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1 INTRODUCTION

1.1 Concepts of allergy

Upon encounter with a normally harmless substance, an individual can in response produce antibodies. When the individual is exposed to that substance again, the immune system is triggered and an allergic reaction occurs (Janeway 2001c).

1.1.1 The immune system

The immune system works as a defence against infectious micro organisms. It consists of the innate immune system and the adaptive immune system. The innate immune system prevents organisms from entering the body by means of physical barriers such as skin and mucous secretions. Pathogens that get through the physical barriers are dealt with by chemical and cellular defences. The adaptive immune system consists of cells and soluble proteins (antibodies) with specificity for microbial structures (antigens). The different cells of the immune system are derived from a common stem cell in the bone marrow. Mast cells, eosinophils and basophils are three important types of cells of the immune system. They induce inflammation and are involved in allergic reactions (Janeway 2001a).

1.1.1.1 The antibody

An antibody is a protein that binds specifically to a particular substance that is referred to as an antigen. Antigens that cause allergic reactions are termed allergens. Antibodies are also known as immunoglobulins (Igs). All antibodies have the same overall structure, two polypeptide chains termed heavy chain (H chain) and light chain (L chain). An antibody has two identical H chains and two identical L that are linked together with disulfide bonds. The H chains and the L chains consist of protein domains containing about 110 amino acids each. The amino acid sequence of the first domain at the amino terminus of both H and L chain differ between different antibodies, hence these domains are termed variable domains (V domains). The variable domains make up the variable region, which is the antigen-binding part of the antibody molecule. The remaining domains are referred to as constant domains (C domains). The H chain differs between different antibodies and the ones with the same H chains are considered to be members of the same immunoglobulin class. The five classes, or isotypes, are immunoglobulin M (IgM), immunoglobulin D, (IgD), immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin E (IgE). Each class possesses its characteristic functional properties. Most allergies involve production of IgE antibodies (Janeway 2001c).
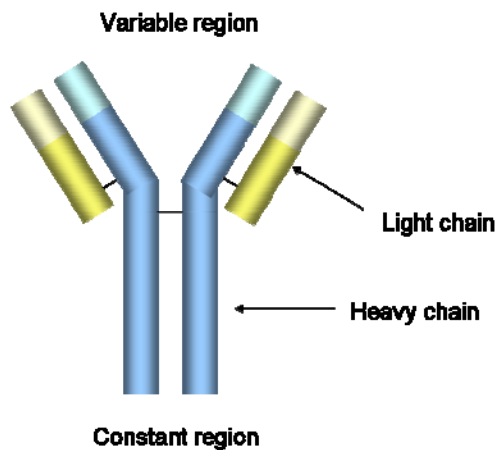
Figure 1.1 Structure of an antibody. Antibodies are composed of two types of polypeptide chains, heavy chains and light chains. The antigen-binding region varies extensively between different antibodies and referred to as the variable region.

1.1.1.2 Hypersensitivity and allergy

Immune responses, involving symptomatic reactions, upon reexposure to a normally innocuous antigen are called hypersensitivity reactions. There are different types of hypersensitivity reactions and they are categorised according to the mechanisms involved. Type I hypersensitivity reactions, often equated with allergy, are mediated by IgE antibodies.

IgE antibodies are located predominately in tissues where they bind to the surface of mast cells through the high-affinity IgE receptor called FcεRI. When antigens bind to IgE the receptors become cross-linked, and chemical mediators, that can cause a type I hypersensitivity reaction, are released from the mast cells. Basophils and activated eosinophils also express FcεRI on their surfaces. Hence they can also be involved in triggering type I hypersensitivity reactions (Janeway 2001c).
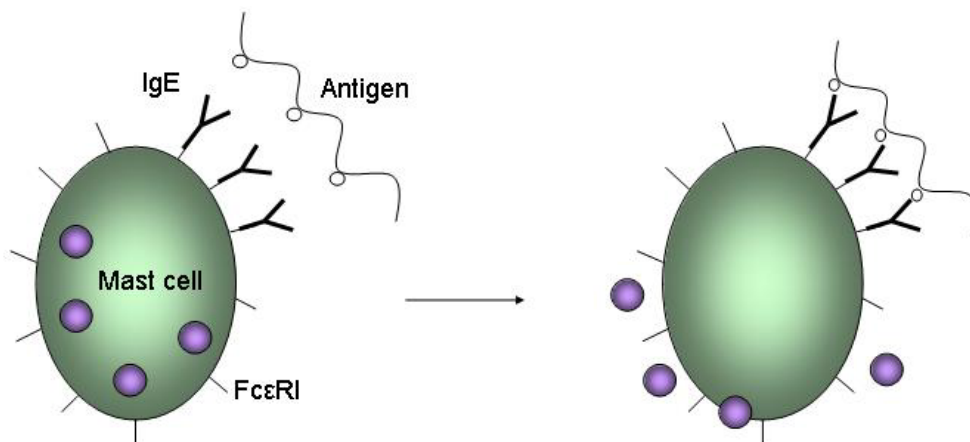
Figure 1.2 Mast cell activation. When antigens bind to antibodies on mast cells, the IgE receptors (FcεRI) become cross-linked and chemical mediators are released.

The consequence of IgE-mediated mast-cell activation depends on the dose of antigen and how the antigen entered the body i.e. which mast cells that are activated. The most common route of entry is inhalation, which leads to activation of mucosal mast cells and symptoms like sneezing and a runny nose can occur. Allergens that are directly introduced into the blood stream or rapidly absorbed from the gut, e.g. drugs, insect venom, can activate the mast cells of connective tissue that are associated with all blood vessels, leading to circulatory collapse and suffocation. This potentially fatal reaction is referred to as anaphylactic shock (Janeway 2001c).

1.2 Food allergy

Food allergy affects many people in the westernized countries and is considered to be a big health problem. Approximately 4% of Americans are thought to suffer from some type of IgE-mediated food allergy (Sampson 2004).

The major food allergens that elicit IgE-mediated reactions in children come from egg, cow's milk, soybean, wheat, fish, peanut and tree nut and among adults fish, shellfish, peanuts and tree nuts (Burks *et al*. 2001). Food allergens are in general water soluble glycoproteins (10 - 70 kDa) that are resistant to proteases, heat, and denaturants hence prevented from destruction during food preparation and digestion (Sampson 2004).

1.2.1 Peanut allergy

Food hypersensitivity, which generally occurs early in childhood, will in most cases resolve. In terms of peanut allergy, it usually persists throughout an individual's lifetime (Lewis *et al*. 2005). Symptoms vary among individuals and range from itchy rashes to anaphylaxis. Due to its persistence and high risk of severe anaphylaxis it is considered to be the most serious food-induced allergic reaction (Scurlock & Burks 2004). The prevalence of peanut allergy in the American and European populations has been estimated to 0.6% and 1%, respectively (Modoulet *et al*. 2005). It has been reported that the prevalence is increasing worldwide (De Leon *et al*. 2007).

1.2.1.1 Diagnosis and treatment

There are different approaches used to diagnose food allergy. Using skin prick tests is a rapid method to screen patients and determine which types of food the patient is reacting to. Another test is the double-blind placebo-controlled food challenge (DBPCFC), which is an oral food challenge. It is also possible to measure an individual's food-specific IgE level and from that predict the probability of

reaction to a specific food (Sampson 2004). Up to date, the only available treatment for peanut allergy is restricted to strict avoidance of all peanut products, recognition of the early symptoms of anaphylaxis, and in case of an accidental ingestion usage of an emergency treatment plan, including treatment with antihistamines and self-administration of epinephrine (Palmer & Burks 2006).

1.2.1.2 Peanut

The peanut plant (*Arachis hypogaea*) is an annual plant native to South America (Scurlock & Burks 2004). It belongs to the legumes and its fruit, the peanut, is matured below the soil surface. Peanuts are most commonly consumed as peanut butter, snacks, and as an ingredient in candy and pastry (InVitroSight, 2007a).

1.2.1.3 Peanut allergens

Peanuts contain up to 32 different proteins and a number of them are identified as allergens (Dean 1998). The first main peanut allergen that was identified is Ara h 1. It has a molecular weight of 63.5 kDa and an isoelectric point of 4.55. Ara h 1 shares sequence homology with plant seed storage proteins in the vicilin family. Ara h 1 is a homo-trimer composed of three monomers that are linked together with hydrophobic interactions and has at least 23 specific IgE-binding epitopes (Burks *et al.* 2001, Scurlock & Burks 2004). Ara h 2 was the second major peanut allergen to be identified. Its molecular weight is 17.5 kDa and it has an isoelectric point of 5.2. Ara h 2 reveals sequence homology for the conglutin family of seed storage proteins and has at least 10 specific IgE-binding epitopes (Burks *et al.* 2001, Scurlock & Burks 2004).

Other officially recognised peanut allergens are Ara h 3, Ara h 4, Ara h 5, Ara h 6, Ara h 7, and Ara h 8 (De Leon *et al.* 2007). Ara h 3 and Ara h 4, nowadays considered to be the same allergen and often denoted Ara h 3/4, has a molecular weight of 40 kDa (20 kDa after reduction), Ara h 5 (14 kDa), Ara h 6 (16 kDa), Ara h 7 (14 kDa), and Ara h 8 (17 kDa) (Lewis *et al.* 2005, Mills 2006). Ara h 1, Ara h 2, and Ara h 3 are considered to be major peanut allergens. They are all seed-storage glycoproteins that are water soluble, resistant to heat and digestion, and present in high concentration (Lee & Scheffer 2003). Ara h 1 and Ara h 2 are reported to be highly allergenic, approximately 70-90% of all peanut allergic individuals have Ara h 1 and Ara h 2-specific IgE (Palmer & Burkes 2006, De Leon *et al.* 2007). Ara h 3 is recognised by 45% of sensitised individuals (Palmer & Burkes 2006).

Table 1.1 Peanut allergens

Allergen	MW (kDa)
Ara h 1	63.5
Ara h 2	17.5
Ara h 3/4	40
Ara h 5	14
Ara h 6	16
Ara h 7	14
Ara h 8	17

Published reports present different opinions concerning how allergenic the different allergens are i.e. how important role they play in allergic reactions (De Leon *et al*. 200). Koppelman *et al*. (2004) suggest that Ara h 2 is the most important peanut allergen, based on a study on 32 adult peanut-allergic patients. They also found peanut proteins with a molecular weight somewhat smaller than 15 kDa that may be important allergens.

The reason why some individuals develop antibodies upon exposure to peanut and others do not, is currently not well understood but genetic predisposition and exposure at early age is thought to increase the risk of sensitisation (Lee & Sheffer 2003). Although the level of peanut specific IgE can be used to predict the likelihood of clinical reactivity to peanut, it is currently not known how or if an individual's IgE-binding pattern relates to the severity of the allergic reaction. It has however been reported that individuals who bind a higher number of peanut allergens have more severe allergic reactions (Palmer & Burks 2006).

1.2.1.3.1 Thermal processing of peanut allergens

Studies have shown that thermal processing influence the allergenicity of peanut proteins. How an allergen is affected depends on both the three-dimensional structure and the chemical structure of the allergen, the type of thermal processing used, temperature, and the duration of treatment. It is also possible that interactions with other components of the food matrix affect the overall allergenicity of the food (Mondoulet *et al*. 2005). Exactly what happens during thermal treatment is not fully understood. It may change the allergen's susceptibility to digestion, alter the tertiary structure of the protein, or destroy or create new IgE-binding epitopes (Pomés & Chapman 2006). The overall effect on a complex allergen is hard, or even considered impossible, to predict (Mondoulet *et al*. 2005).

It has been suggested that roasting may enhance the allergenic properties of peanut (Scurlock & Burks 2004). According to Mondoulet *et al*. (2005), is the IgE-binding capacity of whole peanut extract prepared from boiled peanut is two-fold lower than that of extract prepared from raw and roasted

peanut. Mondoulet *et al.* (2005) could not observe any significant difference between extracts from raw and roasted peanut. The observed decrease in allergenicity of boiled peanut is explained by the fact that low-molecular-weight allergens are transferred into the water during cooking (Mondoulet *et al.* 2005).

Opinions differ concerning the effects of roasting on antibody binding. Earlier studies propose that thermal processing has no effect on IgE-binding to Ara h 1 or Ara h 2. Increased allergenicity is rather due to a more efficient extraction leading to a higher quantity of allergens available (Pomés & Chapman 2006). Other studies have shown that roasted peanut contains more allergenic components than fried or boiled peanut. Levels of peanut specific IgE to Ara h 1, Ara h 2, and Ara h 3 in serum are higher in roasted when compared to boiled/fried peanut (Scurlock & Burks 2004). More recently it was proposed that by roasting the peanut the allergenicity increases. This was reported in the same context as it was proposed that the IgE-binding epitopes of Ara h 1 and Ara h 2 are altered upon roasting (Pomés & Chapman 2006). Pomés & Chapman (2006) suggest that roasting (10-15 min) increases the efficiency of Ara h 1 extraction, whereas treatment for 20-30 min decreases it. The total peanut protein content decreases with roasting time, indicating that many proteins, unlike Ara h 1, are not heat stable. Additionally, Pomés & Chapman (2006) suggest that it is rather the increase of epitope accessibility to allergens than conformational change on IgE-binding epitopes that leads to an increased allergenicity in roasted peanut. Beyer *et al.* (2001) report that the amount of Ara h 1 is reduced in fried or boiled peanut fractions, hence a reduction of IgE-binding intensity can be observed. It is also reported that the amount of IgE binding to Ara h 2 and Ara h 3 is lower in fried and boiled peanut even though the three fractions (roasted, boiled, fried) contain similar amount of Ara h 2 and Ara h 3 (Beyer *et al.* 2001).

1.3 Food allergy in Asia

The prevalence of food allergy is increasing in the United States, Europe and Australia meanwhile there are very few data on the prevalence and clinical features of food allergy in Asia. For many years, it has been believed that the prevalence of allergic diseases was low in Asian countries. However, recent studies show that allergic diseases are increasing in the area and it has been reported that food is an important cause of severe allergic reactions in Asia (Shek & Lee 2006).

It is noticeable, that peanuts and tree nuts, despite a high rate of consumption, rarely are the cause of allergic reactions in Asian countries (Shek & Lee 2006). In contrast, more than 80% of the anaphylactic shocks occurring in the United States are caused by either peanuts or tree nuts (Scurlock & Burk 2004). The difference in allergic response could be explained by differences in food

preparation, early consumption, differences in maternal diet and other not yet identified genetic and/or environmental factors (Shek & Lee 2006). In the United States, the consumed peanuts are predominantly roasted peanuts, whereas in Asian countries the consumed peanuts usually are boiled or fried (Pomés *et al*. 2006).

