 2018, and the SDS-based buffer was applied to filters collected in April 2018. Therefore, protein detection after using the SDS-based extraction buffer may be attributed to the presence of tree pollen (given that the UK tree pollen season is late March to mid-May), rather than due to the use of the SDS-based method.

I did not know what proportion of the total protein on the filters; I had extracted and detected. In view of this, I set up a controlled experiment using the same Teflon filters which were spiked with a known amount of protein and extracted them using the same five extraction methods. This was to determine what proportion of protein could be extracted using each buffer. The methods and findings of this are described in the following section.

7.4 Comparing the quantity of protein extracted from spiked Teflon filters using recognised and newly reported extraction methods

7.4.1 Methods

7.4.1.1 Spiking filters with bovine serum albumin (BSA)

Bovine serum albumin (BSA) was prepared as a 10mg/ml solution in dH_2O and when fully dissolved, 100µl (1mg) was used to spike Teflon filters. For each extraction method, the solution was pipetted onto two filters. The BSA solution was left to dry on the filters for approximately 1 hour before extraction.

7.4.1.2 Filter extraction

Once dry, each filter was placed into an Eppendorf and one of the following five extraction methods in Table 7-3 below was used, each with a 1ml volume of buffer. All filters were extracted overnight at RT on a rock 'n' roller, heating at 60°C was for 15 minutes and sonication was for 30 minutes.

Table 7-3 Five methods used to extract protein from Teflon filters spiked with BSA. All filters were extracted overnight at RT on a rock 'n' roller and only filters extracted in SDS-based buffer were heated at 60°C for 15 minutes. Sonication was for 30 minutes.

Extraction method number	Extraction buffer	Filter cut-up into 1cm^2 pieces	Heated at 60°C	Sonication	Rock 'n' roll overnight
1	PBS-T				✓
2	PBS-T			✓	✓
3	Methanol			✓	✓
4	SDS-based		✓		✓
5	SDS-based	✓	✓		✓

7.4.1.3 Protein quantification

To quantify the protein extracted from these spiked filters I performed a protein quantification assay using the *DC*™ Protein Assay kit as already described. Using the protein concentration as determined by the assay, the mass of protein (mg) in the filter extract was calculated as the:

Concentration of protein (mg/ml) × volume of extraction buffer (ml).

Each filter extract solution was tested in triplicate and values are given as an average.

7.4.1.4 Statistical analysis

To determine if the protein detected from spiked filters differed between extraction methods, we applied a non-parametric Mann-Whitney U-test using STATA (version 15).

7.4.2 Results

7.4.2.1 Comparison of five protein extraction methods

Protein was detected on filters spiked with 1mg of BSA using four of the five extraction methods by the *DC*™ Protein Assay. These results are shown in Table 7-4, with two filters used for each of the five extraction methods.

Table 7-4 Mass of protein detected (mg) from a total of 10 filters spiked with 1mg of BSA, extracted using one of five methods. Extraction buffers and methods used were as follows, 1: PBS-T buffer, 2: PBS-T buffer and sonication, 3: Methanol buffer and sonication, 4: SDS-based buffer and a 60°C incubation and 5: filter cut into 1cm^2 pieces, SDS-based buffer and a 60°C incubation. All filters were extracted overnight at RT on a rock 'n' roller in a 1ml volume of buffer. Two filters were used for each extraction method and each filter extract was tested in triplicate by DC™ Protein Assay with values given as an average. An average of the two filter extracts used for each extraction method is also given.

Extraction method number	1	2	3	4	5
Mass of protein in each filter extract (mg)	0.74	0.69	No protein	0.8	1.29
	0.71	0.68	No protein	No protein	0.77
Average of 2 filters	0.73	0.68	No protein	0.80	1.03

The highest quantity of protein was detected from filters that were cut into 1cm^2 pieces, extracted in SDS-based buffer and incubated at 60°C (extraction method number 5).

Protein was also detected when using a PBS-T buffer (methods 1 and 2), but this small experiment provided little evidence that sonication increased the mass of protein extracted from the filter (method 2 used sonification, method 1 did not). No protein was detected from filters extracted with methanol.

7.4.2.2 Validating the results

To validate these results, I repeated the test using more filters (n=10), for each of the three extraction methods, that detected the highest quantity of protein in the previous experiment. Filters were again spiked with 1mg of BSA and extracted either using (1) PBS-T buffer, (2) SDS-based buffer and incubation at 60°C, or (3) cutting the filter into 1cm^2 pieces using an SDS-based buffer and incubation at 60°C. The results are shown below in Table 7-5 and in Figure 7-2 (on page 288).

Table 7-5 Mass of protein detected (mg) from a total of 30 filters spiked with 1mg of BSA, extracted using one of three methods. Extraction buffers and methods used were as follows, 1: PBS-T buffer, 2: SDS-based buffer and a 60°C incubation and 3: filter cut into 1cm^2 pieces, SDS-based buffer and a 60°C incubation. All filters were extracted overnight at RT on a rock 'n' roller in a 1ml volume of buffer. Ten filters were used for each extraction method and each filter extract was tested in triplicate by the DC™ Protein Assay with values given as an average. An average, range and coefficient of variation of the ten filter extracts used for each extraction method is also given.

