# The Impact of Elevated Carbon Dioxide Concentration and Other Environmental Conditions on the Allergenicity of Peanuts

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DOCTOR OF PHILOSOPHY



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# **Abstract**

Several parameters associated with climate change, in particular elevated atmospheric $CO_2$ concentrations, influence peanut plant growth. Commercial peanut plants grown in different regions of Australia are exposed to different environmental conditions. After extensive evaluation of methods to analyse the allergenicity of peanuts on a protein level, projected $CO_2$ concentrations and different growth regions in Australia were tested for their impact on the abundance of peanut allergens. While the impact of elevated $CO_2$ could not be determined conclusively, different environmental growth conditions did not appear to have an impact on the allergenicity of peanuts. Peanuts from different growing regions in Australia had a vet similar allergen content. The extraction method strongly influenced allergen extraction efficiency and their subsequent detection, which shows that the standardisation of peanut protein extraction is essential to quantify peanut allergenicity and understand its molecular and physiological basis.

# Declaration

The research reported in this thesis contains original material, except where clearly acknowledged within the text. No part of this thesis has been submitted for any other award to any other university or institute.

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# Conference abstracts, submitted book chapters, oral presentations and prizes

## Conference abstracts

**Nicole E. Walczyk**, Penelope M.C. Smith, Euan R. Tovey, Paul J. Beggs, Thomas H. Roberts "The Impact of Climate Change on the Allergenicity of Peanuts" Poster for presentation at the 4th International Symposium of the SFB 429 in Potsdam, Germany (October, 2010)

**Nicole E. Walczyk**, Penelope M.C. Smith, Euan R. Tovey, Paul J. Beggs, Thomas H. Roberts "The Impact of Climate Change on the Allergenicity of Peanuts" Poster for presentation at the 4th International Symposium Of Molecular Allergology in Munich, Germany (October, 2010)

**Nicole E. Walczyk**, Euan R. Tovey, Penelope M.C. Smith, Paul J. Beggs, Thomas H. Roberts "The Impact of Climate Change on the Allergenicity of Peanuts" Oral presentation at the 4th annual meeting of EuroBAT- The European consortium on application of flow cytometry in allergy in Munich, Germany (November, 2010)

## Submitted book chapters

Lena Y.C. Soo[§], **Nicole E. Walczyk**[§] and Penelope M.C. Smith (in press 2012) *Using genome-enabled technologies to address allergens in seeds of crop plants: Legumes as a case study;* §: Both authors contributed equally to this work. In: Seed Development: OMICS Technologies toward Improvement of Seed Quality and Crop Yield; G.K. Agrawal R. Rakwal (eds.); (26) Springer, Berlin (Appendix 5).

## Oral presentations

Department of Plant and Food Sciences, Faculty of Agriculture and Environment, University of Sydney, Australia. "Peanut Allergenicity, Climate Change and Plant Growth Conditions" (May, 2012)

4th annual meeting of EuroBAT- The European consortium on application of flow cytometry in allergy in Munich, Germany. "The  Impact of Climate Change on the Allergenicity of Peanuts" (November, 2010)

Department of Molecular and Clinical Allergology, Borstel, Leibnitz Centre for Medicine and Biological Sciences, Germany. "The  Impact of Climate Change on the Allergenicity of Peanuts" (October, 2010)

Peanut Company Australia (PCA) in Kingaroy, Australia. "The Impact of Climate Change on the Allergenicity of Peanuts " (February (2010)

Woolcock Institute of Medical Research, Sydney, Australia. "Climate Change, Allergens and Peanuts" (April, 2009)

Postgraduate Conference at Macquarie University in Sydney. Australia. "Climate Change and Human Health: Impacts of $CO_2$ on the Allergenicity of Plants"

University of Cologne, Germany. "Climate Change and Human Health: Impacts on the Allergenicity of Plants " (September, 2008)

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# Abbreviations

| | |
|---|---|
| 1D-gel | one-dimensional SDS-PAGE gel |
| 2D-gel | two-dimensional SDS-PAGE gel |
| 1D Western blot | one-dimensional Western blot |
| 2D Western blot | two-dimensional Western blot |
| 2D-DIGE | two-dimensional difference gel electrophoresis |
| ACN | acetonitrile |
| Amb a 1 | *Ambrosia artemisiifolia* allergen 1 |
| ANOVA | analysis of variance |
| Ara h 1, 2, 3, etc. | *Arachis hypogaea* allergen 1, 2, 3 etc. |
| ATP | adenosine 5'-triphosphate |
| BSA | *bovine serum albumin* |
| °C | degrees Celsius (temperature) |
| C3 | sugar containing three carbon atoms |
| C4 | sugar containing four carbon atoms |
| CH4 | methane |
| CHAPS | 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate |
| $C_6H_8O_7$ | citric acid |
| $C_6H_{12}O_6$ | glucose |
| $CO_2$ | carbon dioxide |
| d | day(s) |
| DAP | days after planting |
| DBPCFC | double-blind placebo-controlled food challenge |
| DEEDI | Department of Employment, Economic Development and Innovation |
| DTT | Dithiothreitol |
| ELISA | enzyme-linked immunosorbent assay |
| FACE | free-air CO2-experiments |
| GRDC | Grains Research and Development Corporation |
| h | hour(s) |
| $H_3BO_4$ | boric acid |

| | |
|---|---|
| HCl | hydrochloric acid |
| H$_2$O | water |
| HRP | horseradish peroxisase |
| IEF | *isoelectric focusing* |
| IgE | immunoglobulin E |
| IPCC | International Panel on Climate Change |
| IPG | immobilized pH gradient |
| KCl | potassium chloride |
| kDa | kilo Dalton |
| KH$_2$PO$_4$ | potassium-di-hydrogen-orthophosphate (anhydrous) |
| kVh | kilo Volt hours |
| lux | light intensity |
| mm | milli meter |
| M; mM | molar; milli-molar |
| mA | milli Ampere |
| MES | 2-(N-morpholino) ethanesulfonic acid buffered saline |
| Milli-Q | ultrapure water |
| MOWSE | molecular weight search |
| MS | mass spectrometry |
| MW | molecular weight |
| NaCl | sodium chloride |
| Na$_2$CO$_3$ | sodium carbonate |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NaHCO$_3$ | sodium hydrogen carbonate |
| Na$_2$HPO$_4$ | di-sodium hydrogen orthophosphate (anhydrous) |
| nan | not a number (Matlab function; The Mathworks Inc., 2009) |
| NaOH | sodium hydroxide |
| NCBI | National Centre for Biotechnology Information |
| NCBI acc. no. | NCBI accession number |
| NCBInr | NCBI non redundant |
| NH$_4$HCO | ammonium bicarbonate |
| N$_2$O | nitrous oxide |
| NSW | New South Wales, Australia |

| | |
|---|---|
| PAR | photosynthetically active radiation |
| PBS | phosphate buffered saline |
| PCA | eanut Company Australia |
| pI | isoelectric point |
| ppm | parts per million |
| p-value | probability value |
| QLD | Queensland, Australia |
| $r_a$ | aerodynamic resistance |
| RAST | radioallergosorbent test |
| rAra h 1, 2, 3 etc. | recombinant Arachis hypogaea allergen 1, 2, 3 etc. |
| $r_b$ | boundary-layer resistance |
| $r_m$ | mesophyll resistance |
| RO water | reverse osmosis |
| $r_{st}$ | stomatal resistance |
| RuBisCO | RuBP-Carboxylase/Oxygenase |
| RuBP | ribulose-1,5-bisphosphate |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SPT | skin prick test |
| SRES | Special Report on Emission Scenarios |
| TBP | tributyl phosphine |
| TBS | Tris buffered saline |
| TBS-T | TBS buffer with Tween-20 |
| TH2 | T helper cells |
| Tris | Trizma base; Tris base; Tris buffer |
| V | Volt(s) |
| (v/w) | (volume/weight) |

# Chapter 1

# Introduction

## *1.1    Increasing atmospheric CO$_2$ and climate change*

### *1.1.1. Rising greenhouse gases since industrialisation*

The key factor triggering current climate change is the increase in the concentration of global atmospheric greenhouse gases. According to the "Intergovernmental Panel of Climate Change" report from 2007, global atmospheric concentrations of carbon dioxide and other greenhouse gases have increased markedly as a result of human activities since 1750, due to high usage of fossil fuels, which came along with industrialisation and changes in land use and agriculture (Figure 1.1; (IPCC 2007). The main anthropogenic greenhouse gases are carbon dioxide (CO$_2$), methane (CH$_4$) and nitrous oxide (N$_2$O), each of which showed an increased in concentration by 70% between 1970 and 2004. Carbon dioxide is the most important anthropogenic greenhouse gas. The pre-industrial CO$_2$ concentration was, according to IPCC (2007), relatively stable for the past 650,000 years at around 280 parts per million (ppm) and increased to a mean value of around 385 ppm in 2008 (http://www.esrl.noaa.gov/gmd/). The current concentration of carbon dioxide exceeds by far the natural range over the last 650,000 years (180 to 300 ppm CO$_2$) as determined from ice cores (IPCC 2007). The increase in global atmospheric CO$_2$ concentration since 1750 has not been linear.  Mauna Loa data (http://www.esrl.noaa.gov/gmd/) show an 18% increase in the mean annual CO$_2$ concentration since these records began in 1959, when it was 316 ppm. This suggests that approximately two-thirds of the increase in atmospheric CO$_2$ concentration since the Industrial Era began has occurred over the last 50 − 60 years or so. Its primary source since the pre-industrial period results is due to anthropogenic activities, mainly from fossil fuel use, with land-use change providing another significant but smaller contribution (IPCC 2007).

### *1.1.2. Projections for atmospheric CO2 concentrations and climate change*

According to IPCC (2007), the atmospheric CO$_2$ concentration is projected to reach 550 to 950 ppm (depending on the SRES scenario: see Appendix 6) by 2100 (Figure 1.1).
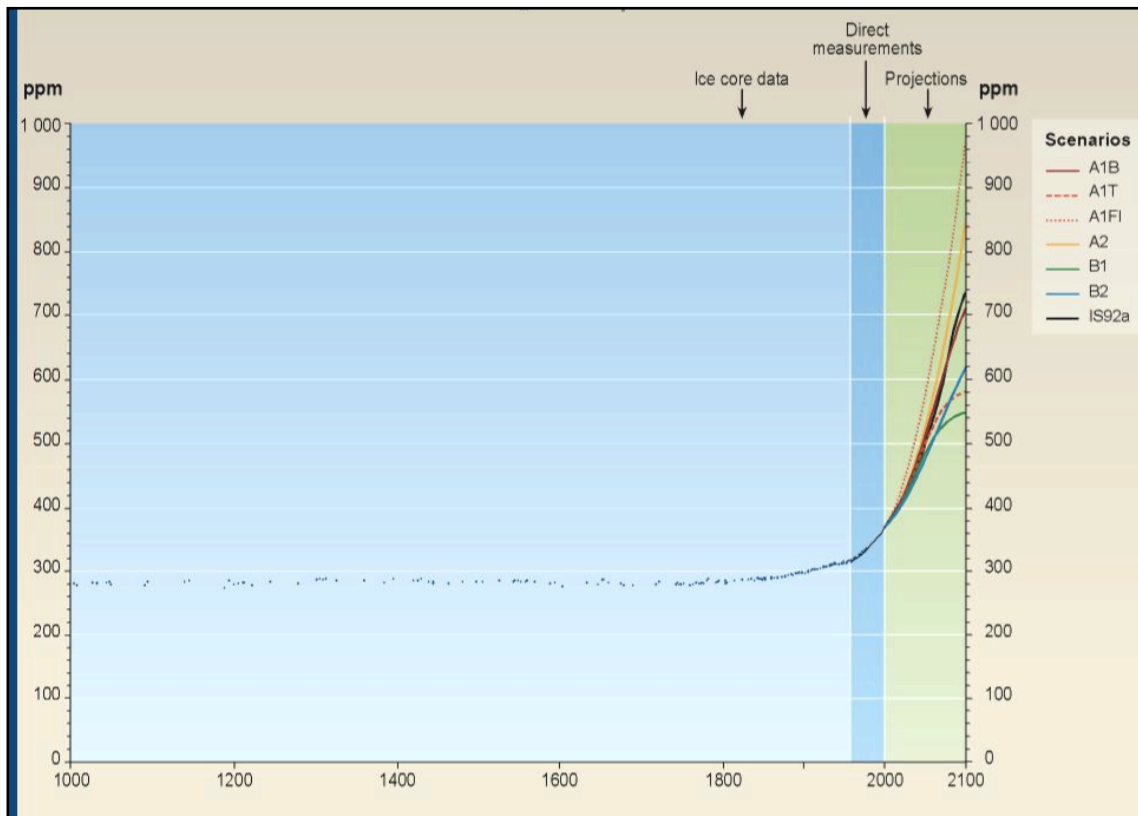
**Figure 1.1 Past and projected future for global atmospheric CO$_2$ concentration**

The graph was published in the report of the International Panel on Climate Change (IPCC) in 2007, and shows the atmospheric carbon dioxide concentrations between 1000 and 2100. The data are based on ice core records and direct measurements as well as estimated future projections (IPCC 2007). The future projections are based on seven different future Special Report on Emission Scenarios, which take into account population growth, economic growth and the use of technologies (see Appendix 6).

Current global climate change is the direct consequence of an increasing global average temperature. Most of the observed increase in global temperature is very likely caused by the rise in atmospheric greenhouse gas concentrations, in particular CO$_2$, as they can trap the heat in the atmosphere. The global average surface temperature is projected to rise between 1.8 and 4°C by 2100 (relative to 1999), according to IPCC (2007), depending on the SRES (Special Report on Emission Scenarios) marker scenario. This is also consistent with the projections for the atmospheric CO$_2$ concentration. It is important to note that even when the average temperature increases only slightly, peak temperatures can increase significantly. This also has an important impact on other aspects of climate, including ocean warming, precipitation and wind patterns. Extreme weather events like cyclones have already become and will become more frequent in the future (IPCC 2007).

Although the rising atmospheric CO$_2$ concentration is just one aspect of global climate change, it is however important, because of its direct role in plant photosynthesis and production of biomass. Furthermore, rising CO$_2$ is unique in being globally almost uniform (in contrast to the rising temperature) and so denying spatial proxies for temporal trends.

# 1.2.   Plants and CO$_2$

## 1.2.1. Assimilation of CO$_2$ by plants

The most important atmospheric gas for all plants is $CO_2$, as plants assimilate it directly from the atmosphere and incorporate it into their biomass, via photosynthesis. Plants are therefore regarded as a major carbon sink for sequestration. The assimilation of carbon dioxide and the synthesis of sugars, fatty acids and amino acids, are crucial for the survival of plants. It is the basis for plant growth, reproduction and storage of high-energy molecules, such as starch. Atmospheric carbon dioxide molecules reach the stroma by diffusing into stomatal pores on the plant surface (Figure 1.2).
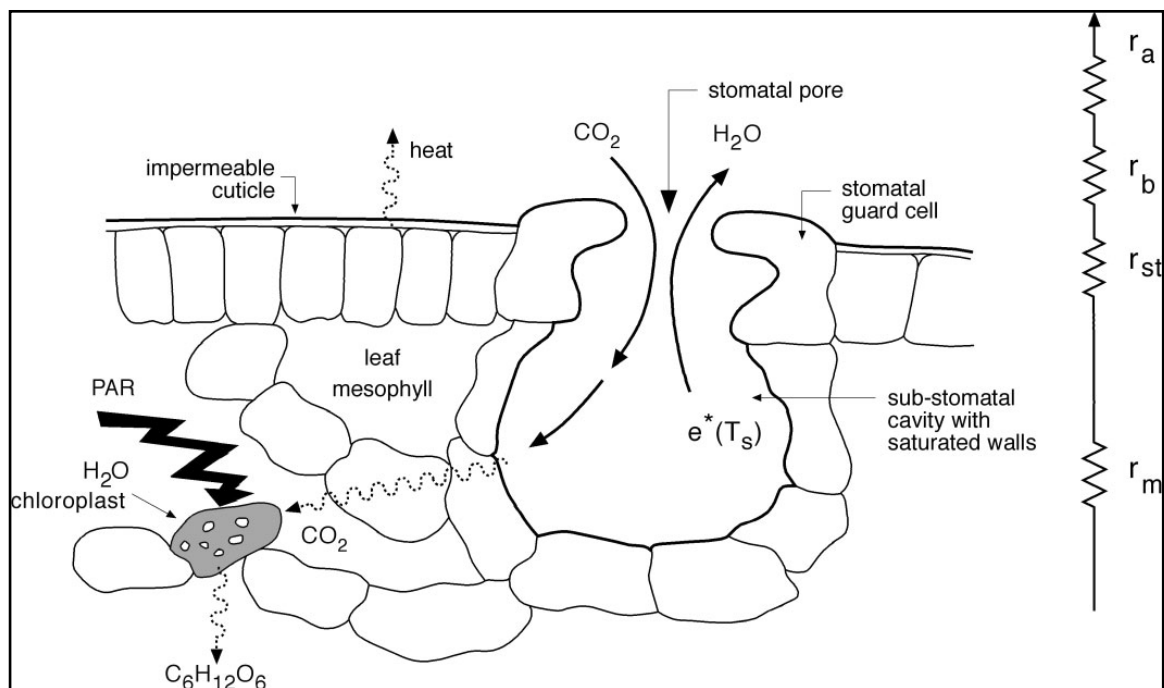


**Figure 1.2 Schematic of a cross-section of a leaf**

As the stomatal pore opens on the leaf surface, atmospheric carbon dioxide diffuses into the plant and reaches the chloroplasts. During photosynthesis, photosynthetically active radiation (PAR) combines water ($H_2O$) and carbon dioxide into high-energy sugar molecules. As the stomata open transpiration of water vapour takes place. This has major impacts on the water balance of the plant and also functions as a mechanism to cool the plant's surface. The degree of swelling of guard cells is highly regulated, as they control the carbon dioxide and water balance of the plant ($r_a$ aerodynamic resistance; $r_b$ boundary-layer resistance; $r_{st}$ stomatal resistance; $r_m$ mesophyll resistance) (Pitman 2003).

The assimilation of atmospheric carbon molecules is performed by the Calvin Cycle, the so-called "dark reactions" of photosynthesis (Figure 1.3). Carbon assimilation occurs in the stroma of the chloroplasts and is driven by the light reactions of photosynthesis, which provides ATP (adenosine 5'-triphosphate) as a source of energy and reductive capacity in the form of NADPH (nicotinamide adenine dinucleotide phosphate). The Calvin Cycle can be

divided into three phases: carbon assimilation, reduction and regeneration of the $CO_2$ acceptor molecule. During the first phase, $CO_2$ is bound by the high-affinity carbon dioxide acceptor molecule, ribulose-1,5-bisphosphate (RuBP). The enzyme that catalyses this reaction is the RuBP-Carboxylase/Oxygenase, or RuBisCO. In the second phase, the resulting molecule undergoes several reactions, including reduction, until a C3 sugar (glyceraldehyde-3-phosphate) is generated after the cycle has run three times to assimilate three atmospheric carbon dioxide molecules. While the $CO_2$ acceptor molecule is regenerated in the following reactions of the Calvin Cycle, this sugar molecule is transported out of the chloroplast. It then undergoes glycolysis, enters the Krebs cycle and undergoes oxidative phosphorylation, while ATP, sugars, fatty acids and amino acids are generated. Amino acids are then transported via the phloem to other organs, such as the seeds, where storage proteins and other proteins, such as defence proteins, are generated.
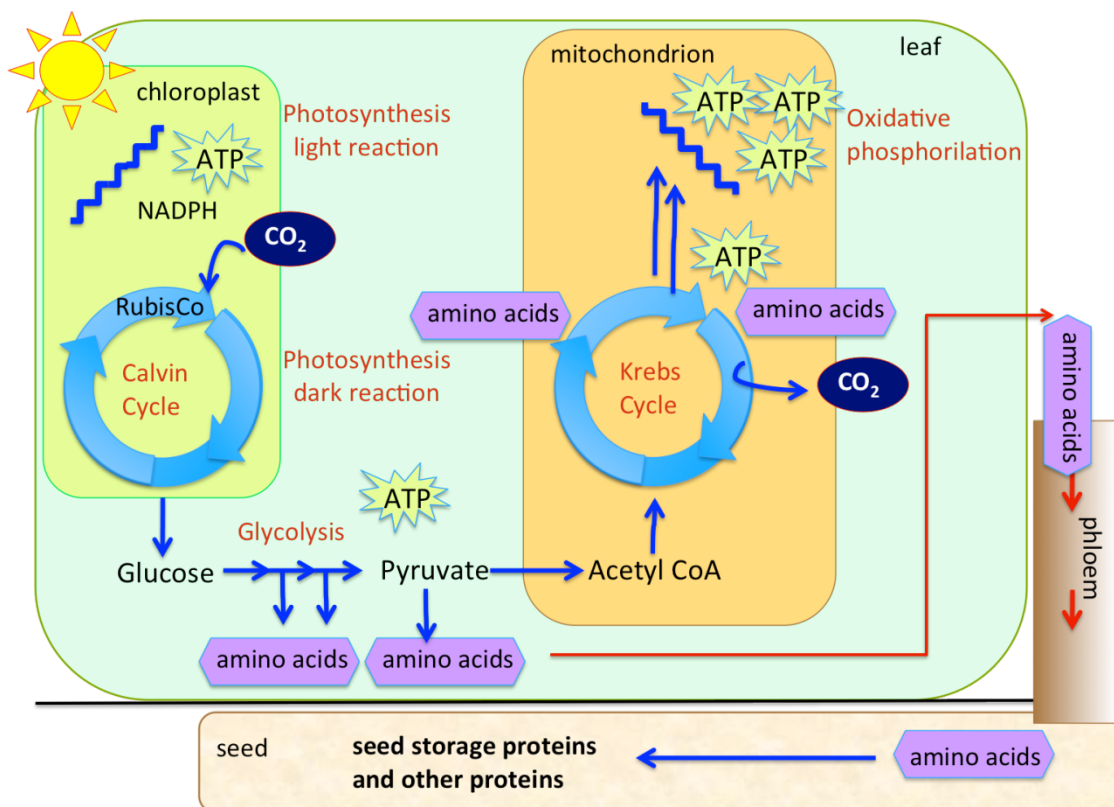


**Figure 1.3 From photosynthesis to proteins in seeds**

A simplified scheme of photosynthesis and oxidative phosphorylation, showing the synthesis of amino acids and their path to the seeds, where they become seed storage proteins and other proteins.

RuBisCO incorporates a dual functionality, as it is unable to avoid fixing oxygen into RuBP (Andrews and Lorimer 1987, Bowes et al. 1971), resulting in the production of phosphoglycolate, which is in turn the substrate for the photorespiratory cycle. This pathway functions as a valve for too high oxidative stress. Although RuBisCO favours carbon dioxide to oxygen, oxygenation of RuBisCO occurs frequently in current atmospheric levels of

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atmospheric carbon dioxide oxygen (approximately one oxygenation per 3 carboxylations). This can cause a substrate limitation for the Calvin Cycle, and hence $CO_2$ assimilation by RuBisCO (Badger 1992). The pattern of RuBisCO limitation differs between two photosynthetic types of plants. In C4 plants (~5% of all plant species), RuBisCO is localised in the bundle-sheath cells, where carbon dioxide is concentrated and higher than in the surrounding atmosphere (Edwards G. et al. 1985 ), thus eliminating the inhibitory effects of oxygen (Badger 1992). At moderate temperatures (20–30°C), it therefore encounters carbon dioxide concentrations close to saturation, so the potential for photorespiration is low (von Caemmerer and Furbank 1999). This is an adaption to high temperatures and high sunlight. In contrast, RuBisCO is exposed to ambient carbon dioxide concentrations in C3 plants (~95% of all plants, including peanut plants), and therefore reaches only approximately half of its saturation level at 20–30°C (Jordan and Ogren 1984, Sharkey 1988). However, it has been shown that $CO_2$ assimilation in C3 plants increases at higher atmospheric concentrations (Klus et al. 2001). Net photosynthesis per leaf unit area is raised under these conditions partly due to a decrease in photorespiration, partly due to an increased substrate supply (Poorter 1993). Increased $CO_2$ has the potential to increase the photosynthetic capacity and efficiency of leaves both in light-saturated and light-limited conditions (Badger 1992), which incorporates potential benefits regarding plant performance at high $CO_2$ concentrations, such as growth and harvestable grain production, specifically of C3 crops (Ainsworth Elizabeth A. and Long 2005, Drake et al. 1997, Kimball et al. 2002, Long Stephen P. et al. 2006, Nowak et al. 2004). In contrast, C4 plants generally show little response of photosynthesis when exposed to an elevated atmospheric carbon dioxide environment (Poorter 1993), unless they are exposed to drought stress (Leakey et al. 2006, Ottman et al. 2001).

### *1.2.2. Impact of increased atmospheric $CO_2$ concentrations on plants*

It has been shown in previous studies that C3 plants show a range of responses to elevated atmospheric $CO_2$ concentration. This includes changes in plant elemental composition, as plants typically show increased concentrations of carbon in their tissues, with correspondingly reduced concentrations of other elements, including nitrogen, phosphorus (Cotrufo et al. 1998, Gifford et al. 2000) and several trace elements (Loladze 2002) when atmospheric $CO_2$ increases. As a result, high atmospheric $CO_2$ can directly lead to alterations in plant development and metabolism. An increased $CO_2$ concentration can affect plant performance in either direction. If resources are favourable with sufficient nutrients, water and sunlight, an increased atmospheric $CO_2$ concentration can lead to better plant performance, with higher yields, increased plant growth and a higher reproductive capacity. A meta analysis by

Jablonsky et al. verifies this across 159 $CO_2$ enrichment papers that provided information on 79 species $CO_2$ enrichment (500–800 ppm [$CO_2$]) resulted on average in more flowers (+19%), more fruits (+18%), more seeds (+16%), greater individual seed mass (+4%), greater total seed mass (+25%) and lower seed nitrogen concentration (−14%) in all species tested. Additionally it was found that crops generally show a higher allocation of additional biomass into reproductive tissues, such as fruits and seeds, than wild species when grown at high $CO_2$. However, Jablonsky et al. also demonstrated important differences among individual taxa and among functional groups (Jablonski et al. 2002). In a compilation of literature sources Poorter et al. showed similar results. In this meta-study it was found that the growth stimulation of 156 plant species by elevated atmospheric $CO_2$ concentrations was, on average, 37%. Moreover, it was concluded that within the group of C3 species differences exist in the growth response to high $CO_2$ (Poorter 1993). Additionally other responses of climate change have been taken into account such as droughts, temperature rise and the presence of other greenhouses gases may have a negative impact on the plant performance. Due to these adverse effects, it is generally thought that the yield of most crops will only change a little as a result of elevated CO2 and climate change (Lopes and Foyer 2011). Most of the studies aimed to determine the effect of elevated $CO_2$ on general phenotypical plant responses, such as biomass, plant weight and height, yield, harvest index, root / shoot ratio, leaf area or growth rate (Edwards Grant R. et al. 2003, Maestre et al. 2007, Mortensen Leiv M. 1994, Mortensen L. M. and Sæbø 1996, Mortley D. G. et al. 1997, Paterson et al. 1996, Retuerto and Woodward 1993, Rogers et al. 1986, Sionit et al. 1980, Stanciel et al. 2000, Uprety et al. 2001, Vara Prasad Pagadala V. et al. 2000). A few studies additionally proceeded on a biochemical level, and examined the effect of elevated $CO_2$ on photosynthetic rates, $CO_2$ assimilation rates and alterations of the total protein concentration within the grains (Sæbø and Mortensen 1996, Stanciel et al. 2000, Vara Prasad P. V. et al. 2003, Vu 2005, Zhu et al. 2008). Taub et al. showed in a meta-analysis with 228 major food crops including wheat and soy that species showed consistently lower protein concentrations in elevated $CO_2$ (540–958 ppm) compared with ambient $CO_2$ (315–400 ppm). In a different study it was shown that the protein content of the grain decreased by 8% in barley in elevated $CO_2$ (Sæbø and Mortensen 1996). Furthermore it has been demonstrated that environmental conditions can significantly influence the seed protein content in some legumes (Burstin et al. 2011, Frimpong et al. 2009, Oluwatosin 1997, Saxena et al. 2002). Moreover, some of the studies concentrate on effects on the allergenicity of the plants. Such studies mostly give attention to $CO_2$ responses of allergenic tissues, such as pollen production, which is increased in elevated $CO_2$ (Ziska Lewis H. and Caulfield 2000). Only one study characterised the allergenicity on biochemical level

after the plants were exposed to elevated $CO_2$ (Singer, 2005) and is discussed after introducing allergy more in detail (Chapter 1.6).

### *1.2.3. Plant responses to other climate change factors*

As the $CO_2$ concentration of soils is increasing along with the increase of atmospheric $CO_2$, they play an important role in future plant performance. Long-term elevated $CO_2$ has major impacts on the soil's pH and increases its capacity for nutrients, like organic carbon, total nitrogen and net mineral-N mineralisation (Burstin et al. 2007). These findings may affect how plants respond to an elevated $CO_2$ atmosphere and therefore alter the potential of allergenic plants. It is important to mention that other climate change factors, especially temperature, also have an important impact on plant metabolism and development.  As a higher rise in temperature is projected for the next decades (Chapter 1.1.2), this effect might speed up in the future. Extreme weather events, like heat waves, cyclones and precipitation events may also alter plant performance.

## *1.3.    The peanut plant*

Peanut plants (*Arachis hypogaea*; Figure 1.4) are C3 plants and belong to the family of legumes (*Fabaceae*), which also include peas, beans and soy. The term "hypogeaea" means below the earth and describes the geocarpic development of the ripening fruit beneath the ground. After self-pollination the peanut flowers whither and gynophores (also called pegs) elongate below the flower stalk and then push underground where the fruit develops into a legume pod, containing 1–3 seeds. Peanut seeds provide a rich source of oil (44–56%) and proteins (22–30%), and represent a valuable source of energy, which makes them economically a very important crop, especially in developing countries (Atasie et al. 2009, Koppelman et al. 2001).