1.4 Aim

It has been reported that the prevalence of peanut allergy in Asia is not as high as in westernized countries, and it has been discussed whether this is due to the differences in how peanuts are prepared before consumption. In Asian countries the consumed peanuts are usually boiled or fried whereas in westernized countries they predominantly are roasted. The aim of this master's degree project was therefore to compare the proteins and allergenic components in differently treated peanuts. Peanuts were heated, boiled, roasted, and defatted. Allergenic components in the different treated peanuts were studied by use of sera from American, European and Japanese patients with suspected peanut allergy.

2 EXPERIMENTAL TECHNIQUES

2.1 Electrophoresis

Electrophoresis is a technique used for separating molecules in a mixture under the influence of an applied electric field. When dissolved molecules are exposed to an electric field, they migrate towards an electrode at a speed determined by their charge-to-mass ratio. Proteins that differ in size and shape have nearly identical charge-to-mass ratios hence they separate poorly in liquid solutions. To overcome this and be able to separate different proteins from each other, various gels are used (Lodish *et al*. 2001). The most commonly support media for carrying out electrophoresis nowadays is either agarose gels or polyacrylamide gels (Wilson & Walker 2005c). Agarose is a polysaccharide originating from seaweed. The higher the agarose concentration in the gel, the narrower the gel is. Polyacrylamide is composed of cross-linked acrylamide and the length of the polymer is depending on the acrylamide concentration used. Long polymers give rise to a narrow gel (Janson 2004). The smaller the protein, the faster it will migrate through the gel. The rate of migration is depending on the pore size of the gel i.e. the concentration of agarose/polyacrylamide in the gel and the strength of the applied electric field (Lodish *et al*. 2001). After electrophoresis the separated proteins can be visualised using different staining methods, see paragraph 2.3 *Gel staining* below.

2.1.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The most widely used method for separating proteins according to their size is sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Wilson & Walker 2005c). Proteins are exposed to sodium dodecyl sulphate (SDS) both before and during gel electrophoresis. Sample preparation involves boiling proteins together with SDS and the gels used for electrophoresis contain SDS. SDS is an anionic detergent that binds to and denatures proteins, causing multimeric proteins to dissociate into their subunits (Lodish *et al*. 2001). Proteins open up and are forced into extended conformations with a series of SDS molecules along the polypeptide chain. Approximately one SDS molecule binds for every two amino acid residues resulting in different proteins having similar charge-to-mass ratios (Wilson & Walker 2005c). SDS treatment consequently eliminates the effect of differences in shape so that chain length, which reflects mass, is the sole determinant of the migration rate of proteins in SDS-PAGE (Lodish *et al*. 2001).

2.1.2 Isoelectric focusing (IEF)

Proteins of similar mass require another approach to be separated and most commonly the physical characteristic electric charge is utilized. A protein's electric charge is determined by its number of

acidic and basic residues (Lodish *et al*. 2001). Proteins are amphoteric substances, i.e. they possess either a positive, negative, or zero net charge, depending on their surrounding pH. The pH at which a protein's net charge is zero, is referred to as the protein's isoelectric point (pI) (Wilson & Walker 2005c). Proteins carry a positive charge in environments with a pH below the protein's pI, meanwhile proteins are negatively charged at pH values above their pI (Görg 1998). In isoelectric focusing (IEF), which is an electrophoretic technique, proteins are separated according to their pI. The gels used for IEF contain a pH gradient and under the influence of an electric field the proteins will migrate toward their pI (Wilson & Walker 2005c).

2.1.3 *Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)*

In two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), proteins are separated in two sequential steps. In the first step (first dimension) the proteins are separated by means of IEF, i.e. the proteins are separated according to their electric charge (pI). In the second step (second dimension) the principle of gel electrophoresis is utilized and the proteins are size separated (Wilson & Walker 2005c). The result of a 2D-PAGE is a series of spots, each one potentially corresponding to a single protein in the sample. 2D-PAGE is a very powerful and widely used method in which thousands of different proteins can be separated (Görg 1998).

2.2 Gel staining

The most commonly used protein stain for visualising proteins separated by gel electrophoresis is Coomassie Brilliant Blue (CBB). CBB binds to proteins and gives rise to blue bands (in one-dimensional gel electrophoresis) or spots (in two-dimensional gel electrophoresis). Another method used is silver staining. When silver ions encounter proteins the ions are reduced to metallic silver and the protein is black or brown coloured. Silver staining is at least 100 times more sensitive than CBB hence silver staining is a method used when detecting low amounts of protein (Wilson & Walker 2005c).

2.3 Immunoblotting

After separating proteins, either one- or two-dimensionally, proteins can be transferred from the gel to nitrocellulose in the presence of an electric field. This is a technique known as electroblotting. A sandwich of electrode papers, gel and nitrocellulose is placed between two parallel electrodes. Applying a current between the electrodes causes the proteins to migrate out from the gel and into the nitrocellulose membrane. A nitrocellulose membrane that contains transferred proteins is referred to as

a blot. When proteins are transferred to the nitrocellulose, the proteins of interest can be examined further. If the blot is incubated with sera containing antibodies that will bind specifically to proteins of interest, this technique is referred to as immunoblotting. To be able to visualise the protein-antibody interactions, the blot is further incubated with a secondary antibody. The secondary antibody, which binds specifically to the primary antibody, is labelled with for example radioactive molecules (Wilson & Walker 2005c). Radioactively labelled molecules or fragment of molecules can be detected using autoradiography (Wilson & Walker 2005d).

2.3.1 Traditional autoradiography using X-ray film

An X-ray-sensitive photographic film is placed close to a sample of interest and the film is exposed at a certain temperature and time. After exposure, the film is developed for visualising the radioactively labelled molecules (Brown 2002a).

2.3.2 Autoradiography using the Typhoon™ System

The Typhoon™ System (GE Healthcare) contains among others the Typhoon™ variable-mode imager that produces digital images of radioactive by scanning exposed storage phosphor screens. In terms of detecting radioactive markers the radiation energy in the sample creates an imprint on the phosphor screen. The Typhoon™ variable-mode imager then converts the stored information on the screen to a digital image.

The phosphor screen used is composed of fine crystals of $BaFBr:Eu^{2+}$ with an organic binder. When the screen is exposed to a radioactive sample the Eu^{2+} electrons are excited by the radiation energy. Sequentially Eu^{2+} is oxidized to Eu^{3+} whereas BaFBr is reduced to $BaFBr^{-}$. When the screen and the sample are separated, the ions remain oxidized and reduced and in this way the phosphor stores the energy from ionizing radiation. When the screen is exposed to light of appropriate wavelength (during the scan of the phosphor screen) stored energy is released. The Typhoon™ variable-mode imager scans the phosphor screens using a red laser at 633 nm. Complexes of $BaFBr^{-}$ absorbs light and free electrons that reduce the Eu^{3+} to Eu^{2+}, which contains an electron in an excited state. When the excited electron of Eu^{2+} falls to ground state, energy is emitted as blue light. A detector measures the light intensity, which is proportional to the amount of radioactivity in the sample and a digital image is created (Typhoon, User's guide 2002).

2.4 Inhibition

The level of allergen-specific IgE antibodies present in a serum sample can be evaluated by the use of an inhibition assay, as described by Yman *et* al. (1975). When using this method serum is mixed with an allergen extract. This will make the IgE antibodies that bind specifically to allergens in the extract partly or completely blocked/inhibited by the added allergens. This preinhibited serum is then used for incubating a nitrocellulose membrane where allergens of interest have been attached. If there are any free IgE antibodies left in the inhibited serum sample, they will bind to the allergens (see figure 2.1 panel B). The amount of bound IgE can then be detected by use of radioactively labelled secondary antibodies.
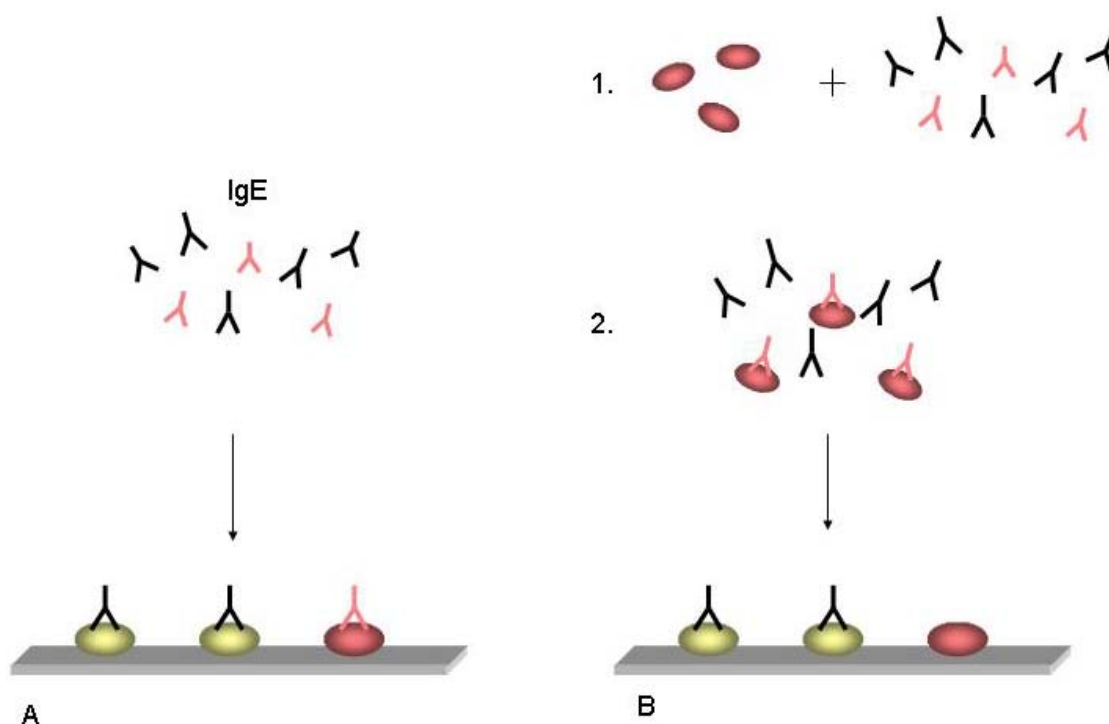
Figure 2.1 Panel A shows how IgE antibodies in a serum sample bind specifically to their corresponding antigens. In panel B, antibodies in a serum sample are inhibited with allergens and only the free allergen-specific IgE antibodies are able to bind to allergens during the second serum incubation.

2.5 Bicinchoninic acid (BCA) protein assay

Bicinchoninic acid (BCA) protein assay is a method used for determining the protein concentration in a solution. The principle is based on the conversion of Cu^{2+} to Cu^{+}. Under alkaline conditions peptide bonds of proteins react with Cu^{2+} and produces Cu^{+}. Produced Cu^{+} then reacts with BCA and gives rise to an intense purple colour with an absorbance maximum at 562 nm. The coloured product is

spectrophotometrically measured and related to the amount of coloured product in a sample with known protein concentration (Wilson & Walker 2005a).

2.6 ImmunoCAP™ Specific IgE

By means of the *in vitro* test ImmunoCAP™ Specific IgE (Phadia), it is possible to measure allergen-specific IgE antibodies in human sera or plasma. The allergen of interest is covalently coupled to a solid phase, the ImmunoCAP™, and when patient serum is added the allergen-specific IgE will bind to the coupled allergens. Non-specific IgE is washed away and thereupon a secondary enzyme-labelled antibody is added. A developing agent that gives rise to a fluorescence signal is added and the intensity of the signal is measured. The higher the flourescense signal, the more allergen-specific IgE present in the serum (InVitroSight 2007b).

2.7 Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS)

The result of a two-dimensional gel electrophoresis is a series of spots, each one potentially representing a single protein that can be further examined. One method for identifying proteins is mass spectrometry. There are different types of mass spectrometers but they are all basically similar and built up of an ion source, an analyser and a detector. The ion source converts molecules into gas-phase ions, the analyser separate the formed ions according to their mass-to-charge ratios and the detector monitors the ion current. In matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS), MALDI and TOF correspond to the ion source and the analyser respectively.

After a two-dimensional gel electrophoresis the spots of interest are excised from the gel and treated with a protease that cleaves proteins into peptides (Brown 2002b). The peptides are mixed with a UV absorbing matrix and loaded onto a target-plate. The plate is introduced into the ion source whereupon it is radiated with a laser beam. The energy in the laser is transformed to excitation energy that ionizes the peptides. The ionized peptides are then accelerated within a flight tube to a reflector and then onto a detector (see figure 2.2 on the next page). The time it takes for the ionized peptide to travel from the plate to the detector is referred to as 'time-of-flight'. The 'time-of-flight' corresponds to a mass-to-charge ratio from which the molecular weight of a peptide can be determined. Low mass ions travel faster than high mass ions. When the molecular masses of the peptides are worked out, these are

compared to peptide masses in a database and the most likely source protein is identified (Wilson & Walker 2005b).

1.

2.

Reflector

Analyser

Ion source

Laser

Detector

3.
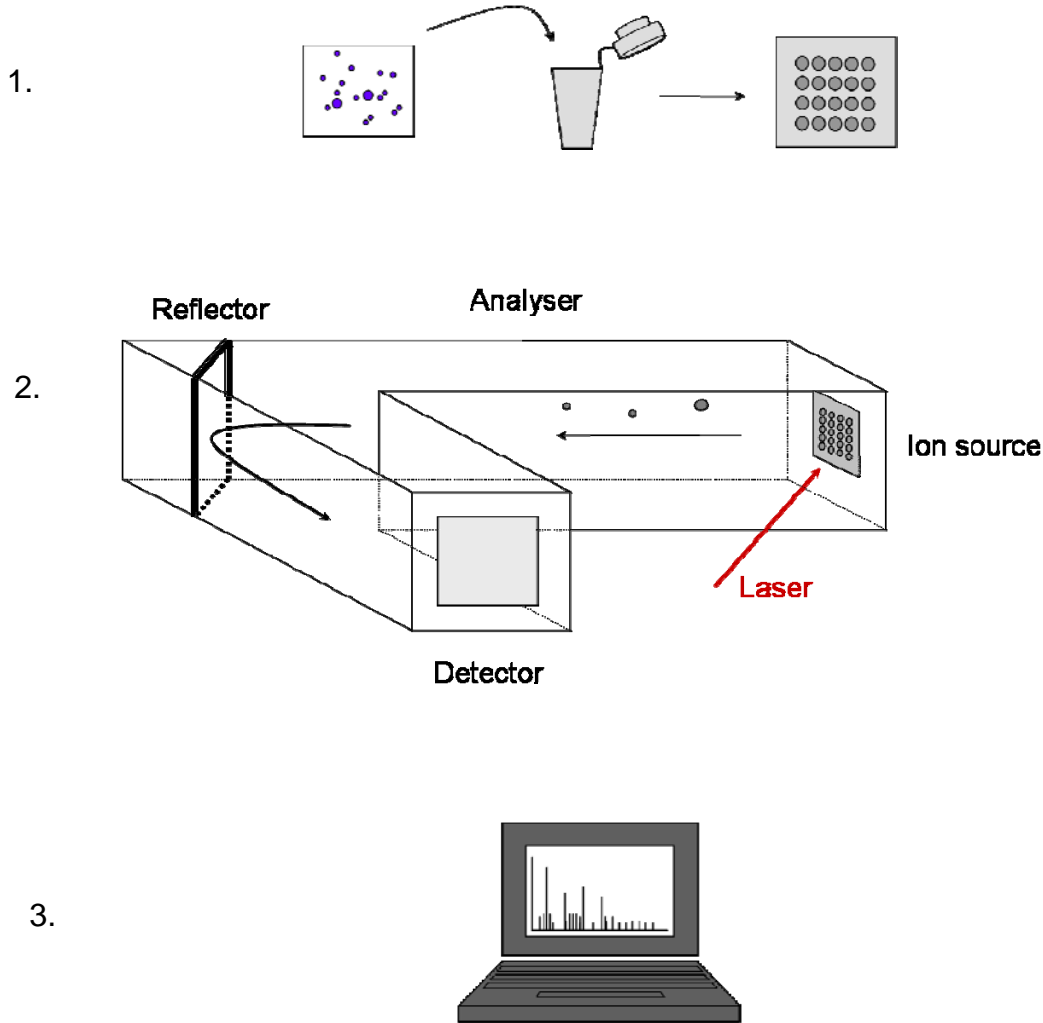
Figure 2.2 Principle of Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS). (1) Sample preparation. Proteins are cleaved in to peptides and placed on a target plate. (2) Laser ionizes the peptides that are accelerated within the flight tube to a reflector and then onto a detector. Time-of-flight is measured and the molecular weight of a peptide can be determined (3) Database search. The most likely source protein is identified.