Extraction method number	1	2	3
Mass of protein in each filter extract (mg)	0.90	1.04	0.97
	1.07	1.29	1.18
	0.88	0.81	0.84
	0.96	1.12	1.04
	0.32	1.13	0.72
	0.95	1.01	0.98
	0.84	0.60	0.72
	0.98	1.15	1.06
	0.62	1.06	0.84
	0.52	1.05	0.79
Average of 10 filters	0.80	1.03	0.92
Range of measures	0.32-1.07	0.6-1.29	0.72-1.18
Coefficient of variation	29.6%	18.8%	17.0%

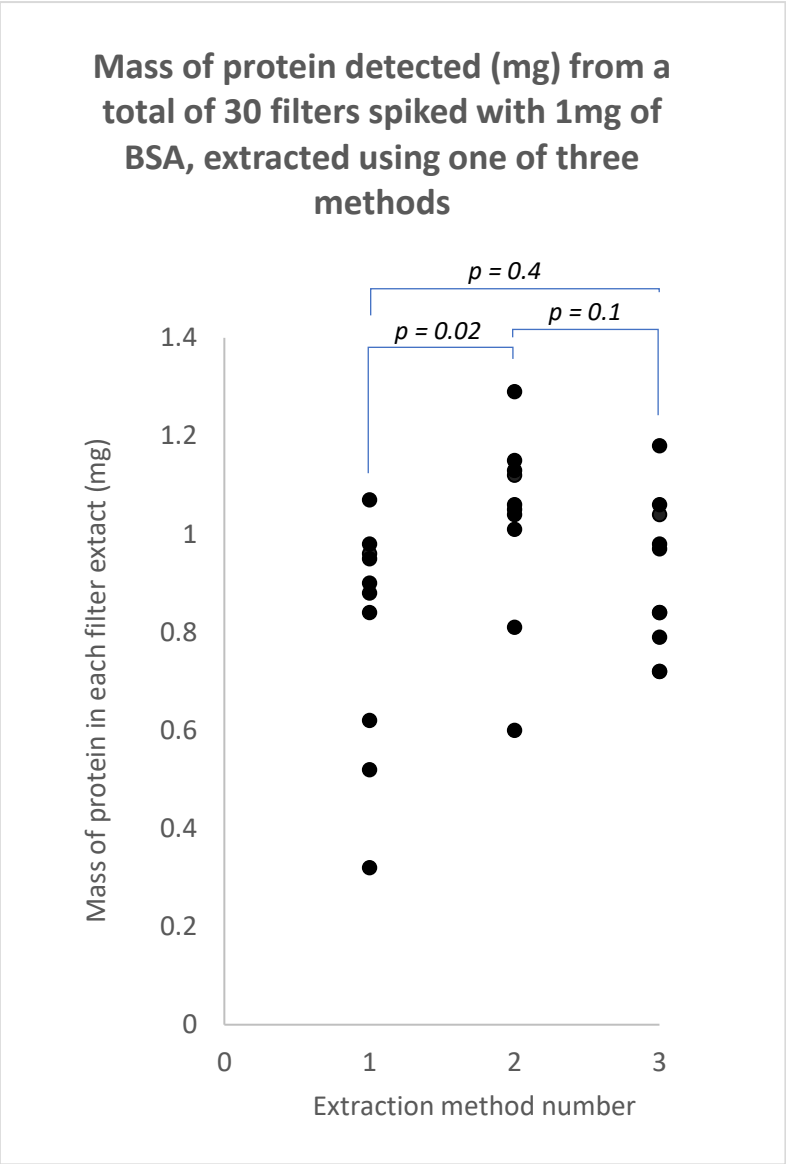


Figure 7-2 Scatter plot showing the mass of protein detected (mg) from a total of 30 filters spiked with 1mg of BSA, extracted using three different methods (p values from Mann Whitney U-test).

Our statistical analysis showed that the quantity of protein detected by method number 2 was significantly higher than method 1 ($p = 0.02$). Method 3 was not significantly different than method 1 ($p = 0.4$) or 2 ($p = 0.1$). Method 1 also showed the greatest variation in the quantity of protein detected amongst 10 filters.

7.4.3 Interpretation

This controlled experiment using BSA to spike Teflon filters showed that the SDS-based buffer could be used to detect the highest content of protein when compared with PBS-T buffer. Furthermore, there was no significant difference in the protein content detected from Teflon filters if the filter was cut up into 1cm^2 pieces.

Having shown that protein can be extracted and detected using this method, I decided to apply it to a similar filter that is used to sample outdoor air at a composting facility. The methods and findings of this are described in the following section.

7.5 Detecting protein from fresh polycarbonate filters collecting PM at an outdoor composting facility, using recognised and newly reported methods.

The aim of this work was to

- determine whether airborne protein could be detected from air samples collected at a composting facility using the SDS-based extraction method.
- assess the minimum sampling time required to detect protein.
- assess the maximum distance from the composting source at which protein could be detected.

The fieldwork was conducted, at a single composting facility by Nor Amirah Nawawi as part of her MSc in Environmental Technology at Imperial College London. I trained and supervised her in the laboratory methods described here and helped plan the study with co-supervisor Dr. Pippa Douglas. I have used the raw results from the students' laboratory work to conduct my own analysis described as follows.

7.5.1 Methods

7.5.1.1 Air sampling

Air sampling was conducted during three visits to a 'open-windrow' composting facility in Crews Hill, Hertfordshire. Airborne PM of no specific aerodynamic diameter was collected using six TUFF 4 Plus I.S. air sample pumps with IOM sampling heads (Casella London Ltd., Kempston, Bedford) and polycarbonate membrane filters (SKC Ltd, Dorset, UK).

Filters were placed inside sampling heads, connected to the pump via plastic tubing then attached to a tripod and run at a flow rate of 3.33×10^{-5} m^3/s, for either 30, 60, 150, 180 or 300 minutes. After air sampling, the filters were stored at 4°C until extraction was performed the next day. The following methods were used for the three visits to the composting facility:

- On visit 1, three air samplers were set up to run twice for 300 minutes, located as close as possible to the source of composting activity.

- On visit 2, two air samplers were set up to run for 30, 60 150 and 180 minutes during different times on the same day, located as close as possible to the source of composting activity.
- On visit 3, two air samplers were set up to run for 60 minutes, each located at 5 sites at the composting facility as follows:
    1. 100 metres (m) upwind the of composting activity,
    2. as close as possible to the source of composting activity or
    3. 100m downwind,
    4. 150m downwind (positioned at an elevation) or
    5. 250m downwind of the composting activity.

It should be noted that when air sampling was conducted, the activity at the composting site was extremely varied, both during the day and from one site visit to the next. We were also limited in the number of air sampling pumps available, meaning we could not always collect air samples during the same time interval.