**Figure 1.4 Peanut plant with details of specific organs and growth stages**

(1) Longitudinal section of flower (2) gynophore or peg (3) mature pod, which is the fruit of the plant (4) pod contains two to three seeds; two seeds are by far more common (5) seed with brown fine seed coat (6) seedling without seed coat (7) seedling after removal of one cotyledon; adapted from (Köhler 1897). The bottom left shows the three stages of gynophore development: (a) Before fertilization (the flower parts have been removed); (b) A few days after fertilization the gynophore starts to grow towards the soil; (c) Two weeks after fertilization, with developing immature fruit (peanut pod containing seeds) buried underground; bar = 1.5 mm (http://www.amjbot.org/content/85/10/1369/F1.large.jpg).

Hundreds of peanut cultivars exist, but four of them are most popular as snack food and peanut butter sources: Spanish, Runner, Virginia and Valencia. Despite their taxonomic relation (Figure 1.5), they are commonly regarded as individual varieties (Koppelman et al. 2001).



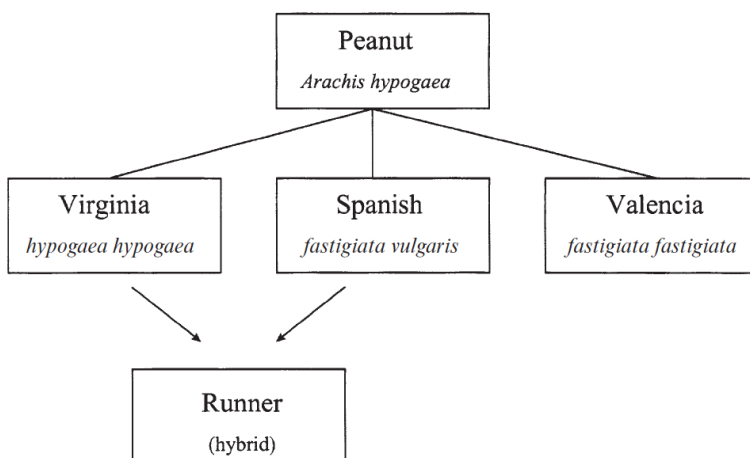**Figure 1.5 Taxonomic classification of common peanut species**

The peanut species *Arachis hypogaea* contains two subspecies: *hypogaea* and *fastigiata*. The latter includes two varieties: *fastigiata vulgaris* and *fastigiata fastigiata*. The runner type is a hybrid of *fastigiata* and *hypogaea* subspecies but is often regarded as an individual variety (Koppelman, 2001 with data from (Krapovickas 1969).

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Some varieties for the confectionery and snack market have been bred for high oleic oil content, such as the variety Walter, a Spanish size peanut kernel variety (Queensland Department of Employment, Economic Development and Innovation (DEEDI), Kingaroy and the Grains Research and Development Corporation (GRDC). Walter peanuts are ultra-early in maturing, which makes them ideal as a model plant.

### 1.3.1. Peanuts grown in elevated $CO_2$

When atmospheric $CO_2$ concentration was increased from ~350 to ~800 ppm, the following plant performance parameters were found to increase in peanut: leaf area, branch length, foliage fresh and dry weight, fresh and dry weight of roots, plants and pods, number of pods and seeds, harvest index and net photosynthetic rate increased (Mortley D. G. et al. 1997, Stanciel et al. 2000, Vara Prasad P. V. et al. 2003).

### 1.3.2 Peanuts and other climate change factors

Generally, the number of flowers, proportion of fruit-set, number of pegs and pods per plant, the seed yield, harvest index and seed size and amount of soluble sugars and starch in leaves of peanuts decreased in elevated temperatures. However, responses depend on the temperature, day-length and length of exposure to the elevated temperature. Although elevated atmospheric $CO_2$ concentrations is known to increase the plant performance, it did not compensate for the decreased plant performance in elevated temperatures (Vara Prasad Pagadala V. et al. 2000, Vara Prasad P. V. et al. 2003, Vu 2005). Being a cultivated plant, peanuts will be likely to be exposed to optimal quantity of water and nutrients throughout plant growth.

## 1.4. Allergy

### 1.4.1. What is allergy?

Allergy is a disorder of the immune system, which occurs as a hypersensitive response to exposure to innocuous environmental proteins (antigens), which are present in a range of food stuffs and airborne particulates (Nauta et al. 2008). Classic immunoglobulin-E (IgE)-mediated food allergies are classified as type-I immediate hypersensitivity reactions upon a classification from (Gell and Coombs 1963). In contrast to a toxic reaction or intolerance, allergies always employ an immune response. An allergic disease develops normally in two temporally discrete stages: the induction or sensitization phase and the elicitation phase. Hypersensitivity is not manifested on first contact with the antigen (induction phase), but usually appears on subsequent encounter (elicitation phase) (Corsini and Kimber 2007). The

allergic reaction is initiated when an antigen recognises and binds specific epitopes in the allergen molecule and then crosslinks immunoglobulin E (IgE) antibodies bound to their high-affinity receptor on tissue mast cells or blood basophils (Sutton and Gould 1993). The immediate reaction, taking effect within minutes of allergen provocation, results in the release of mediators that lead to symptoms characteristic of the target organ. A late-phase response associated with the influx of T cells, monocytes, and eosinophils may ensue some hours later (Gould et al. 2003).

The daily burden of allergy to the individual patient can range from frequently local mucosal symptoms such as mild oral allergy syndrome, itching, sneezing, coughing and watery eyes to more devastating symptoms such as eczema, hives, hay fever, asthma, anaphylaxis, caused by mast cell activation in mucosal tissues of, respectively, the nose, lung, gut and skin. As a result the patients' quality of life is decreased (Gould et al. 2003, Sohi and Warner 2008). Moreover, an anaphylactic shock can lead to death within a few minutes if it is not treated quickly. As yet, there is no cure for peanut allergy. Therapy focuses primarily on peanut avoidance, early recognition of symptoms brought on by accidental ingestion, and pharmacologic treatment of adverse reactions (Bock et al. 2001, Roberts 2007, Skolnick et al. 2001).

### 1.4.2. Major food allergies and prevalence

Despite the enormous diversity of food antigen exposure, only a few foods account for 90% of food allergic reactions. Therefore the major allergenic foods are identified by law and food companies are obliged to label a list of potential allergenic ingredients on the package of various food stuffs. This list differs slightly across different countries, due to differences in prevalence of allergic reactions to food, caused by genetic variation, differences in consuming behaviour and environmental factors. According to the U.S. (United States) Food and Drug Administration, the law identifies the eight most common allergenic foods in the U.S.A., often called "the big eight" as milk, eggs, fish (e.g., bass, flounder, cod), crustacean shellfish (e.g. crab, lobster, shrimp), tree nuts (e.g., almonds, walnuts, pecans), wheat, soybeans and peanuts. The Food Standards Australia New Zealand additionally declares sesame as a major allergen. In the European countries the list of potential allergenic ingredients to be labelled also includes celery and mustard, as stated by the European Commission (from 29. September 2003).

The prevalence of food allergy is greatest during the first years of life, affecting about 6% of children (Grundy et al. 2002), however most infants develop clinical tolerance and epidemiological studies suggest that about 2% of the adult population in the U.S. has IgE-mediated food allergy (Sampson 2004). This is the case for other food allergens than peanut such as milk and egg. Peanut allergy is however thought to persist beyond childhood (Bock and Atkins 1989). A later study suggested that the prevalence of food allergy in the U.S. is estimated to be 6% in young children and 3.7% in adults (Sampson 2004). A study based in the U.K. (United Kingdom) showed that the prevalence of asthma, allergic rhinitis and eczema increased from 1964 to 1999 (Figure 1.6) (Ninan et al. 2000). Furthermore, it has been reported that the prevalence and severity of allergies have increased, particularly in developing countries in recent decades (van Ree and Yazdanbakhsh, 2007).
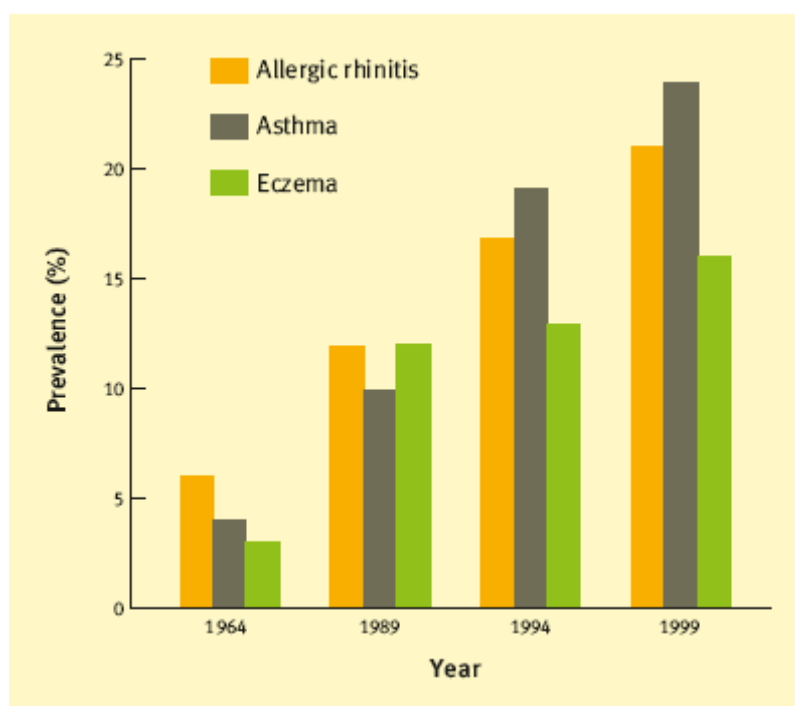


**Figure 1.6 The rise in prevalence of paediatric allergic diseases in Aberdeen, U.K.**
The increase of allergic rhinitis, asthma and eczema of children in Aberdeen aged 9–11 over a 30-year period. Reproduced by Sohi et al. (Sohi and Warner 2008) with data from Ninan (Ninan et al. 2000) .

The apparent increasing global prevalence of allergy is likely due to a range of factors, such as a higher diagnosis rate, changes in lifestyle (including diet), infections, the process of urbanisation and economic growth, pollutants and the introductions of allergens to non-native areas, which has been shown to contribute to the development of allergic disease (Sohi and Warner 2008). Furthermore different hypotheses have been postulated to explain this increase in prevalence, such as the hygiene hypothesis, dietary fat hypothesis and the vitamin D hypothesis. The hygiene hypothesis suggests that improved hygiene, with improved public practices and the use of vaccines and antibiotics, has reduced the incidence of infections that

would normally stimulate the immune system. This causes a lack in stimulation of the immune system, which results in a lack of protection against the development of allergic responses against innocuous environmental substances (Strachan 1989). The dietary fat hypothesis states that increase in the use of margarine and vegetable oils in the past decades and the simultaneous reduction in consumption of animal fats have led to the increase in allergies (Black and Sharpe 1997). The vitamin D hypothesis takes two forms, depending on whether a deficiency or an excess of vitamin D is thought to lead to increased allergies (Milner et al. 2004, Wjst 2005). However, little work has been done with respect to food allergens and there is limited evidence that strongly support any one of the hypotheses (Lack 2008).

### 1.4.3.   *Allergy to peanuts*

Among the allergenic foods, peanut is the leading cause of anaphylactic fatalities worldwide (Bock et al. 2007, Burks 2008). Peanut allergy is a major health care problem affecting 1% to 2% of the population in U.K., U.S.A. and Australia and this prevalence appears to be increasing (Burks , Emmett et al. 1999, Grundy et al. 2002, Hourihane et al. 2007, Mullins et al. , Osterballe et al. 2005, Sicherer S. H. et al. 2003, Sicherer Scott H. et al. 1999). Unlike egg and milk food allergy, which presents in infancy and typically resolves by school age, peanut allergy persists through adulthood in 80% of cases, significantly impairing the quality of life of afflicted individuals and their families (Bock et al. 2007, Kemp and Hu , O'Hehir and Douglass , Yun and Katelaris). Peanut allergies are of particular concern, because of the extreme hypersensitivity of some individuals. Threshold doses for eliciting allergic reactions can be as low as 30–100 μg peanut protein up to a dose of 300–1000 mg of peanut protein (Wensing, et al., 2002). It was estimated that 50% of a population with peanut allergy will have (subjective) allergic reactions after a dose of 3 mg of peanut protein (Wensing et al, 2002). Given that Ara h 1 is 12–16% and Ara h 2 was around 6% of total protein (De Jong et al. 1998, Koppelman et al. 2001) it can be estimated that 50% of the people who are allergic to peanuts will react to around 360–480 μg Ara h 1 and/or 180–270 μg Ara h 2. It is therefore likely that a magnitude of three-digit microgram of allergens Ara h 1 and 2 will have an impact on many peanut allergic patients. The thesholds for an allergic reaction are however very individual. Generally patients with potentially life-threatening reactions appear to be more sensitive than patients with milder symptoms (Wensing et al. 2002). Furthermore, peanut allergy is special because the routes of exposure are not only through ingestion, but also skin contact and inhalation (Sicherer Scott H. et al. 1999). It has been proposed that the

sensitization to peanut allergens does not occur as a result of consumption but can occur through other ''environmental food exposure'' (Lack 2008). This is substantiated by the fact that 72–81% of allergic reactions to peanut occurs at first known contact, so that the sensitisation is likely to occur through route other than ingestion (Sicherer, 1998). Furthermore it has been shown that exposure of peanut to abraded skin of mice leads to significant IgE responses (Strid et al. 2005).

Despite proposed guidelines recommending avoidance of peanuts during infancy in countries such as the U.K., Australia, and, North America, there is evidence that peanut allergy prevalence increased in these countries (Sicherer Scott H. et al. 2010). It has been observed that Israeli infants consume peanut in high quantities in the first year of life and have an extremely low prevalence in peanut allergy. These findings suggest that early introduction of peanut during infancy, rather than avoidance, can cause a tolerance to peanut (Du Toit et al. 2008). One study recorded the maternal peanut consumption during pregnancy, breast-feeding, and the first year of life, as well as peanut consumption of all household members, allowing quantification of environmental household exposure to peanuts. It showed that that there is a dose response relationship between environmental (=non-oral) peanut exposure and the development of peanut allergy. Based on these findings, it has been suggested that early high consumption of peanuts by infants leads to tolerance (Fox et al. 2009). This has led to an additional hypothesis: the dual-allergen-exposure hypothesis, which states that timing and balance of cutaneous and oral exposure determines whether child will have allergy or tolerance (Lack 2008).

As yet, there is no cure for peanut allergy. Therapy focuses primarily on peanut avoidance, early recognition of symptoms brought on by accidental ingestion, and pharmacologic treatment of adverse reactions (Bock et al. 2001, Roberts 2007, Skolnick et al. 2001). However, it has been shown that oral immunotherapy resulted in a 48 to 478-fold threshold increase of peanut tolerance in four children (Clark et al. 2009).

## 1.5.  Allergens

### 1.5.1.  What are allergens?

The susceptibility of an allergic immune reaction of a patient is based on environmental factors, host related factors, like an genetic preposition, the mode of the immune response and the allergic response; i.e. the symptoms and of course the exposure to allergenic material. This includes the dose of the exposed allergen, the pattern of its exposure, its duration, route

(i.e. how it enters the patients' body, by ingestion, inhalation or skin contact) and the extent of exposure (Corsini and Kimber 2007). In order to be able to predict the severity of an allergic reaction it is therefore crucial to identify the potency and dose of the exposed allergenic material by quantification of the exposed allergens.

Allergens have three distinct molecular properties. First is the property to sensitize, i.e. induce the immune system to produce high-affinity antibodies, particularly of the IgE class. These are capable of inducing IgE immunogenicity (or "true allergenicity"). The interactions of the potential allergen involve therefore (among others) the key cells of the immune system: antigen presenting cells, various types of T cells and B cells. Second is the ability to elicit an allergic reaction; i.e. to trigger allergic symptoms in a sensitized subject. And thirdly, an allergen must have the property to bind IgE antibodies (Aalberse 2005) in Type I allergic reactions (Chapter 1.4.1). The ''allergenic potential'' of a novel allergen is therefore a reflection of these three distinct protein properties.

The related term 'allergenicity' can either be interpreted as the capacity of an antigen to induce symptoms, or to the capacity of a protein to induce IgE antibodies or to bind to such antibodies. To date it is not possible to predict which proteins are potentially allergenic. Within this constraint, any antigen may be allergenic. A few characteristics can give an indication of a potential allergen. These include the capability to avoid an activation of immune response suppressor mechanisms (e.g. mechanisms that suppress TH2 cell expansion, which leads to development of IgE antibody production and an allergic phenotype) or other regulatory T cells and downstream mechanisms (such as for cytokines) (Aalberse 2000, 2005). In order to trigger an immune response, allergens must also be able to be transported over mucosal barriers (mucosal tissues include the organs, where the allergic reaction occurs, like nose, lung, gut, and skin). Therefore the protein structure and its characteristics, including the compactness of the overall fold, are very likely to be important. Additionally, cross-reacting allergens are able to react with usually pre-existing IgE antibodies. In most cases these proteins show immunogenicity, but in some cases they are not able to sensitize (Aalberse 2005). This is due to sequence homologies between the two allergens (Aalberse 2000), but the accuracy of prediction is very low if only linear sequences are used. This is because cross-reactivity is largely determined by structural aspects of the protein, which is the most relevant for antibody binding, particularly the epitopes, which on a molecular level interact with the antibody: two proteins are cross reactive (almost) only if they share structural features (Aalberse 2005), meaning that cross reactions between allergens may occur if they

share similar epitopes critical to the binding of the IgE formation of complete allergens (Corsini and Kimber 2007). A potential allergen can therefore be active at two levels: influencing the immune response in a quantitative or qualitative way or, more commonly, interacting with cell-bound IgE antibodies and triggering the effector phase of the allergic reaction (Radauer and Breiteneder 2006).

Despite the huge diversity of allergens, most of them can be grouped into just a few protein families by comparing their structural and functional properties (Bateman et al. 2002). Allergens from the allergen database AllFam (www.meduniwien.ac.at/allergens/allfam/, July 2007), which is available online, were found in only around 2% of all sequence-based and 5% of all structural protein families within a total number of 9318 families from the general protein families database Pfam (Radauer et al. 2008). All known plant food allergens belong to only 27 families of a total of 2615 seed plant protein families (out of 7868 protein families in Pfam), representing around 0.26% of the total number of families in the Pfam database. The four most important families for food allergens are the prolamin, cupin, profilin and Betv1 family. The fact that allergens belong to only a small number of protein families and the constricted functional distribution of most allergens validate the existence of yet unknown factors that determine their allergenicity (Sohi and Warner 2008).

### 1.5.2. Peanut allergens

To date 11 peanut allergens have been identified, which are expressed in peanut seeds and belong mainly to four protein groups and superfamilies: the cupin superfamily (including the 7S and 11S globulins); prolamins, which are (by definition), grain proteins that are insoluble in water or dilute salt solutions but soluble in aqueous ethanol (including the nonspecific lipid transfer proteins-nsLTPs, and the 2S storage albumins); profilins, which contain globular proteins (123 — 139 amino acids) that prevent the polymerization of actin; and the larger group of pathogenesis-related proteins (mostly composed of homologues of the major birch pollen allergen, Bet v 1) (Riascos et al. 2010).

**Table 1.1 Summary of known peanut allergens**

The protein families, protein classifications, common sizes on SDS-PAGE and epidemiology are summarised for the known peanut allergens. Importantly the band sizes depend on the degree of denaturation before electrophoresis.

| Allergen | Protein family | Protein classification | Band size (SDS-PAGE) | Epidemiology[1] | Cohort[2] | Country |
|---|---|---|---|---|---|---|
| Ara h 1 | Cupins | 7S vicilins | 63.5 (monomer) 145 (trimer) | 80% (Vereda et al.2011) >62% (Vereda et al. 2011) 30% (Vereda et al. 2011) 75% (Codreanu et al. 2011) | Symptoms, IgE Symptoms, IgE Symptoms, IgE Provocation test | US Sweden Spain France |
| Ara h 3/4 | | 11S legumins | 60 (precursor) 36 | >56% (Vereda et al., 2011) >37% (Vereda et al., 2011) 16% (Vereda et al., 2011) 45% (Rabjohn et al. 1999) > 61% (Condreanu et al. 2011) 53% (Kleber-Janke et al. 1999) | Symptoms, IgE Symptoms, IgE Symptoms, IgE Symptoms, SPT, IgE Provocation test Symptoms, SPT, IgE[3] | US Sweden Spain Spain France Not defined |
| Ara h 2 | Prolamins | 2S albumins | 17.3 | 90% (Vereda et al., 2011) >37% (Vereda et al., 2011) 16% (Vereda et al., 2011) >81% (Koppelman et al. 2004) >95%(Condreanu et al. 2011) 43% (Kleber-Janke et al., 1999) | Symptoms, IgE Symptoms, IgE Symptoms, IgE Symptoms, SPT, IgE Provocation test Symptoms, SPT, IgE[3] | US Sweden Spain Netherlands France Not defined |
| Ara h 6 | | | 14.5 | 69% (Koppelman et al. 2005) 80% (Flinterman et al., 2007) 94% (Bernard et al , 2007) >91% (Condreanu et al. 2011) 38% (Kleber-Janke et a., 1999) | Symptoms, IgE Provocation test Symptoms Provocation test Symptoms, SPT, IgE[3] | Netherlands Netherlands France France Not defined |
| Ara h 7 | | | 14–17 | >43% (Condreanu et al. 2011) 43% (Kleber-Janke et al., 1999) | Provocation test Symptoms, SPT, IgE[3] | France Not defined |
| Ara h 9 | | nsLTPs | 9 | >7% (Vereda et al., 2011) >14% (Vereda et al., 2011) 60% (Vereda et al., 2011) 24% (Lin et al. 2012) | Symptoms, IgE Symptoms, IgE Symptoms, IgE Symptoms, IgE[3] | US Sweden Spain Taiwan |
| Ara h 5 | Profilins | | 14 | 13% (Kleber-Janke et al. 1999) | Symptoms, SPT, IgE[3] | Not defined |
| Ara h 8 | PR proteins | PR-10 | 17 | 19% (Vereda et al., 2011) >65% (Vereda et al., 2011) 2% (Vereda et al., 2011) | Symptoms, IgE Symptoms, IgE Symptoms, IgE | US Sweden Spain |
| Ara h 10 | Oleosines | | 16 | No data | | |
| Ara h 11 | | | 14 | No data | | |
| 18kDa oleosin | | | 18 | 36% (Pons et al. 2002) | Provocation test | Not defined |
| Agglutinin | Legume lectines | | 29 | No data | | |

[1] The epidemiology depends on the method of allergenicity testing and may vary substantially between cohorts and regions.

[2] A cohort of peanut allergic patients or sera was selected with various criteria: known symptoms after food challenge or convincing history of acute reactions; positive SPT; positive for peanut IgE; allergic reaction after provocation test (DBPCFC)

[3] Only patients with high IgE in cohort

More important allergens such as Ara h 1–3, Ara h 6, and Ara h 7 are seed storage proteins, which belong to the cupin and prolamin superfamilies. Most peanut allergic patents have specific IgE against the major peanut allergens Ara h 1, 2 and 3. Around 90 – 95% of peanut allergic individuals have specific IgE to Ara h 1 and Ara h 2 (Burks et al. 1995, Scurlock and Burks 2004). However, Ara h 2, which functions as trypsin inhibitor (Maleki et al. 2003) was found to be a more potent allergen than Ara h 1 (Koppelman et al. 2004). Around 50% of patients so also have IgE against Ara h 3/4 (Kleber-Janke et al. 1999, Rabjohn et al. 1999),

while the recognition of other allergens is less common. Ara h 2 and 6 show a high sequence homology of 59% and native Ara h 2 and Ara h 6 have virtually identical allergenic potency. The sequence identity between Ara h 2 and Ara h 7 is 35% (Lehmann et al. 2006). Despite their high sequence homology to Ara h 2, little is known about Ara h 6 and 7.

The allergens Ara h 5, 8, 9,10, and 11, as well as agglutinin and 18-kDa oleosin are non-storage proteins, but are also present in the peanut seeds. Ara h 5 is an actin-binding protein that is also known as profilin. Ara h 8 is a pathogenesis-related protein, also called PR-10 and known to cross-react with the homologous Bet v 1, the major allergen in birch pollen. Although 85% of patients recognised Ara h 8, it has a very low stability upon roasting and gastric digestion (Mittag et al. 2004), which is probably why it is not regarded as a major allergen. Ara h 9 is a lipid transfer protein, while Ara h 10 and Ara h 11 are oleosins (Table 1.1). Agglutinin is a lectin involved in cellular recognition (Mari et al. 2006 ).

## *1.6.*   *Can allergenicity of peanuts change in different climatic conditions?*

Up to now, there is only one study, which examined the direct effect of elevated $CO_2$ on the allergen content in the plant *per se*. Singer et al. was able to show that ragweed (*Ambrosia artemisiifolia*) contained 1.8 times more allergen Amb a 1 when grown at 370 ppm $CO_2$, compared to the pre-industrial concentration of 280 ppm $CO_2$ and 1.6 times more Amb a 1 in 600 ppm $CO_2$ compared to 370 ppm $CO_2$ (Singer et al. 2005). The fact that peanut plants are C3 plants, which are not saturated with ambient $CO_2$ concentrations, and because the major peanut allergens are seed storage proteins (such as Ara h 1–3, Ara h 6, and Ara h 7; see Chapter 1.5.2), which are primary metabolites that derive directly from the $CO_2$ assimilating pathways, this suggests that the abundance of individual peanut allergens might be altered when the peanuts plants are exposed to elevated atmospheric $CO_2$ concentrations. This means that the allergenicity of peanuts might change in the future with the steadily increasing atmospheric $CO_2$ concentration in the atmosphere, which triggers climate change. This hypothesis has not been described previously and there are no studies on impacts of elevated $CO_2$ on food allergens.

Because other climatic factors, such as changes in temperature, have been shown to influence peanut plant development, peanuts from different growing regions might also show different allergen abundances. To date only Koppelman et al., compared the amount of allergens Ara h 1 and 2 in runner peanuts grown in two regions: U.S.A. and Argentina and showed that there are in fact no differences (Koppelman et al. 2001). The methods used, however, are now

outdated and it is necessary to confirm the findings as the outcome has both scientific and potentially commercial value for the peanut industry. Therefore the allergen content of peanuts from different growing regions in Australia was compared. For peanuts, even trace amounts of residual allergens can elicit reactions in a very atopic patients (Dodo et al. 2008, Flinterman et al. 2006, Morisset et al. , Wen et al. 2005, Wensing et al. 2002), which is underlined by the fact that the IgE in patients' sera still detected the allergens in Ara h 2 and 6-silenced peanuts (Chu et al. 2008, Dodo et al. 2008). The transformed peanuts therefore still present a risk for patients allergic to the allergens. However, for some allergenic proteins, a reduction of allergen may provide some benefit and should be at least attained.

## 1.7. *Extraction methods and quantification of peanut allergens*

To be able to quantify all abundant peanut proteins, including the allergens, from the different growth conditions, very comprehensive and replicable methods should be used for their extraction. A literature search resulted in a large number of different methods to extract the peanuts proteins that have been used in the past two decades. It is not only the extraction buffers and their pH values that differ vastly in the literature, but also the extraction protocol including de-fatting procedure, extraction times and temperatures, centrifugation speeds as well as the starting material (with or without seed coat). Some publications discuss the influence of buffers and their pH values as well as other experimental conditions on the amount of extracted proteins. They demonstrate that extraction of proteins is highly dependent on the extraction procedure and buffers (Kain et al. 2009, Kim et al. 2011, Poms et al. 2004, Sathe et al. 2009). However, there is no empirical study that compares the most commonly used extraction conditions and the extractability of allergens. The extraction method and solubility and conformation of the proteins is the basis for all subsequent measurements regarding the allergen content of peanuts grown in different conditions. It is therefore crucial to evaluate the most commonly used extraction methods quantitatively and qualitatively for their protein and allergen content before testing peanut samples from different growth conditions.

## *1.8.* *Research questions*

1. Does the extraction buffer composition and its pH value influence the extraction yield of crude protein and Ara h 1 and 2?

2. Does the extraction buffer composition and its pH value influence the outcome of subsequent methods of analysis, such as SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), ELISA (enzyme-linked immunosorbent assay) and Western blots?

3. Does the de-fatting reagent and extraction protocol influence the extraction yield of crude protein and Ara h 1 and 2?

4. Should seed coats be used when extracting allergens from peanuts?

5. Does the amount of Ara h 1 and 2 and other allergens change when the peanut plants are grown in elevated $CO_2$?

6. Is the amount of crude protein and Ara h 1and 2 and other allergens different when peanuts of the same variety are grown in different growing regions in Australia?

## *1.9. Thesis structure*

The first results chapter (Chapter 3) is divided in two parts: A) Effect of different buffers and their pH values on extraction yield of peanut proteins and allergens Ara h 1 and 2; B) Evaluation of other common peanut protein extraction parameters and determination of Ara h 1 and 2 content. Using the most suitable methods validated in Chapter 3, crude proteins and allergens Ara h 1 and 2 of peanuts were grown in different atmospheric $CO_2$ concentrations (Chapter 4) and in different growing regions in Australia (Chapter 5) and were compared quantitatively and qualitatively.