2.8 Defatting by means of aerosil

In laboratory work it is sometimes preferable to use defatted products, since too much fat can disturb the analysis results. Different methods and chemicals can be used. One method to remove fat from peanut is to use aerosil (SiO_2). Aerosil effectively removes fat from peanut but noticeable is that it also affects the protein composition. It has been reported that the proportion of certain allergens in aerosil treated peanut is larger whereas the proportion of other allergens is smaller when compared to raw peanut (in-house laboratory investigations, Phadia).

3 MATERIALS AND METHODS

3.1 Sera

Sera from European, American and Japanese patients with suspected peanut allergy were used.

3.1.1 European/American sera

European and American sera with specific IgE (sIgE) response to the peanut ImmunoCAP™ were provided by an in-house sera bank. Measured IgE levels are listed in table 3.1 below.

Table 3.1 European/American sera used in the study.

Serum sample	IgE level (kU/l)	Clinical history
49071	2.26	
27591	2.79	
31919	2.94	
51071	3.08	Reaction to peanut
49072	4.05	
31910	4.44	
39061	4.49	
48748	4.58	
44383	7.05	
51425	3.8	
51424	7.6	Reaction to peanut
48307	20	
51074	46	Reaction to peanut
51078	72	Reaction to peanut
51079	98	Reaction to peanut

3.1.2 Japanese sera

The Japanese sera with sIgE response to peanut ImmunoCAP™ used in the study are listed in table 3.2 below.

Table 3.2 Japanese sera used in the study.

Serum sample	Clinical history
03NA AS-001	Reaction to peanut
03NA AS-024	Reaction to peanut
03NA AS-023	No documented reaction to peanut
03NA AS-026	No documented reaction to peanut
03NA AS-028	No documented reaction to peanut
03NA AS-040	No documented reaction to peanut

3.2 Peanut samples

Jumbo Runner peanuts coming from Argentina and USA were used in this study. The peanuts were provided by Allergon, a company that is world leader in research and manufacturing of allergen source materials. The peanuts are exposed to heat during removal of the seed coats but in this context the nuts are still considered as untreated.

3.3 Treatments

Whole peanuts coming from Argentina were heated, boiled and roasted and also used untreated. Whole peanuts from USA were used untreated. Already defatted grinded peanuts were used untreated and further defatted with aerosil. Throughout the report the different peanut samples are referred to as peanut sample 1, 2, 3 etc. according to table 3.3 below.

Table 3.3 The different peanut samples.

Peanut sample	Peanut product	Origin	Treatment
1	Defatted grinded peanut	USA	Untreated
2	Defatted grinded peanut	USA	Aerosil treated
3	Whole peanut	Argentina	Untreated
4	Whole peanut	Argentina	Heated (90°C, 20 min)
5	Whole peanut	Argentina	Boiled (20 min)
6	Whole peanut	Argentina	Roasted (170°C, 20 min)
7	Whole peanut	USA	Untreated

3.3.1 Heating

An oven was heated to 90°C and 50 g of peanut was evenly distributed on a preheated oven tray and treated for 20 minutes.

3.3.2 Boiling

50 g of peanut was put in 0.5 litres of boiling water for 20 minutes.

3.3.3 Roasting

An oven was heated to 170°C and 50 g of peanut was evenly distributed on a preheated oven tray and treated for 20 minutes.

3.3.4 Aerosil treatment

Aerosil was added to defatted grinded peanuts during preparation of peanut extract, see paragraph 3.4.2 below.

3.4 Preparation of peanut extract

Peanut proteins were extracted from seven different kinds of raw material, see details in table 3.3 above.

3.4.1 Peanut sample 1. Defatted grinded peanut, USA

3 g of defatted grinded peanut was mixed with 60 ml of extraction buffer (0.1 M phosphate buffer, pH 7.4) and placed on a shake board (2 h, 4°C). The protein mixture was subsequently centrifuged (4200 rpm, 30 min, 4°C) and the supernatant was collected. The supernatant was then stepwise filtered, first through filter 5 and 00H Munktell (5: pore size 1-2μm, 00H: pore size >20μm) and then through filters with the pore sizes 5 μm, 0.45 μm, and 0.22 μm respectively. Buffer change was carried out by using PD-10 desalting columns (GE Healthcare). A volume of 2.5 ml of the filtered protein solution was loaded on the column and eluted with 3.5 ml of water. The eluate was collected and fractionated in portions of 2 ml into glass flasks appropriate for lyophilization. Lyophilization was performed in a HETO Dry Winner system as per the manufacturer's instructions.

3.4.2 Peanut sample 2. Defatted grinded peanut, aerosil treated, USA

Preparation of extract was performed as described in paragraph 3.4.1 above with the exception that 1.12 g of aerosil was added to the mixture before it was placed on the shake board.

3.4.3 Peanut sample 3, 4, 5, 6. Whole peanut (untreated, heated, boiled, roasted), Argentina

Prior to the extraction, filtration and lyophilization that was performed as described in paragraph 3.4.1 above, 3 g of whole untreated, heated, boiled, and roasted peanut, respectively, was mixed with 60 ml of extraction buffer (0.1 M phosphate buffer, pH 7.4) and grinded by use of an Ultra Turrax (Janke & Kunkel, IKA-WERK).

3.4.4 Peanut sample 7. Whole peanut, USA

Preparation of extract was performed as described in paragraph 3.4.3 above.

3.5 BCA protein assay

Protein concentration in peanut sample 1 – 6 was determined using a BCA™ Protein Assay kit (Pierce, Perbio). Frozen allergen extract was thawed and diluted 1:10 and 1:40 in 0.9% NaCl. To 50 μl of each sample 1 ml of kit reagent was added. Next, the samples were heated (80°C, 30 min) and then, to stop the reaction, centrifuged (15°C, 30 min, 14000 rpm) as per the manufacturer's instructions. The absorbance was spectrophotometrically measured at 562 nm. Samples were measured in triplicates and standards of diluted bovine serum albumin, BSA, (500 μg/ml, 250 μg/ml, 125 μg/ml, 50 μg/ml, 25 μg/ml, 10 μg/ml) were measured in duplicates. Measured absorbances for the standards were used to create a second order equation curve, from which the protein concentration in the samples were determined.

3.6 One-dimensional gel electrophoresis

One-dimensional gel electrophoresis was carried out on peanut sample 1-7.

3.6.1 Sample preparation

Lyophilized peanut extract was dissolved in sample buffer (250 mM Tris, 5% SDS, 5 mM EDTA-Na_2, 0.05% BFB, 2.6 M DTT), heat treated (95°C, 5 min) and centrifuged (4°C, 14000 rpm, 5 min) before use.

3.6.2 SDS-PAGE

Peanut proteins were separated by SDS-PAGE under reducing conditions using a Multiphor II Electrophoresis System (GE Healthcare). The gel used was a homogenous polyacrylamide gel (ExcelGel™ SDS homogenous 12.5, GE Healthcare) with precasted sample wells. Anode and cathode buffer strips (ExcelGel™ Buffer strip, GE Healthcare) were positioned on each side of the gel and 14 μl of sample was loaded in each well. In one well a low molecular weight (LMW) marker, heat-treated and centrifuged as the other samples, was loaded. Run parameters for the electrophoresis were 600 V, 50 mA, 30 W, 15°C and the electrophoresis was stopped when the front zone reached the anode buffer strip.

3.6.3 Gel staining

Gels containing separated proteins were stained in a Hoefer Automated Gel Stainer apparatus (Amersham Biosciences). The gels were stained using either Coomassie Brilliant Blue (CBB) or silver (see the solutions used in table 3.4 and 3.5 below).

3.6.2.1 Coomassie Brilliant Blue

Table 3.4 Program for CBB gel staining.

Step	Solution	Content	Time (min)
1	Fix	40% EtOH, 10% HAc	20
2	Destaining	25% EtOH, 8% HAc	2
3	CBB staining solution	67.5% Fix solution, 2.5% HAc, 2% CBB*	60
4	Destaining	-	10
5	Destaining	-	30
6	Destaining	-	30
7	Preserving	25% EtOH, 8% HAc, 15.3% Glycerol	30

* 1 tablet PhastGel BlueR (Pharmacia Biotech) dissolved in 80 ml of water (Milli-Q) and 120 ml of ethanol.

3.6.2.2 Silver staining

Table 3.5 Program for silver staining.

Step	Solution	Content	Time (min)
1	Fix	40% EtOH, 10% HAc	30
2	Incubation	30% EtOH, 0.73 M $NaC_2H_3O_2$, 8 mM, $Na_2S_2O_3$ x $5H_2O$, 0.5% glutaraldehyde	30
3	Wash	Water (Milli-Q)	5
4	Wash	-	5
5	Wash	-	5
6	Silver staining	6 mM $AgNO_3$, 0.02% formaldehyde	20
7	Wash	-	1
8	Wash	-	1
9	Developing	0.24 M Na_2CO_3, 0.01% formaldehyde	5
10	Stop	50 mM EDTA-Na_2 x $2H_2O$	10
11	Wash	-	5
12	Wash	-	5
13	Wash	-	5
14	Preserving	15.3% Glycerol	30

3.7 Two-dimensional gel electrophoresis

3.7.1 IEF

Proteins from peanut sample 2 (aerosil treated), 3 (untreated), and 6 (roasted) were separated by IEF using Ettan IPGphor II Isoelectric Focusing system (GE Healthcare). Lyophilized peanut extract was dissolved in rehydration buffer (8 M Urea, 2% Triton X-100, 0.002% BFB) mixed with 0.5% IPG Buffer (pH 3-10 NL, GE Healtcare) and 0.2% DTT. The dissolved extract and Immobiline™DryStrips™ (pH 3-10, NL 7 cm, GE Healthcare) were placed in IPG Strip Holders as

per the manufacturer's instructions and the strips were rehydrated over night at room temperature. After rehydration IEF was carried out with the parameters listed below.

Table 3.6 Run parameters for isoelectric focusing.

Step	Voltage (V)	Time (h:min)
1	500	00:30
2	1000	00:30
3	5000	01:40

3.7.2 SDS-PAGE

After IEF, the Immobiline™ DryStrips™ were equilibrated for 15 min in equilibration solution 1 (75 mM Tris-HCl, 6 M Urea, 30% Glycerol, 2% SDS, 0.002 BFB, 65 mM DTT) and for 15 min in equilibration solution 2 (75 mM Tris-HCl, 6 M Urea, 30% Glycerol, 2% SDS, 0.002 BFB, 0.14 M IAA) prior to the second-dimension separation. The separation was carried out in a Multiphor II Electrophoresis System (GE Healthcare) using a homogenous polyacrylamide gel (ExcelGel™ 2-D homogenous 12.5, GE Healthcare) and buffer strips (ExcelGel™ Buffer strip, GE Healthcare). The gel strips were positioned on the polyacrylamide gel and electrophoresis was carried out in two steps. The first step (600 V, 20 mA, 30 W, 15°C) was finished when the sample had left the gel strip and had wandered into the gel. The gel strips were then removed and run parameters were set to 600 V, 50 mA, 30 W, 15°C. The electrophoresis was stopped when the front zone reached the anode buffer strip.

3.7.3 Gel staining

Gels containing two-dimensionally separated proteins were stained with CBB in a Hoefer Automated Gel Stainer apparatus (Amersham Biosciences) see table 3.4 and 3.5 for solutions.

3.7.4 MALDI-TOF MS

Proteins from peanut sample 2 (aerosil treated peanut) and 6 (roasted peanut) were studied by MALDI-TOF MS. Tryptic in-gel digestion was performed according to an adapted method from Shevchenko *et al*. (1996). After two-dimensional gel electrophoresis, the gel was stained with CBB as described in paragraph 3.6.3, with the exception that water was used instead of preserving solution. Gel pieces containing spots of interest were excised from the gel and washed two times in water and one time in a solution of 10 µl of water and 50 mM NH_4HCO_3/acetonitrile 1+1 (v/v). Gel pieces were dehydrated by adding 10 µl of acetonitrile that subsequently was removed, rehydrated in 10 µl of 50 mM NH_4HCO_3, and dehydrated once again by adding 10 µl of acetonitrile. All liquid was removed and dehydration and rehydration, as described above, was repeated once. After the washing step, gel particles were dried in a vacuum centrifuge. Next, 1 µl of enzyme solution (25 mM NH_4HCO_3, 5 ng/µl

trypsin) was added and enzymatic cleavage was carried out over night at 37°C. Enough 25mM NH_4HCO_3 to cover the gel was added to prevent the gel from drying during cleavage.

Next, peptides were extracted by ultrasound sonication and 0.5 μl of the sample was mixed with 0.5 μl of a-Cyano-4-hydoxy-cinnamic acid, saturated in 50% acetonitrile, deposited on a MALDI target-plate and left to dry. The plate was then inserted into a mass spectrometer (Autoflex II MALDI-TOF MS; Bruker Daltonics) and radiated with laser. The obtained mass spectra were used to search the NCBInr database.

3.8 Immunoblotting

3.8.1 European/American sera

Allergenic components in peanut sample 1-6 were studied by means of immunoblotting.