7.5.1.2 Filter extraction

After sampling, each filter was placed into an Eppendorf. For comparative results, we decided to extract using both 1ml of PBS-T or SDS-based buffer. The filters, which were primarily designed for microscopy of biological contaminants were thin and transparent, and so we were unable to cut them into 1cm^2 pieces as part of the method developed in the previous section. Filters extracted in SDS-based buffer were incubated at 60°C for 15 minutes and all filters were left overnight at RT on a rock 'n' roller.

7.5.1.3 Protein quantification

To quantify the protein extracted from these filters a protein quantification assay using the *DC*™ Protein Assay kit was used, as already described. Using the protein concentration as determined by the assay, the mass of protein (mg) in the filter extract was calculated as the:

Concentration of protein (mg/ml) × volume of extraction buffer (ml).

Each filter extract solution was tested in triplicate and values are given as an average.

7.5.1.4 Calculating airborne protein concentration

Using the calculated mass of protein (mg), the flow rate of the pumps used (l/min) and sampling duration (min), the airborne protein concentrations sampled at the composting facility were estimated as follows:

1. The volume of air sampled (m^3) through the filter was calculated by:

   (Flow rate of pump (l/min) × sampling duration (min)) ÷ 1000 and

2. The mass of airborne protein in volume of air sampled (mg/m^3) was calculated by:

   Mass of protein (mg) ÷ volume of air sampled (m^3).

7.5.2 Results

7.5.2.1 Visit 1 – Detection of protein

Using PBS-T and SDS-based buffers for protein extraction we detected protein by the *DC*™ Protein Assay, from polycarbonate filters used to collect airborne PM from three air samplers set up to run twice for between 150 and 300 minutes, during one day, located as close as possible to the source of composting activity at a composting site. The results are shown below in Table 7-6.

The average mass of airborne protein detected in the volume of air sampled (mg/m^3), using the PBS-T and SDS-based extraction buffers was 1.37 and 1.36mg/m^3, respectively.

Table 7-6 Mass of airborne protein (mg/m^3) detected from a total of 6 filters sampling PM at a composting facility, extracted with either PBS-T or SDS-based buffer. Each filter was extracted in 1ml of buffer overnight at RT on a rock 'n' roller. Filters extracted in SDS-based buffer were also incubated at 60°C. Three filters were used for each of the two extraction buffers and each filter extract was tested in triplicate by the DC™ Protein Assay with values given as an average. An average of the three filter extracts used for each extraction method is also given.

Extraction method	PBS-T	SDS-based
Mass of airborne protein detected (mg/m_3)	1.04	1.29
	1.36	1.21
	1.71	1.58
Average of 3 filters	1.37	1.36

7.5.2.2 Visit 2 – The quantity of protein detected for different sampling times

Using the SDS-based buffer for protein extraction, we detected protein by the *DC*™ Protein Assay, from polycarbonate filters used to collect airborne PM from two air samplers set up to run twice during one day, located as close as possible to the source of composting activity at a composting site, for a minimum of 30 minutes. The results are shown below in Table 7-7 and include the mass of protein detected for longer sampling times.

At 30 minutes we were able to detect an average of 0.6mg of protein. At the longest sampling time used (180 minutes), this was increased to an average of 1.05mg of protein. The mass of protein did not increase proportionally to the sampling time used (i.e. doubling the sampling time did not double the mass of protein detected).

Table 7-7 Mass of protein (mg) detected from a total of 8 filters sampling PM at a composting facility, for either 30, 60, 150 or 180 minutes. Each filter was extracted in 1ml of SDS-based buffer, incubated at 60°C and left overnight at RT on a rock 'n' roller. Two filters were used for each sampling time and each filter extract was tested in triplicate by the DC™ Protein Assay with values given as an average. An average of the two filter extracts used for each sampling time is also given.

Sampling time (minutes)	30	60	150	180
Mass of protein detected (mg)	0.48	0.78	0.65	0.8
	0.71	0.41	0.9	1.3
Average of 2 filters	0.6	0.6	0.78	1.05

7.5.2.3 Visit 3 – The quantity of protein detected at different sampling distances

Using the SDS-based buffer for protein extraction we detected protein from polycarbonate filters used to collect airborne PM at a composting facility for ~60 minutes, at 5 sites located at different distances from the source of compositing activity, by the *DC*™ Protein Assay. The results are shown below in Table 7-8.

The highest average mass of airborne protein detected was 2.04mg/m^3, from filters sampling at the source of composting activity. The lowest average mass of airborne protein detected was 0.25mg/m^3, from filters sampling 250m downwind of the composting activity. A decrease in the mass of airborne protein was observed from the source of composting activity to sites both upwind and downwind.

Table 7-8 Mass of airborne protein (mg/m^3) detected from a total of 10 filters sampling PM at a composting facility, at different sampling distances. Each filter was setup for sampling either at source, 100 metres upwind, or at 100, 150 or 250 metres downwind. All filters were extracted in 1ml of SDS-based buffer incubated at 60°C and left overnight at RT on a rock 'n' roller. Two filters were used for each sampling distance and each filter extract was tested in triplicate by the DC™ Protein Assay with values given as an average. An average of the two filter extracts used for each sampling distance is also given.

Sampling distance (metres)	100 upwind	Source	100 downwind	150 downwind	250 downwind
Mass of airborne protein detected (mg/m^3)	2.23	2.15	0.6	0.19	0.08
	0.43	1.92	0.55	0.38	0.41
Average of 2 filters	1.33	2.04	0.58	0.29	0.25

7.6 Discussion

7.6.1 Overview

Burney et al., (2008), were able to detect outdoor airborne protein from fresh PM_{10} glass fibre filters after high volume sampling, by the binding of PM_{10} to specific-IgE in serum from patients who experienced asthma exacerbations.

My initial plan was to use the MS-based method (applied in the previous three chapters), to identify allergenic proteins on stored glass fibre filters, from an air sampling study (ALLOHA) that had been conducted several years earlier. However, I could not detect protein on the stored PM_{10} glass fibre filters and so I investigated how I could optimise extraction methods using filters widely available and locally used for air pollution monitoring. These differed in that they came from a low-volume sampler and were Teflon filters.