# Chapter 2

# Methods and general optimisations

## *2.1. Peanut samples*

All peanuts used in experiments reported in this thesis were runner peanuts of the variety "Walter", which were kindly provided by the PCA and stored at 4°C until use. Walter is one of many varieties of Spanish peanuts. This variety was chosen, because, it is a relatively small, compared to other Spanish size peanut kernels and ultra-early in maturing, which makes it ideal for greenhouse experiments, where space and time are limiting. Furthermore Walter peanuts are widely used as an important marketing trait of "Hi-Oleic" oil chemistry amd suitable for the confectionery and snack market. For Chapters 3 and 4, two batches of Walter peanuts, which were grown close to Kingaroy in Queensland, Australia, were obtained. The first batch was used for all experiments in Chapter 3, for which peanut flour was prepared by homogenising 100–120 peanuts and de-fatting with hexane (Chapter 3.8.3). For some experiments in Chapter 3B, 10 peanuts were pooled instead of using the prepared defatted peanut flour. In Chapter 4 the second batch of peanut kernels were used, which was covered with fungicide (Captan and Quntiozene) were planted and grown in greenhouses with ambient and elevated $CO_2$.

For Chapter 5, the Walter peanuts came from a variety evaluation trial performed by PCA from November 2009 to March 2010 in three peanut-growing regions in Queensland, Australia: two regions in South Queensland, one close to Kingaroy and the other around 250 km north-east of Kingaroy, close to Bundaberg, and a third region in North Queensland close to Kairi, around 1500 km north of Bundaberg (Table 2.1; Chapter 5.2). In Kingaroy two sites were set up at the Redvale and Taabinga Research Stations. In Bundaberg one site was located at the Bundaberg Research Station and another site at Russo Farms close to Childers, around 50 km south-west of Bundaberg. In Kairi only one site was set up at the Kairi Research Station (Figure 5.1). To obtain triplicate samples for each of the five locations, three plots were set up at each site in a randomised block design, each of which was approximately 5 m long by 2 rows and contained approximately 150 plants in total. The entire row was harvested at full maturity. After this the samples were dried and stored at safe moisture (~10% kernel moisture), until the peanuts from each plot were pooled by PCA and a subsample was allocated for the study reported in this chapter.

**Table 2.1 Locations of peanut cultivation and closest weather station**

Five locations across Queensland were used to grow peanuts. Some of the locations shared the same weather station. The three weather stations indicate the three regions used for growing peanuts.

| Label on map[1] | Location of plot | Postcode, State | Nearest weather station (ID number) |
|---|---|---|---|
| A | Redvale Research Station | 4610 QLD | Kingaroy Airport (040922) |
| B | Taabinga Research Station | | |
| C | Russo Farms, Childers | 4660 QLD | Bundaberg Airport (039170) |
| D | Bundaberg Research Station | 4670 QLD | |
| E | Kairi Research Station | 4872 QLD | Kairi Research Station (031034) |

[1] See map in chapter 5.2

## *2.2. Lipid removal*

Peanuts were pooled and homogenised with a mortar and pistil or an automatic coffee grinder. The homogenate was defatted tree times by adding at least 1:3 (w/v) n-hexane. The solution was vortexed or stirred and left in the fume hood until the defatted peanut flour precipitated for 10 – 60 min, depending on the quantity of peanut material. The lipid-containing supernatant was then discarded. After the third step the peanut flour was left overnight in the fume hood, so the rest of the hexane could evaporate. The defatted flour was then used immediately for peanut protein extraction or stored at 4°C using a plastic tube and parafilm until further use. To test different defatting reagents (Chapter 3.8.3.) diethyl ether was used instead of hexane. Each sample was prepared in triplicate.

## *2.3. Crude peanut protein extractions*

After homogenisation and defatting, exactly 45 mg of peanut flour was placed in a tube before adding 1350 µl of buffer (1:10 (v/v); Table 2.2). After vortexing for 30 s the proteins were extracted under constant agitation according to the conditions mentioned in the chapters:

- Chapter 3A: 1 h at 40°C with various buffers

- Chapter 3B: for various times and temperatures with 20 mM Tris (pH 8.5)

- Chapter 4 and 5: 30 min at 21°C with 20 mM Tris (pH 8.5)


After the extraction, the samples / supernatants were centrifuged 3x at 12,600 g for 2 min, while the pellets were discarded. Finally, the supernatant containing the proteins was vortexed, aliquoted and stored at –80°C. The extractions were done in triplicate with three identical but independently prepared buffers.

**Table 2.2 Composition and pH values of crude protein extraction buffers**

The listed buffers have been used to extract crude peanut proteins from defatted peanut flour.

PBS (Phosphate buffered saline); TBS (Tris buffered saline); DTT (Dithiothreitol); SDS (*sodium dodecyl sulphate)*; HCl (hydrochloric *acid*); NaOH (sodium hydroxide)

| Prepared buffer | Composition |
|---|---|
| 10 mM PBS (pH 2.1) | 130 mM sodium chloride NaCl<br>0.0045 mol potassium-di-hydrogen-orthophosphate (anhydrous) $KH_2PO_4$<br>0.0054 mol di-sodium hydrogen orthophosphate (anhydrous) $Na_2HPO_4$<br>adjust to pH with HCl |
| PBS (pH 6.7, 8.0) | 130 mM sodium chloride NaCl<br>1.7 mM potassium-di-hydrogen-orthophosphate (anhydrous) $KH_2PO_4$<br>10 mM di-sodium hydrogen orthophosphate (anhydrous) $Na_2HPO_4$<br>2.7 mM potassium chloride KCl<br>adjust to pH with HCl |
| Citrate (pH 4.5) | 50 mM citric acid $C_6H_8O_7$<br>adjust with HCl or NaOH to pH 4.5 |
| Urea | 6 M Urea |
| $H_2O$ | Milli-Q (ultrapure) water |
| TBS (pH 7.2 or 8.5) | 50 mM Tris (Trizma base; Invitrogen Life Technologies Co.)<br>150 mM NaCl<br>adjust pH with HCl |
| 20, 50 or 100 mM Tris (pH 7.5 or 8.5) | 20, 50 or 100 mM Tris<br>adjust pH with HCl |
| 50 mM Tris (pH 8.5) + DTT and/or SDS | 50 mM Tris<br>10 mM DTT and/or 2% SDS<br>adjust pH with HCl |
| 50 mM Tris (pH 8.5) + Urea | 50 mM Tris<br>6 M Urea<br>adjust with HCl to pH 8.5 |
| Ammonium bicarbonate (pH 7.8 or 8.5) | 100 mM ammonium bicarbonate $NH_4HCO_3$<br>adjust pH with HCl or NaOH |
| Sodium borate (pH 9.2) | 50 mM boric acid $H_3BO_4$<br>adjust with NaOH or HCl to pH 9.2 |
| Sodium carbonate (pH 10.6) | 50 mM sodium hydrogen carbonate $NaHCO_3$<br>adjust pH with NaOH |

## *2.4. Exhaustive extraction*

Exhausting extractions were done with the hexane-defatted peanut flour by extracting for 30 min at 21°C with 20 mM Tris or TBS (pH 8.5). Instead of discarding the pellet after the first centrifugation, it was re-used for another five extractions at 30 min extraction at 21°C with 1,350 µl buffer. Before the last extraction, 6 M urea was added to the buffers. The exhaustive extraction was done in triplicate.

## *2.5. Protein extraction from seed coats*

After removal from the peanut kernels, 45 mg seed coats were placed in a mortar. In one experiment the seeds coats were treated as peanut kernels, and homogenised for approx. 2

min, placed into a tube, 1,350 µl buffer - citrate (pH 4.5), urea (pH 6.7), 50 mM Tris (pH 8.5) or sodium borate (pH 9.2), was added and extracted for 30 min at 21˚C, before centrifuging three times at 12,600 g for 2 min.

In another experiment 1,350 µl of buffer was added to the seed coats in the mortars and the seed coats were homogenised vigorously for approx. 15 min using a pestle until the homogenate became a creamy liquid. In order to be able to transfer most of the liquid out of the mortar into a tube, 300 µl of buffer was added and the homogenate pipetted in a tube, so that the end volume was around 1,500 µl. The samples or supernatants were then centrifuged three times at 12,600 g for 2 min. In both experiments the supernatant was vortexed, aliquoted, and then stored at –80˚C, while the pellet was discarded. The seed coat extractions were done in duplicate.

## 2.6. *Storage and usage of protein samples*

All protein extracts were vortexted, aliquoted and stored at –80 ˚C. The samples were thawed and used for any subsequent experiments a maximum of four times. Tubes that were thawed at least once were either stored at –80 or –20˚C.

## 2.7. *Total protein quantification with 2D Quant kit*

Protein concentration was determined using the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden), which is based on the specific binding of copper ions to proteins. This method has the advantage that it precipitates the proteins and removes interfering compounds before the protein concentration is measured. The 2D Quant kit was used according to the manufacturers' instruction manual. Dilutions were generally done with a minimum pipetting volume of 10 µl to ensure reproducibility. In order to increase the accuracy, each standard, blank and sample was measured in duplicate. The compatibility of the different buffers in Chapter 3A was tested by running two standard curves, one without buffer, and one with the same amount of buffer as used in the samples. None of the buffers affected the protein measurement significantly (data not shown).

## 2.8. ELISA Ara h 1 and 2

The Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.) were used according to the manufacturers' instruction manual with the following additions: The Ara h 2 assay was preformed according to the protocol. Because the optical density was too low in the Ara h 1 ELISA assay, the streptavidin-peroxidase was diluted 1:200 instead of the in the instructions

suggested dilution of 1:1000. Each extraction sample (present in triplicate) was diluted in duplicate with a minimum pipetting volume of 10 µl to ensure reproducibility. In order to find the dilutions that showed an optical density in the linear part of the standard curve, various dilutions – 1:100 to 1: 2,500 for Ara h 1 and 1:100 to 1:40,000 for Ara h 2 – were tested prior to the actual measurements. To lower the pipetting error, the last dilution step was performed on microtiter mixing plates or the final dilution was added onto a microtiter mixing plate, before adding each dilution in duplicate onto the microtiter sample plates. Thus every triplicate extraction sample was present in two duplicate dilutions and measured in duplicate.

| Prepared buffer | Composition |
|---|---|
| Carbonate/Bicarbonate buffer (pH 9.6) | 15 mM sodium carbonate $Na_2CO_3$<br>35 mM sodium hydrogen carbonate $NaHCO_3$<br>adjust with NaOH or HCl to pH 9.6 |
| PBS (pH 7.4) | 130 mM sodium chloride NaCl<br>1.5 mM potassium-di-hydrogen-orthophosphate (anhydrous) $KH_2PO_4$<br>16 mM di-sodium hydrogen orthophosphate (anhydrous) $Na_2HPO_4$<br>2.7 mM potassium chloride KCl<br>adjust with KCl or HCl to pH 7.4 |
| PBS-T (pH 7.4) | PBS buffer<br>0.5% (v/v) Tween-20 |
| PBS-BSA (pH 7.4) | PBS buffer<br>1% (w/v) BSA |
| Citrate-phosphate buffer (pH 4.2) | 7.3 mM citric acid (anhydrous) $C_6H_8O_7$<br>10 mM di-sodium hydrogen orthophosphate (anhydrous) $Na_2HPO_4$<br>adjust with NaOH or HCl to pH 4.2 |

## *2.9. 1D- gel electrophoresis*

1D-SDS gel electrophoresis was performed under reducing conditions using 4–12% NuPAGE Novex Bis-Tris Mini gels (Invitrogen Life Technologies Co.) according to the manufacturers instructions. The samples were always prepared immediately prior to loading on the gel. 80 mM DTT was added to the 4x NuPAGE® LDS Sample Buffer (Invitrogen Life Technologies Co.). Then the protein samples were added to the appropriate amount of sample buffer with DTT the mixture denatured at 70˚C for 10 min prior to loading on the gel as stated in the instruction manual. To increase the reproducibility usually 10% more than the anticipated loading volume of 10 µl containing around 0.25 µg were prepared, so that exact amounts could be loaded onto the gels. In order to show differences of efficiency between the different extractions, the same volume was added to the lanes for each sample, instead of calculating equal amounts of protein. The gels were run at constant 100 V for exactly 55 min in an XCell SureLock Mini Cell (Invitrogen Life Technologies Co.). The gels were then stained with

either Sypro Ruby (Invitrogen Life Technologies Co.), Coomassie Brilliant Blue G-250 or R250 (Sigma-Aldrich Co.), depending on the desired sensitivity and subsequent applications.

## *2.10.  Optimised 2D-gel electrophoresis*

Crude protein was extracted from hexane-defatted peanut flour and the protein concentration determined. 25 µg (+ 10%) proteins were pipetted to 220 µl IPG (immobilized pH gradient) rehydration buffer, vortexed and centrifuged briefly at 22˚C. The rehydration buffer had been optimised prior to the experiments by testing various reducing agents, such as 65 mM DTT and/or 2 mM TBP (tributyl phosphine). DTT gave the best results and was used in all 2D-gels and 2D-Western blots presented (data not shown). The IPG strips (11 cm; pH 3-10 NL; Bio-Rad Laboratories Inc.) were rehydrated exactly with 200 µl containing 25 µg protein according to the manufacturers instructions. Great care was taken in rehydrating the whole sample equally across the strip, before adding 2 – 3 ml of mineral oil. The rehydration was done overnight at 16˚C.

To run the first dimension of the 2D-gel, according to the pI (isoelectric point) of the proteins, wet wicks were placed over the electrodes in the IEF (*isoelectric focusing*) transfer chamber, before placing the rehydrated IPG strips into the IEF cell (Bio-Rad Laboratories Inc.). The strips were covered with mineral oil and the IEF focussing was performed at 200 V for 3 h, 1000 V for 1h, 3000 V for 1 h and 5000 V for 90,000 – 100,000 kVh. This had been shown to give the best separation and focussing of protein spots on the gels compared to 30,000, 50,000 and 70,000 kVh (data not shown). Although it was mentioned in the manufacturers' instructions that the protein spots stay focussed when they run under a constant 100 V after the last focussing step and that IPG strips can be frozen at –80˚C after the focussing step before continuing the protocol, it was observed that this caused the protein spots to dissociate and to largely decrease the quality of the 2D-gels (data not shown). To avoid defocusing of the protein spots the IPG strips were removed while the voltage was still at 5000 V and used immediately for the second dimension.

For the second dimension the IEF strips were equilibrated for 15 min in SDS equilibration buffer with DTT, dabbed and incubated for 15 min in carbamidomethylation buffer under constant agitation. This had been shown to give better results than using the SDS equilibration buffer containing TBP rather than DTT (data not shown). The IEF strips were placed onto Criterion XT Bis-Tris gels (12%; 11 cm IPG + 1 well; Bio-Rad Laboratories Inc.) and covered with 500 µl heated MES (2-(N-morpholino) ethanesulfonic acid buffered saline)

heated Agarose (95% for 15 min to become fluid). After placing the gels in the Criterion cell (Bio-Rad Laboratories Inc.), MES buffer (Bio-Rad Laboratories Inc.) and protein marker (P7703S; 10–250 kDa; New England BioLabs Inc.) was added. The second dimension was run at 180 V for 55 min. The gels were then stained with Sypro Ruby (Invitrogen Life Technologies Co.), which is visible only upon scanning in a fluorescence imager, and Coomassie Brilliant Blue G-250 (Sigma-Aldrich Co.), or used for Western blots. The gels were scanned using the Typhoon FLA 9000 scanner and software (GE Healthcare Life Sciences; General Electric Company).

| Prepared buffer | Composition |
|---|---|
| IEF rehydration buffer (pH 9.6) | 5 M urea<br>2 M thiourea<br>2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)<br>2% (w/v) sulfobetaine 3-10 (SB 3-10; Bio-Rad Laboratories Inc.)<br>40 mM Tris (pH 8.8)<br>1% (v/v) Carrier Ampholytes (SB 3-10 Bio-Rad Laboratories Inc.)<br>0.05% (w/v) Bromophenol blue<br>65 mM DTT (add just before needed) |
| SDS equilibration buffer (pH 8.8) | 375 mM Tris (pH 8.8)<br>6 M urea<br>20% (v/v) glycerol<br>2% (w/v) SDS<br>2.5% (v/v) acrylamide solution<br>0.5% DTT |
| Carbamidomethylation buffer (pH 8.8) | 375 mM Tris (pH 8.8)<br>6 M Urea<br>20% (v/v) glycerol<br>2% (w/v) SDS<br>2.5% (v/v) acrylamide solution<br>2.5% iodacetamide |
| MES-agarose | MES running buffer (Bio-Rad Laboratories Inc.)<br>0.5% agarose<br>Bromophenol blue |

## 2.11.  Protein gel staining methods

Depending on the necessary sensitivity and subsequent applications various protein gel-staining methods were used.

### 2.11.1. Sypro Ruby

Sypro Ruby (Invitrogen Life Technologies Co.) is a fluorescent stain with a sensitivity of 1–2 ng protein per spot. Because of its high sensitivity and because it is invisible to the naked eye it was typically used on all 2D-gels and 1D-gels with very low protein abundances (e.g. protein extracts from seed coat), prior to the less sensitive but visible (and therefore useful for spot picking prior to mass spectrometry) Coomassie Brilliant Blue G-250 stain. The protein gels were placed in trays with fixing solution for 20 − 60 min. After discarding the fixing solution, 100 ml Sypro Ruby (Invitrogen Life Technologies Co.) was added and incubated

overnight under constant agitation. The Sypro Ruby solution was re-used a maximum of three times. After adding destain solution for 1 – 6 h the gels were equilibrated in Milli-Q water for 10 min and scanned using a Typhoon FLA 9000 scanner (GE Healthcare Life Sciences; General Electric Company). The gels were scanned at the Sypro Ruby fluorescence setting at a resolution of 100 μm and PMT voltage of 450 – 650, using the Typhoon FLA 9000 software (GE Healthcare Life Sciences; General Electric Company). The protein gels were then typically stained with Coomassie Brilliant Blue G-250 (Sigma-Aldrich Co.) before scanning them again and the images edited using Image Quant software (GE Healthcare Life Sciences; General Electric Company; version TL 7.0). The analysis of all 2D-gels was performed using Progenesis software (Nonlinear Dynamics Ltd.).

| Prepared buffer | Composition |
|---|---|
| Fix/destain solution | 10% (v/v) methanol<br>7% (v/v) acetic acid |

## 2.11.2. Coomassie Brilliant Blue G-250

Coomassie Brilliant Blue G-250 (Sigma-Aldrich Co.) is a reversible staining solution with a sensitivity of 500 ng per protein spot and ideal for subsequent spot picking procedures for mass spectrometry.

| Prepared buffer | Composition |
|---|---|
| Coomassie Brilliant Blue<br>G-250 staining solution | 0.1% (w/v) Brillant Blue R (Sigma-Aldrich Co.)<br>5% (v/v) acetic acid<br>25% (v/v) methanol (add just before use) |

After washing the protein gels in Milli-Q water for 10 min, they were incubated in 50–100 ml Coomassie Brilliant Blue G-250 staining solution (Sigma-Aldrich Co.) overnight. Destaining with Milli-Q water was then performed very briefly to remove excess Coomassie stain particles on the gels before the gels were scanned on a GS-800 calibrated densitometer using the PD Quest software (Bio-Rad Laboratories Inc.). The filter was set on red, transmissive and the resolution was typically 42.3 μm. Images were either taken using the Typhoon FLA 9000 software (GE Healthcare Life Sciences; General Electric Company) or a G:Box using the GeneSnap software (Synoptics Ltd.)

## 2.11.3. Coomassie Brilliant Blue R-250

Coomassie Brilliant Blue R-250 (Sigma-Aldrich Co.) is an irreversible protein stain with a sensitivity of 100 ng per protein spot and was used for a fast staining of protein gels. After washing the protein gels in Milli-Q water for 10 min, 50 – 100 ml of Coomassie Brilliant Blue

R-250 staining solution (Sigma-Aldrich Co.) were given to the protein gels and heated in the microwave for approx. 1 min just until the staining solution started to boil. The staining solution was replaced by destain solution and also heated for 30 s – 1 min in an microwave. The protein gels were then incubated in destain solution for another few minutes under constant agitation until the desired background was achived. The gels were finally washed with Milli-Q water for 10 min and pictures taken in a G:Box using the GeneSnap software (Synoptics Ltd.)

| Prepared buffer | Composition |
|---|---|
| Coomassie Brilliant Blue R-250 staining solution | 0.1% (w/v) Brillant Blue R-250 (Sigma-Aldrich Co.)<br>10% (v/v) acetic acid<br>40% (v/v) methanol (add just before use) |
| Destain solution | 10% (v/v) ethanol<br>7.5% (v/v) acetic acid |

## *2.12. Storage of protein gels*

The gels were sealed in plastic foil with 1% acetic acid and stored at room temperature until further use; i.e. for identification of protein spots via mass spectrometry.

## *2.13. Progenesis software for 2D gel analysis*

The 2D-gels were analysed using the Progenesis SameSpot software (Nonlinear Dynamics Ltd.) according to the manufacturers instructions. To overcome gel-to-gel variation and to allow statistical analyses, all 2D-gels were analysed in three or more biological replicates per treatment.

## *2.14. 2D-DIGE*

The crude peanut protein extracts were prepared with 20 mM Tris at 21°C for 30 min and the 2D-DIGE (two-dimensional difference gel electrophoresis) was performed according to the manufacturer's instructions (GE Healthcare Life Sciences; General Electric Company). Earlier, it had been shown repeatedly that only around ~10% of crude proteins could be recovered after using the 2D-Clean up kit (data not shown). To change the buffer, but avoid losing protein in the 2D Clean up kit, 625 µg protein (24 – 38 µl sample) samples were concentrated using a speedy vac (Eppendorf AG) and resuspended in DIGE buffer to a concentration of 5 mg/ml (as stated in the manufacturer's instructions for the 2D-DIGE kit). The molarity of Tris in the concentrated sample was so low compared to the resuspension buffer that it could be neglected.

For the peanut samples in Chapter 4, eight biological replicates for each treatment were used: ten peanuts each from eight different plants grown at ambient $CO_2$ (four from Greenhouse 1 and four from Greenhouse 3) and ten peanuts each from eight different plants grown at elevated $CO_2$ (all from Greenhouse 2, because Greenhouse 4 could not be used as a replicate; see Chapter 4). For the peanuts in Chapter 5, all three replicates for each of the five locations were used for the 2D-DIGE. To avoid bias towards upon labelling with CyDye; i.e. in case CyDye 3 or 5 might have a higher affinity for the proteins, half of the biological replicates were labelled with CyDye 3 and the other half with CyDye 5. All samples for 2D-DIGE in Chapters 3 and 4, were mixed in equal volumes and labelled with CyDye2. Equal volumes of 2x sample buffer were then added to the labelled protein samples. Finally, 8.3 µg of a CyDy3- and CyDye5-labelled sample were combined with 8.3 µg of mixed standard to a total protein quantity of 25 µg and the standard procedure for 2D-gel electrophoresis followed. The gels were kept in the dark during the procedure.

The 2D-DIGE gels were scanned using the 2D-DIGE setting on the Typhoon FLA 9000 scanner and software (GE Healthcare Life Sciences; General Electric Company) with different PMT (photomultiplier tube) voltages at a resolution of 100 µm and edited using Image Quant software (GE Healthcare Life Sciences; General Electric Company). They were then stained as usual with Sypro Ruby (Invitrogen Life Technologies Co.) and Coomassie Brilliant Blue G-250 (Sigma-Aldrich Co.) and analysed using the Progenesis software (Nonlinear Dynamics Ltd.).

| Prepared buffer | Composition |
|---|---|
| DIGE buffer (pH 8.8) | 10 mM Tris (pH 8.8)<br>7 M urea<br>2 M thiourea<br>4% CHAPS<br><br>2% DTT |
| 2x sample buffer (pH 8.8) | 10 mM Tris (pH 8.8)<br>7 M urea<br>2 M thiourea<br>4% CHAPS<br><br>1% (v/v) carrier ampholytes (SB 3-10 Bio-Rad Laboratories Inc<br>2% DTT |

## 2.15. Acquisition of peanut allergic human sera for Western blotting

The ethics application for the collection of human blood for this thesis was reviewed and approved by the Human Ethics Research Committee (Human Research) at Macquarie

University under the title "The impact of global climate change on the allergenicity of plants" (HE30OCT2009-D00168). Peanut allergic human blood was kindly collected by Dr. Med. Karl Baumgart in his private allergy practice or the Douglass Hanly Moir Pathology. According to the ethics approval, the recruitment of peanut allergic patients was done through information sheets in the waiting room of Dr. Baumgart's private practice, or through private contacts. The ethics approval allowed the recruitment of children and adults. Despite great efforts to recruit volunteers, only six patients with history of peanut allergy participated. Only one patient showed a high signal on a 1D Western blot and was tested positive for Ara h 1, 2 and 3 in Phadia ImmunoCap 1000 (Thermo Fisher Scientific Inc.) serum no. 2, Appendix 1), a blood test used to identify substances to which a person is allergic. This serum and a serum that had been tested negative (as a control) were used throughout the thesis for Western blotting.

## 2.16. Western blots

After running a SDS-PAGE, the gel and nitrocellulose membrane (0.45 µm, Bio-Rad Laboratories Inc.) were equilibrated in transfer buffer for 15 min and placed into a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories Inc.) according to the manufacturer's instruction manual. The transfer cell was surrounded with ice and placed on a magnetic stirrer. The transfer was done in a coolroom at 4°C at 100 mA for 2 h at 100 V and the 10 V over night, using a programmable power pack (Bio-Rad Laboratories Inc.).

In 1D-gels the presence of all protein bands from the prestained molecular weight marker (P7709; New England BioLabs Inc.) on the membrane was a first indicator for a successful transfer. To make sure the blotting of the peanut proteins was successful, the membrane was washed three times for 5 min in Milli-Q water and stained with Ponceau S until the protein spots were clearly visible on the membrane. The membrane was then rinsed with RO (reverse osmosis) water to remove background and a picture taken using the G:Box and GeneSnap software (Synoptics Ltd.). If applicable the blot was cut into pieces for different treatments. The Ponceau S stain was then removed by washing the membrane with TBS (pH 7.4). The membrane was then incubated in blocking buffer for 2.5 – 7 h and washed four times for 10 min in TBS-T.

To minimise the amount of serum (the sera used are described in Appendix 1) used as the primary antibody, but maximise its exposure to the membrane, the membranes were sealed on three sides in foil, before adding 1.5 – 3 ml serum (diluted 1:10 in antibody buffer) and sealing the foils entirely. The membranes were incubated under constant agitation at 4°C over

night. After washing the membranes four times for 10 min in TBS-T they were incubated with the secondary antibody (Anti-Human IgE (ε-chain specific)−Peroxidase antibody produced in goat; cat no. A9667; Sigma-Aldrich Co.; diluted 1:20000 with antibody buffer) for a maximum of 2 h at 21˚C. The membrane was washed four times for 10 min with TBS-T.

The detection of allergens on the membrane was conducted with the BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit; cat no. 11520709001; Roche Diagnostics) according to the manufacturer's instructions. To minimise the amount of substrate and assure that the whole membrane was exposed equally to it, 2 ml of substrate was constantly applied to the membrane using a pipette for exactly 2 min. The luminescence was usually captured ten times at 30 s-intervals (the exposure time was additive; e.g. two times 30 s exposure means 1 min exposure) in a G:Box using the GeneSnap software (Synoptics Ltd.). The increasing exposure times gave a time scale for the development of spot signals and allowed from strong (early) signals to be distinguished from weak (late) signals. The detection of all major protein spots was usually complete after 1 min. Longer exposure times sometimes resulted in additional very faint signals, which were also visible on negative controls and regarded as background. Therefore the Western blot images in this thesis are mostly taken at 30 s to 1 min exposure. Finally images were taken with visible light, to be able to identify the spot or band pattern in relation to the membrane and the molecular weight marker (P7703; New England BioLabs Inc.), which had not been destained after incubation with Ponceau S.

| Prepared buffer | Composition |
|---|---|
| Transfer buffer | 10 mM Tris<br>100 mM glycine<br>10% (v/v) methanol |
| Ponceau S stain | 1% (v/v) acetic acid<br>0.05% (w/v) Ponceau S (Sigma-Aldrich Co.) |
| TBS | 20 mM Tris base<br>500 mM NaCl<br>adjust to pH 7.5 |
| TBS-T | TBS buffer<br>0.1% (v/v) Tween 20 |
| Blocking buffer | TBS buffer<br>0.2% (w/v) BSA (*Bovine serum albumin*) |
| Antibody buffer | TBS-T buffer<br>0.2% (w/v) BSA |

## *2.17. Mass spectrometry*

The protein bands or spots on 1- and 2D-gels were identified with mass spectrometry (nanoLC electrospray ionisation MS-MS). After their preparation the samples were provided to Ms. Victoria Clarke at University of Sydney for analysis in a Q-Star Elite mass spectrometer (Applied Biosystems). The peptide masses were then used for identification using a custom-prepared peanut allergen database and the public NCBInr database, using Mascot (Matrix Science Ltd.).

### *2.17.1. Trypsin digest and preparation of samples for mass spectrometry*

Protein gels that were stained with Coomassie Brilliant Blue G-250 were equilibrated three times in Milli-Q water for 5 min each. The gels were placed on a clean foil and approx. $1cm^2$ cut out and finely chopped using clean razor blades. The chopped plugs were then placed in microtiter plates and destained immediately by pipetting up and down 100 µl of 50% acetonitrile (ACN). The ACN solution was replaced a few times until the gel plugs were completely destained. The ACN was removed with a pipette and the residual solution evaporated after a few minutes at 21˚C. Then 15 µl of 25 mM ammonium bicarbonate containing 15 ng/µl trypsin solution (pH 7.8) was added to the gel plugs and incubated for 1 h at 4˚C. Excess solution was then removed with a pipette and 25 mM ammonium bicarbonate (pH 7.8) added and incubated at 37˚C overnight.