3.8.1.1 SDS-PAGE

Lyophilized peanut extract was dissolved in sample buffer (250 mM Tris, 5% SDS, 5 mM EDTA-Na_2, 0.05% BFB, 2.6 M DTT), heat treated (95°C, 5 min) and centrifuged (4°C, 14000 rpm, 5 min). SDS-PAGE was carried out as described in paragraph 3.6.2 above with the exception that the gel used was homogenous polyacrylamide gel (ExcelGel™ SDS homogenous 12.5, GE Healthcare) without precasted sample wells. Instead, a sample holder was used for loading the sample (2μl of sample /mm holder length). When the sample had wandered into the gel, electrophoresis was paused and the sample holder was removed. Electrophoresis continued with the same parameters (600 V, 50 mA, 30 W, 15°C) until the front zone reached the anode buffer strip.

3.8.1.2 Transfer to nitrocellulose

A sandwich of electrode papers, gel and nitrocellulose was stepwise composed on the anode tray of a Multiphor apparatus (GE Healthcare). First, six electrode papers were soaked in 250 ml of anode solution 1 (0.3 M Tris-(hydroxymetyl)-aminomethane, 20% methanol) and placed on the anode plate. The gel containing the separated proteins was equilibrated in anode solution 2 (0.03 M Tris-(hydroxymetyl)-aminomethane, 20% methanol) for seven minutes whereupon the gel was separated from its plastic support with a film remover (Pharmacia LKB). A 250 mm x 10 mm nitrocellulose membrane was soaked in anode solution 2 and thereafter placed on the gel and left to attach when three electrode papers were soaked in anode solution 2 and added to the sandwich. The gel with attached nitrocellulose was placed on the sandwich with the nitrocellulose facing the anode plate. The plastic support was removed from the gel and nine electrode papers were soaked in cathode solution

(0.04 M 6-aminohexanoic acid, 20% methanol) and placed on the gel. Throughout the procedure air bubbles between the different layers were removed. The cathode plate was placed upon the sandwich and the apparatus was connected to a power supply. Blotting was run for 1 hour (10 V, 200 mA, 5 W). When blotting was completed, the part of interest was cut out from the nitrocellulose. In order to block the part of the membrane that does not contain bound proteins, the membrane was placed in RASTno5/0.1% tween (in-house buffer containing 0.05 M phosphate, 0.9% NaCl, 0.3% Dextran T10, 0.1% (v/v) Tween-20, pH 7.4) for 1 h in room temperature on a shake board. The molecular weight marker was cut out and stained with amido black to check for good transfer. Subsequently the weight marker was used to determine molecular weights of separated proteins.

3.8.1.3 Sera incubation

After blocking, the membrane was cut into 4 mm wide strips. Each strip was incubated in 1.5 ml of serum from patients with suspected peanut allergy. In the study, 14 different patient sera, diluted with RASTno5/0.1% tween to appropriate concentrations, were used. Incubation took place over night, in room temperature, on a tilting plate.

3.8.1.5 Wash, secondary antibody incubation and visualisation of allergenic components

After incubation, each strip was washed 3 x 5 min in 2.5 ml of wash solution (0.9% NaCl, 0.5% Tween-20). Next, 1.5 ml of ^{125}I-labelled anti-IgE was added to each strip and left to incubate for 4 hours in room temperature on a tilting plate. After incubation the strips were washed 3 x 5 min with wash solution as described above with an additional wash step 2 x 5 min with water (Milli-Q). Strips were then left to dry on a filter paper and thereupon attached to a sheet of paper, which was placed in a cassette for exposure either on a storage phosphor screen (GE Healthcare) or on an X-ray sensitive photographic film. The phosphor screen was scanned in a Variable Mode Imager apparatus (Typhoon™ 9410, GE Healthcare) and allergenic components were studied using the Image Quant Tools v2003.02 software. Photographic film was developed in a film developing apparatus (Agfa).

3.8.2 Japanese sera

3.8.2.1 Specific IgE concentration determination

The peanut-specific IgE concentration in sera from Japanese patients was measured by use of an ImmunoCAP™100 apparatus (Phadia) as per the manufacturer's instructions. Both a regular peanut-

specific ImmunoCAP™ and an in-house prototype containing roasted peanut, produced only for Phadia's own laboratory research, were used.

3.8.2.2 Sera incubation and visualisation of allergenic components

Preparation of 4 mm wide blots with one-dimensionally separated proteins, from peanut sample 3 (untreated) and peanut sample 6 (roasted) were performed as described in paragraph 3.8.1.2. Sera were diluted with RASTno5/ 0.1% tw and each strip was incubated with a total volume of 1 ml instead of 1.5 ml as for European/American sera. Wash, secondary antibody incubation and visualisation of allergenic components were performed as described in paragraph 3.8.1.5.

3.9 Inhibition (one dimension)

Inhibition studies were performed using three sera from patients with suspected peanut allergy (sample ID: 51074, 51424, 51425) and extract from three different peanut samples: peanut sample 2 (aerosil treated), 3 (untreated peanut), and 6 (roasted peanut). Lyophilized protein extract was dissolved in 0.1 M phosphate buffer, 0.03% BSA, pH 7.4. Blots with size separated proteins from peanut sample 2, 3 and 6, respectively, were prepared as described in paragraph 3.8.1.2. Blots, cut into 4 mm wide strips, were then incubated with 1.5 ml of diluted serum each. Prior to incubation, sera were crosswise inhibited with extracts from peanut sample 2, 3 and 6 as described in figure 3.1 below. A serum volume of 750 µl was mixed with 750 µl of dissolved extract and left to incubate on a shake board for 1 h in room temperature.
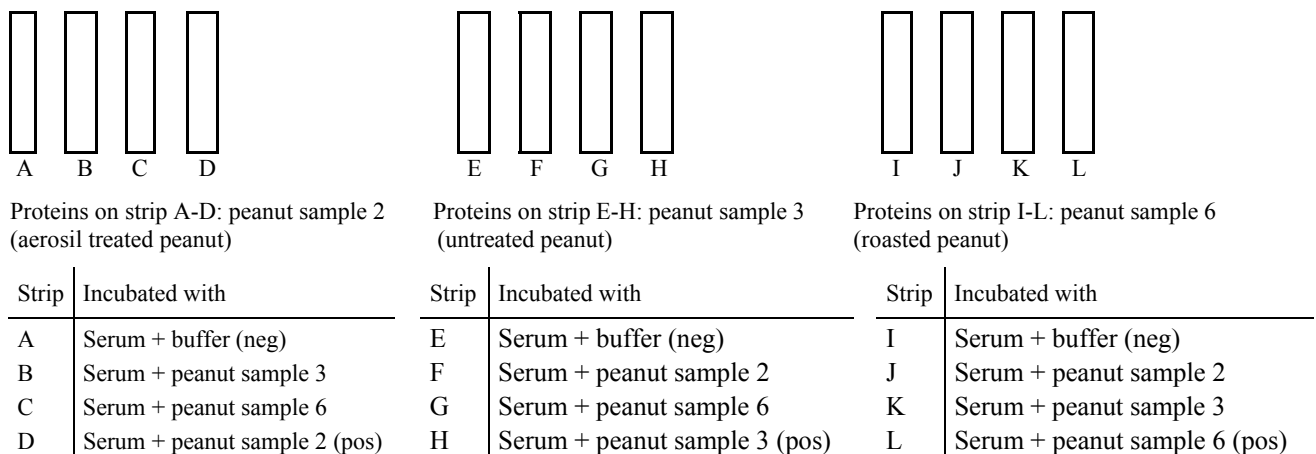
A   B   C   D
Proteins on strip A-D: peanut sample 2 (aerosil treated peanut)

E   F   G   H
Proteins on strip E-H: peanut sample 3 (untreated peanut)

I   J   K   L
Proteins on strip I-L: peanut sample 6 (roasted peanut)

Strip	Incubated with	Strip	Incubated with	Strip	Incubated with
A	Serum + buffer (neg)	E	Serum + buffer (neg)	I	Serum + buffer (neg)
B	Serum + peanut sample 3	F	Serum + peanut sample 2	J	Serum + peanut sample 2
C	Serum + peanut sample 6	G	Serum + peanut sample 6	K	Serum + peanut sample 3
D	Serum + peanut sample 2 (pos)	H	Serum + peanut sample 3 (pos)	L	Serum + peanut sample 6 (pos)

Figure 3.1 Inhibition scheme
Strips containing one-dimensionally size separated proteins were incubated with inhibited sera. Strip A-D contained proteins from peanut sample 2, strip E-H contained proteins from peanut sample 3, and strips I-L contained proteins from peanut sample 6. Strip A, E, I and D, H, L were used as negative and positive control, respectively.

Wash, secondary antibody incubation and visualisation of allergenic components were performed as described in section 3.8.

3.10 Inhibition (two dimensions)

Inhibition studies in two dimensions were performed using extract from aerosil treated peanut (peanut sample 2) and roasted peanut (peanut sample 6). Proteins were separated with two-dimensional gel electrophoresis and transferred to nitrocellulose as described in paragraph 3.7 and 3.8. After blocking of the blot, the part of interest ($\sim$56 cm^2) was cut out and placed in a plastic bag for sera incubation. Prior to incubation, serum (sample ID: 51074) was inhibited with dissolved lyophilized extract from peanut sample 2 and peanut sample 6 as described in figure 3.2. Lyophilized peanut extract was dissolved in 0.1 M phosphate buffer, 0.03% BSA, pH 7.4 and mixed with sera to a total volume of 6 ml.
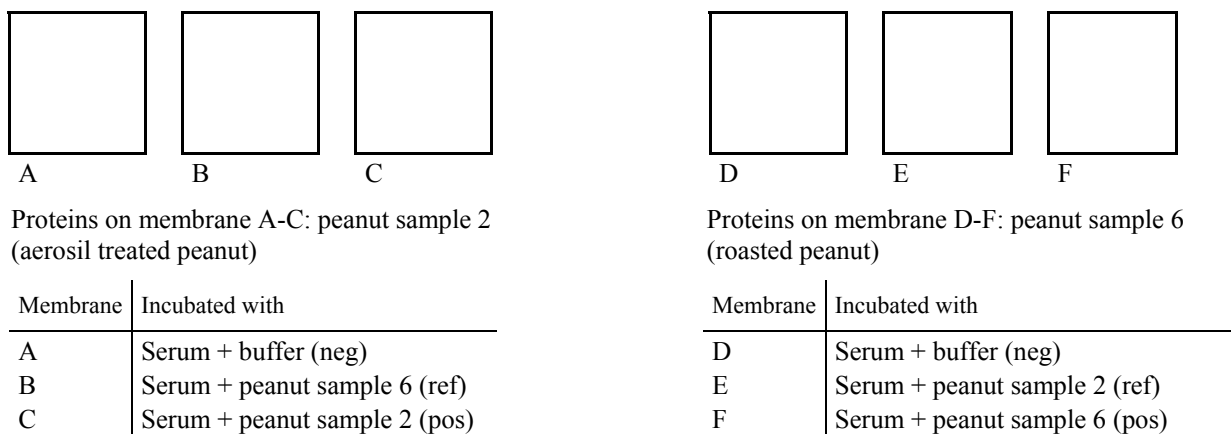

Proteins on membrane A-C: peanut sample 2
(aerosil treated peanut)

Membrane	Incubated with
A	Serum + buffer (neg)
B	Serum + peanut sample 6 (ref)
C	Serum + peanut sample 2 (pos)

Proteins on membrane D-F: peanut sample 6
(roasted peanut)

Membrane	Incubated with
D	Serum + buffer (neg)
E	Serum + peanut sample 2 (ref)
F	Serum + peanut sample 6 (pos)

Figure 3.2 Inhibition scheme
Membranes containing two-dimensionally separated proteins were incubated with inhibited sera. Membrane A-C contained proteins from peanut sample 2, and membrane D-F contained proteins from peanut sample 6. Membranes A, D and C, F were used as negative and positive control, respectively.

Wash, secondary antibody incubation and visualisation of allergenic components was performed as described in section 3.8.1.5 with the exception that the volumes used for washing and secondary antibody incubation were increased to 15 ml and 10 ml, respectively.

4 RESULTS AND DISCUSSION

4.1 One-dimensional gel electrophoresis

To get an overview of the protein composition in the differently treated peanuts, a one-dimensional gel electrophoresis was carried out with all seven peanut samples. Proteins were stained with Coomassie Brilliant Blue (CBB). Samples were loaded in three concentrations: 2 mg/ml, 4 mg/ml and 8 mg/ml. Concentrations were calculated from the dry weight of lyophilized extract.
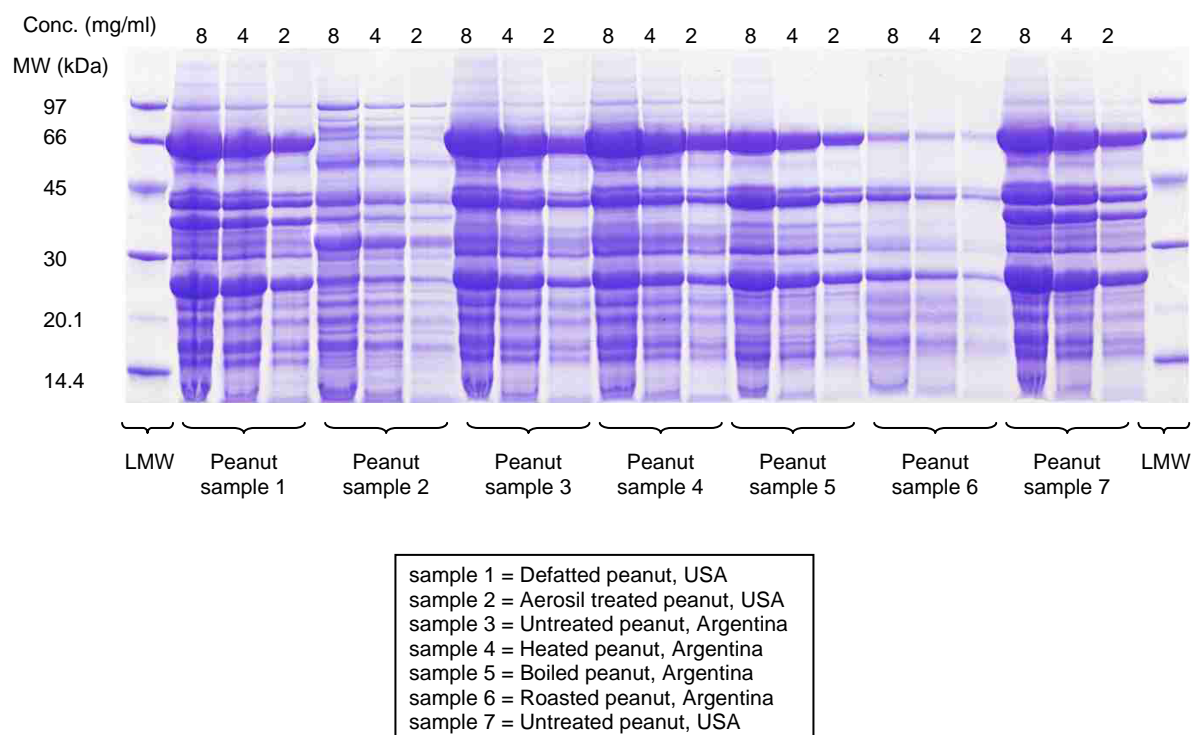

sample 1 = Defatted peanut, USA
sample 2 = Aerosil treated peanut, USA
sample 3 = Untreated peanut, Argentina
sample 4 = Heated peanut, Argentina
sample 5 = Boiled peanut, Argentina
sample 6 = Roasted peanut, Argentina
sample 7 = Untreated peanut, USA

Figure 4.1 Size separated peanut proteins coming from seven different extracts stained with CBB. Each extract was loaded in three concentrations: 2 mg/ml, 4 mg/ml and 8 mg/ml.