After the initial experiments on stored PM_{10} glass fibre filters, I hypothesised that the most likely reason protein was not detected was due to the prolonged storage (~8 years) of the filters. But I could not rule out that there may have been no protein collected when sampling took place between July 2008 and July 2010. This seems unlikely given that I selected filters for extraction that collected PM_{10} during the grass pollen season and would have expected some grass pollen proteins to be present.

As well as the duration for which filters were stored, the conditions in which filters were stored may have impacted on the presence of protein. Storage conditions, such as temperature, humidity and the duration of storage are known to have an impact on allergenic pollen proteins (Anderson & Baer, 1982), with increasing temperature shown to cause degradation of some proteins. The filters were stored for approximately 8 years, at RT in individually labelled A4 plastic envelopes, in an office cupboard. These are less than optimal conditions and are likely to have impacted on proteins that may have been present on the filters.

It is possible that the extraction and detection methods used were not adequate to detect protein from the filters. This also seems unlikely given that the buffers I used, make up some of the common solvents for extraction of allergenic proteins (Mansouritorghabeh et al., 2017). Moreover, it is unlikely given that we tested for protein in the filter extracts by nano

LC-MS/MS, which is a highly sensitive method for protein detection (Schuchardt and Sickmann 2007).

Despite not detecting protein from the stored glass fibre filters, protein was detected from fresh Teflon filters sampling outdoor PM_{10} from a pollution monitoring site, using a newly reported extraction method described by Chen and Hilderman (2009). Chen and Hilderman (2009) had previously collected indoor and outdoor $PM_{2.5}$ and PM_{10}, using low-volume sampling (3.5×10^{-4} m^3/s and 2.7×10^{-4} m^3/s respectively), onto 47mm Teflon filters, through cyclones running between 9 and 12 hours. After sampling, they stored their filters at 4°C for no more than 3 months before protein extraction using the SDS-based buffer, described in this chapter. They were able to detect protein by a bicinchoninic acid protein assay, up to 2.5µg/m^3 from Teflon filters that were extracted using this SDS-based buffer.

Protein was only detected from fresh Teflon filters collecting PM_{10} after using the SDS-based method and not with dH2O, AHC, PBS-T or methanol buffers. This was unexpected given that the other buffers had been used in studies elsewhere to successfully extract protein. AHC was used by Burney et al., (2008) to detect protein from PM_{10} glass fibre filters. Methanol with sonication was used more recently by Roper et al., (2015) to extract protein from $PM_{2.5}$ collected on Teflon filters. PBS-T has been used to extract airborne cat and mite allergen from glass fibre filters (de Blay et al., 1991), to extract crude allergen from fresh black tiger prawn (Rahman et al., 2010) and to extract *Platanus* pollen allergen for immunochemical quantification (Fernández-González et al., 2010).

In addition, we applied the fresh PM_{10} Teflon filters that were extracted with the SDS-based buffer and which we had detected protein from, to silver-stained SDS-PAGE to separate the proteins detected according to their molecular weight. However, despite detecting protein by the *DC*™ Protein Assay in these filter extracts, protein was not observed by silver-stained SDS-PAGE. According to the manufacturer's instructions the detection limits of both methods are 0.25ng (0.00025µg) per band in silver stained SDS-PAGE and 0.1mg/ml of protein in solution for the *DC*™ Protein Assay. These results are difficult to explain, but it may be the case that enough total protein was present in the extract to be detected by the *DC*™ Protein Assay but not enough protein per band was present to be detected by silver stained SDS-PAGE.

The use of the SDS-based method to extract protein was further demonstrated by filter spiking experiments. These results showed the highest quantity of protein was extracted from Teflon filters when using the SDS-based method when compared with other more recognised and newly reported extraction methods using PBS-T or methanol. Although using a controlled experiment with spiked filters to compare the different methods for protein extraction is of benefit to optimise the extraction methods, it does not truly reflect the detection of airborne protein sampled from outdoor air.

The SDS-based extraction method was also successfully applied to detect airborne protein from fresh polycarbonate filters collecting outdoor PM by low-volume sampling at a composting facility. We showed that using this buffer, protein could be detected at a sampling time of 30 minutes and at a distance of 250 metres downwind from the source of composting activity. The air sampling techniques previously used at composting facilities have focused on measuring bacteria, fungi species such as *Aspergillus fumigatus*, dust and endotoxin (Pearson et al., 2015) and have not measured total protein.

The allergenic protein *A. fumigatus* is often measured at composting facilities using a direct impaction method. Direct impaction works by sucking air into sampling ports and onto agar plates. The plates are removed and incubated to allow colonies of the fungi to form. These are measured as colony forming units per meter cubed (CFU/m^3) (Morris et al., 2000). Although this is an efficient method to measure species of known airborne fungi such as *Aspergillus fumigatus*, it is not suitable for detecting other potentially allergenic proteins.

In the systematic review by Pearson et al., (2015), mean concentrations of *A. fumigatus* were reported between 10^1 to 10^7 cfu/m^3, while organic dust measures were reported between 0.1 to 56.14 mg/m^3 and were usually under 3mg/m^3. If total protein is thought to represent organic dust (or part of), then the results I have shown here would agree with the reviewed literature on airborne PM detected at composting facilities.

Studies have been performed to determine the optimal extraction method for protein (Douwes et al., 1995), but no defined user protocol exists to efficiently extract and detect total airborne protein from filters sampling outdoor PM.

7.6.2 Limitations

As previously mentioned, I was unable to apply all extraction methods to Teflon filters that had collected PM_{10} at the same time. Instead, the filters were collected in the months of December 2017, January 2018 and April 2018. As the SDS-based buffer was applied to those collected in April and the other buffers were applied to those collected in December and January, the detection of protein may be attributable to the increase in airborne protein from tree pollen present in April, rather than to the use of the SDS-based buffer. In retrospect, I would have ensured that I could collect filters during the same period, so the different buffers could have been more reliably compared.

When air sampling was conducted at the composting site, the composting activity varied substantially, with periods where there was complete inactivity. Therefore, it was likely that the airborne protein levels would have also varied substantially during air sampling. This would explain variation in the mass of protein detected, between filters sampled for the same duration and at the same distance from the source of composting activity. Moreover, this would explain why the mass of protein detected for different sampling durations may have not increased at the same rate as the sampling duration. It was also not known whether these polycarbonate filters had a maximum saturation point - whereby after a certain sampling duration, no more airborne protein would be sampled.