After approx. 12 – 16h h, 1 – 3 µl 2.5% formic acid was added to each sample, so that the pH value in was < 4.0. PerfectPure C18 tips (Eppendorf AG) were wetted by aspirating/expelling 10 µl of wetting solution (0.1% (v/v) formic acid, 50% (v/v) ACN) and equilibrated using 10 µl of equilibration solution (0.1% (v/v) formic acid). The samples were bound to the columns by aspirating/expelling ten times and washed three times with 10 µl of washing solution (0.1% (v/v) formic acid). The samples were eluted with 10 µl elution solution (0.1% formic acid, 70% ACN). After adding 50 µl equilibration solution (0.1% (v/v) formic acid) to increase the volume for the mass spectrometry, the samples were centrifuged at 10,000 g for 10 min at 16˚C and 55 µl pipetted into the low-binding microtiter plates for the mass spectrometry.

### *2.17.2. Mass spectrometry*

The prepared samples were provided to Ms. Victoria Clarke for nanoLC electrospray ionisation MS-MS in a Q-Star Elite mass spectrometer (Applied Biosystems). Samples were separated by liquid chromatography (LC) and analysed on an Analyst QSTAR ESI-QUAD-TOF mass spectrometer (Applied Biosystems). The LC component consisted of a 150 mm

separation column (Zorbax Column 300SB C18) driven by Agilent Technologies 1100 series nano/capillary liquid chromatography system. Peptides were separated over 1 h (5% Acetonitrile – 40% Acetonitrile) and eluted directly into the mass spectrometer. The mass spectrometer was run in positive ion mode and MS scans ran over a range of m/z 400-1500 and at four spectra s$^{-1}$. Precursor ions were selected for auto MS/MS at an absolute threshold of 500 and a relative threshold of 0.01, with a maximum of three precursors per cycle. Precursor charge-state selection and preference was set to 2+ and then 3+ and precursors selected by charge then abundance.

## 2.18.  Protein identification

### 2.18.1. Creation of a peanut allergen database

To be able to identify the peanut allergens more effectively, a database containing a list of non-redundant allergen sequences was created and used for the protein identification. The list contained allergen sequences for Ara h 1 – Ara h 11, which were acquired in FASTA format from the allergome webpage (www.allergome.org, December, 2011), which links to uniprot (www.uniprot.org December, 2011) and NCBI (National Center for Biotechnology Information) Entrez (http://www.ncbi.nlm.nih.gov/, December 2011) and sorted according to the respective allergen. An NCBI Entrez search to identify a first sequence was preformed. This sequence was then used in BLAST to find similar sequences in NCBInr database that may be considered isoforms of the allergen. For all allergens, except Ara h 8 and 10, proteins with 90–100 % amino acid identity to the original sequence were included in the peanut allergen database. For Ara h 8 proteins with sequence identities 56–100% were used, while for Ara h 10  isoforms, the identity range was 50–100%. The sequences were aligned with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/, February 2012) to ensure there were no redundant sequences and list of sequences (Appendix 3) provided to Dr. Ben Crossett, who made the database available through ProteinPilot (Applied Biosystems, version 2.01).

### 2.18.2. Mascot search

The peptide masses obtained from the nanoLC electrospray ionisation MS-MS data, were opened with Analyst QS 2.0 software, and exported to Mascot (Matrix Science), where first the created peanut allergen database was searched for peptide matches. Only peptides that could not be matched to the peanut allergen database were then compared to NCBInr (NCBI non redundant) database in order to identify peanut proteins that are not allergens. The parameters for the Mascot search specified Trypsin as the cleavage enzyme, allowed up to one missed cleavage, and identified peptides with variable modifications such as carbamidomethylation (C) and methionine oxidation (M). The peptide charge was set to 2+

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and 3+ (monoisotopic) and the MS/MS tolerances to 0.2 Da. When using the NCBInr database the taxonomy was set to viridiplantae ("green plants").

The protein matches were regarded as positive if the Mascot search marked them as significant and if at least two different peptides could be matched. Appendix 2 contains a table with all sequenced protein spots on 1- and 2D-gels, their MOWSE (molecular weight search) score, the number of queries matched, the sequence coverage, a list of the matched peptides, the molecular mass and pI values of the protein, their NCBI accession number and protein name, and the name of the respective allergen. Percent coverage was calculated based on coverage of protein sequence by matched peptide queries. For each protein spot only the first listed most significant match with the highest MOWSE score was listed and the number of present isoforms were given. If more two allergens were present in one protein spot, both were listed and the number of their isoforms given.

## 2.19. Glycosylation analysis

After running 2D-gels using the standard procedure, gel plugs corresponding to the protein spots were provided in duplicate to Dr. Morten Andersen and Prof. Nicki Packer at Macquarie University for glycosylation analysis. The spots were analysed for N-glycan content by treating the excised gel plugs separately with deglycosylation enzyme (PNGase F) and analysing the released fraction using LC-MS/MS.

## 2.20. Peanut planting and optimised greenhouse work

After running a trial in growing peanuts in the greenhouses (Chapter 4.2.1.) the experimental procedure was improved by (1) adjusting the solar radiation in Greenhouse 2, which had more sunlight than the other greenhouses in the trial and improved monitoring of environmental conditions; (2) using soil with higher drainage capacity and better-draining pots; (3) increasing the number of plants to raise the sample size; (4) monitoring of moisture in the lower part of the pots and watering appropriately and supplying nitrogen fertilizer earlier, and (5) improving the procedure to increase yield of mature seeds upon harvesting.

### 2.20.1. Improved monitoring of environmental conditions

The position and orientation of the used greenhouses is described in detail in Chapter 4.2.2.1. Prior to the experiment the solar radiation in the greenhouses was adjusted by fitting a shade cloth to the left wall of Greenhouse 2 to adjust for the additional sun this greenhouse otherwise received in the afternoons. Greenhouses 1 and 2 were fitted with independent (from the controllers) $CO_2$ data loggers (Telaire, CA), which were mounted to the wall of the

greenhouses, close to the peanut plants and calibrated by the operator before the experiment. In Greenhouses 3 and 4 the $CO_2$ concentration was checked manually using independent sensors throughout the experiment and was always very close to the desired values. All greenhouses were fitted with independent (from the controllers) temperature data loggers (Telaire, CA), which were placed in the shade in order to measure the temperature accurately (without any interference from solar radiation). In Greenhouses 1–3 these measured temperature every 5 min during the entire experiment. Due to technical issues beyond the author's control the temperature data in Greenhouse 4 were recorded only from 101 DAP (days after planting) onwards. Additionally, smaller HOBO data loggers (Onset Computer Cooperation) were placed on the table with the peanut plants to measure the relative humidity and light intensity to which the plants were exposed. During the entire growth period (03.07 – 13.08.2010) the solar radiation and relative humidity (and temperature) were measured every 3 min in each greenhouse.

The HOBO data loggers also contained temperature sensors but, because they had to be placed in full sunlight to allow measurement of light intensity, the values were elevated due to solar radiation and were therefore not "true" temperature measurements. In order to estimate the temperature in Greenhouse 4 in the first 101 DAP, the measurements recorded by the HOBO loggers had to be calibrated with the measurements of the temperature sensors in the shade. For that purpose the temperature data measured after 101 DAP with both temperature sensors in Greenhouse 4 were compared. Unfortunately, the differences between the temperature measurements in the shade and sunlight were so large that it was not possible to calibrate the temperature data with the HOBO data loggers and extrapolate the calibration to the first 101 DAP.

### 2.20.2. Improved drainage capacity of the soil

Good quality loamy soil was obtained from Kangaroo Valley (NSW) and mixed with 50% river sand and 10 g of Osmocote (Scotts Australia Pty Ltd) to ensure a high drainage capacity and efficient long-term fertilisation. To enhance drainage the soil was placed in the pots without newspaper.

### 2.20.3. Increased sample size

Due to the limited availability of space in the greenhouse facilities, only 10 pots and five smaller backup pots could be fitted per greenhouse. Three seeds were planted approximately 8 cm apart into each pot (including the backup pots). The pots were kept at ambient $CO_2$ to ensure consistent germination conditions and were moved immediately to the respective

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greenhouse after the first shoots were visible in all pots (3 DAP). Based on the presumption that the peanut plants in each pot were competing for space and nutrients and to ensure that the sample size was as large as possible, only the two tallest, greenest and most vigorous plants per pot were left to grow at 10 DAP.

### 2.20.4. Improved watering and fertilisation

The peanut plants were watered every day until germination at 4 DAP and then approximately every 3 – 4 d with 800 ml of water per pot. The exact frequency of watering was dependent on the weather (more often on very sunny days). The short-term fertiliser Aquasol (Yates, Australia) was added once a week and, instead of nodulating the peanut plants, soluble nitrogen (urea) was applied every few weeks.

### 2.20.5. Improved harvesting

Mature and immature peanuts can appear very similar. Therefore indicators for immaturity and maturity of peanut pods were defined before harvesting, in order to remove bias from the peanuts plants grown in ambient and elevated $CO_2$.

Indicators for maturity of peanuts:
- The peanut pods are dry and have distinct "wrinkles".
- The peg to which the pods are attached is dry.
- The pods fall off the stem easily and so de-attached mature pods can be found in the soil.
- In some cases the fibres of the pegs are too strongly attached to the pod, so the pod will not fall off even if mature. However, if the peg is extremely fibrous and can be broken off, the pods are likely to be mature.
- The seed and the pod are not attached to each other anymore (can only be determined after opening a pod).
- There is a brown-red seed coat around the peanut seeds.

Indicators for immaturity:
- Although the peanuts might look like mature peanuts they are still immature if they are smaller than other mature pods and appear to be moist.
- The peg to which the pods are attached still appears moist and alive.
- The pods cannot be broken off the peg easily, because the stem is still moist and alive.
- The seeds are still attached to the pod (in this case it is very hard to open the pods).
- The brown-red seed coat is not developed completely and appears to have "wrinkles".

It was observed that not all seeds in one plant were mature at the same time. To maximise yield of mature peanut kernels, the plants were checked every 1 – 2 weeks for mature peanut pods. The dry soil (before watering) was combed very gently around 10 – 13 cm through the soil by hand without destroying the roots. Mature pods were easy to identify because they were split loose from the pegs or came off very easily. Some peanut pods were opened during each harvesting session, to make sure the peanut kernels were mature. Pegs with attached immature pegs were covered with soil and the plants watered and left to grow. Using this gentle technique, the peanut pods were harvested on three different dates, presuming the plants would not be disturbed and more seeds would reach maturity. The seeds were cleaned of soil (by gentle brushing with water) and stored at 4˚C until further use.

### 2.20.6. Analysis of data for environmental conditions in the greenhouses

The data for the environmental conditions (atmospheric $CO_2$ concentration, temperature, solar radiation and relative humidity) in the greenhouses were selected and analysed using the Matlab software (The Mathworks Inc., 2009). The $CO_2$ and temperature data were already calibrated and were separated into day (06:00–18:00) and night (18:00–06:00) measurements by using the time series as an indicator. Data for each greenhouse were plotted and checked for any obvious errors. Some data points, as recorded by the data loggers, represented values of zero $CO_2$ and temperature (always around the time when the data were retrieved from the loggers) and were filtered from the data (using the "nan" function (not a number) on Matlab) before calculating any subsequent functions. The averages and standard deviations of the day and night $CO_2$ concentration were calculated and listed in Table 4.1, Chapter 4.2.2.2.. Individual date and weekly running averages for day and night were plotted over time, red being the day and blue the night data (Chapter 4.2.2.2.).

Matlab software (The Mathworks Inc., 2009) was used to calibrate the data that were measured with the HOBO data loggers using the regression formula to an averaged standard. The measurements for solar radiation and humidity were taken at exactly the same time, making it possible to separate the data into day and night by filtering. This was done by defining night as a light intensity of 0 lux and day as a light intensity of >0 lux. The individual data for each greenhouse were plotted over time to identify potential false measurements, which were filtered from the dataset if applicable using the "nan" function in Matlab, before using the data for any subsequent calculations or plots. Filtering was conducted on relative humidity and light intensity measurements, which was to exclude false values obtained around the time when data were retrieved from the loggers (eight times throughout the

experiment). Additionally, humidity measurements from 19.7. – 14.8.10 for greenhouse data showed an error and were removed from the data. The averages and standard deviations for total light intensity and relative humidity, as well as day (06:00–18:00) and night (18:00–06:00) temperatures and $CO_2$ concentrations, were calculated for the entire period of plant growth (Table 4.1).

Graphical time-series were generated with Matlab software (The Mathworks Inc., 2009) by plotting weekly running averages of day and night temperature, relative humidity and solar radiation over time and comparing them to weekly running averages of average values among the greenhouses (Chapter 4.2.2.3.). The quantification of differences between the conditions in the greenhouses were illustrated by plotting anomalies, which were obtained by subtracting the average values from the data points in the individual greenhouses (Chapter 4.2.2.3.; Figure 4.3). To plot the daylight intensity in all greenhouses, values below 400 lx (the light intensity between sunset and sunrise) were ignored (using the "nan" function in Matlab) before calculating and plotting running weekly averages (Chapter 4.2.2.3.; Figure 4.3).

## 2.21. *Obtaining and plotting environmental conditions for the three peanut growing regions in Australia*

In order to obtain data on the climatic conditions of the various peanut-growing regions, the closest weather stations to the peanut field sites were identified. The climatic conditions in Taabinga and Redvale were monitored by the weather station at Kingaroy Airport (1.5 km from Taabinga and 7.5 km from Redvale). Childers and Bundaberg were monitored by the weather station at Bundaberg Airport (37 km from Childers and 15 km from Bundaberg research station). For Kairi the weather was monitored directly at the research station. Average monthly maximum and minimum temperatures, total monthly rainfall and monthly average solar exposure measured in the weather stations were obtained from the Bureau of Meteorology, Australia (www.bom.gov.au, January 2012) and plotted using Excel (Microsoft Office, 2008). The monthly averages of the conditions measured in each station as well as the overall averages were plotted against time. Anomalies were plotted so that the individual averages could be compared easily with the overall average of the conditions (Chapter 5.3.1.; Figure 5.2).

## *2.22. Statistical analysis*

Graphics and statistical analysis was performed using the EcStat software and "student t-test" function in Excel (Microsoft Office, 2008).

## *2.23. Image editing*

Images were edited using Image Quant software (Image Quant TL 7.0; GE Healthcare Life Sciences; General Electric Company; version TL 7.0), Gimp (GNU Image Maniplation Program, version 2.6.8) and PowerPoint (Microsoft Office, 2008).

# Chapter 3A

# Effect of different buffers and their pH values on extraction yield of peanut proteins and allergens Ara h 1 and 2

## *3.1. Summary*

The impact of different buffers and pH values on the extraction yield of crude peanut protein and Ara h 1 and 2 content was investigated. Hexane-defatted peanut flour was used for extraction with a range of buffers with pH values ranging from 2.1 to 10.6. The crude protein was quantified and Ara h 1 and 2 content determined using ELISA kits. Some protein extracts obtained with various buffers were used for Western blotting. Furthermore, a preliminary glycosylation analysis was conducted for some proteins spots on 2D gels. The buffer composition and pH value had an effect on the crude protein and Ara h 1 and 2 extraction efficiency. When extracts were analysed by ELISA, buffers conferring denaturating conditions increased the detection of Ara h 1, and buffers conferring reducing conditions influenced the detection of Ara h 2. Protein extracts prepared with TBS (pH 7.2) resulted in more spots on 2D-Western blots compared to extracts prepared with PBS (pH 8.0) and Tris (pH 8.5).

## *3.2. Peanut samples*

The peanuts used in this chapter were runner peanuts (variety "Walter"), which were grown close to Kingaroy in Queensland and acquired from PCA. A single batch of peanut flour, which was prepared by homogenising around 100 g of peanuts and de-fatting with hexane, was used throughout these experiments (Chapter 2.1).

## *3.3.* *Results*

### *3.3.1.* *Quantification of crude protein and Ara h 1 and 2 in peanut protein extracts prepared with different buffers*

The peanut protein extraction efficiency of various buffers with pH values ranging from 2.1 to 10.6, including their influence on crude protein and Ara h 1 and 2 extraction, was tested (Table 3.1 and Figure 3.1). The extractions of hexane-defatted peanut flour were performed for 1 h at 40˚C, using the standard procedure (Chapter 2.2. –2.3.). All extractions were performed in triplicate using replicate preparations of the respective buffer with the same batch of defatted peanut flour (Chapter 2.1). The 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) was used to quantify the extracted total protein and commercial Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.) were used to quantify Ara h 1 and 2. All protein amounts reported are an average of the triplicates (± standard deviation). Subsequently the protein extracts were run on 1D-gel electrophoresis and used for Western blotting with serum from a single human allergic to peanuts (Chapter 2.16 and Appendix 2).

Two distinct factors influenced the extraction yield of crude peanut protein: the pH and the type of buffer. Buffers with higher pH values (pH 8.5 and above) resulted in a high crude peanut protein extraction efficiency (Table 3.1 and Figure 3.1). This pH effect was observed with a range of buffers: PBS at pH 8.0 resulted in 32% more crude protein extraction than at pH 6.7, while TBS and Tris at pH 8.5 had 27% and 45% more crude protein, respectively, when compared to the same buffers at pH 7.2. Moreover, ammonium bicarbonate at pH 8.5 had 23% more crude protein when compared to pH 7.8. The extraction efficiency with PBS (pH 2.1) (149 ± 12 mg) was statistically similar ($p < 0.5$) to that with PBS (pH 6.7) (167 ± 24 mg). The lowest extraction efficiency obtained was with citrate (pH 4.5), for which only 45 ± 4.8 mg per g peanut flour was extracted (92.5% less than with 50 mM Tris). Citrate was not tested at other pH values, and so it is not entirely clear if the low extraction efficiency is due to the pH value or the buffer composition.

Chemically different buffers with the same pH resulted in great differences in extraction efficiency. At pH 7.2, Tris had on average 36% more crude protein than TBS at the same pH, and 25% more protein than ammonium bicarbonate at pH 7.8. At pH 8.5, these effects were even stronger, with Tris being 52% more efficient in extracting crude proteins than TBS and

46% more efficient than ammonium bicarbonate. Pure Milli-Q water appeared to extract the proteins 20–50% more efficiently than any buffer in the pH range 6.7–8.0 (except urea), including ammonium bicarbonate and TBS at pH 8.5. The pH value of the water changed from 7.2 to pH 6.5 immediately after adding the peanut flour and stayed the same even after 1 h of extraction at 40˚C. The extraction "buffer" therefore had effectively a pH value of 6.5.

**Table 3.1 Efficiency of buffers differing in pH and ionic components on the extraction of total protein and allergens Ara h 1 and Ara h 2 from defatted peanut flour**

The crude protein and Ara h 1 and 2 yield per g of defatted peanut flour after extraction with 20 different buffers. Each extraction derived from a pool of 10 seeds per treatment and was performed in triplicate. The amount of crude protein and Ara h 1 and 2 was measured in triplicate with the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and commercial Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.; after diluting significantly with ELISA buffer), respectively. All values are averages (± standard deviation) based on mg per g of defatted peanut flour.

| Buffer | pH value | Crude protein [mg] | Ara h 1 [mg] | Ara h 2 [mg] |
|---|---|---|---|---|
| PBS | 2.1 | 149 ± 13 | 561 ± 266 | 55 ± 4.0 |
| Citrate | 4.5 | 45.5 ± 4.8 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| PBS | 6.7 | 167 ± 24 | 3.9 ± 1.2 | 14 ± 0.8 |
| Urea | 6.7 | 606 ± 5.3 | 84 ± 13 | 15 ± 0.6 |
| $H_2O$ | 7.2 | 401 ± 29 | 2.4 ± 0.4 | 16 ± 2.3 |
| TBS | 7.2 | 202 ± 1.7 | 5.2 ± 0.6 | 17 ± 3.0 |
| 50 mM Tris | 7.2 | 313 ± 25 | 2.8 ± 0.3 | 14 ± 1.9 |
| Ammonium bicarbonate | 7.8 | 235 ± 25 | 7.1 ± 2.8 | 39 ± 8.0 |
| PBS | 8.0 | 246 ± 42 | – | 41 ± 10 |
| Ammonium bicarbonate | 8.5 | 305 ± 6.3 | 13 ± 1.8 | 71 ± 23 |
| TBS | 8.5 | 275 ± 12 | 2.5 ± 0.7 | 89 ± 28 |
| 20 mM Tris | 8.5 | 586 ± 44 | 1.7 ± 1.5 | 66 ± 24 |
| 50 mM Tris | 8.5 | 570 ± 44 | 1.0 ± 0.6 | 32 ± 4.2 |
| 100 mM Tris | 8.5 | 601 ± 55 | 3.3 ± 0.5 | 23 ± 3.1 |
| 50 mM Tris + 10 mM DTT | 8.5 | 566 ± 20 | 0.5 ± 0.2 | 77 ± 1.0 |
| 50 mM Tris + SDS | 8.5 | 604 ± 25 | 65 ± 3.9 | 34 ± 1.8 |
| 50 mM Tris + DTT / SDS | 8.5 | 589 ± 48 | 73 ± 1.5 | 8.6 ± 0.1 |
| 50 mM Tris + Urea | 8.5 | 536 ± 36 | 64 ± 2.4 | 41 ± 10 |
| Sodium borate | 9.2 | 555 ± 7.4 | 3.6 ± 0.4 | 44 ± 4.1 |
| Sodium carbonate | 10.6 | 530 ± 13 | 23 ± 6.6 | 32 ± 1.8 |
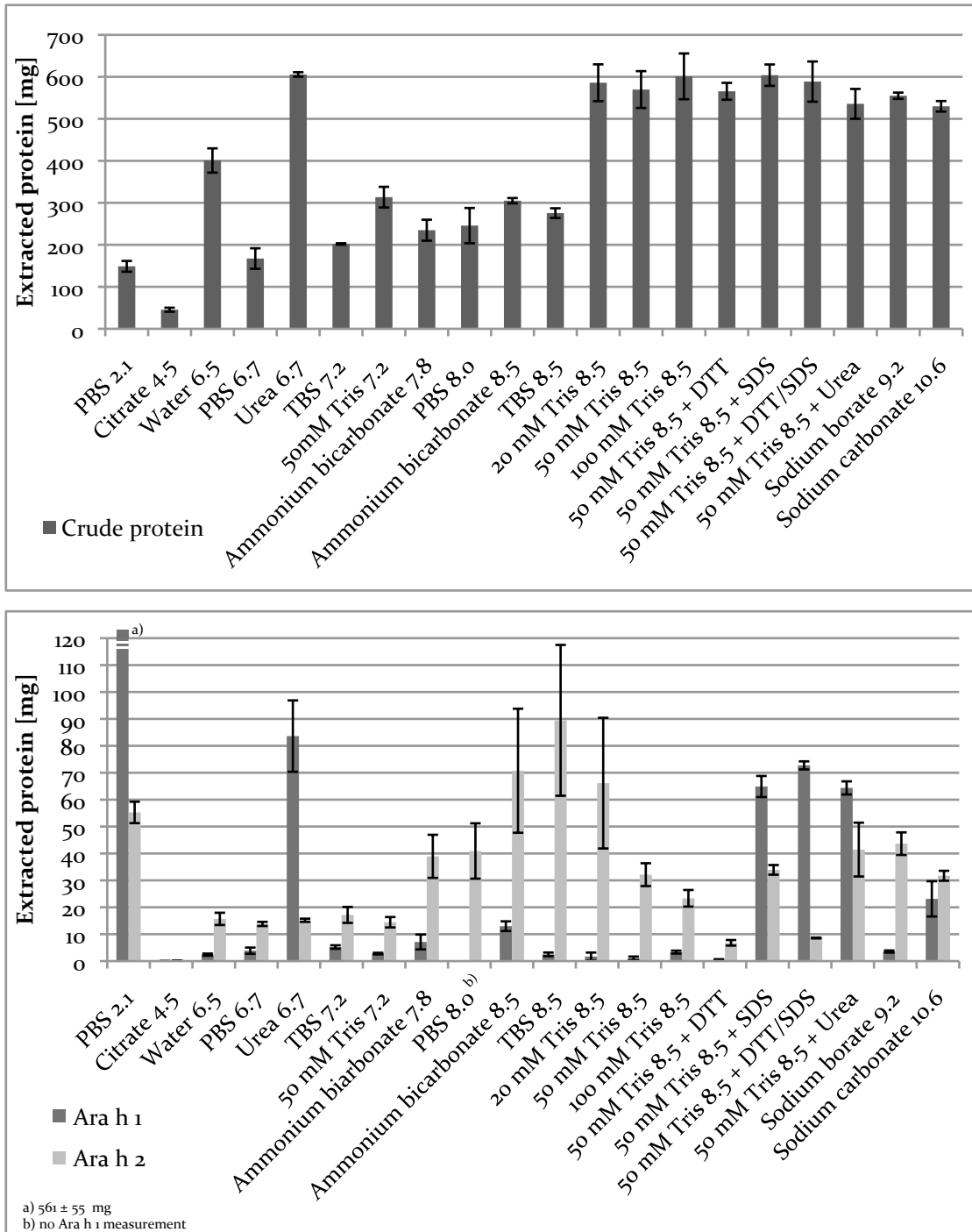
**Figure 3.1 Effect of various buffers and their pH values on peanut protein and allergen extraction efficiency**

Crude protein and Ara h 1 and 2 yield per g of defatted peanut flour were determined after extraction with 20 different buffers (shown with pH increasing from left to right). Each extraction derived from a pool of 10 seeds per treatment and was performed in triplicate. The amounts of crude protein and Ara h 1 and 2 were measured in triplicate with the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and commercial Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.), respectively. All values are averages (± standard deviation) based on mg per g of peanut flour.

Buffers with a pH of 8.5 or higher appeared to give the highest protein extraction efficiency with values around 530 – 606 mg protein per g peanut flour. The buffer 20 mM Tris (pH 8.5) resulted in around 585 mg per g peanut flour. Neither higher pH value, nor increased Tris molarities (50 or 100 mM), nor the addition of SDS, DTT and/or urea, which are used to denature and solubilise proteins, were able to increase this efficiency significantly. Furthermore, pure urea resulted in a similar extraction yield to that of Tris. In conclusion, 20 mM Tris (pH 8.5) gave maximal extraction efficiency for aqueous-soluble crude protein of peanut under non-denaturing conditions and with the lowest molarity, which is expected to result in the least interference with subsequent experiments.

The extraction yield of Ara h 1 was dependent on the buffer composition (Figure 3.1). The concentration of Ara h 1 was apparently very high in the extracts prepared with PBS (pH 2.1), and the values exceeded the amount of crude protein in the sample, indicating that the low pH value affects the detection of Ara h 1. This was the case for all three replicates and two duplicate ELISA tests. Since the extracts were diluted 1:800 with ELISA buffer prior to the ELISA measurements (Chapter 2.8.), it seems highly unlikely that the extraction buffers themselves interfered with the ELISA measurements. Indeed the dilution of 1:10 should be sufficient to prevent interference with the assay (personal communication with technical support of Indoor Biotechnologies Inc.). Unexpectedly, the Ara h 1 concentration also appeared to be higher when urea or SDS was added to the buffers, but not DTT, which might also be due to effects on the detection of Ara h 1 with the ELISA kit. It is likely that the data indicate a fragmentation of Ara h 1 by PBS (pH 2.1), urea and SDS, which resulted in the exposure of additional epitopes for the primary antibody in the Ara h 1 ELISA kit (Indoor Biotechnologies, Inc.) and a higher detectability of Ara h 1, rather than a higher extraction efficiency (Chapter 3.4.2.). Interestingly, the Ara h 1 concentration was also higher in protein extracts made with sodium carbonate (pH 10.6) (23 ± 6.6 mg) compared to all other buffers (except PBS (pH 2.1) and those containing SDS or urea), which contained 0.0 – 13 mg Ara h 1. This might point to a higher extraction efficiancy with this buffer, but it is shown later that it is more likely that it is due to the formation of aggregates and a higher detectability of Ara h 1 in the ELISA assay (Chapter 3.3.2 and 3.4.2.). Notably, 20 mM Tris (pH 8.5) had a very low extraction yield of 1.7 (± 1.5 mg) Ara h 1. As shown in the following chapters (Chapter 4 and 5) the extraction yield of Ara h 1 with 20 mM Tris was between 10.7 and 16.5 mg per g of peanut flour for extractions performed for 30 min at 21°C (rather than for 1 h at 40°C, the conditions used in this experiment). Although the extraction yield of ammonium bicarbonate

(pH 7.8) (7.1 ± 2.8 mg) was 45% less that ammonium bicarbonate (pH 8.5) (13 ± 1.8 mg), there was no further evidence for the dependence of Ara h 1 yield on the pH value.

The extraction yield of Ara h 2 depended mainly on the pH value of the extraction buffer, the molarity of some of the buffer components and the presence of DTT and urea (Figure 3.1). The quantity of extracted Ara h 2 was highest with TBS (pH 8.5) for which an average yield of 89 mg ± 28 mg Ara h 2 per g peanut flour was obtained. Protein extracts prepared with buffers at the same pH as TBS (pH 8.5), such as ammonium bicarbonate (pH 8.5) and 20 mM Tris (pH 8.5) appeared to contain only slightly lower (not significantly different) Ara h 2 quantities. Furthermore, it was observed that the molarity of Tris had a significant impact on the extraction efficiency of Ara h 2. Accordingly, extraction with 20 mM Tris (pH 8.5) resulted on average in 51% more Ara h 2 compared to 50 mM Tris, and 65% more Ara h 2 than 100 mM Tris at the same pH. The addition of DTT to Tris buffer greatly decreased the extraction efficiency of Ara h 2, while SDS and urea did not have a significant effect. Extraction efficiency was also high (55 mg Ara h 2 per g peanut flour) when PBS (pH 2.1) was used. As found for the crude protein, the type of buffer appeared to have no substantial influence on Ara h 2 extraction efficiency.