Many similarities can be observed when studying the protein composition of the different peanut samples. The sample that differed noticeable from the other samples was peanut sample 2 (aerosil treated peanut).In the wells corresponding to peanut sample 2 a different pattern of bands can be observed when compared with the other samples. More stained protein bands are visible in the area ~50-97 kDa and ~15 -25 kDa, see highlighted areas in figure 4.2. Bands are also missing or appear weaker when compared to the other samples. For example the prominent band around 66 kDa that can be seen in the other samples is not as prominent in sample 2. Aerosil obviously affects the protein

composition. This result is in accordance with previous investigations where it has been shown that the proportions of certain proteins are changed after treatment with aerosil.

Sample 3 (whole untreated peanut) and 4 (whole heated peanut) are almost identical hence it seems that heating, to 90°C, does not affect the protein composition noticeable.

An extra protein band between the 30 and 45 kDa weight markers can be seen in sample 1 (untreated peanut), 2 (aerosil treated peanut) and 7 (whole untreated peanut), see figure 4.1 and 4.2. The extra band may depend on geographical differences. Sample 1, 2 and 7 come from USA and the other samples from Argentina.

Since sample 3 and 7 showed similar band pattern and both involve whole untreated peanut, sample 7 was from now on excluded from the study.

Peanut sample 5 (boiled peanut) and 6 (roasted peanut) show a different pattern when compared to sample 1, 3 and 4. Weaker bands are visible in sample 5 and 6 indicating that boiled and roasted peanut contain less amount of proteins than untreated and heated peanut. One possible reason to why weaker bands are seen in sample 5 and 6 could be that during boiling and roasting, a lot of fat comes out from the peanut and since a lot of proteins are embedded in the fat a lot of proteins will be removed.

If the band patterns of sample 5 and 6 are compared it is possible to observe some differences. Generally, bands in sample 6 are weaker and some bands that are present in sample 5 cannot be seen at all in sample 6. The results indicate that the proteins are affected to a larger extent by the high temperature used for roasting than the temperature for boiling. In case of sample 5, thin and fairly prominent bands can be seen in the low molecular area, indicating that proteins during boiling fall apart into smaller fragments. This observed pattern cannot be seen in the low molecular area in the other samples containing argentine peanuts (sample 3, 4, 6).

Studying the bands of sample 3-6 it is possible to distinguish three prominent bands that are common for all samples, indicating that these proteins are the major proteins regardless the treatment used, see figure 4.2.
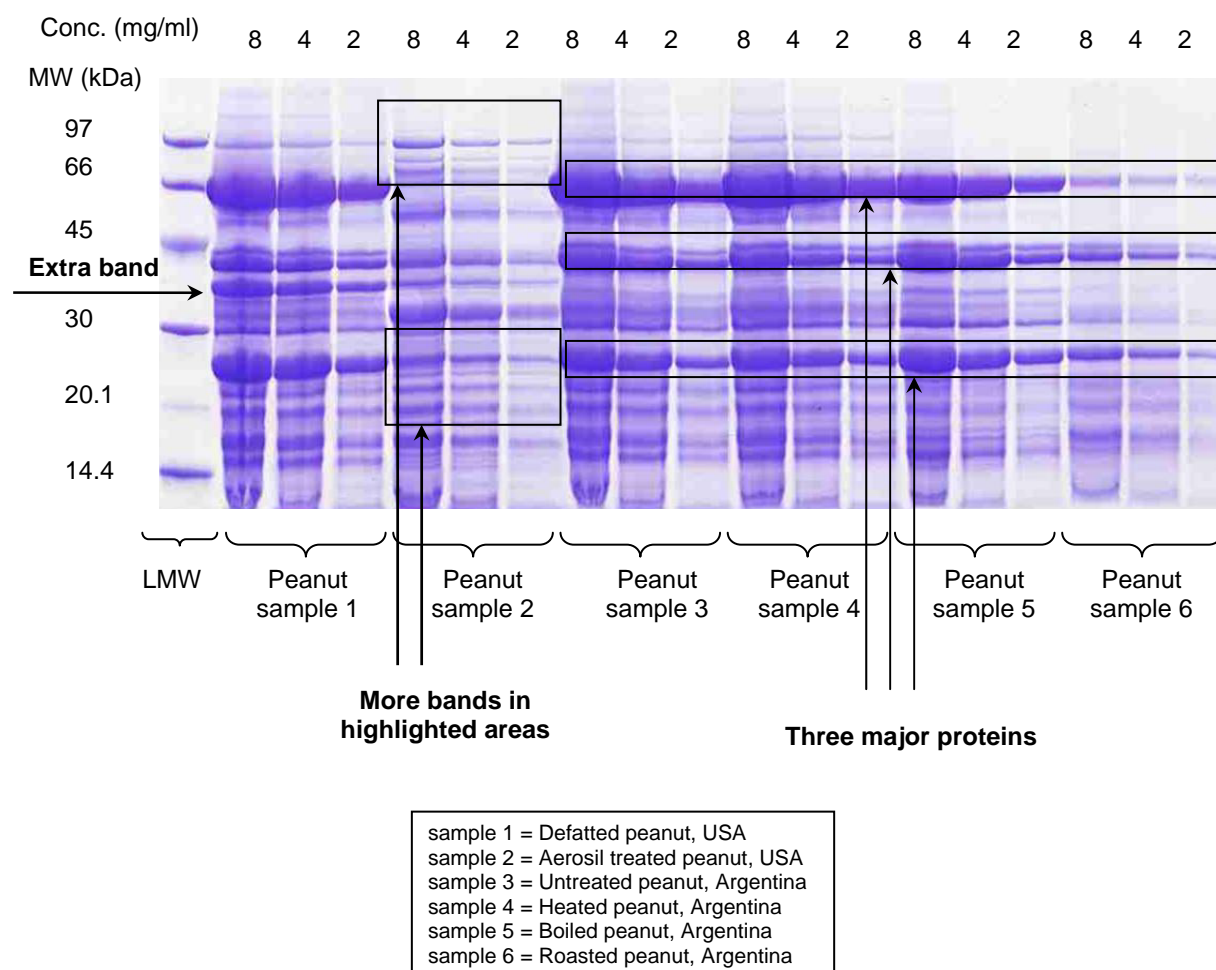
Figure 4.2 Size separated peanut proteins coming from six different peanut samples stained with CBB. Areas of interest are highlighted.

When studying the gel pictures shown in this study it should be taken into account that the sample concentrations have been calculated from the dry weight of lyophilized extract and that this is not an exact method. A colour difference in the stained gel may therefore be due to differences in the amount of protein loaded on the gel. See further comments in paragraph 4.6 BCA protein assay. Differences in colour shade can also be due to the staining method. CBB binds to certain amino acids so if the amino acid sequence is changed during for example heat treatment a different band/colour pattern will be shown.

To be able to detect bands that were weak when stained with CBB, a one-dimensional gel electrophoresis followed by silver staining was performed, see figure 4.3. Silver staining is a more sensitive method than using CBB.
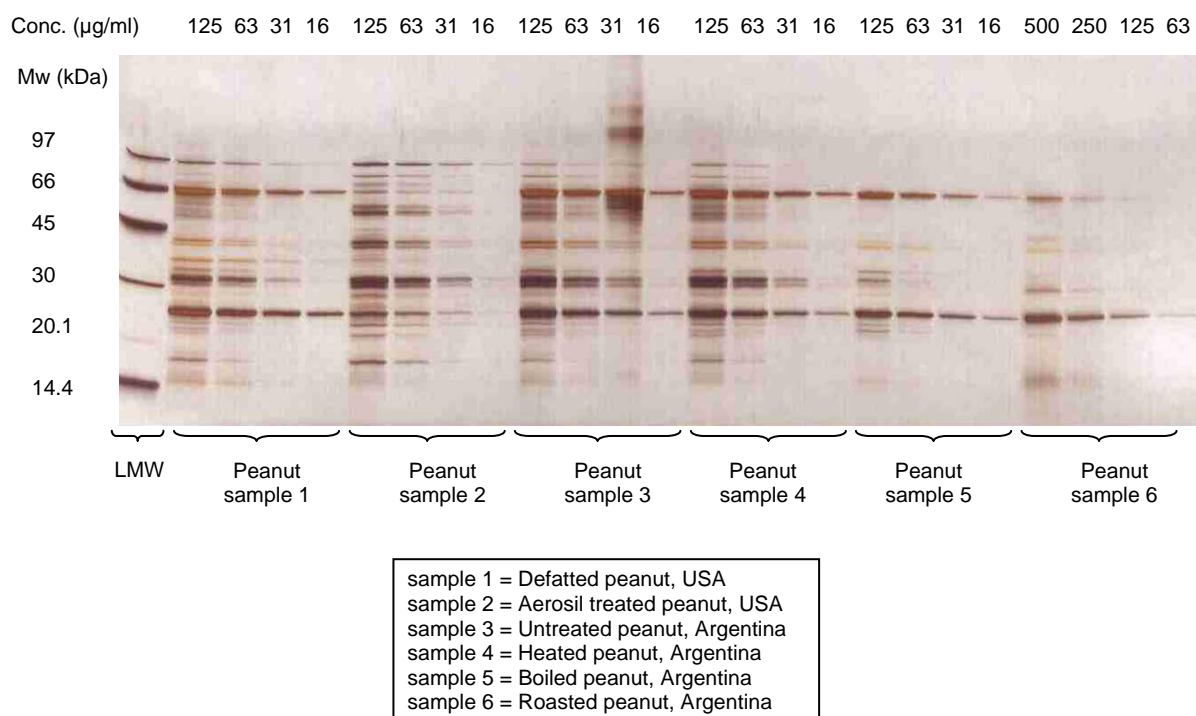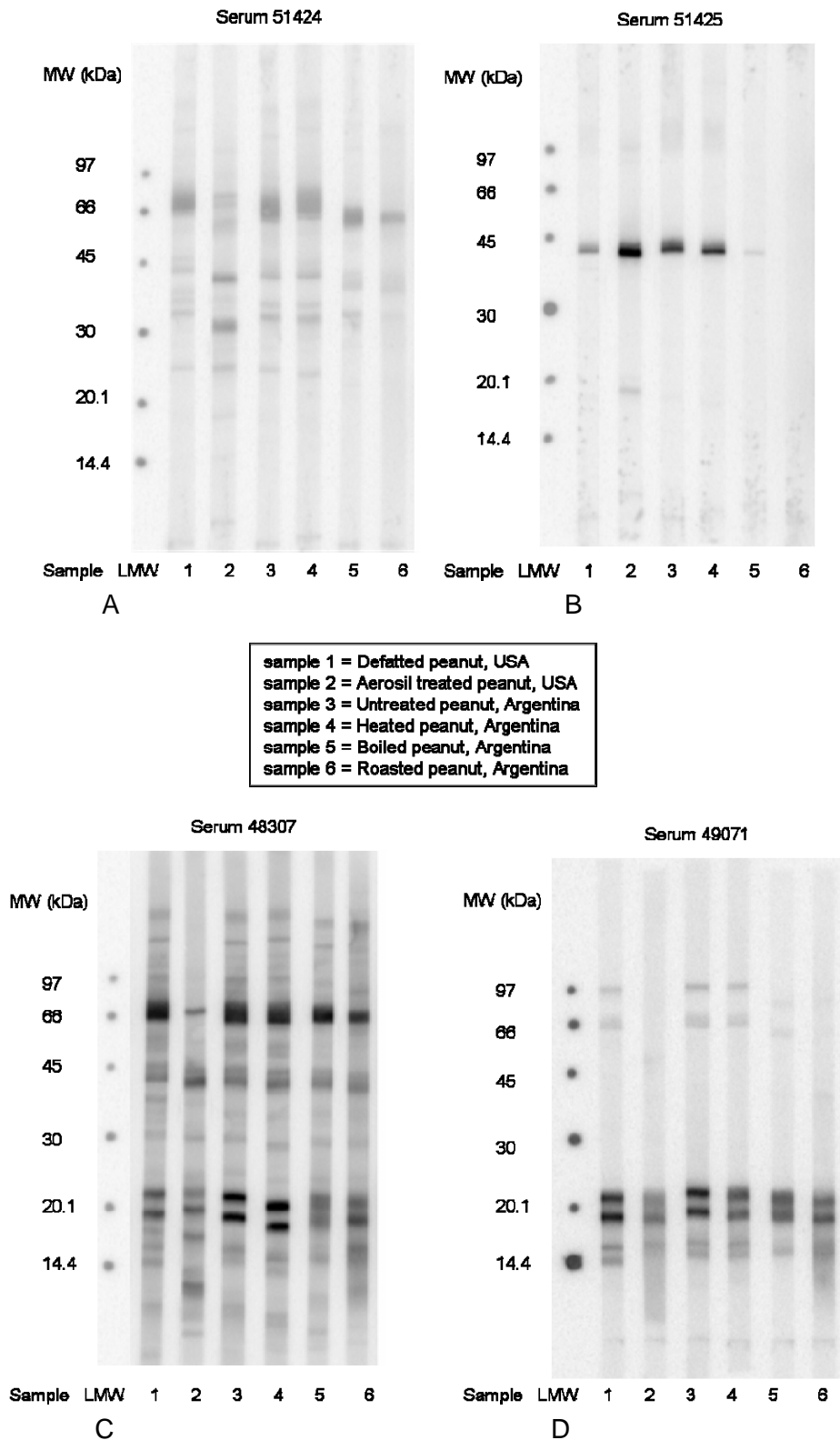
Figure 4.3 Silver stained size separated peanut proteins coming from six different extracts. Each extract is loaded in four concentrations.

Also these results show that it is sample 2 (aerosil treated peanut) that differs most from the other samples. Different amino acids are stained when silver is used instead of CBB, wherefore the protein band pattern shown after silver staining cannot be directly compared to the band pattern achieved after CBB staining.