In this chapter, the methods vary according to the different filters used and the different sampling kits and flow rates, which limits the comparability of the results, both from different sections of the chapter and with the wider literature on outdoor air sampling. Glass fibre and Teflon are commonly used for outdoor airborne sampling as they are known to be the optimal materials for gravimetric analysis and the measurement of chemical pollutants (Mentrez et al., 2007), however, they are known to be less than optimal for biological experiments. Polycarbonate filters on the other hand are suited towards microscopic examination of biological contaminants but were too thin to be cut into $1cm^2$ pieces. The filters also differed in cost and this had to be considered when conducting the experiments in this chapter.

We were also limited with regards to accessing a high-volume sampler during this work. The sampling conducted during the study by Burney et al., (2008) and the unpublished works of

the ALLOHA study were both using high-volume sampling equipment, with a flow rate of 1.2m^3/s. This was compared to flow rates of 2.7×10^{-4} and 3.33×10^{-5} m^3/s used respectively for the Teflon filters collected from the London air pollution monitoring site and the polycarbonate filters from the composting site. Although setting up a high-volume sampler would have meant an increase in outdoor air that was sampled per filter, the equipment needed would have been bulky and likely in need of frequent maintenance, compared to equipment used for low-volume sampling which we found to be easily portable, easy to use and relatively inexpensive.

7.6.3 Summary and future works

In summary, the initial results of this chapter suggested that the storage duration and conditions of filters sampling outdoor air must be considered if protein analysis is intended. The more significant results of this chapter were that an SDS-based extraction method was successful in detecting outdoor airborne protein from Teflon and polycarbonate filters.

Future work in the long-term should be directed at investigating the presence and identity of airborne proteins at outdoor composting sites, which may have the potential to cause adverse health effects. However, more imminent future work should be directed at conducting a series of experiments to validate the detection of protein from filters sampling outdoor air at an urban pollution monitoring site. For this, I have provided a rough outline of how the experiments should be run, which is as follows.

1. Collection of filters 3x/week during 3 months in the pollen season and 3 months out of pollen season.
2. This would be using (as before) a Thermo Scientific Partisol sampler collecting outdoor PM_{10} onto a 47mm Teflon filter for a 48 hour continuous sampling period (flow rate 2.7 x 10^{-4} m^3/s, low-volume). Polycarbonate filters could be used in parallel experiments to compare with Teflon filter.
3. Filters collected should be extracted on the same day of collection. Filters collected could also be immersed immediately in buffer after collection, to determine if this affects extraction efficiency.
4. SDS-based extraction as before: 2% sodium dodecyl sulphate (SDS), 0.4mM ethylenediamine tetra-acetic acid (EDTA) and 10mM Tris-HCl. The role of detergent could be assessed by using the SDS detergent at various concentrations, to examine whether there is an effect on extraction efficiency, e.g. 1%, 2%, 4%, 8%.
5. As before protein quantification would be performed using a *DC*™ Protein Assay kit.

If protein is detected, defined allergens will be detected by mass spectrometry. For example, Ph p 1 from Timothy grass pollen or Bet v 1 from Birch pollen could be identified, by using mass spectrometry and the protein database Uniprot.

8 Overall discussion

Allergens that provoke respiratory symptoms are commonly derived from animals, plants, mould and fungi and routine allergy diagnosis is performed by skin prick testing and/or commercial blood tests to a panel of these known allergens. However, some individuals experience allergic symptoms to airborne substances which have not yet been identified as allergens. These 'unknown allergens' have been identified as the causative agent most often, in the occupational setting but have also been identified in localised outbreaks of asthma (Anto et al., 1989; Burney et al., 2008).

In this PhD, I have reviewed methods that have been used to identify outdoor airborne allergens in asthma outbreaks; developed a unique mass spectrometry (MS)-based method to identify airborne allergens and identified potential 'new' allergens present within three different occupational settings. I have shown that sampling of outdoor air and suitable extraction methods can be used to detect airborne protein – but that this protein could not be detected in filters which were collected in one large epidemiological study and stored for several years. This latter observation was not foreseen and altered the initial focus of this PhD from outdoor allergens to occupational allergens.

8.1 Overview

The purpose of the systematic review in Chapter 2 was to describe which laboratory methods had previously been used to investigate outbreaks of asthma that were associated with peaks in outdoor airborne allergens. This was to inform which methods should then be used in this thesis, to identify potential unknown airborne allergens.

The systematic review protocol was written according to the PRISMA-P 2015 Statement and registered on PROSPERO. The review suggested that detection of serum specific-IgE, either by SPT or *in vitro* tests, such as western blot, RAST and ELISA was the most common laboratory method used to identify the allergen responsible in outbreaks of asthma.

Articles reviewed were published between 1928 and 2016 and so there was significant variation in the laboratory and clinical methods used; making it possible to 'track the history' of experimental method development. The review showed that the 'scratch method', now referred to as SPT has remained a cornerstone method for allergy diagnosis, from its use in castor bean, soybean and thunderstorm associated asthma outbreaks. Some authors developed their own SPT reagents. For example, Weill et al., (1964a; 1964b) used high-volume sampling to collect air samples at strategic locations where asthma-outbreak patients resided and made extracts from the air samples which they used for scratch testing on asthma-outbreak individuals. This enabled them to identify a point-source responsible for outbreaks of asthma.

The soybean associated asthma outbreaks occurring in Barcelona, were the most well-described, accounting for 45 out of the 85 articles that were reviewed. Epidemiological methods were first used by Anto and Sunyer (1986) to identify a point source (the local harbour), responsible for the outbreaks of asthma in Barcelona. In subsequent years, many authors including Sunyer et al., (1989), Anto et al., (1993), Rodrigo et al., (1990), Swanson et al., (1991), Pinto et al., (1994) and Soriano et al., (1995), used laboratory methods such as western blot, ELISA and RAST, to further characterise the soybean allergens responsible for asthma-outbreaks. However, the most advanced characterisation of soybean allergens was conducted by Codina et al., (1997), in the form of N-terminal sequencing. This was an early method used to determine the partial amino acid sequence of a protein and then identify a protein match by comparison with other known protein sequences in a stored database.