The highest amount of crude protein and Ara h 2 under was achieved with buffers around pH 8.5, including TBS and 20 mM Tris. The extraction yield of Ara h 2 was not statistically significant different in these buffers, but the crude protein extraction yield was significantly higher with 20 mM Tris, which had an efficiency of almost 100%. This buffer was therefore likely to contain a range of other allergens, making it a good candidate for extractions in the following chapters. Although the Ara h 1 extraction yield was low with 20 mM Tris (pH 8.5), it was shown in later experiments that an extraction at room temperature for 30 min could increase the Ara h 1 extraction efficiency substantially (Chapter 3.8.2).

### 3.3.2. 1D-gels, mass spectrometry and Western blots of peanut protein extracts prepared with various buffers

The qualitative analysis with 1D-gel electrophoresis (Figure 3.2) was consistent with the quantitative results, with band intensities being equally dependent on the overall protein concentration in the samples. In accordance with this, the citrate extract contained very few visible bands due to the low protein concentration in the samples. With the exception of the acidic buffer PBS (pH 2.1), which had a distinct band pattern, with more protein bands below

20 kDa, all extracts made with buffers with pH values 6.7 and higher appeared to display the same major bands on 1D gels.
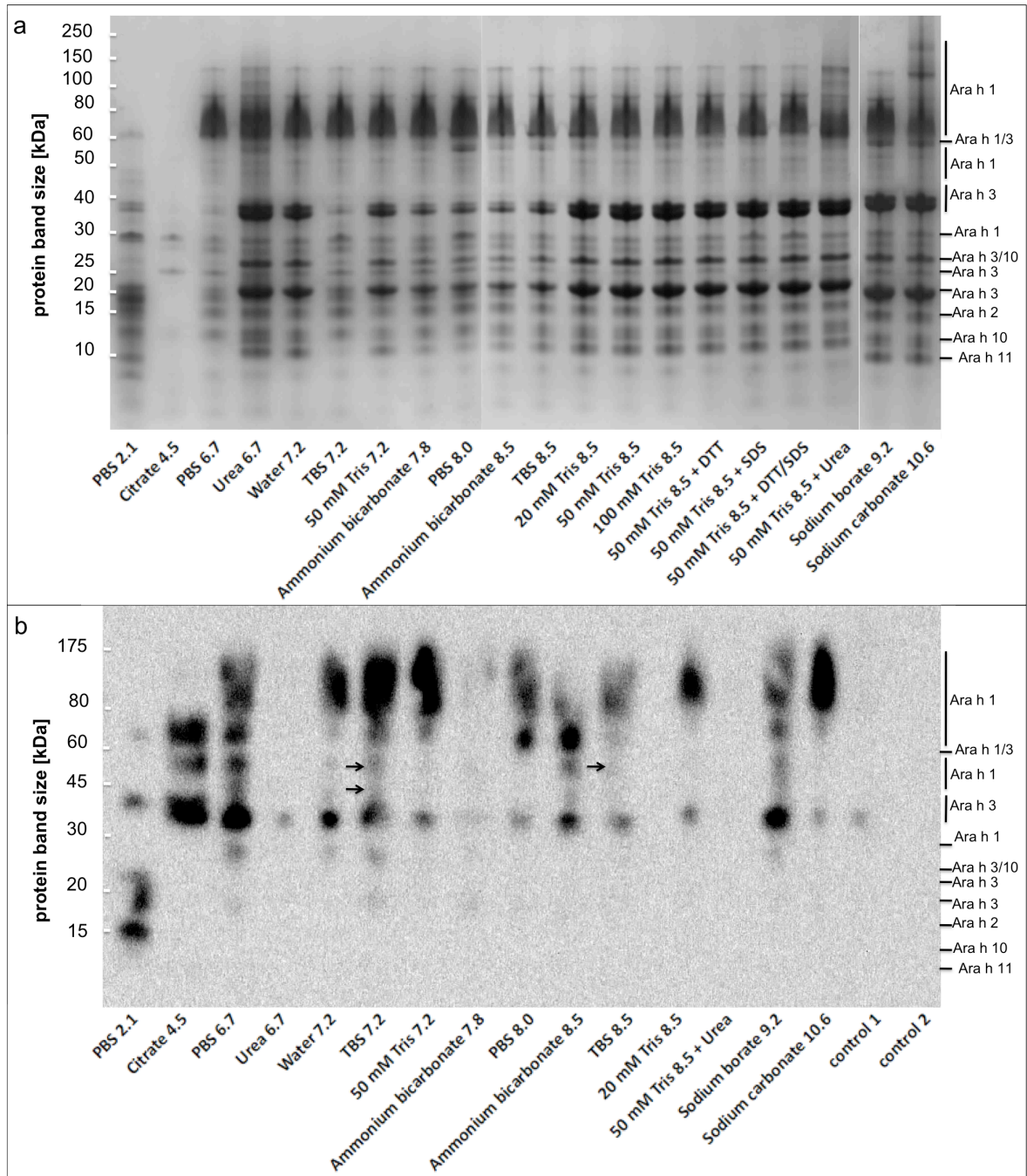


**Figure 3.2 Effect of various buffers and their pH values on protein separation on 1D-gel electrophoresis and allergen detection with Western blotting**

(a) Equal volumes of the protein extracts obtained with the 20 different buffers were run on SDS-PAGE (Invitrogen, Carlsbad, CA). (b) A subset of extractions was used for Western blotting with serum from a human individual allergic to peanut. Negative control 1 was performed with non-allergic serum while negative control 2 was performed in the absence of primary antibody. The protein identifications derive from mass spectrometry and are described below (further details in Figure 3.3).

The protein bands were identified, by via mass spectrometry. It was revealed that Ara h 1 was abundant in a range of protein bands on the 1D-gel, ranging from 140–32, including a ~64 kDa band (Figure 3.3; details Appendix 2). The extracts prepared with PBS (pH 2.1) lacked these high molecular weight Ara h 1 bands. Instead PBS 2.1 extracts contained Ara h 1 in a smeary band with lower molecular weight, which indicated that Ara h 1 might be fragmented in the acidic extraction buffer. Ara h 3 was present in protein bands between 60–22 kDa, also suggesting its fragmentation. Furthermore, two protein bands at around 28 and 14 kDa contained Ara h 10 and a protein band at around 12 kDa Ara h 11. Surprisingly, Ara h 2 was found in only one protein band and Ara h 6 could not be detected on the 1D-gel (see Chapter 3.4.7.).
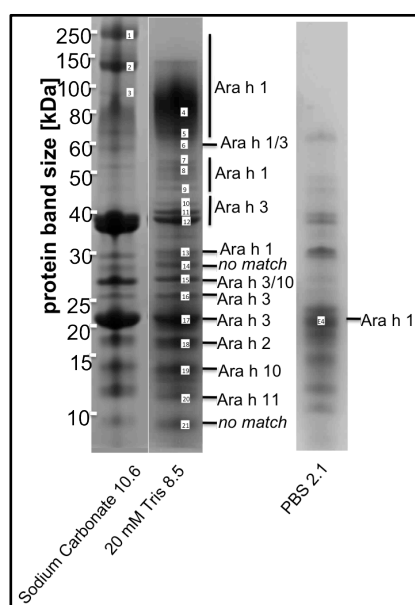


**Figure 3.3 Identities of proteins on 1D-gels via mass spectrometry**

The high molecular weight bands in the protein extracts prepared with sodium carbonate were used for mass spectrometry, while protein bands lower than 90 kDa were picked from 20 mM Tris gels. One protein band was taken from a gel prepared with PBS (2.1). For the identification an in-house peanut allergen database was used. Appendix 2 contains detailed results for the corresponding protein spots.

The 1D-gel patterns obtained with sodium carbonate extracts (Figure 3.2) contained an additional band at 230 kDa, which was not present in any other extracts. It was identified as Ara h 1 with mass spectrometry (Figure 3.3; details in Appendix 2). In a later experiment it was observed that the intensity of this band was dependent on the extraction temperature. At 4°C the band was absent but the band intensity steadily increased as temperatures of 21, 40 and 60°C were compared (data not shown). Although this band was identified as being Ara h 1 with mass spectrometry it appeared negative on a Western blot with peanut allergic serum (not shown).

Despite the relatively poor quality of the Western blot in Figure 3.2 (this Western blot could not be repeated due to a shortage of peanut allergic serum), it was observed that the extraction buffer strongly influenced the protein band pattern on the Western blot with a serum from an individual allergic to peanut (Appendix 1). However, there did not seem to be a correlation between increasing pH and band pattern intensity. Most peanut protein extracts resulted in protein bands between 28 and 100 kDa; nevertheless there were big differences between extracts in the presence of individual bands and their intensities. For example, extracts made with TBS had two additional bands at 42 and 50 kDa (see arrows in Figure 3.2) when compared with the band pattern with Tris. In accordance with the previous results, PBS (pH 2.1) had a distinct pattern, with bands of 15 and 23 kDa being unique and 18 kDa having a particularly strong intensity. Finally, only a very weak signal at 40 kDa signal was obtained when pure urea was used for extraction or when urea was added to Tris buffer. This suggests that urea interferes with recognition of allergens by IgE antibodies in the patient's serum.

### 3.3.3. *Maximal extraction efficiency: exhaustive extraction with Tris and TBS*

Experiments were conducted to test whether a single extraction was sufficient for extracting most of the protein from peanut flour or whether the extraction process should be repeated. Proteins were extracted from the same peanut flour six times for 30 min using the standard procedure (Chapter 2.4). The denaturant urea was added to the buffers before the sixth extraction in order to (potentially) enhance the extraction process. The protein concentration and Ara h 1 and 2 abundance were determined using the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.), respectively.

The first extract with Tris (pH 8.5) contained 85% of all total extracted protein (622 ± 18 mg) and 82% of all extracted Ara h 1 (13.4 ± 1.6 mg; Figure 3.4). All major bands were present on a 1D-gel (Figure 3.5). In the following extractions a rapid (but gradual) decrease in extraction efficiency of all protein bands was observed. Only an additional 11% of crude protein (87.4 ± 7.7 mg) and 16% of Ara h 1 were extracted in the second extraction, which accounts for a subset of less intensive protein bands that were already present in the first extraction. These included a 70 kDa protein band, a double band at around 39 and 41 kDa, a 28 and a 18 kDa band.
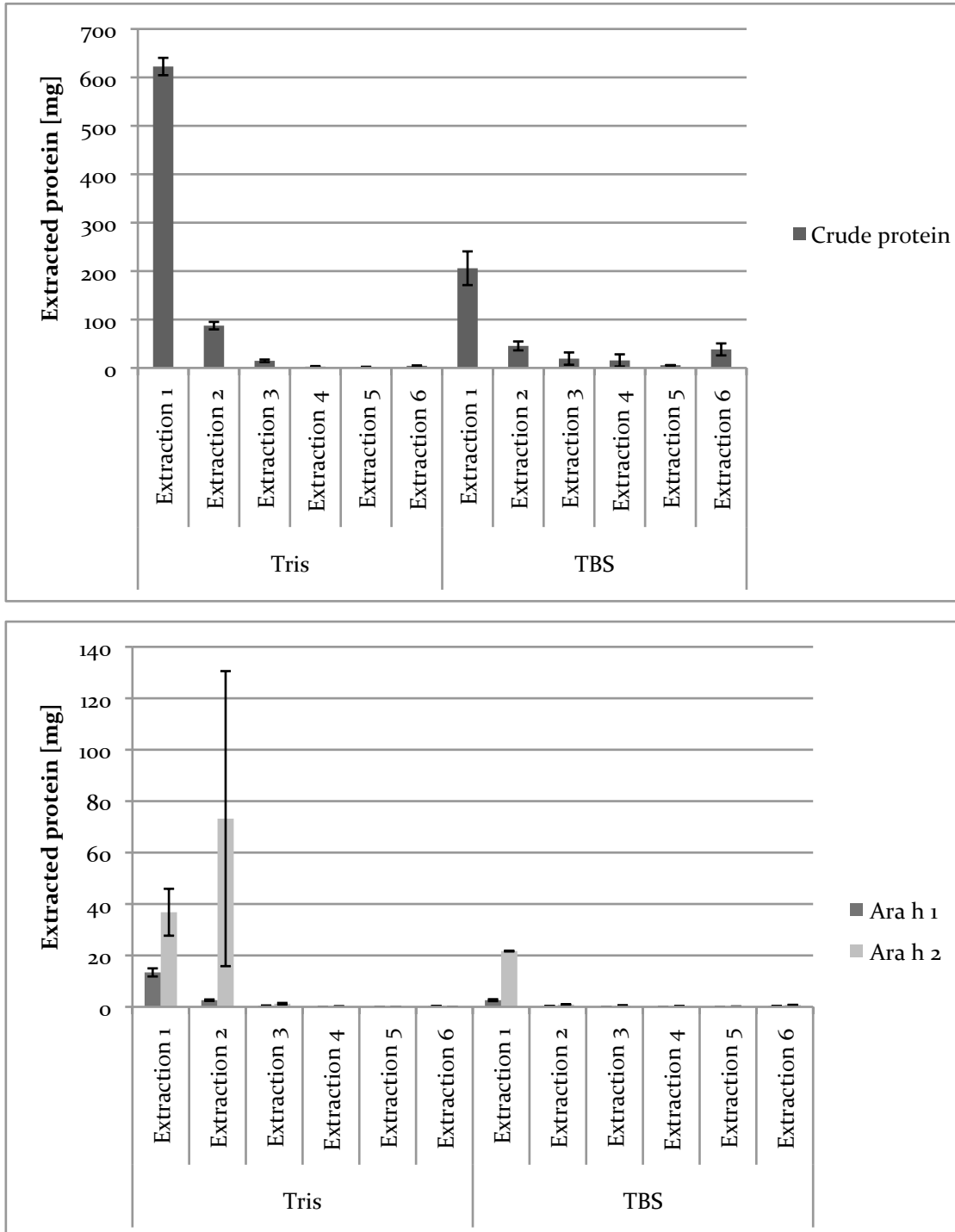
**Figure 3.4 Protein and Ara h 1 and 2 content after exhaustive extraction with Tris (pH 8.5) and TBS (pH 8.5) per g of defatted peanut flour**

Protein yield per g of defatted peanut flour after extracting the pellet one to six times with TBS and Tris. In the sixth extraction urea was added to the buffers. The 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.) were used for the measurements. All values are averages (± standards deviations) based on mg per g of peanut flour.

Mass spectrometry results suggested these bands are mostly fragments of Ara h 3, but also one faint Ara h 1 band is visible (Figure 3.5; Appendix 2). The same protein bands were also visible in the third extraction, after which the amount of additional protein eventually dropped

below 0.5 µg, which is the threshold for visualisation with Coomassie Brilliant Blue G-250. The remaining 4% of protein was extracted in the last four extraction steps. Urea did not enhance the extraction process, suggesting that very little or no protein remained in the pellet after the fifth extraction. Due to the high error in Ara h 2 measurements in the second extraction, it was unclear whether most Ara h 2 was extracted in the first or second extraction; however, a gradual decrease of Ara h 2 in the following extractions was observed, with only 1.2 mg (± 0.4 mg) detected in extraction 3. The protein bands at ~20 and 17 kDa, are likely to contain Ara h 2 and the ~20 kDa band also contains Ara h 3 (Chapter 3 and Appendix 2). Notably the intensity of the protein bands also decreased gradually in the subsequent extractions. The ~20 kDa band intensity only decreased slowly in the TBS extractions.
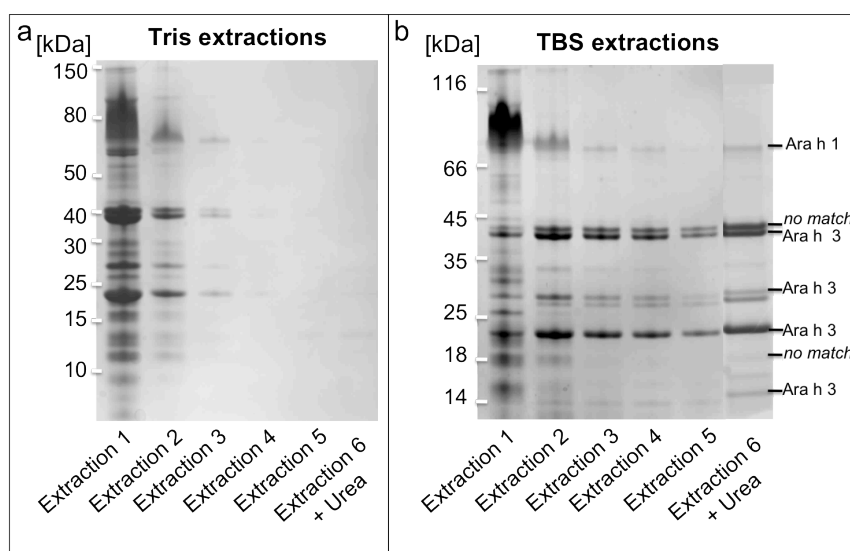


**Figure 3.5 1D-gel electrophoresis after exhaustive extraction with Tris (pH 8.5) and TBS (pH 8.5)**

After extracting the pellet one to six times for 30 min with Tris and TBS (in the sixth extraction urea was added to the buffers), 1D-gel electrophoresis was performed using precast 4–12% Bis-Tris gel (NuPAGE, Invitrogen, Carlsbad, CA) with equal volume run on each lane for Tris protein extracts in (a) and TBS protein extracts in (b). Gel electrophoresis was performed with all three extracts per treatment and each extraction was derived from a pool of 10 seeds per plant. Only one sample is shown in the figure. The protein identities derive from mass spectrometry experiments (details in Appendix 2).

With TBS (pH 8.5), 62% (206 ± 35 mg) of all extracted protein could be detected in the first extraction (Figure 3.4). In this fraction the Ara h 1 and 2 extractability was highest, but less than in Tris. This fraction also contained the majority of individual proteins, whose abundances decreased gradually in the subsequent extraction fractions (Figure 3.4). However, some bands such as a double band at 39 and 41 kDa, a 28, 27 and a 18 kDa band appeared to be more abundant in the second extraction, where an additional 13% of protein (45.5 ± 9.21 mg) could be detected. These proteins exhibited only a slight gradual decrease in the following extractions. This result was consistent in all three replicates. The remaining 12% of

protein was extracted in the last three extraction steps. An additional 11.6% was solubilised by urea in the last extraction step. The same result was obtained with a mixture of SDS and DTT (data not shown).

Notably, the sum of all extracted proteins reached a total of 733 ± 31 mg with Tris (pH 8.5) and only 330 ± 82 mg in total when TBS (pH 8.5) was used, even though urea was used in the last extraction. Thus the total protein yield with TBS was only 45% of that with Tris (pH 8.5). This also applies to the extracted total amounts of Ara h 1 and 2 with TBS, which were, respectively, 80% and 78% lower than the total amount extracted with Tris.

In conclusion, the first peanut protein extraction is highly efficient for most proteins when Tris (pH 8.5) is used as extraction buffer. The combination of the first and the second extraction would increase the overall quantity of the peanut proteins in the extracts but add only a small amount of proteins already present and at the same time reduce the overall concentration of the proteins in the extracts. Because of this and out of convenience, only one extraction per pellet was performed when extracting proteins from peanuts in work reported in the following chapters of this thesis.

### 3.3.4. 2D gels and western blots peanut protein extracts prepared with various buffers

In order to investigate the effect on extraction efficiency of some of the most common peanut extraction buffers more closely, 2D-gel electrophoresis and Western blots with peanut allergic serum were performed with peanut protein extracts made with TBS (pH 7.2), PBS (pH 8.0) and Tris (pH 8.5).

Subsequent staining with the highly sensitive fluorescent dye Sypro Ruby (Invitrogen, Carlsbad, CA) and analysis with Progenesis software revealed 45 protein spots that differed significantly ($p < 0.05$) in spot volume between the peanut protein extracts tested (Figure 3.6 significantly different spots are marked). These spots represented proteins with sizes 12–100 kDa and pI values of 4.7–9.3. The protein spots with significantly different volumes were distributed throughout the gel and included both major and minor protein spots. The identity of most protein spots was determined via mass spectrometry and detailed results are listed in Appendix 2.

Although differences in spot volumes were significant, most spots differed only quantitatively in volume rather than in presence/absence (a complete list of spots with details of those with

significantly different spot volumes is given in Appendix 4). To ensure the differences in spot volume presented here were not due to gel-to-gel variation but rather to real differences in extraction, selection criteria were established after evaluating the gel-to-gel variation on 2D-gel and 2D-DIGE data in Chapters 4 and 5. Hence, the only protein spots selected were those that differed significantly with p-values below 0.01 and had average normalised volume differences of >12,000 (in order to overcome gel-to gel variation the Progenesis software (Nonlinear Dynamics Inc.) uses this normalisation method, in which each spot on a gel image is expressed relative to the total volume of all spots on that image). Since this tends to produce extremely small values, the ratio can be multiplied by a user-defined scaling factor, usually 100), a minimum of 3-fold difference in mean abundance of protein spots and clearly visible different spot volumes (on detailed pictures) were regarded as different between the TBS, PBS and Tris extracts (highlighted in yellow in Figure 3.6 and listed in Table 3.2; detailed images and graphics with normalised volumes are given in Appendix 4).
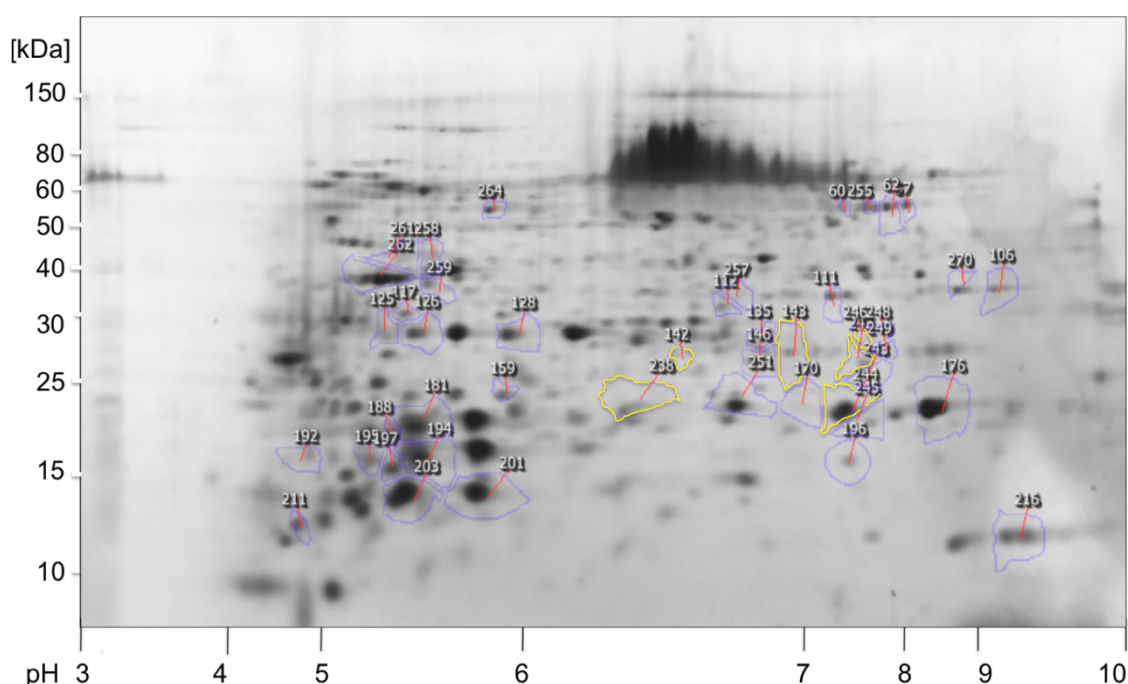


**Figure 3.6  2D-gel of crude peanut proteins extracted with TBS, PBS and Tris**
Protein spots with significantly different spot volumes (p < 0.05) between crude proteins samples extracted with TBS (pH 7.2), PBS (pH 8.0) and Tris (pH 8.5) are highlighted on the 2D-reference gel shown. Spots highlighted in yellow differed significantly with p-values below 0.01, average normalised volume differences of >12,000, a minimum of 3-fold difference in mean abundance of protein spots and clearly visible different spot volumes (see Table 3.2). 2D-gels were performed with three replicates. IPG strips (pH 3–10 non-linear, Bio-Rad Laboratories Inc.) were rehydrated with 25 µg protein and focussed up to 100,000 kVh with a maximum of 5,000 on a Protean IEF cell (Bio-Rad Laboratories Inc.). The second dimension was run on 12% Bis-Tris gels (12% Criterion XT Bis-Tris precast gels, Bio-Rad Laboratories Inc). The 2D-gels were stained with Sypro Ruby and scanned using a Typhoon FLA 9000 laser scanner (GE Healthcare, General Electric Company, 2011) before analysis using the Progenesis Same Spot software (Nonlinear Dynamics Ltd.). Protein identities of most protein spots, including not marked spots, are listed in detail in Appendix 2.

**Table 3.2 Protein spots with significantly different spot volumes on 2D-gels of peanut extracts prepared with TBS, PBS and Tris**

Identity, significance, magnitude and averaged normalised volumes of protein spots with significantly different spot volumes on 2D-gels of protein extracts prepared with different extraction buffers: TBS, PBS and Tris. Of the 45 significantly different spots, only protein spots that differed significantly with p-values below 0.01, average normalised volume differences of >12,000, a minimum of 3-fold difference in mean abundance of protein spots and clearly visible different spot volumes (on detailed pictures; Appendix 4) are listed. Spot number, p-values and averaged normalised volumes of protein spots were acquired using Progenesis software (Nonlinear Dynamics Ltd.). The molecular weight and pI values were estimated from the 2D-gels. The spot number corresponds to Figure 3.6, while the MS spot number refers to the protein identities determined by mass spectrometry listed in detail in Appendix 2.

| | Identification MS/MS | | | | | | | | Average normalised Volumes | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Spot no. | NCBI acc. no.[a] | Isoallergen or other protein | Fragment (full length) [kDa] | MS spot no.[b] | Anova [p] | Fold[c] | pI | MW [kDa] | TBS | PBS | Tris |
| 142 | gi\|9864777 | Ara h 3 | Yes (60) | 71 | 0.000 | 4.7 | 6.5 | 26 | 4421 | 8766 | 2.1e+004 |
| 143 | gi\|9864777 | Ara h 3 | Yes (60) | 47 | 0.000 | 4.2 | 7 | 26 | 2.4e+004 | 4.2e+004 | 9.9e+004 |
| 238 | gi\|37789212 | Ara h 3 | Yes (61) | 39 | 0.000 | 4.2 | 6.4 | 22 | 4.3e+004 | 7.5e+004 | 1.8e+005 |
| 242 | gi\|9864777 | Ara h 3 | Yes (60) | 79 | 0.000 | 4.5 | 7.6 | 27 | 9608 | 2.0e+004 | 4.3e+004 |
| 244 | gi\|9864777 | Ara h 3 | Yes (60) | 41 | 0.000 | 3.1 | 7.4 | 22 | 8.9e+004 | 1.5e+005 | 2.8e+005 |
| 246 | gi\|9864777 | Ara h 3 | Yes (60) | 83 | 0.000 | 3.1 | 7.6 | 28 | 3874 | 6429 | 1.6e+004 |

[a] The first hit with highest score is given (Appendix 2)
[b] Spot number in mass spectrometry table (Appendix 2)
[c] Fold difference in mean abundance of protein spots

Markedly, all spots with significantly different volumes were identified as fragments of Ara h 3, with high scores for a range of Ara h 3 isoforms (in Table 3.2; details are in Appendix 2). Four of the protein spots had greater volumes in the Tris samples compared to the TBS and PBS samples. Protein spot 238 had a greater volume in the PBS extracts and spot 244 had a greater volume in the TBS extracts.