4.2 Immunoblotting (one dimension)

Allergenic components in peanut sample 1-6 were studied by use of 15 different sera coming from European/American patients with suspected peanut allergy. Not all data shown.
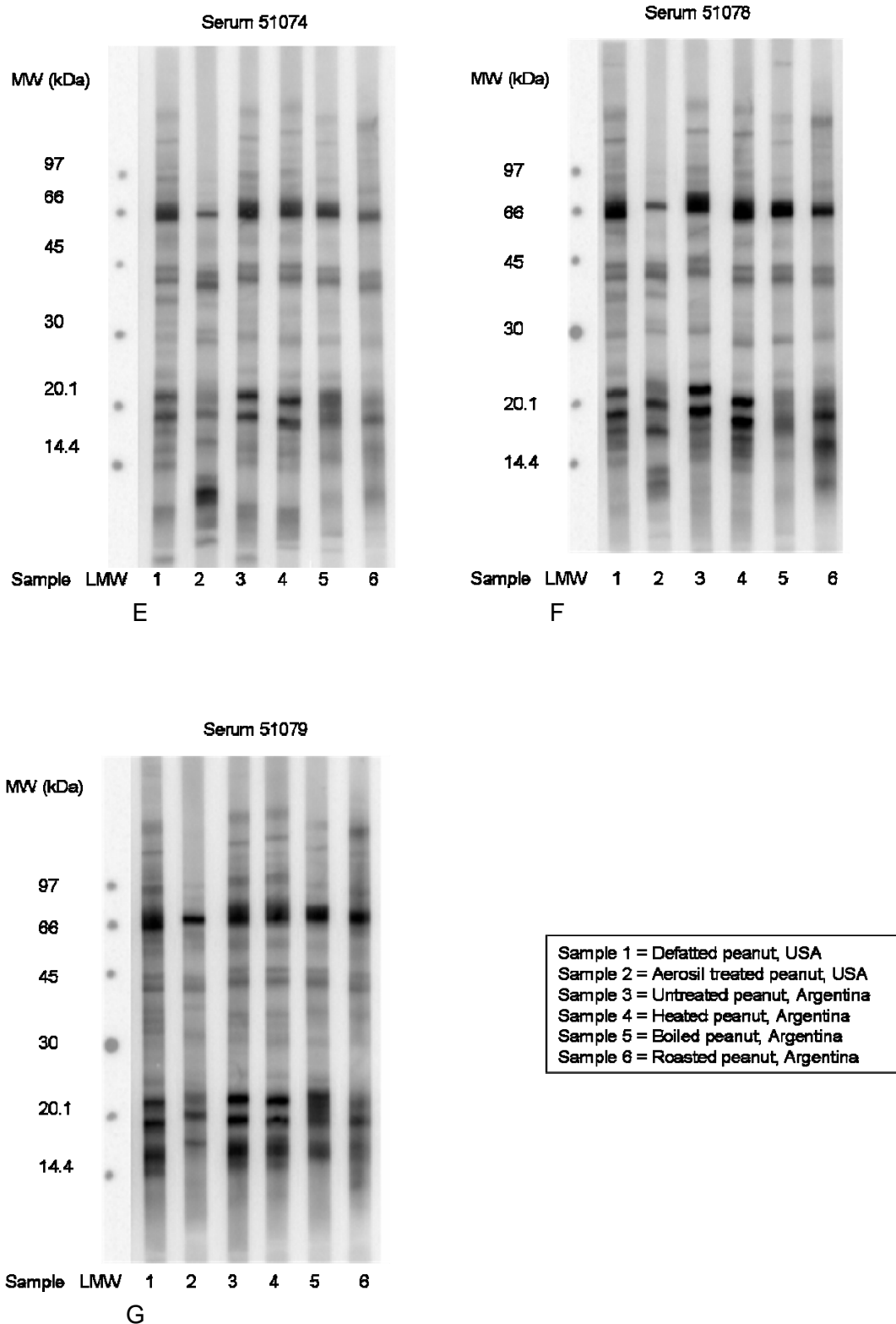

sample 1 = Defatted peanut, USA
sample 2 = Aerosil treated peanut, USA
sample 3 = Untreated peanut, Argentina
sample 4 = Heated peanut, Argentina
sample 5 = Boiled peanut, Argentina
sample 6 = Roasted peanut, Argentina

Figure 4.4 Allergenic components in six different peanut samples studied with European/American sera.

Different patients react to different allergenic components, and different allergenic components are present in the different peanut samples. Studying serum 51425, see panel B, it can be seen that the

component that the patient has antibodies against is not present in peanut sample 5 (boiled peanut) and 6 (roasted peanut), indicating that this component is either changed or destroyed during boiling and roasting. The same pattern can be observed in serum 49071 ~66-97 kDa, see panel D, where components that are present in the other samples are lacking in peanut sample 5 and 6.

In most cases it is peanut sample 2 (aerosil treated peanut) that differ the most. For example the protein band around ~66 kDa that is seen in all samples, is less prominent in peanut sample 2 if compared with the other samples. This band most likely correspond to Ara h 1 thus Ara h1 is affected by aerosil treatment. Some components are also enhanced when treated with aerosil, see serum 51074, panel E, in the area somewhat smaller that 14 kDa where a fairly prominent band can be observed. The other peanut samples look very similar, indicating that the allergenic components in these samples are not affected noticeable by the different treatments.

4.3 Two-dimensional gel electrophoresis

To get a more detailed overview of the protein composition in peanut sample 2, 3 and 6 a two-dimensional gel electrophoresis was carried out. The gel was stained with CBB. Sample 2, 3 and 6 was chosen since these are the extremes in this context.
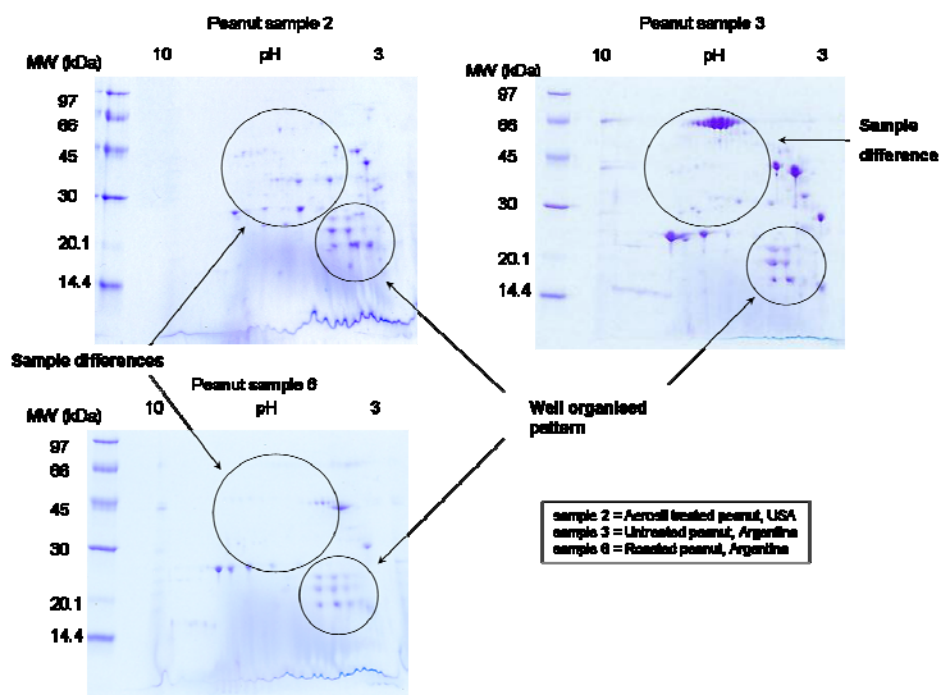

Figure 4.5 Two-dimensionally separated peanut proteins coming from three different peanut samples, aerosil treated peanut, untreated peanut and roasted peanut.

A big difference can be observed when comparing sample 2 (aerosil treated peanut) and sample 6 (roasted peanut). The most noticeable difference is seen in the area 30-45 kDa, low pH. A lot of spots are lacking in the roasted peanut extract, see figure 4.5. The same well organised pattern in the area 20.1-30 kDa, low pH, is found in all three samples, encircled in figure 4.5. The well organised pattern indicates that the different spots represent isoforms of the same protein.

It should be taken into account that peanut sample 2 originates from USA and did show an extra band between the 30 and 45 kDa weight markers in one-dimensional gel electrophoresis compared with the peanut samples originating from Argentina, wherefore extra spots can be observed on the gel corresponding to sample 2.

4.4 Inhibition (one dimension)

To easier discover differences in which allergenic components that are present in peanut sample 2, 3 and 6, inhibition studies were carried out.
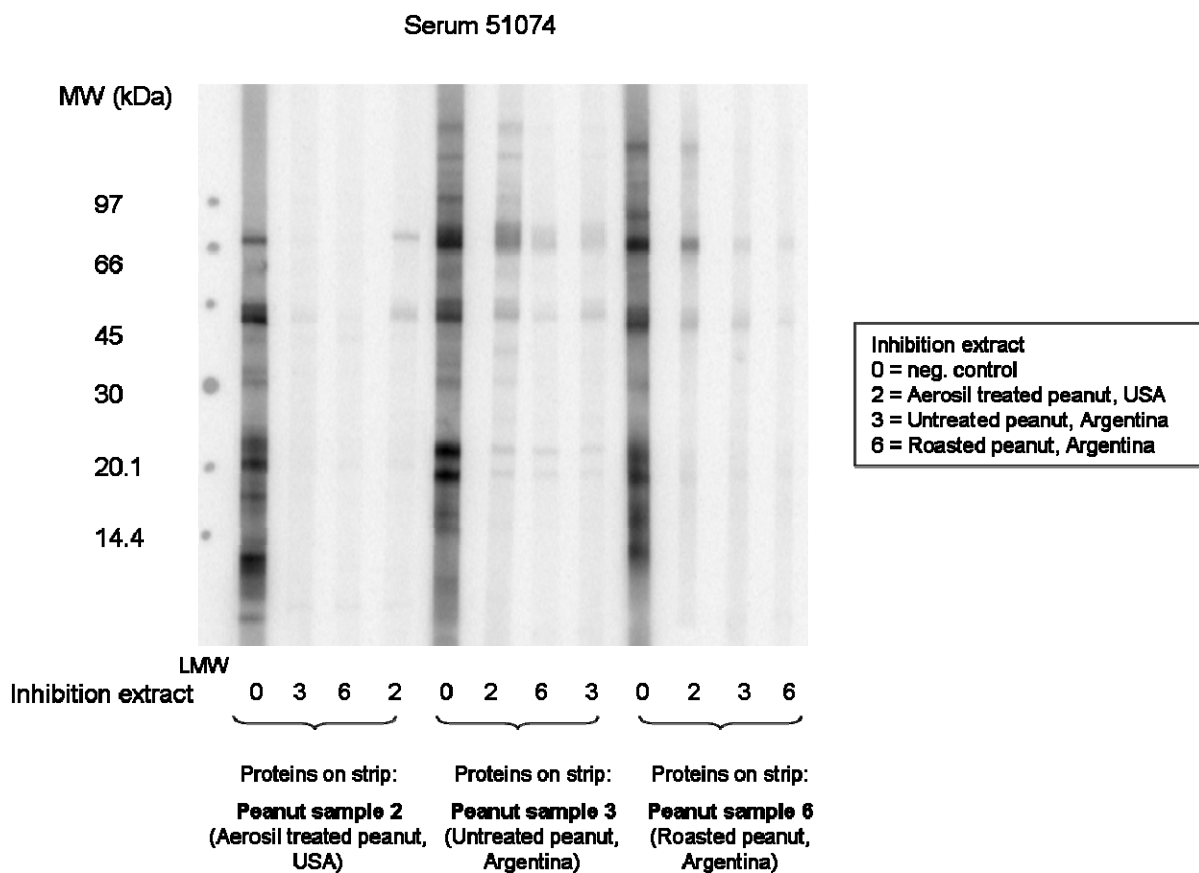

Figure 4.6 Inhibition. Serum 51074.

Whether an extract is a strong or a weak inhibitor is serum specific hence each serum must be studied separately. When serum 51074 is studied, see figure 4.6, strips that contain proteins from peanut sample 3 (untreated peanut) and 6 (roasted peanut), respectively, and are incubated with sera inhibited with peanut sample 2 (aerosil treated peanut) still show fairly prominent bands. The extract coming from aerosil treated peanut works as a weak inhibitor. When extracts from untreated or roasted peanut are used as inhibitors, less and weaker bands can be observed and the extracts can be considered as rather strong inhibitors. When the strips contain proteins from peanut sample 6 (roasted peanut), all three peanut extracts inhibit to the same extent in the area 14-20 kDa, whereas in the area 40-66 kDa, it is the proteins from roasted peanut that are the best inhibitors. Proteins from untreated peanut inhibit almost as good, and proteins from aerosil treated are the worst inhibitors.
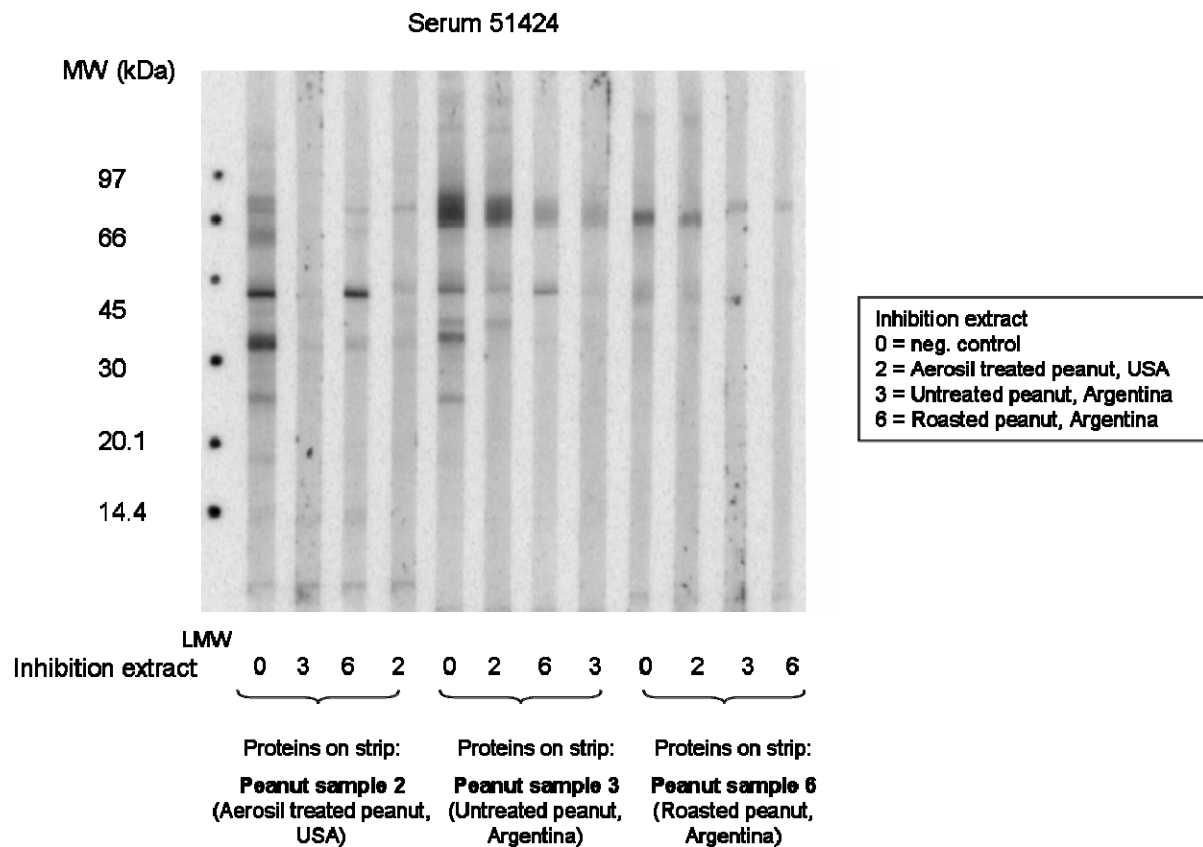

Figure 4.7 Inhibition. Serum 51424.