All the outbreaks reviewed concerned sudden life-threatening cases of asthma and a considerable number were seen during thunderstorm associated asthma outbreaks. One of the world's largest outbreaks of thunderstorm asthma occurred during the conduct of this review, in Melbourne in November 2016, causing 10 deaths and 3500 presentations to emergency departments (Lee et al., 2017; The Chief Health Officer's Report, April 2017; Then et al., 2018). Tests on outbreak patients to detect serum specific-IgE, suggested rye grass pollen was the airborne allergen responsible (Lee et al., 2017).

In addition to these large-scale events, the review gave evidence for smaller asthma outbreaks, occurring more frequently at lower levels. Newson et al., (1998) identified 56 regionally based asthma epidemics between 1987-1994 in a comprehensive analysis of hospital asthma admissions, not all of which could be predicted by either a thunderstorm or elevated pollen levels. Using similar epidemiological methods, Marks et al., (2001) identified 48 asthma epidemic days from 1995-1998.

More recently, Burney et al., (2008) showed that in patients with asthma exacerbations attending a London GP practice, there was an association with serum specific-IgE binding to airborne PM_{10} which was collected at the time of patient asthma exacerbation.

In retrospect, and in light of the subsequent direction of the thesis, this systematic review would have been better directed at methods used to identify occupational allergens. However, when I planned the systematic review it was in preparation for work to detect proteins, from stored filters that had previously collected outdoor airborne PM_{10} by high-volume sampling (Chapter 7, section 7.2). During my PhD I could not detect protein from these filters, so my attention shifted to how the methods identified in the systematic review (e.g. detection of serum specific-IgE binding by western blot), could be applied to identify airborne allergens in the occupational setting.

I was able to develop and optimise a methodology for the identification of potential unknown allergens. This was evidenced through identification of the known allergen, Mus m 1, from mouse urine. I detected serum specific-IgE binding proteins in both mouse urine and mouse epithelium crude extracts, by using an enhanced chemiluminescence (ECL) method, which showed greater sensitivity than a chromogenic method of detection. Using a *DC*™ protein assay, I showed that PBS-T extraction buffer yielded the highest protein content

from a crude mouse epithelium extract. Moreover, I optimised the serum and anti-human IgE antibody incubation times and dilutions for the methodology.

During method development in Chapter 3, I used tryptic digestion of specific-IgE binding proteins and MS methods from both Steven Lynham at King's College London and Prof. Peter Briza at the University of Salzburg. For the subsequent Chapters, I chose to adopt methods for MS used by the research group based at the University of Salzburg as they offered feedback and expertise specifically relevant to the field of allergen proteomics.

Unexpectedly, my western blot results for mouse urine showed an uncharacterised specific-IgE binding protein at approximately 35kDa. Using MS, I putatively identified this as the potential mouse urine allergen – kallikrein. There has been only one report by Hollander et al., (1997) of serum specific-IgE binding to a mouse urine protein with a molecular weight close to 35kDa. However, the researchers did not conduct any further characterisation. Kallikrein has been previously identified as a major allergen from dog urine and is registered within the WHO/IUIS Allergen Nomenclature as the dog allergen - Can f 5 (Mattsson et al., 2009).

When using the optimised method to identify proteins from a crude mouse epithelium extract, I identified known allergen, Mus m 4 (also known as serum albumin), in addition to another uncharacterised specific-IgE binding region. Using MS, the muscle protein Titin was identified as a potential novel allergen from this uncharacterised specific-IgE binding region. Titin has been identified previously as an allergen from other species, namely black tiger prawn by Kamath et al., (2014), who showed that 11 out of 16 shellfish allergic patients had specific-IgE binding to the titin muscle protein.

Moreover, I compared the serum specific-IgE binding proteins present within a SPT mouse epithelia solution with those present within a crude mouse epithelium extract and found that while Mus m 4 and the putatively identified titin protein were present in both solutions, there were three additional uncharacterised specific-IgE binding regions (25-50kDa) in the crude mouse epithelium extract. This adds to research by Canizales et al., (2015), who showed that some laboratory animal workers have specific-IgE binding to mouse epithelium but not to mouse urine. This suggests there are allergens present within

mouse epithelium that are not present in mouse urine and which are therefore relevant for laboratory animal allergy (LAA).

I went on to successfully apply the optimised methodology to identify potential allergenic proteins from both the common fruit fly, *Drosophila melanogaster* and from improver enzymes used in the UK supermarket scratch baking industry.

In a serum specific-IgE binding region (~10kDa) from fruit fly, MS identified the potential novel allergenic protein, fructose-bisphosphate aldolase. I found that this protein has been identified as an allergen from other species, such as cod, salmon and tuna and is registered on the WHO/IUIS Allergen Nomenclature, for these species (Kuehn et al., 2013; Sookrung et al., 2014). In addition, a larval serum protein was identified as a potential allergen from the ~10kDa serum specific IgE binding region. This protein has been previously identified as a potential fruit fly allergen in a recent publication by Colomb et al., (2017).

There are no allergens registered in the WHO/IUIS Allergen Nomenclature for *D. melanogaster* and the literature available is limited to only a few publications (Spieksma et al., 1986; Jones et al., 2017; Colomb et al., 2017). I was able to not only detect serum specific-IgE binding regions to fruit fly proteins using western blot, but I also putatively identified specific-IgE binding proteins using MS. This is an extension to the current literature that is available on occupational allergy caused by exposure to fruit fly.

I set up the same MS-based method as a 'proof of principle' application to determine whether unknown allergens from improver enzymes could be identified. A very high number of proteins were identified from specific-IgE binding regions in two improver enzyme extracts that were analysed by MS. Proteins were most commonly derived from *Aspergillus*, *Bacillus* and *Triticum* and although the correct proteins - bacterial xylanase and glucose oxidase were identified, these were amongst a list of many other highly likely protein candidate identifications.