Following 2D-gel electrophoresis, Western blotting was performed to test the recognition of allergens in peanut protein extracts prepared with TBS, PBS and Tris by IgE antibodies in the serum of a peanut allergic patient (Appendix 2). The limited amount of available serum allowed only two biological replicates to be run for each of the extracts. Surprisingly, the Western blots performed with proteins extracted with TBS (pH 7.2) resulted in more protein spots than PBS (pH 8.0) and Tris (pH 8.5) extracts (Figure 3.7). This unexpected finding was nevertheless consistent between the duplicates.
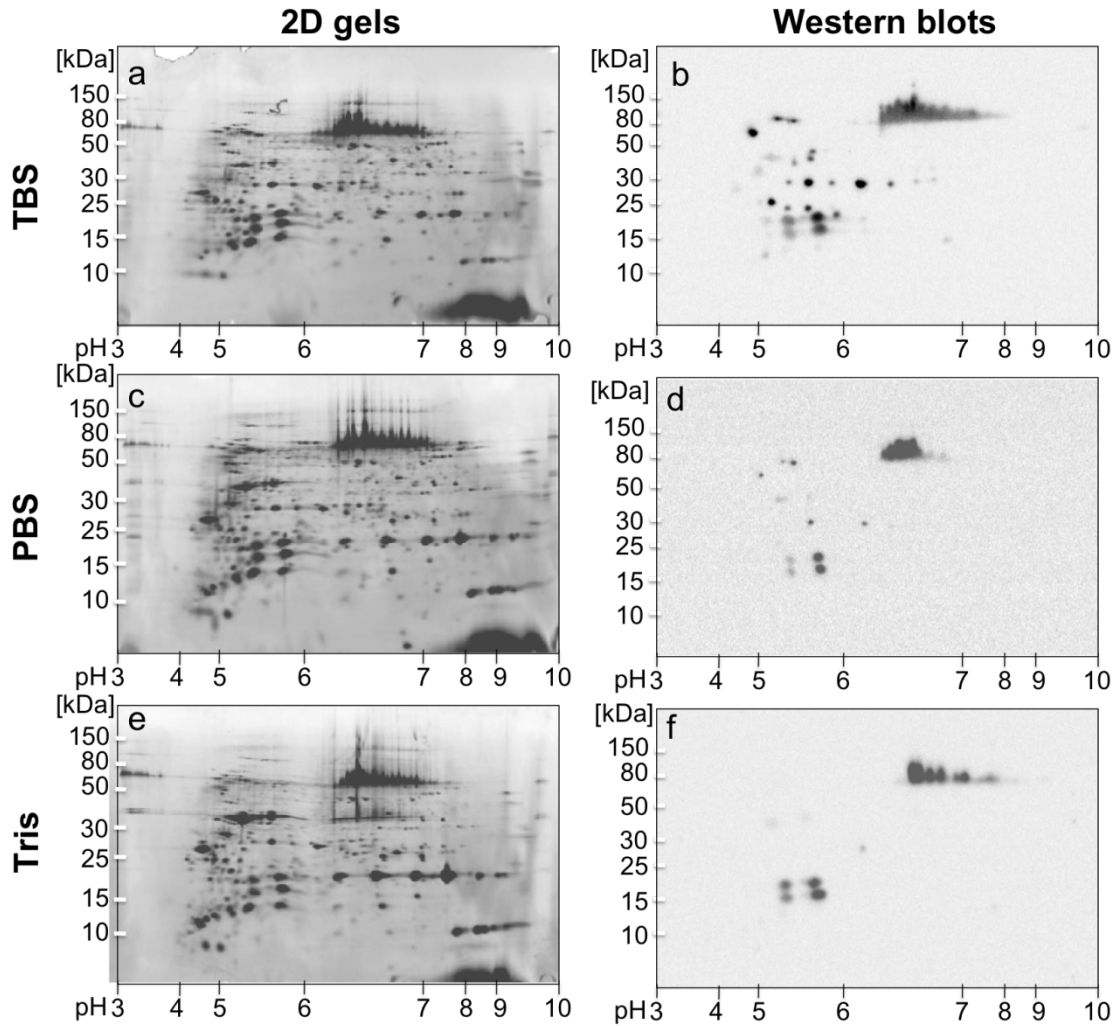
**Figure 3.7 2D-gels and Western blots of peanut proteins extracted with TBS, PBS and Tris buffers.**
Patterns of peanut protein extracted with (a,b) TBS (pH 7.2); (c,d) PBS (pH 8.0); (e,f) Tris (pH 8.5); in total the Western blot assays were performed in duplicate. IPG strips (pH 3–10 non-linear, Bio-Rad Laboratories Inc.) were rehydrated with 25 µg protein and focussed up to 100,000 kVh with a maximum of 5,000 on a Protean IEF cell (Bio-Rad Laboratories Inc.), run on 12% Bis-Tris gels (12% Criterion XT Bis-Tris precast gels, Bio-Rad Laboratories Inc) and blotted onto a nitrocellulose membrane (Bio-Rad Laboratories Inc.). The Western blots were exposed to serum of a peanut-allergic patient (containing very high titres of IgE to Ara h 1 and 2 and moderate titres of IgE to Ara h 3. No IgE against Ara h 8 and 9 was detected; see Appendix 1) and secondary antibody conjugated with horseradish peroxidase and developed using the ImmunStar HRP Chemiluminescence kit (Bio-Rad Laboratories Inc.). The blots were scanned using a G box (Synoptics Ltd.). The identities of most protein spots on 2D gels are listed in Appendix 2.

The Western blot prepared with TBS extracts contained 18 high-volume spots, identified as fragments of Ara h 1 (one spot), fragments of Ara h 3 (four spots), a mixture of Ara h 1 and 3 (one spot), Ara h 7 presursor (one spot, but the Mascot match was not significant) and thioredoxin (one spot). Five protein spots could not be matched using either the in-house peanut allergens database or the NCBInr database and three protein spots were not subjected to mass spectrometry (Table 3.3). PBS extracts resulted in eight intense protein spots, identified as a fragment of Ara h 1 (one spot), fragments of Ara h 3 (two spots), a mixture of Ara h 1 and 3 (one spot), the putative Ara h 7 presursor (one spot) and thioredoxin (one spot).

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Three protein spots could not be matched to the databases employed and one spot was not subjected to mass spectrometry. Tris resulted in only five protein spots, which were identified as Ara h 3 spot (one spot), a mixture of Ara h 1 and 3 (one spot), a thioredoxin fold (one spot) and a protein spot that did not match any protein in the databases employed. Moreover, all extracts had a number of physically close protein spots of ~65 kDa spread between pI 6.5 and 8.0, which were identified as Ara h 1 isoforms via mass spectrometry. Although always present, the dispersion of these protein spots differed in the replicates in all Western blots, including the blots discussed in the following chapters.
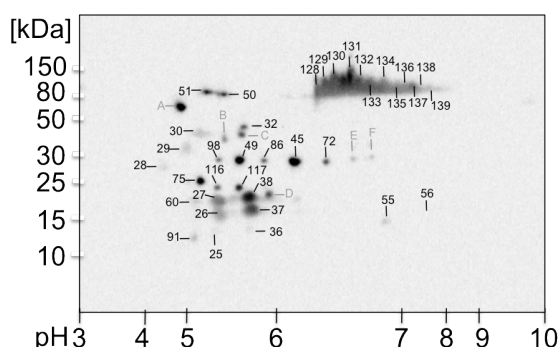


**Figure 3.8 Labelling of protein spots on Western blot of peanut proteins extracted with TBS for identification with mass spectrometry**

Protein spots recognized on a Western blot of peanut proteins extracted with TBS (pH 7.2) are labelled for identification via mass spectrometry in Table 3.3 (as well as Table 4.5 in Chapter 4 and 5.3 in Chapter 5). Protein extracts prepared with PBS and Tris show a subset of the marked protein spots. The labelling corresponds to the detailed mass spectrometry results in Appendix 2.

Additionally to the intense spots on the Western blots, nine faint protein spots were detected in the TBS extracts, while three faint protein spots were detected in the Western blots after extraction with PBS and Tris (Figure, 3.7; Table 3.3). One protein spot was found to be a mixture of Ara h 6, 3 and 8 and its relatively low intensity may be due to that some of the Ara h 3 binding sites were masked by the unrecognised Ara h 6 and 8. Although the negative controls (Western blots with serum from a patient without IgEs against peanut allergens) resulted in clear blots, it could not be clarified whether the majority of faint protein spots were actual signals on the blots or increased background. However, it is most likely that the protein spots that had high spot volumes in the TBS extracts (e.g. some of the Ara h 3 fragments in Table 3.3) but were less intense in Western blots performed with PBS and Tris extracts, were actual positive signals. This is substantiated by the fact that Ara h 3 was recognised in extracts with all three buffers.

**Table 3.3 Identities and intensities of protein spots detected in Western blots of peanut proteins extracted with TBS, PBS and Tris buffers**

The proteins matching the spot pattern in the Western blots in Figure 3.7 and labelling in Figure 3.8 were subjected to mass spectrometry (Appendix 2). The identity and intensity of the protein spots are listed below. (+++) indicates high intensity and (+) low intensity of the protein spot. If the intensity is not marked, the protein spot was absent in the respective Western blot (Figure 3.7). No match refers to peptides that did not show a match in the databases employed.

| Spot no.[a] | Identification MS/MS | | Intensity of protein spot | | |
|---|---|---|---|---|---|
| | Isoallergen or protein | Fragment | TBS | PBS | Tris |
| 128-139 | Ara h 1 | Yes | +++ | +++ | +++ |
| 37 | Ara h 1 and 3 | Yes | +++ | +++ | +++ |
| 38 | Ara h 3 | Yes | +++ | +++ | +++ |
| 45 | Ara h 3 | Yes | +++ | +++ | + |
| 32 | Ara h 3 | Yes | +++ | + | + |
| 72 | Ara h 3 | Yes | +++ | | |
| 30 | Ara h 3 | Yes | + | + | + |
| 28 | Ara h 3 | Yes | + | | |
| 29 | Ara h 3 | Yes | + | | |
| 25 | Ara h 6, 3, 8 | Yes | + | | |
| 36 | Ara h 5[b] | No | + | | |
| 91 | Ara h 6[b] | Yes | + | | |
| 50 | Ara h 7 precursor[b] | Yes | +++ | +++ | |
| 60 | Ara h 7 precursor[b] | Yes | + | | |
| 55 | Ara h 9[b] | Yes | + | | |
| 26 | Thioredoxin fold[c] | No | +++ | +++ | +++ |
| 27 | *No match* | | +++ | +++ | +++ |
| 49 | *No match* | | +++ | +++ | |
| 51 | *No match* | | +++ | + | |
| 75 | *No match* | | +++ | | |
| 98 | *No match* | | +++ | | |
| 86 | *No match* | | +++ | | |
| 116 | *No match* | | +++ | | |
| 117 | *No match* | | +++ | | |
| 107 | *No match* | | + | | |
| A | - | | +++ | +++ | |
| B | - | | +++ | | |
| C | - | | +++ | | |
| D | - | | + | | |
| E | - | | + | | |
| F | - | | + | | |

[a] Spot number in mass spectrometry in Appendic 2
[b] Not significant, no other match
[c] Result from NCBInr database; no match from in-house peanut allergen database

### 3.3.5. *Preliminary glycosylation analysis*

The four distinct spots on the Western blots at ~17 and 21 kDa (Figure 3.7.; spot numbers 26 and 27) are known to contain Ara h 2 (Chapter 3.4.7.) and showed distinct intensities, with the spots at the lower pI values (~5.3) being more faint than the spots at the higher pI values of around ~5.8 (spot numbers 37 and 38). Therefore the glycosylation status of the proteins from

these four spots on the 2D-gel was determined to test whether the different intensities on a Western blot were the result of differential glycosylation. After running the 2D-gels, gel plugs corresponding to the protein spots were provided to Dr. Morten Andersen and Prof. Nicki Packer at Macquarie University for analysis. The spots were analysed for N-glycan content by treating the excised gel plugs separately with deglycosylation enzyme (PNGase F) and analysing the released fraction using LC-MS/MS. In spots 27 (no match; possibly Ara h 2 fragment) and 38 (Ara h 3 fragment; possibly Ara h 2 fragment), N-glycans were detected that are significantly different from each other. The glycosylation of these peanut proteins appeared to be unusual and different from that suggested in the literature for peanut allergens (data not shown due to preliminary nature of the results). No protein glycosylation could be detected for spots 26 (Thioredoxin; possibly Ara h 2 fragment) and 37 (Ara h 1 and 3 fragments; possibly Ara h 2 fragment).

## *3.4.* **Discussion**

### *3.4.1. The extraction yield of crude protein depends on the pH and chemical composition of the extraction buffer*

Buffers with higher pH values (pH 8.5 and above) resulted in high crude protein extraction yield. The lowest crude protein extraction yield of all buffers tested, including PBS (pH 2.1), was obtained with citrate pH 4.5. One factor affecting solubility of proteins is the pI of the buffer solution, such that proteins are typically less soluble when in a solution whose pH is close to their pI as their net charge is zero. However, their solubility can also be dependent on other factors, including the mixed ionic and cationic composition of the buffer and saturation of the solution, e.g. when adding salt. According to the 2D gel images, most extracted peanut proteins (and their fragments) have a pI value between pH 4.5 and 8.5. Hence, theoretically the extraction yield of individual peanut proteins might increase depending on the pH value of 4.5 or lower, or pH 8.5 or higher. Nevertheless, the acid extraction buffers, such as citrate (pH 4.5), resulted in a low extraction yield of peanut proteins. Poms et al. obtained the lowest tested protein concentration when citrate (pH 4.0) was used, while citrate (pH 3.0) had a higher extraction efficiency. However, from the results it was not clear whether the difference was significant (Poms et al. 2004). Using HCl or NaOH to adjust to various pH values between pH 2 and pH 11 in water, Kain et al. showed that the lowest protein solubility was at pH 4.5 (Kain et al. 2009). These observations suggest that although it is possible that there is impact of buffer composition on the solubility of the proteins the pH value is likely to be the decisive factor resulting in the low extraction yield. This might be due to fragmentation (Chapter 3.4.2) and denaturation (Fink et al., 1994) of proteins by the acid, which might cause their precipitation as well as shifts in pI values. The high extraction efficiency with buffers with pHs values ≥ 8.5 is consistent with the results of earlier studies by Poms et al. and Kain et al., who concluded that the most efficient peanut protein extraction was obtained with buffers in the range of pH 8 − 11 (Kain et al. 2009, Poms et al. 2004). Kain, however, gives only relative values for the protein yield, so that the magnitude of extraction efficiency is not evident. Although the same trend to higher pH values is clearly noticeable, Poms et al. extracted only up to approximately 75 − 80 mg crude protein per g peanut flour with 6 M urea (pH 8) and sodium borate (pH 9), while the most efficient buffers in this chapter (including urea and sodium borate) resulted in an extraction yield of approximately 600 mg per g of peanut flour. This difference is most likely due to the extraction protocol of Poms et al, who extracted from ground peanuts either at 60˚C for 20

min or 4˚C overnight, and defatted the protein extracts with hexane after centrifugation. Most extraction protocols involve defatting the ground peanuts before extraction (Chen et al. 2011, Mondoulet et al. 2003, Mondoulet et al. 2005, Porterfield et al. 2009, Schmitt et al. 2010). It is likely that the unusual hexane-treatment is responsible for the low extraction efficiency reported by Poms et al., but the extraction temperatures and times in combination with the buffers used might also have made a contribution. A similar observation was made when comparing the presented results to Sathe et al. (2009), where Tris buffer (pH 8.1) resulted in an extraction efficacy of 280 mg per g of defatted peanut flour (Sathe et al. 2009). The highest amount of protein (400 mg per g of peanut flour) could be extracted when adding EDTA (among other ingredients) to the Tris buffer. In this case the difference in extraction efficiency compared to this chapter is likely to be due to the different de-fatting protocol diethyl-ether at 40˚C. In fact it is shown later in this thesis that ether resulted in significantly lower crude protein extraction compared to hexane (Chapter 3.8.3.). Sathe et al. did not specify the extraction temperatures and times used, but they are likely to have influenced the extraction efficiency as well. In accordance with Kain et al., the solubility of total protein in this chapter appeared to be lowest at pH 4, and increased slightly at pH 2.1. The higher abundance of protein bands below 20 kDa with PBS (pH 2.1) substantiates findings of Krause et al. (2009), who found that acidic extracts resulted in an enrichment of Ara h 9 in lower molecular weight bands (Krause et al. 2009). However, Krause used ammonium acetate buffer (pH 5.0) to achieve these results, whereas the buffer with the most similar pH value, citrate (pH 4.5), used in this chapter did not have increased lower molecular weight bands. Therefore the differences in extraction efficiencies might not only be a result of the pH values but also the different buffer compositions (citrate at pH 4.5 and PBS at pH 2.1).

Use of chemically different buffers with the same pH resulted in great differences in crude protein extraction yield; for example, Tris was about 50% more efficient than TBS. The total protein yield with TBS was only 45% of that with Tris (pH 8.5) when the pellet was extracted multiple times. This suggested that the buffer component sodium chloride, interfered with the extraction process, as sodium and chloride ions are the only additional components of TBS (50 mM Tris pH-adjusted with hydrochloric acid and 150 mM sodium chloride) as compared to Tris (50 mM Tris pH-adjusted with hydrochloric acid). Water appeared to extract the proteins more efficiently than any buffer in the pH range 6.7–8.5 (except Tris and Urea). It is also a reflection that most of the proteins in peanut seeds are albumins (proteins that by definition are soluble in water) (Osborne 1907). If the major proteins in peanut are

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glycosylated, this would also be expected to result in a high level of solubility in water. The findings in this chapter are consistent with the results of Poms et al., who did similar experiments on a smaller scale and also found that water has a higher extraction efficiency than PBS and TBS at a similar pH and that chemically different buffers at similar pH values, such as TBS and PBS, showed different extraction efficiency (Poms et al.). In this chapter more buffers were included and the extraction efficiency was generally higher, but the results confirm and expand Poms et al.'s findings. This suggested that some of the buffer components, such as sodium chloride, interfered with the extraction process.

The extraction efficiency of the different buffers was tested at only one temperature (40˚C), which was more efficient than lower or higher temperatures according to Kain (2009). Other temperatures or extraction times might lead to different results with the various extraction buffers.

### 3.4.2. Ara h 1 and 2 concentration values obtained using ELISA depend on the number of epitopes and must be distinguished from detection rates

Ara h 1 concentration was highest in the extraction samples prepared with denaturant buffers, including the acidic extraction buffer PBS (pH 2.1) and buffers that contained SDS or urea. In contrast to the crude protein measurements, the detection of allergens depends on the accessibility of IgE-binding epitopes for the antibodies, which is based on the conformation of the antigen. Denaturation would cause a loss of conformational epitopes but might increase the accessibility of linear epitopes within the allergen molecules. Ara h 1 exists as an oligomer upon purification of the native protein from seeds (Shin et al. 1998, van Boxtel et al. 2006) and as a homotrimer (a protein band of ~145 kDa on SDS-PAGE) during recombinant expression, similar to native Ara h 1 (Cabanos et al. 2011, Pomes et al. 2003). This homotrimer is very stable, primarily due to hydrophobic interactions (Maleki et al. 2000b), but due to the treatment with SDS the monomeric band (~64 kDa) is visible on electrophoresis gels (Beyer et al. 2001, Maleki et al. 2001, Mondoulet et al. 2005), such as in the 1D-gels this chapter. Ara h 1 contains 24 known linear IgE binding epitopes (Burks, 1997; Shin, 1998; Shreffler et al., 2004) but three of them are considered as irrelevant because they were found to be located in the N-terminal region of native Ara h 1 (Wichers et al. 2004), which is regarded as the signal peptide. The 21 remaining known epitopes span the entire Ara h 1 sequence. Thirteen of the linear epitopes (Burks et al. 1997, Shreffler et al. 2004) present in the core region of Ara h 1 were mapped on the crystal structure of the protein. Epitope 15 was reported to be significantly (≥50%) buried inside the monomeric form the molecule,

while a further 11 epitopes became partially (<50%) to significantly buried upon trimer formation. The significantly buried epitopes were located in the N and C-terminal extension regions near the regions of Ara h 1 monomer-monomer contact (Cabanos et al. 2011, Maleki et al. 2000b 437). These data indicate that the Ara h 1 molecule has to dissociate into the monomeric form or be completely or partially fragmented to become completely accessible to immunoglobulins (Cabanos et al. 2011), which suggests that Ara h 1 is being fragmented in the gut, making these epitopes accessible to IgEs.

It is likely that the addition of acid, such as PBS (pH 2.1), SDS or urea to the extraction buffer, caused disruption of non-covalent bonds in the proteins of the extracts and a partial or total unravelling of the tertiary structure of Ara h 1. Because SDS and DTT, as well as urea (in the case of 2D-gels), are added to non-native electrophoresis sample buffers, the proteins separated are largely denatured, so that only linear epitopes are available for binding to antibodies on gels (Davis and Williams 1998) and Western blots. This also implicates the fragmentation of the homotrimeric or oligomeric form of Ara h 1 in all 1D and 2D-gels performed, which explains why the Ara h 1 ~143 kDa homotrimer band is only very weak in all extracts on the 1D gels and invisible on 2D gels (the higher resolution causes a "dilution" of the bands). This means that the protein conformation and exposition of Ara h 1 epitopes in the peanut protein extracts (particularly with non-denaturating extraction buffers) can be different from the protein conformation on 1- and 2D gels and Western blots. The fragmentation of Ara h 1 by PBS (pH 2.1) is substantiated by the fact that the monomeric 64 kDa Ara h 1 band was very faint and higher-molecular-weight bands were absent in the 1D-gels, while at the same time mass spectrometry showed that Ara h 1 was present in lower-molecular-weight bands. The latter result suggested that Ara h 1 was split into lower-molecular-weight fragments by the acid hydrolysis, allowing denaturation beyond the monomeric form of Ara h 1. A similar result was obtained by Kim et al. who used a different method to expose peanuts to acids, but nevertheless showed that the density of the 64 kDa Ara h 1 band was slightly reduced in samples treated with acetic acid (pH 3.0 or 5.0), and that the band was completely absent in samples treated with pH 1.0 acetic acid or commercial vinegar (pH 2.3) (Kim et al. 2011). Using a different method, Maleki at al. found that cross-linked Ara h 1 trimers were stable even after incubation at pH 2 and could still bind IgE, although effects of acid hydrolysis could be seen on the integrity of the protein on 1D gels.

It is likely that the fragmentation of Ara h 1 by PBS (pH 2.1) resulted in the exposure of additional epitopes for the primary antibody in the Ara h 1 ELISA kit (Indoor Biotechnologies, Inc.), whose binding site is not known (personal communication with technical support of Indoor Biotechnology Inc.). This would explain the strong signal at a low molecular weight on Western blots with the extracts prepared with PBS (pH 2.1) and the extremely high detection rate of Ara h 1 (higher than the total amount of crude protein) in the PBS (pH 2.1) extract. Unexpectedly, only a very weak signal on a 1D Western blot was obtained when pure urea was used for extraction and no signal, when urea was added to Tris, although the linear Ara h 1 epitopes should be more accessible. This is probably due to the greater disruption of epitopes that are recognised by the patients' IgE, after storing the protein samples in urea, rather than just adding denaturant before conducting 1D-gel electrophoresis. It might also be possible that urea interferes with the binding IgE recognition of allergens on the gel for unknown reasons. Addition of DTT did not have any effect on the Ara h 1 ELISA measurements, presumably due to the lack of stabilising disulfide bonds in the Ara h 1 monomers. The mixture of SDS and DTT resulted in a slightly higher Ara h 1 yield than SDS alone, which might be due to variation in samples (the small number of replicates), or the fact that the DTT might have been able to access buried disulfide bonds after denaturation with SDS.

The Ara h 1 concentration was on average more than twice as high in the sodium carbonate extracts compared to the other non-denaturating extracts. Moreover, an additional Ara h 1 band at around ~190 kDa was observed in protein extracts made with sodium carbonate (pH 10.6). This band was absent in the extracts made at $4 - 21°C$, and became increasing apparent between 40 and 60°C. Ara h 1 is known to form homotrimers and often the trimer and/or possibly other forms of oligomerisation are visible on SDS-PAGE. Although the theoretical molecular weight of the trimeric from of Ara h 1 is 192 kDa ($3 \times 64$ kDa), band sizes between ~ 145 – 180 kDa have been reported in various studies (Beyer et al. 2001, Cabanos et al. 2011, Maleki et al. 2001, Mondoulet et al. 2005, Shin et al. 1998). This is due to a degree of retention of the proteins' conformation, which influences its hydrodynamic size and mobility on the gel. Additionally, it was reported that the allergen upon extraction from peanuts occurs as a large oligomer rather than as a trimer with an apparent molecular weight of 700 kDa and purification techniques have different effects on the allergen's quaternary structure (van Boxtel et al. 2006). Although it has been reported that an alkaline environment was effective in solubilising the storage proteins and other minor seed protein components of legumes to

monomers (Derbyshire et al. 1976), it is likely the observed band represent aggregates of Ara h 1. This is substantiated by the size of the band, which does comply with the observed sizes of the trimeric or oligomeric forms of Ara h 1. The fact that the extracts show an elevated, not reduced, detection efficiency in the ELISA, indicates that the epitopes for the primary antibody are not buried in the molecule (although the oligomeric form is know to do that). This further supports the formation of aggregates (Cabanos et al. 2011, Maleki et al. 2000b). The ~190 kDa band was not visible on the Western blot (data not shown), which shows that some epitopes for IgE recognition are nevertheless buried inside the molecule.

These data show that it is not trivial to distinguish between true higher extraction values of Ara h 1 as measured with an ELISA kit, and an increased detection signal due to the exposition of more epitopes in the denatured antigen. Although it has been reported that Ara h 1 forms a highly stable homotrimer (Maleki et al. 2000b 437), it has been observed that Ara h 1 is less stable than Ara h 2 and particularly vulnerable to denaturation at room temperature (personal communication with Indoor Biotechnologies Inc.). The accuracy of the Ara h 1 ELISA assay might therefore depend on proper storage and the number of frosting/defrosting cycles to which the samples have been exposed. In this study the samples were aliquoted upon extraction and defrosted a maximum of three times, to minimise potential problems. Measurements of protein concentration of the peanut protein extracts, which were prepared in non-denaturing conditions, should therefore be accurate.

The addition of DTT to Tris buffer greatly decreased the extraction yield of Ara h 2, while SDS and urea did not have an effect. The resistance to urea and SDS supports previous findings by Maleki et al., who observed high stability of Ara h 2 against urea, roasting and trypsin digestion and proposed that four disulfide bonds are predominantly responsible for the stabilisation of the molecule (Maleki et al. 2003, Mueller et al. 2011). Moreover it was shown that a partial reduction of Ara h 2 is possible without significant impact on the secondary structure (although a difference in function is noticeable) (Maleki et al. 2003). After DTT treatment overnight, most of the α-helix was lost (~5–10% remaining) and only random coils and some β-sheet remained (Maleki et al. 2003). When Ara h 2 is stored in the presence of DTT, it might aggregate and precipitate upon thawing, which might explain lower concentrations of Ara h 2 in solution. Another plausible explanation involves the detection of Ara h 2 epitopes by the ELISA kit. There are 10 known epitopes in Ara h 2, which are distributed throughout the molecule (Stanley et al. 1997) and, although there is remarkable

heterogeneity in the number and patterns of epitope recognition, there appeared to be four major epitopes present in Ara h 2 (Shreffler et al. 2004). Because Ara h 2 is very stable to denaturants, it is likely that these epitopes are accessible in the native structure. The addition of DTT to the extraction buffer probably resulted in a reduction of the disulfide bonds in the Ara h 2 molecules. This reduction then affected the Ara h 2 structure and disrupted the epitope for the monoclonal antibody in the Ara h 2 ELISA kit, resulting in detection of only a very low level of Ara h 2. The detection of Ara h 2 in the ELISA kit is therefore likely to be accurate, as long as no reducing agents are present.

There is no current standard for the measurement of allergens in ELISA assays but the specificity, sensitivity and reproducibility of ELISA assays is currently being assessed as part of the CREATE project (van Ree R. et al. 2008). The findings in this chapter imply not only that the heterogeneity between allergen standards in commercial ELISA kits should be validated (van Ree R. et al. 2008), but also that the influence of the extraction buffer on the conformation of allergens and its influence on the accessibility of epitopes should be evaluated to allow standardization of allergen ELISA assays. The susceptibility of Ara h 1 and 2 towards reducing agents also implies that its conformation in non-reducing extraction buffers is likely to be denatured subsequently in 1-and 2D gels (DTT is present in the electrophoresis sample buffer). It also implies that epitopes for patients' IgE might be lost in subsequent Western blots. Generally, these data show quantitative results using ELISA kits depend in some cases, such as Ara h 1, on the conformation of the measured molecules in the sample and the standard. A reliable and reproducible ELISA assay will therefore be determined by the exact conditions of extraction and other assay conditions. Because some allergens, such as Ara h 1, are very susceptible to the extraction conditions, both the samples and the standards have to be treated in the same way for reliable quantitative results. This needs to be taken into account when the assays are then used in the public domain, considering that the results (such as those for Ara h 1) are highly context-dependent.

### 3.4.3. The extraction yield of Ara h 1 in non-denaturating conditions was lower than expected

Based on the assumption that the protein content of a peanut is on average 25% (Koppelman et al. 2001) the Ara h 1 in extracts prepared with non-denaturating buffers (except for sodium carbonate) analysed in this chapter contained around ~0.6 − 5.5% Ara h 1 in crude protein (~0.15 − 1.4% of Ara h 1 in peanut kernels). This is much less compared to previously published measurements, where the Ara h 1 is 12 − 16% of total protein (De Jong et al. 1998,

Koppelman et al. 2001). It was later observed that the extraction time used in this chapter (1 h) is sub-optimal for the extraction of Ara h 1 (see Chapter 3.8.2.). Because the extraction yield was very low in almost all samples, differences in extraction efficiency (other than the denaturating conditions) could not be reliably examined. However a few trends were observed, including a higher extraction efficiency of ammonium carbonate at pH 8.5, as opposed to pH 7.8 and other buffers, which might indicate an effect of the chemical composition and pH value of the extraction buffer on the Ara h 1 extraction efficiency. This has to be verified in additional experiments under more optimal conditions for Ara h 1 extraction.

### 3.4.4. The extraction yield of Ara h 2 in non-reducing conditions depends on the pH and chemical composition of the extraction buffer

The extraction yield of Ara h 2 (with non-reducing buffers) was dependent on the composition of the extraction buffer and its pH. It was highest at a pH around 8.5 and higher or lower pH values resulted in less Ara h 2, with lower pH values being particularly inefficient. Although crude protein was quantified after extraction with various buffers (Poms et al. 2004, Sathe et al. 2009), and these extracts have varying protein bands at the known molecular weight of Ara h 2, the extraction efficiency of Ara h 2 with different buffers has not been reported previously.

The extraction yield of Ara h 2 decreased to a great extent with increasing amounts of Tris in the buffer (20, 50 and 100 mM), while the addition of other salts, such as sodium chloride (in the TBS buffer) increased the extraction efficiency. High concentrations of Tris most likely decreased the solubility of Ara h 2 through a salting-out effect. This effect depends on the salt concentration, the temperature and pH, and interference with surface tension, but also on the nature of the protein and its concentration. Moreover, it was observed that the amount of Ara h 1 and 2 measured in the TBS extract was higher than in Tris. However, comparing the results to the exhaustive extractions, which were performed at 21˚C, the Tris extract had around 80% less Ara h 1 and 2 in combined total. This indicates a positive effect of sodium chloride in the TBS at 40˚C on extraction efficiency and a negative effect of higher temperature and extraction time on the extraction efficiency with TBS. Kholief et al., who observed that raising the concentration of sodium chloride decreased the extraction efficiency at pH values from 1–4 but increased it at pH 4–5, reported a similar finding, whereas addition of sodium chloride at pH 6–10 decreased the percentage of extracted protein again (Kholief 1987). This further shows that not only the buffer composition and pH, but also the extraction

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protocol, including extraction temperature and duration, influences the extraction rate of individual proteins, including the allergens of peanut.