Even though each serum are studied separately, and a certain pattern can be observed, all allergenic components within a peanut sample are not equally inhibited. See for example strip number three from the left in figure 4.7. One prominent band ~ 45 kDa can be seen indicating that this component is weakly if at all inhibited by extract from peanut sample 6. On the other hand the component somewhat larger than 30 kDa is strongly inhibited by extract from peanut sample 6.
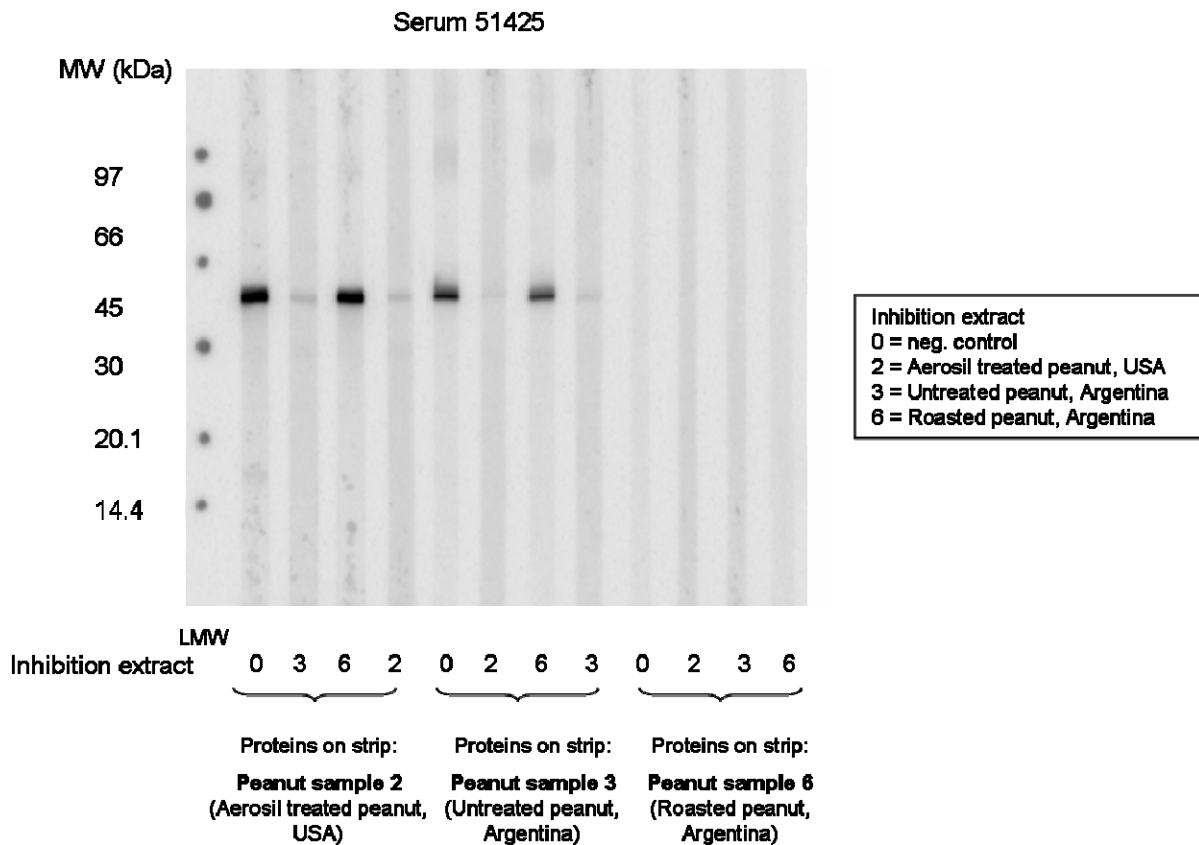
Figure 4.8 Inhibition. Serum 51425.

The only allergenic component seen when studying serum 51425 is strongly inhibited by extract from peanut sample 3 (untreated peanut) and peanut sample 2 (aerosil treated peanut), see figure 4.8. Extract from peanut sample 6 (roasted peanut) works as a weak inhibitor. The four strips to the right containing proteins from peanut sample 6 show no bands at all, thus this serum lack antibodies against allergenic components in extract from roasted peanut, see negative control that is blank.

4.5 Inhibition (two dimensions)

To further study the differences between allergenic components present in different peanut samples, inhibition studies with two-dimensionally separated proteins were performed. Peanut samples 2 and 6 that according to inhibition studies in one dimension can work both as weak and strong inhibitors, depending on the sera and the allergenic components studied, were used. Serum 51074 was used.
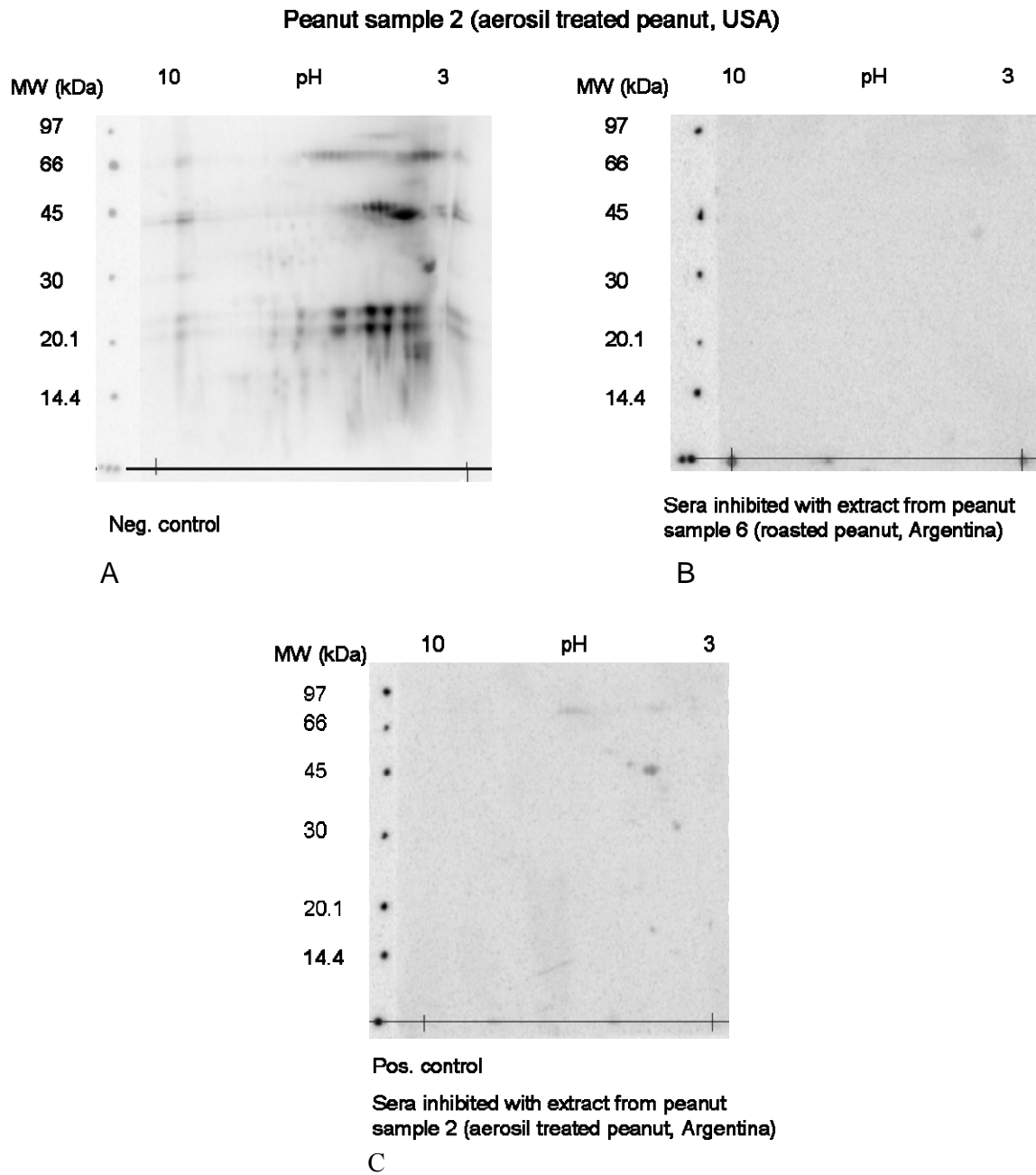
Peanut sample 2 (aerosil treated peanut, USA)

A

Neg. control

B

Sera inhibited with extract from peanut sample 6 (roasted peanut, Argentina)

C

Pos. control

Sera inhibited with extract from peanut sample 2 (aerosil treated peanut, Argentina)

Figure 4.9 Inhibition, two-dimensionally separated proteins from aerosil treated peanut.

No prominent spots can be seen when studying panel B, thus extract from peanut sample 6 works as a strong inhibitor, confirming the results from inhibition studies in one dimension. The positive control contains weak spots, indicating that extract from aerosil treated peanut cannot inhibit all peanut specific antibodies in the serum.
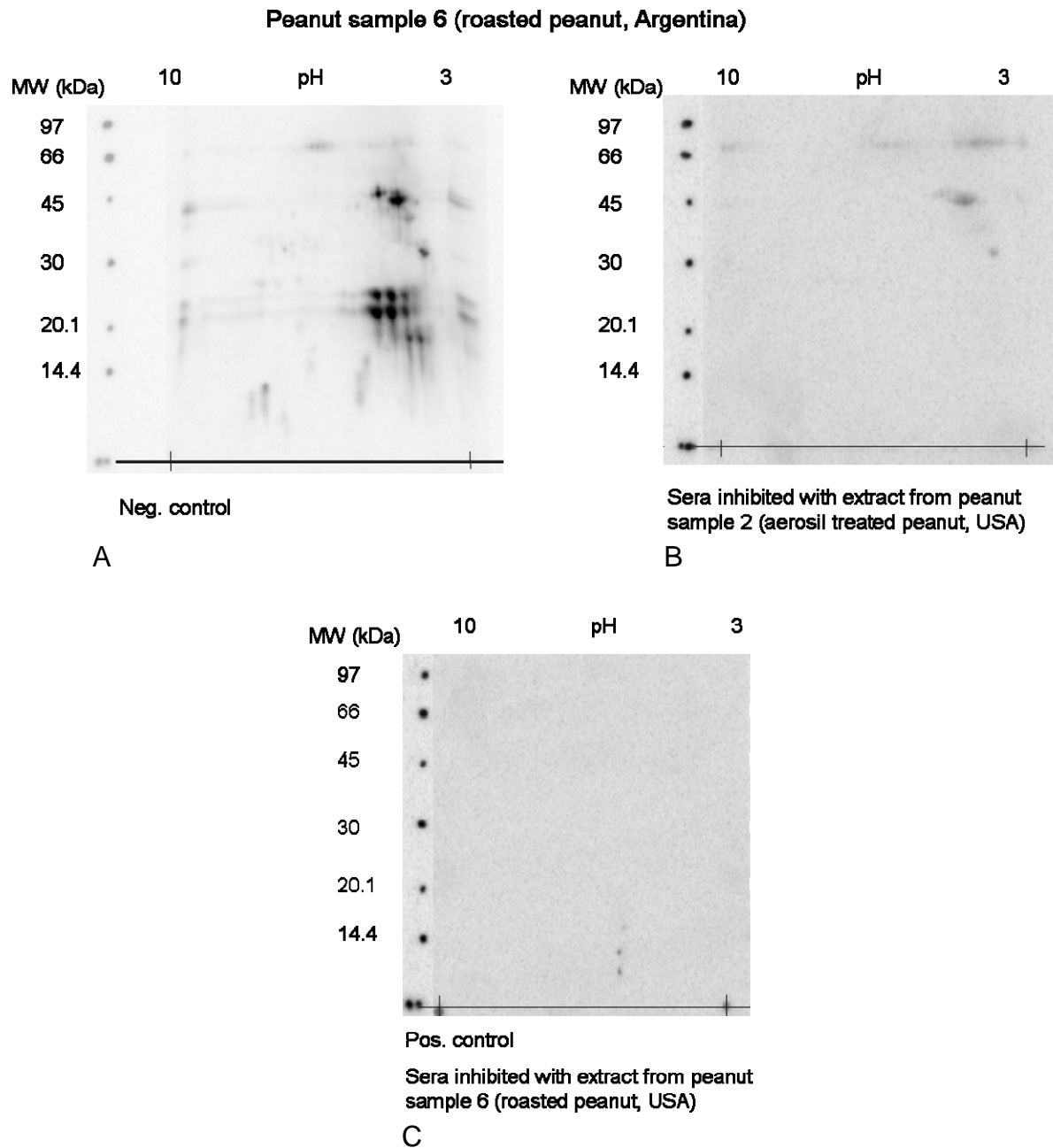
Peanut sample 6 (roasted peanut, Argentina)

Neg. control

A

Sera inhibited with extract from peanut
sample 2 (aerosil treated peanut, USA)

B

Pos. control

Sera inhibited with extract from peanut
sample 6 (roasted peanut, USA)

C

Figure 4.10 Inhibition, two-dimensionally separated proteins from roasted peanut.

In accordance with the result shown in figure 4.6 it can in figure 4.10 above be observed that the extract from peanut sample 6 works as the strongest inhibitor. Studying the negative controls in figure 4.9 and 4.10, it is possible to see that the allergenic components in aerosil treated and roasted peanut show a similar pattern even though some differences can be observed. In the area 30 - 45 kDa, low pH, the same pattern i.e. three prominent spots can be seen. In the area somewhat larger than 20 kDa, low pH, an organised pattern can be observed.

4.6 BCA protein assay

The protein concentration of six peanut samples was determined by means of a BCA protein assay.