In parallel, I attempted to detect protein from stored glass fibre filters which were collected in a previous epidemiological study, that had sampled outdoor particulate matter with an aerodynamic diameter <10μm (PM_{10}). I was unable to detect protein from these filters, and therefore unable to identify proteins binding to serum specific-IgE from patients who

suffered asthma exacerbations when sampling took place. I hypothesised that the prolonged storage of the filters was the most likely reason protein was not detected.

Moving forward, I optimised filter extraction methods to detect outdoor airborne protein, using more recently collected filters. I obtained fresh Teflon filters, collecting outdoor PM_{10} from a pollution monitoring site and used a newly reported method described by Chen and Hilderman, (2009) which utilised an SDS-based extraction buffer. I also used another newly reported method alongside recognised extraction methods on Teflon filters sampling outdoor PM_{10}. Protein was only detected after using the SDS-based method, which was unexpected, given that the recognised methods had proven success for protein extraction in other studies (de Blay et al., 1991; Burney et al., 2008; Fernandez-Gonzales et al., 2010; Roper et al., 2015). Moreover, the filter spiking experiments I conducted showed that the SDS-based method was suitable for protein extraction from a larger sample number of Teflon filters.

This extraction method was then successfully applied to detect airborne protein from fresh polycarbonate filters collecting PM at an outdoor composting facility. I observed that protein could be detected after sampling for a minimum of 30 minutes and at a distance of 250 metres, downwind from composting activity at the facility. Air sampling techniques used at composting facilities previously focused on detecting and measuring specific species such as *Aspergillus fumigatus* (Morris et al., 2000). There isn't a method currently used to detect other potential allergenic proteins present at composting facilities. This thesis gives important preliminary findings, that contribute towards investigating the presence of unknown airborne proteins at composting facilities.

In this thesis, I have presented a new combined western blot and MS-method for the identification of occupational allergens. However, during the time I completed my PhD, I became aware of similar methods being used and developed elsewhere. Moreover, as the field of allergen proteomics has taken a crucial shift towards component resolved diagnosis, there has been a greater need for researchers to identify and obtain the sequence for individual allergenic proteins, in addition to evidencing their allergenicity.

As mentioned in the introductory part of this thesis, a research group in India have also used a combined western blot and MS approach for allergen identification. They detected a

44kDa specific-IgE binding region from *Rhizopus oryzae* (a prevalent fungal species in India) and using MALDI MS identified this as an aspartic protease protein (Sircar et al., 2012). They also went on to assess the allergenicity of this putatively identified protein using *in silico* methods. This meant comparing an 80-amino acid window with other known allergen sequences to find that their *R.oryzae* protein had a significant match (>35% homology) with an aspartic protease allergen - Bla g 2 from *Blatella germanica*. Moreover, they aligned the full amino acid sequence of the *R.oryzae* protein with known allergens: Asp f 10 and Asp sa AP.

In another study, the same research group identified a novel allergen – Cari p 1, from a papaya extract, acting as both a respiratory and food sensitizer (Sarkar et al., 2018). As with the methodology presented in my work, they used serum from sensitised patients to detect a 56kDa specific-IgE binding region which was identified as endopolygalacturonase (Cari p 1) by tandem MS. In this study, they assessed the allergenicity of the novel allergen by producing a recombinant form of Cari p 1. This was done using specifically designed primers in PCR of a papaya pollen RNA extract. Using the recombinant allergen, specific-IgE reactivity was verified by western blot and immunogenicity tested by a basophil activation test. In addition, they used *in vivo* mouse models to demonstrate inflammatory responses in the lung were elicited on exposure to the allergen.

8.2 Limitations

In developing a new method to identify airborne proteins responsible for causing allergic asthma, I was aware of limitations to the method itself as well as limitations present in my research overall.

8.2.1 Limitations of the method

Using filter extracts collected by the same air sampling method used by Canizales et al., (2016) in animal testing facilities I was unable to detect the mouse urine allergen – Mus m 1, by my optimised western blot method. Canizales et al., (2016) had used a specific Mus m 1 ELISA kit to detect Mus m 1 from filters, which is far more sensitive than a western blot method to just detect for Mus m 1. Hence the method proposed in this thesis, is limited in its detection of airborne allergens collected using specific air sampling techniques. It is likely that a higher concentration of airborne allergen is required for detection by western blot. This can be achieved by high-volume air sampling.

A critical limitation of the method for future use is its reliance on serum specific-IgE binding to an allergenic protein, which can be detected via an ECL western blot. In some instances, we observed no binding from sensitised individual serum shown to have elevated levels of specific-IgE (classed as a score of ≥2% binding by RAST). This suggests that some individuals classed as having elevated levels of specific-IgE by RAST to the allergen extract, may not be identified using the method proposed here, especially if these 'elevated' levels are relatively low. However, if individuals with relatively high elevated levels of specific-IgE are used then the allergenic protein responsible for symptoms of allergy, is far more likely to be detected and identified using the method proposed here. Given that the method relies on detection of serum specific-IgE binding, it is not suitable for many low molecular weight allergens that are responsible for causing occupational asthma. For example, specific-IgE is only detected in a small subset of individuals affected with respiratory symptoms that is due to isocyanate exposure in the workplace (Maestrelli et al., 2009).

The immediate practical application of this method into, for example, the occupational asthma clinic, is limited due to the large data output given by MS that requires time-consuming interpretation and allergen proteomics expertise. The data may consist of many significant 'high probability matches', which means the unique peptide count, sequence

coverage and molecular weight of identified proteins all need to be scrutinised. Moreover, if the species taxonomy of the potential allergen is unknown, then it can be difficult to distinguish proteins that have been identified from closely related species. This was the case for proteins identified from the improver enzyme extracts. In most of its different forms, MS is still an expensive tool and the equipment it requires will most often only be found in specialised laboratories.

Researchers should also consider that the molecular weight of a binding region observed by western blot may not match the theoretical molecular weight given from protein databases. As previously explained, this may occur: (a) when larger proteins have degradation products that appear with a lower molecular weight and (b) when lower molecular weight proteins aggregate within an SDS gel and western blot, appearing at a higher molecular weight than is expected.