### 3.4.5. 2D-gel protein profiles of crude peanut proteins extracted with different buffers

Although differences were significant, most protein spots on 2D-gels differed only in volume rather than presence/absence. All significantly different protein spots that met the selection criteria were identified as fragments of Ara h 3 (Table 3.2). The distinction of true protein spot differences between populations and variations in replicates is not simple. Schmidt et al. based the classification of non-equal protein spot volumes on the two-fold square deviation of all included spots, and only included spots that differed more than 12.5-fold (Schmidt et al. 2009). However, this approach includes only mean spot volumes and does not include the variation within a sample population, in terms of a statistical significance. The selection criteria for truely different spots in this chapter was therefore expanded into criteria evaluating the averaged spot volumes, average normalised volume differences of >12,000, a minimum of 3-fold difference in mean abundance of protein spots (and clearly visible different spot volumes) and a high significance level between populations (p-values below 0.01). The cut off values were selected by comparing the 2D gel results in Chapter 4 and 5 with the more reliable 2D-DIGE results. None of the protein spots that fulfilled the criteria showed spot volumes that were $\geq 12.5$ fold; thus, according to the criteria applied by Schmidt et al., there are actually no real qualitative differences in the 2D gels reported here. However, the differences in Ara h 3 protein spots are highly significant indicating the diverse quantity of Ara h 3 fragments and isoforms within the populations. Accordingly, previous publications suggested that diversity in this group of iso-Ara h 3 may be the product of extensive post-translation modification (Koppelman et al. 2003, Liang et al. 2006). The grade of posttranslational modification might vary depending on the active proteases and protease inhibitors in the crude protein. The function of these enzymes and inhibitors is pH dependent and might vary in the different buffer preparations used for the 2D gels (TBS (pH 7.2), PBS (pH 8.0) and Tris (pH 8.5)), which might cause different rates of proteolysis and lead to the observed differences in proteolytic products of Ara h 3. It is therefore possible that the observed differences in abundance of Ara h 3 fragments are real.

### 3.4.6. 2D Western blots with crude protein extracted with various extraction buffers at various pH values resulted in distinct recognition of protein spots by patients' IgE

Although it was observed that a range of extraction buffers strongly influenced the protein band patterns on 1D Western blots with a serum containing IgE against Ara h 1 and 2 and Ara

h 3, some results remain inconclusive, due to the poor quality of the 1D western blot. For example ammonium bicarbonate extracts (pH 7.8) had a very low signal on the Western blots, although buffers with similar pH values and ammonium bicarbonate buffer (pH 8.5) had strong signals. Furthermore, the 1D and 2D Western blots largely correlated, except for the protein spots at 17 – 20 kDa, which were partially identified as fragments of Ara h 1 and 3, but that are also known to contain Ara h 2 (Kottapalli et al. 2008, Schmidt et al. 2009). These were not detected on 1D-Western blots but were clearly visible on 2D Western blots. All proteins bands were transferred successfully from the 1D gel to the nitrocellulose membrane, as determined by reversibly staining the proteins after blotting. Because the same serum was used for both experiments, a plausible explanation could only be drawn from the experimental differences between 1 and 2D gel electrophoresis, such as different treatment of the protein samples with DTT. The samples for the 1D gels were heated to 70˚C after adding DTT. Such treatment might have caused a more advanced denaturation compared to the extracts on the 2D gels, which stayed at 16˚C throughout the experiment. This might have resulted in only partial reduction, leaving the epitopes for the patients' IgE intact. On the other hand in 1D Western blot results to test the sera (see Appendix 1) and an additional Western blot to optimise the Western blot conditions (not shown), a signal at 17 – 20 kDa was clearly visible, indicating an inconsistency in the presented Western blot. It was observed that the exact handling of the chemilumisescence kit used is difficult and that small and unavoidable differences in chemical exposure can result in big differences in the Western blot results. Before conclusions can be drawn as to why the ammonium bicarbonate extract (pH 7.8) did not show any signal, or why the 17–20 kDa bands were not visible in this experiment, the 1D Western blot has to be repeated.

In contrast, the 2D western blots prepared with TBS (pH 7.2), PBS (8.0) and Tris (pH 8.5) showed highly distinct protein patterns that were very similar in the duplicates and are therefore more reliable. The most striking observation was that different extraction buffers resulted in a different number of recognised protein spots on Western blots, although the Western blots were simultaneously exposed to denaturating and reducing conditions. Western blots performed with proteins extracted with TBS (pH 7.2) had (among other non-identified proteins spots) 1–2 more Ara h 1 fragments, and 2–4 more Ara h 3 fragments compared to Western blots with protein extracts prepared with PBS (pH 8.0) and Tris (pH 8.5). The affected protein spots were in fact visible on all 2D gels, although they only appeared on the TBS (or PBS) Western blots. Ara h 3 naturally a hetero-hexamer, formed by two hetero-

trimers contains an interchain disulfide bond between the N-terminal and the C-terminal subunits of each monomer (Jin et al. 2009). The molecule contains four linear epitopes (epitope 1–4) that have been identified on the Ara h 3 sequence (Rabjohn, 1999; Shreffler, 2004). Parts of these epitopes are partially or fully exposed on the surface of the native allergen, which may suggest that they are available for IgE binding even if the intact protein is presented as an allergen. On the other hand, the burial of critical residues of the linear epitopes in the Ara h 3 structure may suggest that the allergen and the linear epitopes are exposed to interaction with the immune system after degradation as a result of digestion. The role of conformational epitopes is unknown (Jin et al. 2009). The striking similarity in the 2D Western blot duplicates suggests that the differences between the samples are not due to general variance between Western blots runs, but to actual differences in the Western blot recognition pattern when different protein extraction buffers are used. It is suspected that the proteins spots show different epitopes in the corresponding protein spots. This has not been observed earlier and needs to be verified in further studies. However, it was shown previously that different membranes types (such as PVDF and nitrocellulose), pore-sizes, different membrane brands, concentrations of blocking reagents and non-ionic detergents, such as Tween, as well as different blocking and incubation times, affected the balance between protein binding capacity, non-specific uptake and the loss of proteins from the membranes. Consequently the immuno-detection of individual components on the Western blots differed significantly (Baldo et al. 1986, Tovey and Baldo 1989 596, Tovey et al. 1987, 1989). It is therefore plausible that not only the Western blot protocol, but also the different extraction buffers in which the proteins are solubilised, may affect the protein binding capacity, the loss of proteins, the as non-specific uptake and possibly the grade of refolding of the proteins on the membranes, resulting in the observed differences of immuno-detection.

### 3.4.7. Mass spectrometry

Rather than using purified allergens run alongside the samples, protein spots were identified via mass spectrometry after gel spots were cut out and digested with Trypsin. This approach has been widely used and has been adopted from Schmidt et al. who also sought to identify peanut allergens after 2D-gel electrophoresis (Schmidt et al. 2009). For the Mascot search an in-house allergen database, which contained 52 available, but not redundant allergen sequences found on the allergome database (www.allergome.org) and NCBI (http://www.ncbi.nlm.nih.gov/protein/) in December 2011, was created (Appendix 3). A similar approach was used previously with a smaller subset of sequences (Chassaigne et al.

2009). Furthermore, the NCBInr database, which has been used regularly in the literature to search for peanut proteins and allergens (Kottapalli et al. 2008, Schmidt et al. 2009) was used to find peanut proteins that were not recognised as allergens. This approach allowed researchers to extend their investigation of peanuts from different regions to include most other extractable proteins that are present in the peanuts, including a range of other major allergens, such as Ara h 3 and 6, which have also found to be important (Lehmann et al. 2006, Rabjohn et al. 1999, Suhr et al. 2004) and all other known peanut allergens. Ara h 1 was mainly identified in a range of neighbouring protein spots at ~64 kDa, which corresponds to the monomeric form of this molecule (Mondoulet et al. 2005). Similar looking protein spots at the same molecular weight were identified previously as Ara h 1 isoforms (Chassaigne et al. 2009, Schmidt et al. 2009), substantiating the results. Ara h 3 was identified in many protein spots between ~25 and 45 kDa and some minor smaller bands. This is in accordance with previous work that identified similar band sizes as Ara h 3 at 45, 42, and 23 kDa and additional minor bands ranging from 12–35 kDa on 1D-gels (Rabjohn et al. 1999). N-terminal sequencing showed that the 45–42 bands are related to the acidic subunit subunit and the band at 23 kDa is related to the basic subunit (Koppelman et al. 2003). The identities or Ara h 1 and 3 were also confirmed on 2D-gels (Chassaigne et al. 2009, Schmidt et al. 2009). The identification of Ara h 6 on 1D-gels turned out to be difficult, which might be due to the large number of proteins in each of the protein bands on 1D-gels.

Other protein bands found in this chapter, such as Ara h 10 or 11 on the 1D-gel or Ara h 8 on 2D-gels (Appendix 2), have, however, not been reported previously in the literature. Ara h 2 is known to be present as two bands of around 17 kDa and 18 kDa MW, corresponding to the two isoforms (Mondoulet et al. 2005). Only one protein band of Ara h 2 was identified on the 1D gel (even though only one peptide was matched with the sequence), which might be due to the fact that the Ara h 3 basic subunit and the Ara h 2 upper bands can co-migrate on gradient gels (personal communication with Dr Soheila Maleki). Nevertheless, Ara h 2 could not be identified in any of the protein spots on 2D-gels. Schmidt et al., from whom the method was adopted, and who also used Trypsin to digest the gel plugs, had, however, identified Ara h 2 in the four distinct spots 26, 27, 37 and 38 on the 2D-gel (Schmidt et al. 2009). Chassaigne et al. ran purified Ara h 2 as a standard on a 2D-DIGE gel and could confirm these four distinct protein spots to be Ara h 2. Subsequent Western blot experiments with antibodies against the native form of Ara h 2 showed binding to spots 37 and 38, which were also recognised by antibodies against a recombinant 40 kDa sub-unit of Ara h 3. In mass spectrometry

experiments, however, neither Ara h 2 nor Ara h 3 could be detected in these protein spots (Chassaigne et al. 2009). In this thesis the presence of Ara h 3 in protein post 37 and 38 could be clearly confirmed.

The detection of Ara h 2 in 2D gels via trypsin digest and mass spectrometry was not possible in this thesis, although a very similar approach was used as Schmidt et al., who was able to identify Ara h 2 in the four distinct spots on the 2D gels at ~17 and 21 kDa (spot numbers in mass spectrometry are 26, 27, 37 and 38, Appendix 2) (Schmidt et al. 2009). However, this is consistent with Chassaigne et al., who also could not identify Ara h 2 using the approach with Trypsin, although Western blots with recombinant Ara h 2 and the utilisation of purified Ara h 2 in 2D DIGE confirmed the presence of Ara h 2. It was shown that Ara h 2 has significant sequence homology with trypsin inhibitors and bifunctional trypsin/α-amylase inhibitors. Due to its inhibitory function the digestion of Ara h 2 with Trypsin is therefore impossible. Chaissagne e al. could show in later experiments that Ara h 2 could be detected on 2D-gels when different peptidases were used, such as pancreatin (Chassaigne et al. 2009). Although this is inconsistent with the results of Schmidt et al. (2009), who might have used a more rigorous trypsin digesting method, it explains why Ara h 2 could not be detected in the mass spectrometry and suggests that Ara h 2 is nevertheless present in the four distinct spots on the 2D gels at ~17 and 21 kDa. This is substantiated by the fact that the four Ara h 2 spots were recognised on 2D-Western blots by patients' serum containing Ara h 2 IgE, indicating a partially or wholly native structure with intact epitopes.

Surprisingly, Ara h 2 was identified in one protein spot in 1D-gels in this thesis, suggesting that its biological function as a trypsin inhibitor was lost during the 1D-gel protocol, which might be due to the treatment with DTT at 70˚C, as opposed to a DTT treatment at 16˚C in 2D-gels. Consequently, the trypsin digest worked when gel plugs from 1D gels were used and Ara h 2 could be detected in the mass spectrometry. The 1D Western blots did not have the Ara h 2 bands, supporting the idea that the 1D gel protocol results in denatured Ara h 2, with disrupted epitopes and lost function. This seems to be variable upon small differences in treatment, which might explain the abundance of the Ara h 2 bands on the 1D Western blots to test the sera (Appendix 1). Generally, this has to be verified in further Western blot replicates, before any substantial conclusions can be drawn.

### 3.4.8. Discussion glycosylation

N-glycans were detected on proteins from 2D gels, which showed different intensities in IgE recognition on Western blots. Two protein spots, namely 27 (weaker intensity; no match; possibly Ara h 2 fragment) and 38 (higher intensity; Ara h 3 fragment; possibly Ara h 2 fragment) contained N-glycans detected were significantly different from each other (the details of the structures are not reported in this thesis). The glycosylation of these peanut proteins appeared to be unusual and different from that suggested in the literature for peanut allergens. No protein glycosylation could be detected for spots 26 (weaker intensity; Thioredoxin; possibly Ara h 2 fragment) and 37 (higher intensity; Ara h 1 and 3 fragments; possibly Ara h 2 fragment). The Ara proteins are decorated with carbohydrates (N-glycans) and this N-glycosylation has been previously linked to peanut allergy (Garcia-Casado et al. 1996, van Ree Ronald et al. 2000). In addition, data were generated indicating that unusual (non-human) glycan structures are present on peanut protein Ara h 2/3 as would be expected from the generation of immuno-responses in some individuals. The observed intensities seem to derive from spots that contained more than one allergen.

Generally, the N-glycosylation in mammals is known to be influenced by physiological and environmental factors have strong influence on the N-glycosylation in mammals (Malhotra et al. 1995, Van Dijk et al. 1995), and although it has been shown that the structure of N-linked glycans varies with different developmental stages, little is known about the influence of plant development and growth conditions on N-linked glycosylation (Elbers et al. 2001). It is therefore possible that the different growth conditions of peanuts in Chapter 3 and 4 might show different glycosylation, which is the reason why these preliminary glycosylation experimnts have been conducted. The possibility was raised recently that galactose-a-1,3-galactose IgE might be the target of reactivity to gelatin and hence red meat (Mullins et al. 2012). On the other hand, it has been previously reported that the antibody-binding glycoproteins do not appear to cause clinical symptoms in most, if not all patients (Altmann 2007). The exact involvement of protein glycosylation in peanut allergy needs to be further investigated to identify and characterise the N-glycans and their protein carriers responsible for peanut allergy and to characterise the molecular mechanisms responsible for triggering allergenic reactions towards peanut exposure. Further experiments are needed to confirm the presence of Ara h 2, which could not be identified in mass spectrometry, possibly due to its

trypsin inhibitory activity. Funding has been acquired to conduct these further experiments in our laboratory.

## *3.5. Conclusion*

Overall, the identity of the buffer, including its pH value, greatly influences the extraction efficiency of crude protein and specific proteins in peanuts. The buffer also has an impact on the outcome of subsequent experiments, such as the recognition of IgE from human serum in Western blotting. Denaturating and/or reducing conditions can affect the number of linear and conformational epitopes on allergens and alter their detection in ELISA assays and make an accurate quantification impossible. The extraction buffer for peanut protein extraction should therefore be selected carefully depending on the research question; i.e. whether the focus is on a specific allergen or a whole range of allergens (most of which are present in the crude protein). Since the aim of the following chapters was to compare the allergen content of peanuts from plants grown under different conditions, a buffer was chosen that allows extraction of as much crude protein (containing most allergens) as reproducibly possible, including Ara h 1 and 2. The extraction buffer should also be compatible with all subsequent experiments, so that further variation of protein amounts that might occur through the use of techniques such as dialysis can be avoided. The extraction buffer chosen for subsequent experiments was thus 20 mM Tris (pH 8.5) because it extracts crude protein (which most likely contains most of the allergens) with high efficiency, including a good yield of Ara h 2. This buffer is also compatible with all subsequent methods used in the research reported in this thesis, such as Western blotting, protein quantification with the 2D Quant kit and 2D-DIGE experiments, meaning that the extracts could be used directly. However, since the abundance of Ara h 1 in the peanut flour was very low, an extraction protocol had to be optimised before deciding on a final protocol to test the allergen content of peanuts from different conditions in Chapters 4 and 5.

# Chapter 3B

# Evaluation of common peanut protein extraction parameters and determination of allergen content

## *3.6.   Summary*

Commonly used peanut protein extraction techniques were tested to determine their peanut protein extraction efficiency. The most efficient and convenient extraction methods for crude protein, Ara h 1 and 2, that is compatible with all employed subsequent experiments is: extraction of n-hexane defatted peanut flour (without seeds coats) with 20 mM Tris for 30 min at 21˚C. This method was used for the peanut samples in the following chapters of this thesis.

## *3.7.   Peanut samples*

The peanuts used in this Chapter were runner peanuts (variety "Walter"), which were grown close to Kingaroy in Queensland and acquired from PCA. In some cases, such as the evaluation of de-fatting methods (Chapter 3.8.3) and determination of the variability of protein content in small sample pools (Chapter 3.8.6), 10 peanuts were pooled and used for the experiments. For all other experiments in this chapter, one batch of peanut flour was used, which was prepared by homogenising around 100 g of peanuts and de-fatting with hexane according to the optimised methods described in the results below.

# 3.8.    Results

## 3.8.1.  Effect of commonly used centrifugation parameters on extraction yield of peanut proteins

Commonly used centrifugation acceleration (g), centrifugation time and number of centrifugations during peanut protein extraction were evaluated for their effect on the extraction yield of crude protein (i.e. the solubilisation of proteins from the peanut kernels) and the overall quality of protein separation by 1D-gel electrophoresis. During protein extraction with TBS (pH 8.5) from hexane-defatted peanut flour, two centrifugation accelerations were tested (3,000 and 12,500 g), as well as three centrifugation times (5, 15 and 30 min). The protein concentrations of the resulting peanut protein extracts were determined using the 2D-Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and qualitative differences in protein abundance were visualised using 1D-gel electrophoresis (Invitrogen, Carlsbad, CA).

Centrifugation accelerations of 3,000 and 12,500 g, centrifugation times of 5–30 min, as well as number of consecutive centrifugations (2–3) did not influence the extraction yield of total peanut protein using defatted flour. All subsequent extractions therefore involved centrifugation at 12,500 g for 5 min. The centrifugations were performed three times in all following experiments because in rare cases a small amount of lipid remained in the tube after only two runs.

## 3.8.2.  Effects of temperature and time on extraction yield of crude peanut proteins and allergens Ara h 1 and 2

To enable extraction of higher yields of Ara h 1 simultaneously with high yields of Ara h 2 and crude protein (containing other allergens), the influence of extraction temperature and time on the extraction yield of total peanut protein and Ara h 1 and 2 was examined (Figure 3.9 and 3.10). The total protein quantity and the Ara h 1 and 2 abundance in the resulting protein extracts were determined using the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and Ara h 1 and 2 ELISA kit (Indoor Biotechnologies Inc.).

The influence of the extraction temperature on peanut protein extraction yield was tested (Figure 3.9), by extracting hexane-defatted peanut flour with 20 mM Tris (pH 8.5) for 1 h at 4, 21, 40 and 60˚C. The total protein quantity and the Ara h 1 and 2 abundance in the resulting peanut protein extracts were determined using the 2D Quant kit (Amersham Biosciences-GE

Healthcare, Uppsala, Sweden) and Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.). The results indicated that the extractions at 4, 21 and 60˚C resulted in very similar crude protein content while, surprisingly, an extraction at 40˚C was less efficient. This result was significant when comparing the extracts made at 40˚C to those made at 4˚C ($p < 0.05$) and 60˚C ($p < 0.01$) but not to those made at 21˚C. Nevertheless, the Ara h 1 and 2 content was similar in all extraction temperatures tested, resulting in around 3.3 mg Ara h 1 and 41 mg Ara h 2 per g peanut flour.



**Figure 3.9 Effect of extraction temperature on extraction yield of crude peanut protein and Ara h 1 and 2 (per g of peanut flour)**

Hexane-defatted peanut flour was extracted with 20 mM Tris (pH 8.5) at 4, 21, 40 and 60˚C for 1 h in triplicate. The 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.) were used to measure crude proteins and the specific allergens, respectively. All values are averages (± standard deviations) based on mg per g of peanut flour.

Extraction times were also tested for their effect on Ara h 1 extraction efficiency (Figure 3.10) All extractions were performed with 20 mM Tris (pH 8.5) at 21˚C from hexane-defatted peanut flour, by vortexing for 30 s and then, extracting for 0.5, 1 and 2 h on a shaker. Additional samples were vortexed for 1 min without further extraction on a shaker.
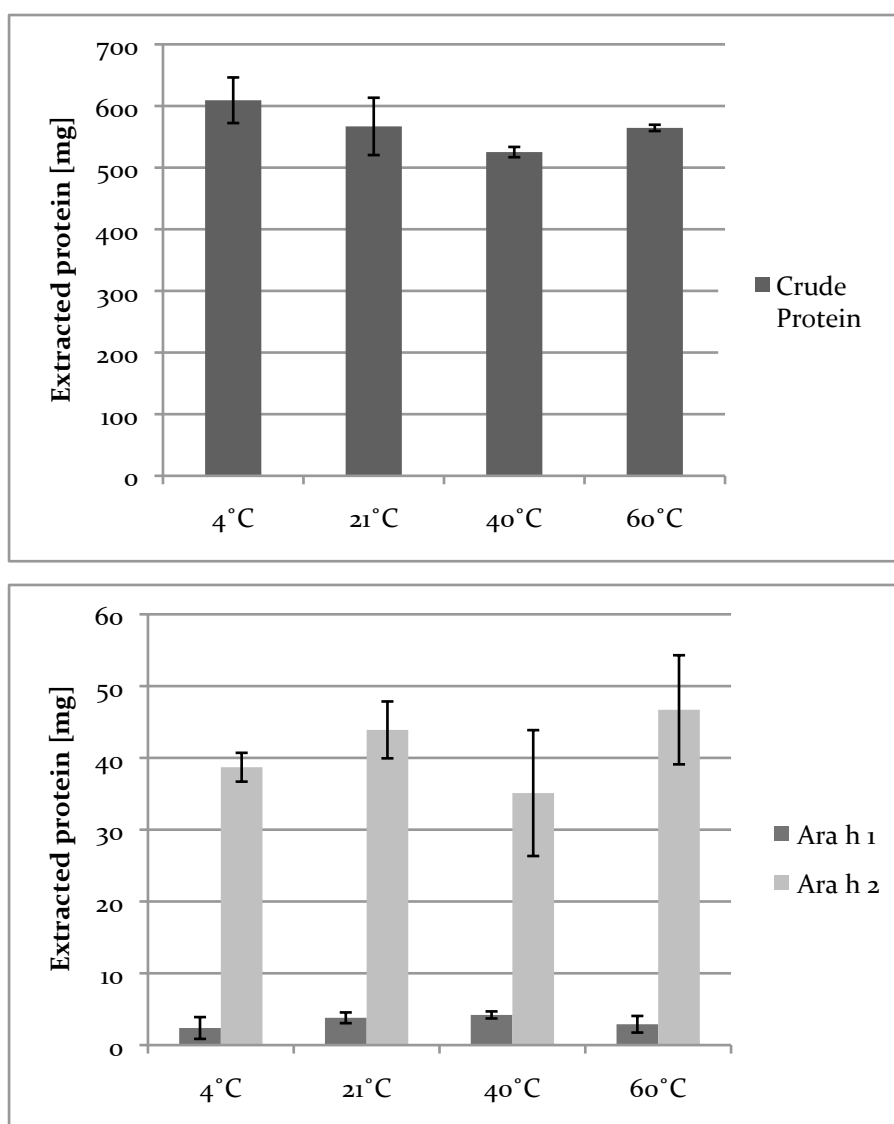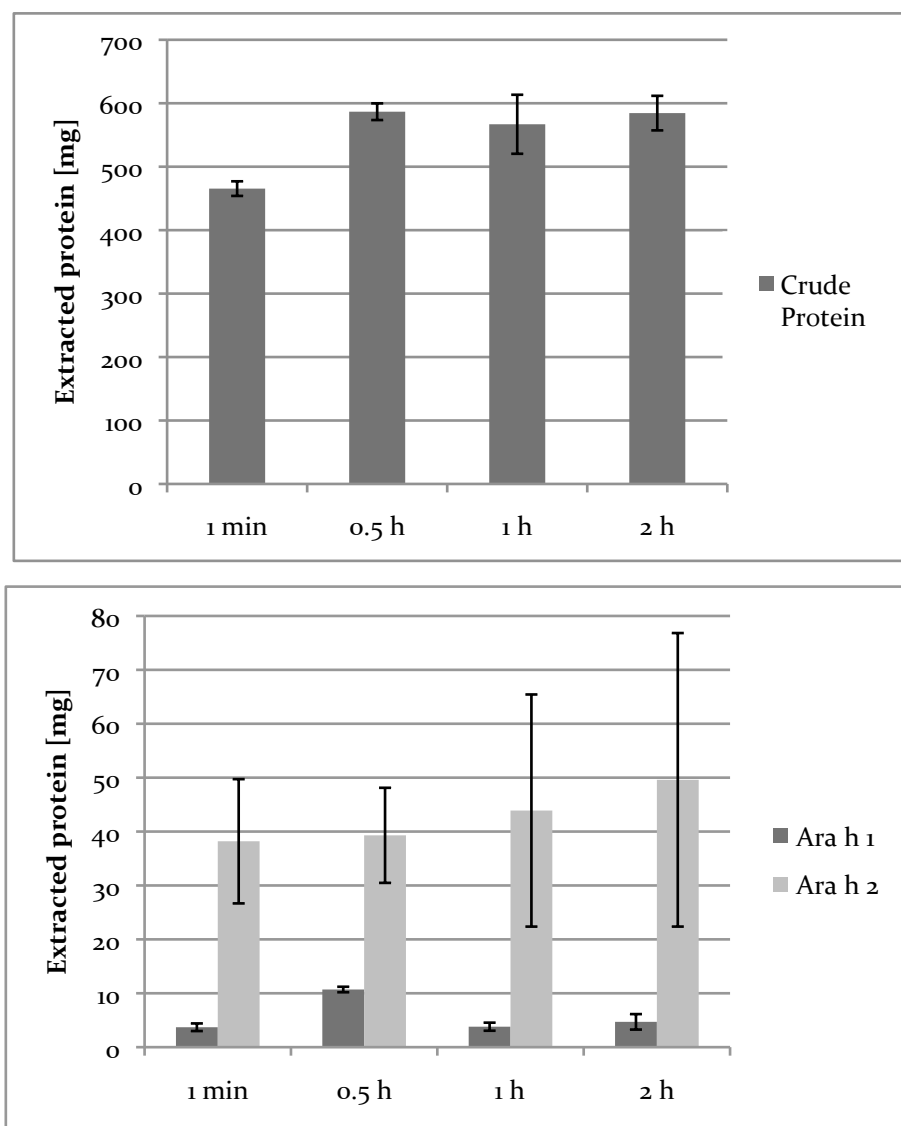


**Figure 3.10 Effect of extraction time on extraction yield of crude peanut protein and Ara h 1 and 2 (per g of peanut flour)**

Hexane-defatted peanut flour was extracted in triplicate with 20 mM Tris (pH 8.5) by vortexing for 30 s and then, extracting for 0.5, 1 and 2 h on a shaker. Additional samples were vortexed for 1 min without further extraction on a shaker. All values are averages (± standard deviations) based on mg per g of peanut flour.

1D-gel electrophoresis showed that there were no major qualitative differences in the band patterns between the extraction time and temperature samples (data not shown).

Crude protein extraction yield was on average 18–20% lower in the samples that were extracted for 1 min compared to the samples that were extracted for longer (0.5 h: $p < 0.01$, 1 h: $p < 0.05$, 2 h: $p < 0.01$; Figure 3.10). This showed that around 80% of all proteins in a single extraction were already solubilised after 1 min. The extractions that were performed for 0.5 h appeared to contain more protein than the 1 h and 2 h samples but the differences were not statistically significant. Surprisingly, the Ara h 1 content was 56–65% higher for extractions performed for 30 min compared to all other extraction times, including 1 and 2 h. In order to verify the results, the ELISA measurements were repeated, and very similar results obtained, showing that the Ara h 1 extraction is statistically more efficient when performed for 30 min. This explains why the Ara h 1 content was low in samples used to test the effect of extraction temperature (Figure 3.9) and to test the effect of extraction buffer identity (Chapter 3.3.1), since in both these experiments extraction was performed for 1 h. Ara h 2 content was very similar in all samples, indicating that Ara h 2 was already extracted during the first minute and stable for at least 2 h at room temperature.

Based on these results the most efficient strategy for crude protein and Ara h 1 and 2 extraction was to extract for 30 min at room temperature. These were therefore the conditions use for experiments reported in the following chapters in this thesis.

### 3.8.3. Comparison of the most common de-fatting reagents, hexane and ether, on extraction yield of peanut proteins and allergens Ara h 1 and 2

The most commonly used de-fatting solvents hexane and ether were tested for their effect on extraction yield of crude peanut protein and Ara h 1 and 2. To obtain three replicates for each condition, three sets of 10 peanuts were homogenised and defatted three times with either hexane or ether using the standard protocol (Chapter 2.2 – 2.3) before protein extraction at room temperature for 30 min with 20 mM Tris-HCl (pH 8.5). The hexane-defatted samples had an extraction efficiency of 587 ± 13 mg protein per g peanut (Figure 3.11) and were significantly (24%) higher than the extraction efficiency for the ether-defatted samples (443 ± 25 mg; $p < 0.05$).