Table 4.1 Protein concentration in six different peanut samples.

Peanut sample	Protein concentration (µg/ml)
1 Defatted peanut, USA	5059
2 Aerosil treated peanut, USA	788
3 Untreated peanut, Argentina	2609
4 Heated peanut, Argentina	2825
5 Boiled peanut, Argentina	998
6* Roasted peanut, Argentina	526

* Sample 6 was a bit diluted during extraction.

Table 4.2 Amount of protein in lyophilized extract.

Peanut sample	Estimated protein concentration (µg/ml)[#]	Measured protein concentration (µg/ml)*	Amount of protein in lyophilized extract (%)
1 Defatted peanut, USA	7555	5059	67
2 Aerosil treated peanut, USA	1930	788	41
3 Untreated peanut, Argentina	3995	2609	65
4 Heated peanut, Argentina	4345	2825	65
5 Boiled peanut, Argentina	2050	998	49
6 Roasted peanut, Argentina	1165	526	45

Concentration is calculated from the dry weight of lyophilized protein extract.
* Concentration is determined with BCA protein assay

The result in table 4.2 shows that lyophilized extract from aerosil treated peanut contains the least amount of proteins.

4.7 MALDI-TOF MS

To identify peanut proteins and possible allergenic components, spots of interest were excised from a gel containing two-dimensionally separated proteins from peanut sample 2 and 6. See picked spots in figure 4.11.
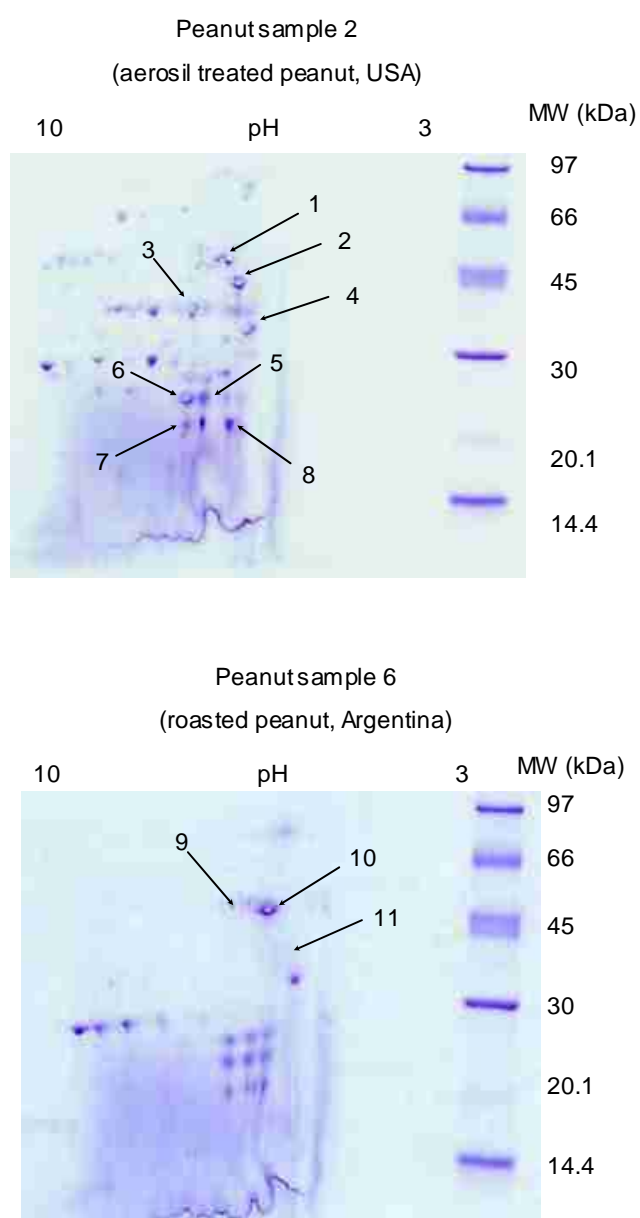
Peanut sample 2

(aerosil treated peanut, USA)

Peanut sample 6

(roasted peanut, Argentina)

Figure 4.11 Spots studied with MALDI-TOF MS

Table 4.3 Search results from NCBInr database.

Spot	Peanut sample	Mascot search result
1	2	Gly 1
2	2	glycinin or iso- Ara h 3
3	2	no hit
4	2	no hit
5	2	Ara h 2
6	2	Ara h 2
7	2	Ara h 2
8	2	no hit
9	6	arachin Ahy-1 / allergen Ara h 3/Ara h 4
10	6	Gly 1
11	6	Ara h 1

Spot 1-11 refers to the spots in figure 4.11.

Spots 5, 6 and 7 did all match with Ara h 2, verifying that these spots represent isoforms of the same protein.

4.8 Determination of specific IgE concentration in Japanese sera

The peanut specific IgE concentration in sera from Japanese patients with suspected peanut allergy was determined by means of the ImmunoCAP™ system (Phadia).

Table 4.4 Specific IgE concentration in six patient sera.

Serum sample	IgE level to coupled antigen on ImmunoCAP™ (kU/l)	
	regular peanut	roasted peanut
03NA AS-001	78.52	68.80
03NA AS-024	2.43	0.27
03NA AS-023	12.65	7.11
03NA AS-026	2.65	1.85
03NA AS-028	6.31	2.05
03NA AS-040	3.51	1.58

4.9 Immunoblotting using Japanese sera

To discover differences in which components that Asian patients react to compared to European/American patients, immunoblotting with six Japanese sera from patients with suspected peanut allergy was carried out. Peanut sample 3 (untreated peanut) and 6 (roasted peanut) was used since these are the variants of peanut that are most commonly consumed.
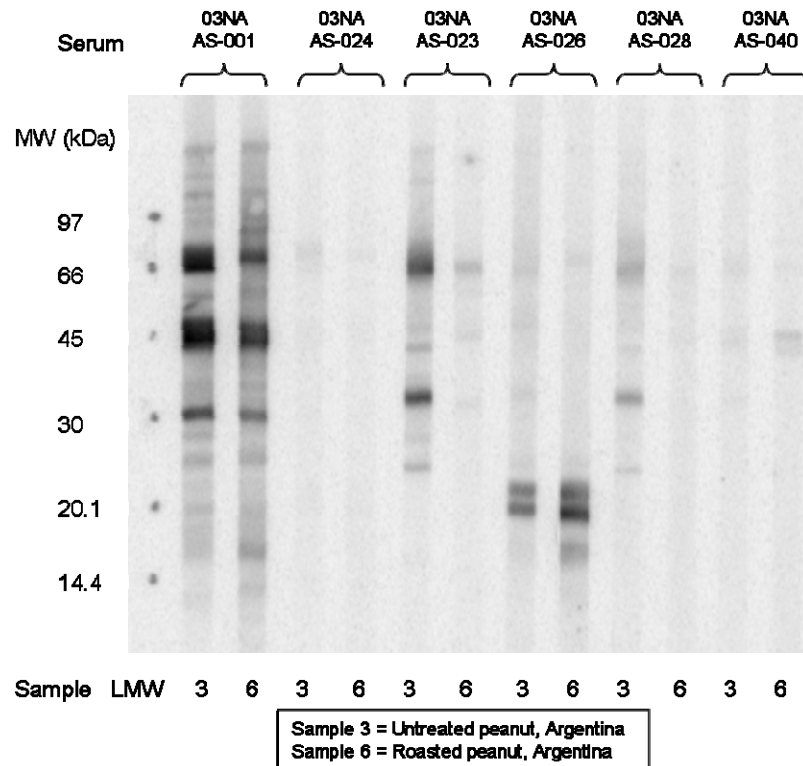

Figure 4.12 Allergenic components studied with Japanese sera. Components exposed on phosphor screen.

To be able to detect allergenic components present in low amounts, components were also exposed on x-ray film. See figure 4.13 below.
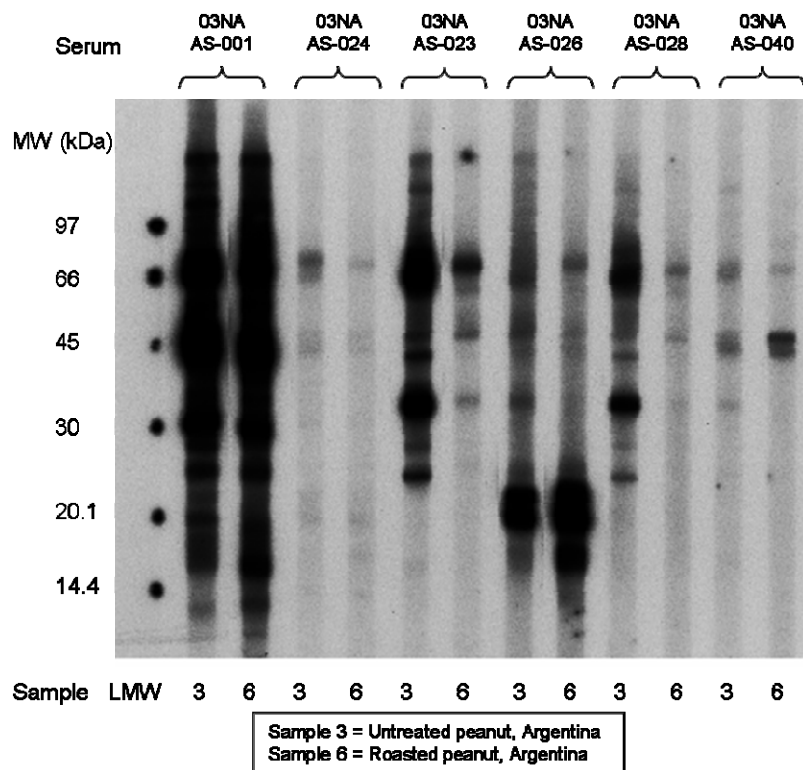

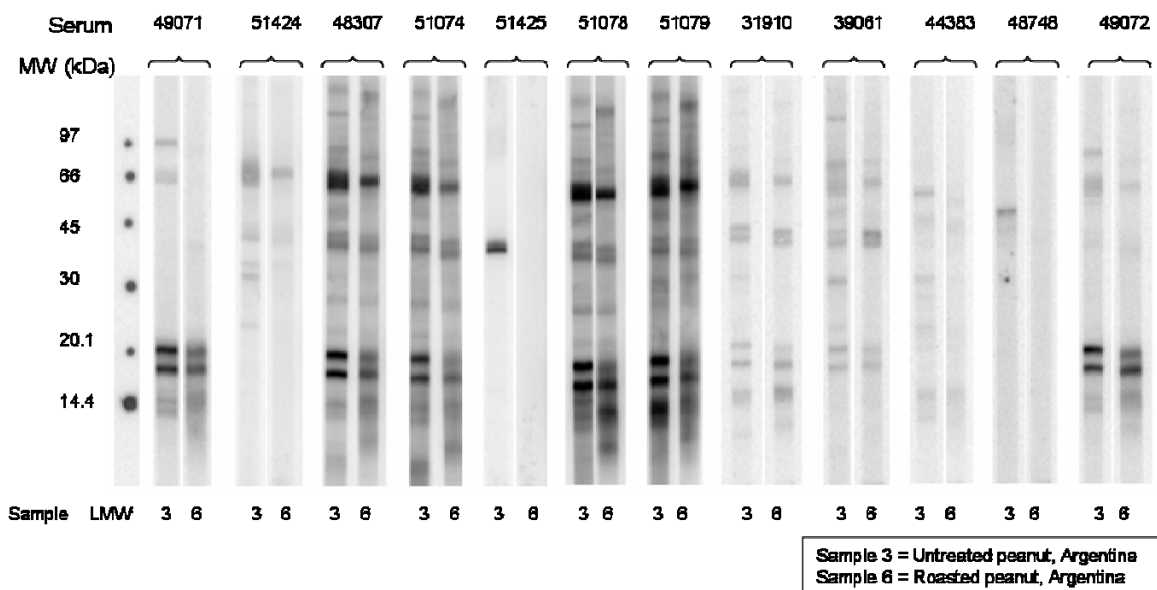**Figure 4.13** Allergenic components studied with Japanese sera. Components exposed on x-ray film.

Figure 4.14 Allergenic components in untreated peanut and roasted peanut studied with European/American sera.

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It is possible to distinguish a somewhat different pattern when studying the allergenic components in untreated and roasted peanut to which Japanese and European/American patients react, respectively.

Both Japanese and European/American sera show a similar pattern around 66 kDa, a prominent protein band most likely corresponding to Ara h 1.

In the majority of strips incubated with European/American sera two prominent bands ~15 - 20 kDa can be seen, see figure 4.14. These two bands are just visible in two out of six Japanese sera used in the study (03NA AS-001 and 03NA AS-026), see figure 4.13.

Sera 03NA AS-001, 03NA AS-024, 03NA AS-023 and 03NA AS-028 give a general lower response to proteins from roasted peanut, see figure 4.12 and 4.13. Serum 03NA AS-026 and 03NA AS-040 give a higher response to a component in roasted peanut located at ~14 kDa and ~45 kDa, respectively.

5 CONCLUSIONS

In this study, the protein content in untreated, defatted, heated, boiled and roasted peanut has been studied. The results indicate that different treatments do affect the protein composition in peanut. The treatment that seems to affect the protein composition the most is defatting by use of aerosil. Some proteins are present in a larger proportion whereas other proteins are present in a smaller proportion after this defatting method. The peanut samples that seem to contain the least total amount of proteins are the boiled and roasted peanuts, indicating that the high temperatures during treatment have a big impact on the protein content.

The allergenic components in untreated, defatted, heated, boiled and roasted peanut are different. Since peanut allergic patients react to different allergenic components, it is difficult to draw any general conclusions concerning how the components are affected by the different treatments. Some allergenic components are enhanced whereas other components are present in a much lower amount or not present at all after each respective treatment. Also in this context it is peanuts defatted by use of aerosil that are most unlike the other peanut samples. One component that is affected by aerosil treatment is Ara h 1, which is present in a lower amount after the treatment. The allergenic components in the other peanut samples look very similar, even though some differences can be observed, suggesting that the allergenic components in these samples are not affected noticeably by the different treatments.

Comparing the allergenic components to which European/American and Japanese patients react, it is possible to observe some differences. For example, two allergenic components that are seen in the majority of the studied European/American patient sera are visible in only two of the studied sera coming from Japanese patients. Whether these observed differences are representative for all peanut allergic patients in Asia remains to be investigated.

To be able to understand why the prevalence of peanut allergy in Asia is not as high as in westernized countries, more studies have to be performed. Future studies should include more peanut allergic patients from both of the studied groups. In this study, differences in protein content in American and Argentine peanuts have been observed. It would be interesting to study different peanut types and peanuts cultivated in both westernized and Asian countries, to be able to conclude whether differences depend on the peanut type or where the peanuts have been cultivated.

6 ACKNOWLEDGEMENTS

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