8.2.2 Limitations of my research overall

I had privileged access to serum samples associated with occupational asthma cases that had been obtained during studies in previous years. However, the small volume and number of serum samples that were available from patients with occupational allergy to the sources I investigated was a limiting factor in the experiments I was able to conduct (Chapters 4, 5 and 6). I chose serum samples based on those with elevated levels of serum specific-IgE to the allergen extract, which meant there was always a limited number available. This was problematic in particular, during the work on supermarket scratch bakers in Chapter 6, where I was unable to conduct any repeat western blot experiments because the number of stored serum samples was more limited.

After protein identification using MS, it is not uncommon to perform further confirmatory tests to ensure the protein identification is accurate. Within the allergy field, this usually involves purifying the protein by producing it in a recombinant form. Doing this requires designing DNA primers for the identified protein and cloning it into an expression vector. When this protein is produced as a separate form from its allergen extract, it can be tested using allergic serum to confirm its allergenicity and immunogenicity. These tests were

beyond the scope of the work presented here, however it is recognised they would make a major contribution to future development of this research.

In Chapter 7, I was able to establish methods for detecting outdoor airborne protein, but there were limitations associated with the equipment and materials I had available. As such, the sampling methods used varied with different filters used (glass fibre, Teflon and polycarbonate) and whether high or low-volume sampling equipment was available.

8.3 Future work

From the results I have obtained on potential novel occupational allergens, there is an opportunity for new allergens to be registered within the WHO/IUIS Allergen Nomenclature. The allergenic proteins should be purified and possibly produced in a recombinant form and be tested for their allergenicity. Methods such as the basophil activation test or the histamine release assay, (both described in Chapter 1, section 1.4.3) are increasingly popular ways to demonstrate immunogenicity and allergenicity of novel allergenic proteins (Mukai et al., 2017; Sarkar et al., 2018; Bahri et al., 2018).

Another popular immunoassay that would offer further characterisation is 2-dimensional SDS-PAGE, whereby proteins are separated by their isoelectric point as well as their molecular weight. This technique is being used in the allergen field (Saha et al., 2015), and although I had the opportunity to observe how the technique is conducted during my research training fellowship in Austria, the costs and time restraints of my PhD meant I was unable to apply this technology in the work presented here. It is an obvious choice for future work of this thesis.

As part of the work conducted in Chapter 4 on mouse allergens, I identified potential novel allergenic proteins from both crude extracts of mouse urine and mouse epithelium. Further work needs to be done to identify those workers who may be currently misdiagnosed, because they do not have specific-IgE to the well-known allergens that are included in SPT and ImmunoCAP testing, but instead are sensitised to uncharacterised allergenic proteins present in mouse urine and epithelium.

Future work investigating allergy to both fruit fly in animal testing facilities and improver enzymes in supermarket bakeries is warranted by the large number of workers that have been and will continue to be exposed in both these industries. There should be larger scale studies which include the collection of clinical samples for specific-IgE detection and gathering of epidemiological data, to assess the extent of the respiratory allergy burden in these occupations.

To complement larger scale studies, it would be of value to develop a ‘method of practice’ that occupational health physicians can use when they encounter a patient experiencing allergic symptoms that they are not able to ascribe to a specific allergen using routine

diagnostic assessments, such as SPT and ImmunoCAP. Based on the results and limitations I have experienced during the course of this PhD, I would suggest for this method of practice that serum should be obtained from the patient, with a volume of at least 5ml and samples should be taken from the workplace; both from suspected sources of allergen and by air sampling, using high-volume equipment if possible. Moreover, it may be possible to develop multiplex chip technology (as described in Section 1.4.2.4) for occupational health clinics, if the allergen components that are included are targeted to those found in workplace environments.

I observed preliminary results for proteins detected at a composting facility. It is important to further investigate the relevance of airborne protein and potential allergens that might be present at composting facilities. Collection of serum and application of the optimised MS based method may be suitable to achieve this.

To disseminate the important findings of this PhD, I have given a strategy for the publication of results from Chapters 2 and 4, as follows.

In Chapter 2, the systematic review of clinical and laboratory tests used for investigating outbreaks of asthma thought to be associated with unusual peaks in outdoor airborne allergens, will be updated for publication into *Clinical & Experimental Allergy*. As the last electronic search was in March 2016, an up-to-date search is required to capture articles that have been published since. This is likely to include articles published on Melbourne thunderstorm asthma, given the significant thunderstorm asthma outbreak that occurred in Melbourne on 21st November 2016. In addition, the figures and tables of the chapter will need to be formatted to suit journal publication (e.g. moving data extraction tables to an appendix).

In Chapter 4, the results showing identification of novel mouse allergens: kallikrein and titin, will be presented for publication into *Allergy*. In this article, I will discuss the importance of Mus m 1 perhaps not being the only relevant allergen in occupational asthma due to exposure to mouse proteins, and compare my findings with the already identified kallikrein allergen from dog (Can f 5). I will include western blot and mass spectrometry results on both kallikrein and titin allergens, providing figures from this thesis that are formatted appropriately for publication.

8.4 Conclusions

I have optimised and developed a successful MS-based method for the identification of airborne allergenic proteins likely to be responsible for causing allergic asthma. In this thesis novel findings are presented on allergenic proteins derived from mouse and fruit fly species used in animal testing facilities and improver enzymes used in UK supermarket scratch bakeries.

Allergen databases, such as the WHO/IUIS Allergen Nomenclature and the Allergome platform rely on researcher-based allergen identifications to record accurate results which can be applied back into the clinical setting for use in allergy diagnosis. Component resolved diagnosis is now at the clinical forefront and therefore the work presented here contributes to a field that is likely to develop rapidly in the coming years.

Further work is needed to confirm the allergenicity of the proteins identified in this work, through recombinant protein production and other cell-based assays. In addition, larger scale studies are necessary to investigate the burden of occupational asthma for those exposed to fruit fly in animal testing laboratories or improver enzymes in supermarket bakeries.

The method has substantial implications to identify potential ‘new’ allergens in other occupational settings, as well as the potential to identify allergens in the outdoor environment.

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