Surprisingly, the Ara h 1 content in the ether-defatted peanut extracts (1.4 ± 0.5 mg) was 90% lower than in the hexane extracts (13.6 ± 1.6 mg; Figure 3.11). This was verified in a single 2D-gel experiment, some protei spots a between were missing when ether was used . Because the 2D-gel experiment was not repeated and most differing protein spots were identified, the

results should be handled cautiously and the data are not shown. The hexane-defatted peanut extracts contained 20% more Ara h 2 (63.2 ± 23.2 mg) than the ether extracts (18.4 ± 2.0 mg).
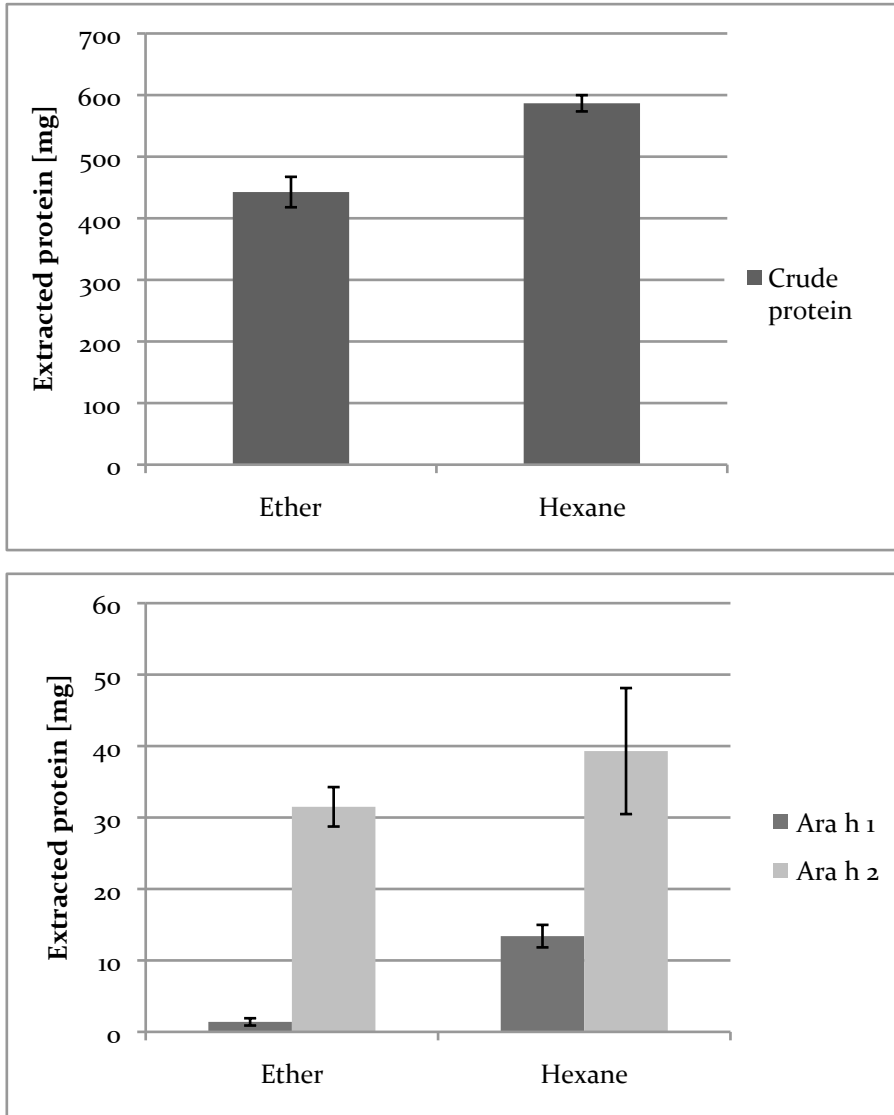


**Figure 3.11 Effect of defatting strategy on crude protein extraction yield and Ara h 1 and 2 abundance (per g of peanut flour)**

Yields of crude peanut protein and Ara h 1 and 2 were determined after defatting peanut homogenate with ether or hexane. Each extraction derived from a pool of 10 seeds per treatment and was performed in triplicate. The amounts of crude protein and Ara h 1 and 2 were measured in triplicate with the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and commercial Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.). All values are averages (± standards deviations) based on mg per g of peanut flour.

After having tested the amount of extracted crude protein and Ara h 1 and 2 in the hexane- and ether-defatted peanut protein extracts, qualitative differences in other proteins between extracts were examined using 1D-gel electrophoresis (Invitrogen, Carlsbad, CA). To test whether the defatting itself would cause the intensity of any of the major protein bands to change or disappear, samples of one set were prepared from non-defatted peanut homogenate, which were used directly for extraction with 20 mM Tris-HCl (pH 8.5). Protein patterns for

the non-defatted, ether-defatted and hexane-defatted extracts subjected to 1D-gel electrophoresis (Figure 3.12) appeared very similar with the same major and minor bands being present.
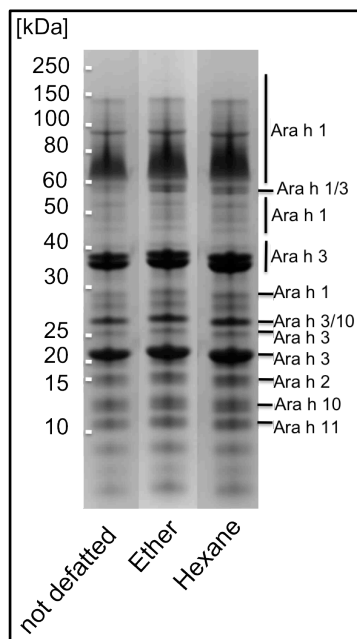


**Figure 3.12 Effect of defatting strategy on crude protein pattern after separation on 1D-gel electrophoresis**

The same volume of extract was run on each lane on a precast 4–12% Bis-Tris gel (NuPAGE, Invitrogen, Carlsbad, CA). Gel electrophoresis was performed with all three extracts per treatment and each extraction was derived from a pool of 10 seeds per plant. Only one sample is shown in the figure. The protein identities derive from mass spectrometry; see Appendix 2 for details.

From these results it was concluded that no proteins represented by clear bands on the 1D gel were removed from the peanut homogenate by de-fatting. Hexane was more efficient that ether and allowed the extraction of a large fraction of crude protein, including the major allergens, Ara h 1 and 2. Hexane was therefore used for defatting in all subsequent experiments.

### 3.8.4. *Effects of roasting and boiling on extraction yield of crude peanut proteins and Ara h 1 and 2*

Experiments were conducted to test the effect of roasting and boiling on the extraction efficiency of crude proteins and Ara h 1 and 2 (Figure 3.13). One set of peanuts used was raw, another roasted for 20 min at 170˚C in a conventional oven and a third set boiled for 20 min in boiling water. The amount of extracted crude protein was significantly higher in the raw peanuts, compared to the roasted (53%; p< 0.01) and boiled (42%; p < 0.01) peanuts. Although the amount of crude protein from the roasted peanuts appeared to be lower than

from boiled peanuts, the difference was not statistically significant. The Ara h 1 content was 40% higher in roasted samples (22.3 ± 2.9 mg) and 70% lower in boiled peanuts (4 ± 0.1 mg), compared to raw peanuts (13.4 ± 1.6 mg). The amount of Ara h 2 extracted was significantly higher (76−87%) in the raw samples compared to roasted (p < 0.05) and boiled peanut extracts (p < 0.05). Additionally, the amount of Ara h 2 was higher in roasted peanuts than in the boiled peanuts (p = 0.01).
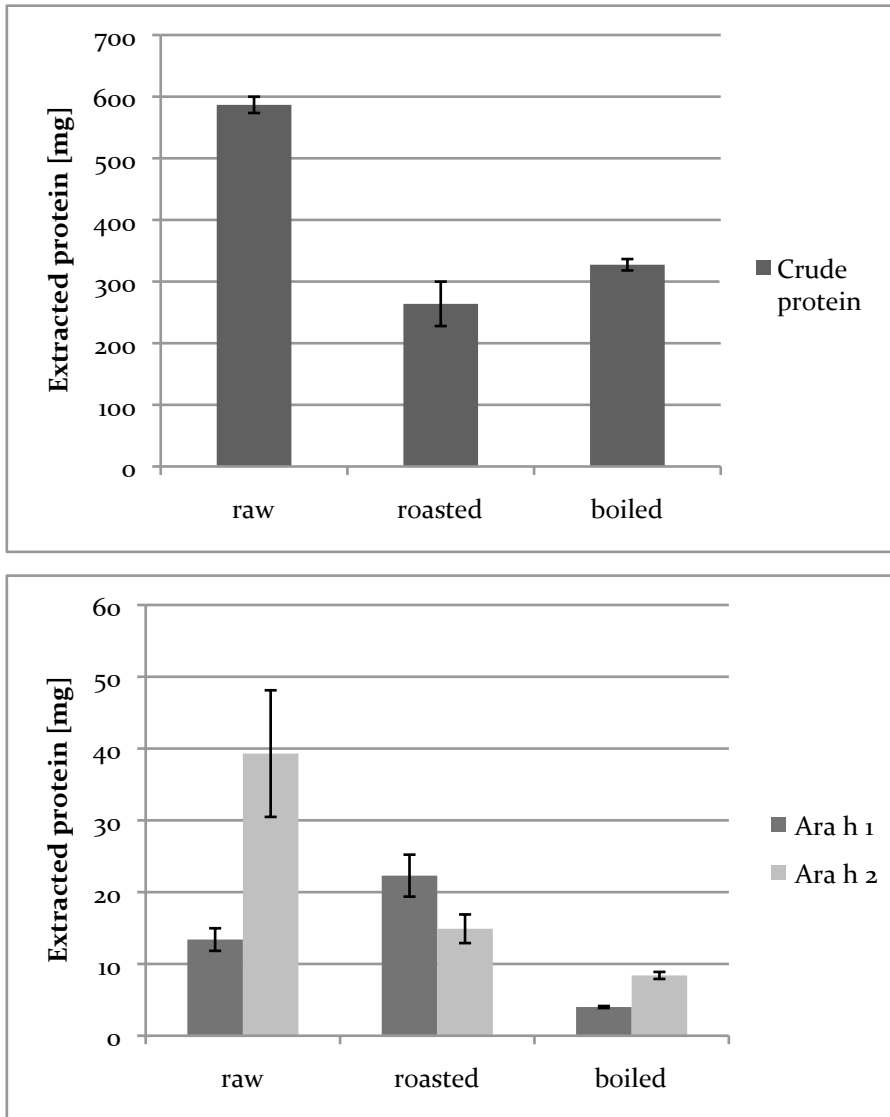


**Figure 3.13 Effect of roasting and boiling of peanuts on extraction yield of crude protein and Ara h 1 and 2 (per g of peanut flour)**

Yields of crude peanut protein and Ara h 1 and 2 were determined from raw, roasted (20 min at 170˚C) or boiled (20 min in boiling water) after defatting with hexane and extracting with 20 mM Tris (pH 8.5). Each extraction derived from a pool of 10 seeds per treatment and was performed in triplicate. The amounts of crude protein and Ara h 1 and 2 were measured in triplicate with the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and commercial Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.). All values are averages (± standards deviations) based on mg per g of peanut flour.

1D-gel electrophoresis (Figure 3.14) demonstrated that the protein extract from raw and boiled peanuts was very similar and all major bands were present in the boiled samples with lower intensity. Notably the low molecular weight bands below 8 kDa were much less abundant in the boiled peanut extracts. The roasted peanut extracts lacked protein bands at 49, 63 and 85 kDa, which are likely fragments, the monomeric form of Ara h 1 and some bands below 8 kDa. The roasted peanut extracts also appeared to be streakier on the SDS-gel.



**Figure 3.14  1D-gel electrophoresis with crude protein extracts from raw, roasted and boiled peanuts**
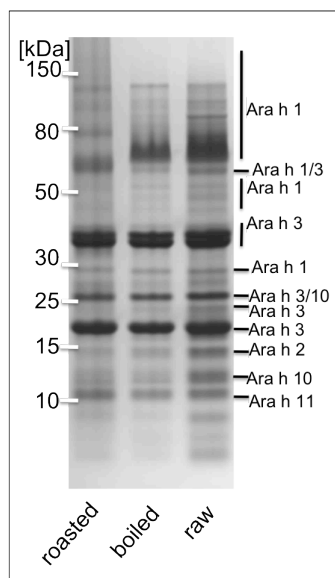Raw, roasted (20 min at 170˚C) or boiled (20 min in boiling water) peanuts were defatted with hexane and extracted with 20 mM Tris (pH 8.5) before running equal volumes of extract on precast 4–12% Bis-Tris gels (NuPAGE, Invitrogen, Carlsbad, CA). Gel electrophoresis was performed with all three extracts per treatment and each extraction was derived from a pool of 10 seeds per plant. Only one sample is shown in the figure. The mass spectrometry results were prepared using peanut protein extracts form raw peanuts (see Appendix 2 for details).

During the extraction of raw, roasted and boiled peanuts it was observed that the consistency of the peanut homogenate differed greatly. Boiled peanuts were more difficult to grind and resulted in small chunks, rather than a homogenous paste, even after using a coffee grinder, vigorous manual grinding in a mortar and using liquid nitrogen to cool the samples. The defatting procedure was difficult, as the peanut chunks did not stir in well with the hexane. On the contrary the roasted peanuts were very easy to grind and resulted in a very smooth homogenate. During defatting the proteins could be stirred in with the hexane easily, before letting the extract divide into two phases. The protein extraction was similar in raw, roasted and boiled peanuts, but the consistency of the roasted pellet was much softer than in raw peanuts, while the pellet from boiled peanuts was much harder. Overall, it was concluded that

the extraction efficiency of treated peanuts is rather an issue of consistency and physical nature of the material than actual protein content.

In summary, the quantity of Ara h 1 was higher in roasted samples, but this is likely the result of a higher accessibility of epitopes, rather than a higher extraction efficiency (see Dicsussion 3.9.4). Because Ara h 2 and crude protein, which most likely contains a number of other allergens, was highest when raw peanuts are used for extraction, raw peanuts have been used in the rest of the thesis.

### 3.8.5. *Peanut protein extraction and allergen content in seed coats*

Experiments were conducted to determine whether the general standard extraction procedure for peanut seeds widely reported in the literature allows extraction of peanut proteins including Ara h 1 and 2 from seed coats. This was done because part of the methods reported in the literature keep the seed coats during the defatting and extraction process, while others remove the seeds coats before this process.

In a first approach the protein extraction yield of peanuts extracted with or without seeds coat was determined. The student t-test was applied and it was found that the extraction efficiencies of crude protein and Ara h 1 and 2 were statistically similar with p >0.05.

In a second experiment 45 mg of seed coats (without the rest of the seed) were defatted as usual and used for extraction at room temperature with either citrate (pH 4.5), urea (pH 6.7), Tris (pH 8.5) or sodium borate (pH 9.2), using the standard procedure as used for seeds. The large number of seed coats and the various buffers and pH values were thought to increase the chance of detecting proteins from the seed coats after performing the standard extraction procedure. However, no protein could be detected in any of the samples. Furthermore, the protein concentration was too low for detection with the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) or with Coomassie G (sensitivity 0.5 µg protein per protein band) or silver stain (0.5 ng protein per protein band) after 1D-gel electrophoresis. Finally, no Ara h 1 and Ara h 2 allergens could be detected using Ara h 1 and 2 ELISA kits  (Indoor Biotechnologies Inc.). This shows that the widely used extraction procedures for peanut seeds do not extract any or extremely low amounts of protein from seed coats.

In a third attempt the respective extraction buffer was added immediately to the seed coats, which were homogenised vigorously using a mortar and pestle until a brown paste was obtained. In these samples, small amounts of protein could be extracted when using Tris (185 ± 3.8 mg per g seed coat), urea (26.3 ± 1.8 mg per g seed coat) and sodium borate (31.9 ± 2.4 mg per g seed coat). This is around 94–97% less protein than in the respective peanut kernel extracts. No protein could be detected when citrate was used for extraction. Commercial ELISA kits (Indoor Biotechnologies Inc.) revealed that no Ara h 1 could be detected in any of the samples. The Ara h 2 content was 0.38–8.4 µg per g of peanut flour in the different seed coat extracts, more than 99% less than in the respective peanut kernel extracts. This might be due to contamination with small peanut kernel pieces and it can be concluded that there was effectively no Ara h 2 present. The proteins could be visualised with SDS-gel electrophoresis using highly sensitive Sypro Ruby stain (Invitrogen, Carlsbad, CA; sensitivity 0.25–1 ng of protein per protein band) with protein bands ranging from 100 to 10 kDa but not with the less sensitive Coomassie G stain (sensitivity 0.5 µg protein per protein band). Due to the very low abundance of the proteins, no further experiments were performed to identify these proteins.

Based on the results above, the seed coats were removed from the peanut kernels before defatting and extractions in the following chapters. This was thought to increase the accuracy of weighing the peanut flour before the protein extraction, as bigger seed coat flakes in the peanut flour can lead to inaccurate measurements.

### 3.8.6. *Variation of crude protein and Ara h 1 and 2 content in small peanut pools*

The variability of the protein amount between small peanut pools was determined in order to be able to test peanuts from different conditions in the following chapters. Four peanut pools containing 10 peanuts each were homogenised individually before defatting and extraction with 20 mM Tris (pH 8.5). The amount of crude protein and Ara h 1 and 2 abundance was determined using the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.) before performing 1D-gel electrophoresis.

The amount of crude protein and Ara h 1 and 2 was very similar in all four peanut pools (Figure 3.15) and statistical analysis using the Student t-test showed that the protein amount in all four pools was similar with $p > 0.05$. Likewise, the Ara h 2 amounts were statistically similar ($p > 0.05$) in all samples. The Ara h 1 abundance varied slightly between the samples,

and pools 2 and 4 had statistically different amounts of this allergen ($p < 0.05$). However, the variance of these two pools differed and the statistical difference might not be valid. The Ara h 1 content in the four pools was between $10.7 \pm 0.5$ mg and $16.5 \pm 2.6$ mg per g of peanut flour. Therefore any Ara h 1 contents around that range were regarded as from similar populations throughout the thesis.
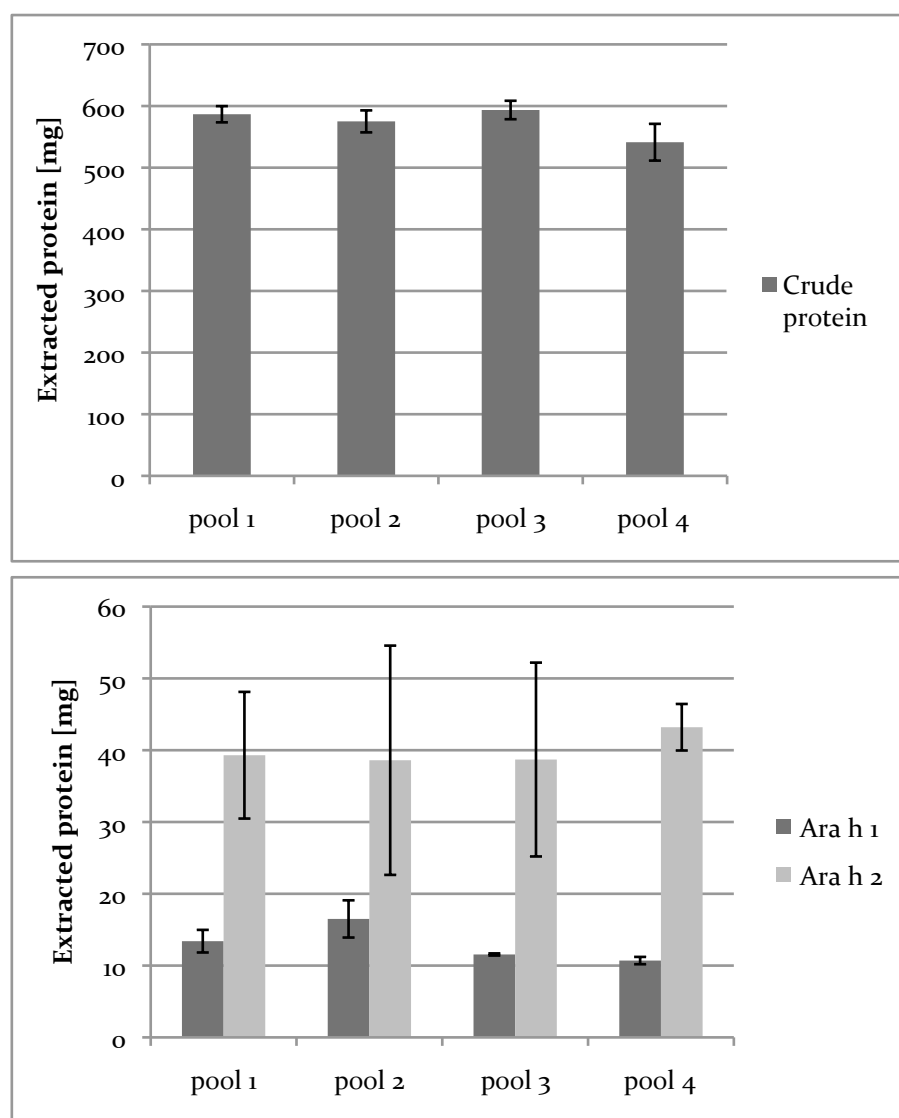


**Figure 3.15 The variation between peanut pools of 10 peanuts each (per g of peanut flour)**

The figure shows that the variation of extracted extracted crude peanut protein and Ara 1 and 2 abundance in four.

Yields of crude peanut protein and Ara h 1 and 2 were determined from four small peanut pools of 10 peanuts each after defatting with hexane and extracting with 20 mM Tris (pH 8.5). The extractions were performed in triplicates. The amounts of crude protein and Ara h 1 and 2 were measured in triplicate with the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and commercial Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.). All values are averages (± standards deviations) based on mg per g of peanut flour.

Additionally, 1D-SDS-gel electrophoresis was performed, showing that the protein band patterns of the peanut pools were indistinguishable (not shown).

It was concluded that small peanut pools of 10 peanuts are sufficient to test peanuts from different conditions, because they do not show any significant differences in protein and Ara h 2 content. Though the Ara h 1 content varied slightly in the samples a statistical difference could not be verified and a range was set that defines Ara h 1 content from similar population.

## *3.9.    Discussion*

### *3.9.1.    Centrifugation accelerations and times did not influence the extraction yield of total peanut protein using defatted flour*

Centrifugation accelerations of 3,000 and 12,500 g, centrifugation times of 5–30 min, as well as number of consecutive centrifugations (2–3) did not influence the extraction yield of total peanut protein using defatted flour. Generally, it would be expected that should be no difference between similar sedimentation rates (i.e., 3000gx 3 x 15 min and 12500g x 2 x 5 min), however, this has tob e verified. For extraction of peanut proteins there is no consensus in the literature regarding extraction accelerations and times. Koppelman et al., used 3000 g in the first centrifugation and then 10000 g for 30 min each (Koppelman et al. 2004), while some publications employ 3000 g for 5 min, then 10000 g for 15 min are used (Dodo et al. 2008, Koppelman et al. 2001). Some groups centrifuge the peanut extracts for 15 min at 10000 g (Boldt et al. 2005, Krause et al. 2010, Schmidt et al. 2009), while others use 10000 g for 10 in (Marsh et al. 2008), 5500 g for 15 min (Schmitt et al. 2010), 3000 g for 20 min (Kim et al. 2011, Poms et al.), 4000 g for 20 min (Mondoulet et al. 2005) or 8500 g for 10 min (Chung S. Y. et al. 2004). Kain et al. compared the centrifugal accelerations, finding that the proteins in the aqueous solution increased the higher the acceleration used. However, only the "rpm" is given and the model of the centrifuge not mentioned, which makes it impossible to calculate the centrifugal force "g". Moreover, the amount of protein was given relatively as % protein yield, and it is not clear how this percentage was defined (Kain et al. 2009).

### *3.9.2.    The extraction time influenced the Ara h 1 extraction efficiency*

The extraction efficiency of crude protein extraction was less efficient after 1 min and at 40˚C, while the Ara h 2 extraction efficiency was not affected by the extraction temperature and time. More Ara h 1 was extracted after 30 min compared to all other tested extraction times. The Ara h 1 abundance was not affected by the extraction temperature but was very low in all samples, probably due to the sub-optimal extraction time (1 h). The lower crude protein extraction efficiency at 40˚C is surprising, but the heterogeneity in the standard deviation compared to the other extraction temperatures, indicates that this result is likely due to the low number of replicates. Extraction times and temperatures vary greatly among the literature. Most common is the extraction of peanut proteins and allergens at 4˚C; e.g. (Burks et al. 1995, Chassaigne et al. 2007, Mondoulet et al. 2005) or room temperature; e.g. (Koppelman et al. 2001, Kopper et al. 2005), but also higher temperatures such as 40 – 60˚C (Kain et al. 2009, Pomes et al. 2006, Poms et al. 2004). Poms et al. observed that the

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concentrations of crude peanut protein obtained after extraction at 4˚C overnight and at 60˚C for 20 min were similar (Poms et al. 2004). Kain et al. tested 20, 30, 40, 60 and 70˚C for the extraction of crude peanut protein, and found that – in contrast to the results in this chapter - 40˚C had the highest efficiency, but the extraction procedure and buffers are not given clearly and extraction yields do not contain error bars and are not comparable to the results in this chapter, as it is not clear how the given percentage is defined (Kain et al. 2009). To date there are no publications that compare the extraction temperature and the extraction efficiency of allergens. The results of this chapter show that besides the extraction buffer and pH, it is not only the extraction temperature but also the time that influences the extraction efficiency of certain peanut proteins, particularly Ara h 1 and 2. The most vulnerable protein tested was Ara h 1, whose extraction was more efficient when done for 30 min than for 1 h. The earlier mentioned susceptibility of the sensitivity of the ELISA assay to the conformation of the Ara h 1 protein might also play a role. A fragmented form of Ara h 1 is thought to increase the detection rate of Ara h 1 due to a higher accessibility of epitopes (Cabanos et al. 2011, Maleki et al. 2000b), showing that an increased extraction time might possibly lead to higher quartiary structures; however, this has to be verified. Nevertheless, it seems surprising that the Ara h 1 extraction efficiency is much higher after 30 min, compared to 1 h.  Future experiments with extraction times of 10, 20, 30, 40, 50 and 60 min are necessary to examine the effect of time on the extraction efficiency of Ara h 1 more closely.

### 3.9.3.   *The concentrations of crude protein and Ara h 1 and 2 were significantly higher when n-hexane was used for defatting rather than diethyl ether*

The Ara h 1 content in the ether-defatted peanut extracts was 90% and the Ara h 2 amount 20% lower than in the hexane extracts. In order to obtain pure protein extracts and to extract lipids from the peanut extracts, both n-hexane (Burks et al. 1995, Cong et al. 2007, Marsh et al. 2008, Poms et al. 2004, Zeleny and Schimmel 2010) and diethyl ether (Kopper et al. 2005, McDermott et al. 2007, Mondoulet et al. 2005, Porterfield et al. 2009, Romano et al. 2009) are used in peanut allergen laboratories. n-Hexane is non-polar and extracts non-polar components such as lipids. Diethyl ether is more polar than n-hexane and can additionally solubilise some polar compounds and might therefore have extracted some protein fragments, including most of Ara h 1 and a large fraction of Ara h 2 into the discarded lipid fraction. Further analysis is necessary to identify the differences in extraction efficiency between defatting techniques and to clarify if these are only present in the lipid fraction of the extracts.

### 3.9.4. The detection of Ara h 1 depends on peanut processing

Overall, it was observed the consistency peanut homogenate was very different after processing, which influenced the solubility of proteins. Most crude protein and Ara h 2 was solubilised from raw peanuts, than from boiled and roasted peanuts. The 1D-gels (in which most proteins are denaturated) showed that some protein bands, including the monomeric form of Ara h 1, as well as one of the Ara h 2 bands and protein bands below 8 kDa were stronger in the raw extracts and very weak in boiled extracts. Accordingly, least crude protein, Ara h 1 and 2 was detected in protein extracts from boiled peanuts. The boiled peanuts resulted in a lower volume of the monomeric form of Ara h 1 on the SDS gels, and a similar abundance of the trimeric form. All protein bands were weaker and bands at around ~70 – 100 kDa were missing or streakier. This is in accordance with previous studies that observed that roasting progressively reduced the solubility of peanuts proteins compared to raw peanuts (Kopper et al. 2005, Maleki et al. 2001). The abundance of Ara h 1 and 2 on SDS-PAGE prepared with proteins from boiled peanuts varies across the literature. Mondoulet et al. observed that the Ara h 1 and 2 bands were less intensive in boiled peanuts on SDS-gels, compared to peanuts extracted from raw and roasted peanuts which had similar patterns (Mondoulet et al. 2005). While the trimeric form of Ara h 1 was faint in boiled extracts, the monomeric form of Ara h 1 was missing. Contrary, Beyer et al. found that the trimeric form of Ara h 1 is missing in boiled peanut extracts, the Ara h 1 monomer was present (Beyer et al. 2001). Generally, thermal processing can cause chemical modifications in proteins leading to their cross-linking, degradation, denaturation and combination of these events. This includes, changes to the conformation of protein including Ara h 1 when exposed to the heat in boiling water (Blanc et al. 2011), suggesting increased accessibility of linear epitopes (Chapter 3.4.2.). Nevertheless, Mondoulet et al. showed that the IgE-binding capacity of peanut protein extracts prepared from boiled peanuts was 2-fold lower than that of the extracts prepared from raw and roasted peanuts. This can be explained with the fact that peanut proteins were found in the cooking water and recognized by the IgE of peanut-allergic patients (Mondoulet et al. 2005). The allergens are therefore thought to be extracted into the water, which reduces the quantity of allergens in the boiled peanuts kernels. The loss of proteins into the water and the decreased solubility of boiled peanut proteins explain why there less protein is extracted from boiled peanuts.

Most Ara h 1 was detected in protein extracts from roasted peanuts. While the trimeric form of Ara h 1 (~145 kDa) was visible on gels with birth raw and roasted peanut extracts, the

roasted peanut extracts lacked the monomeric form of Ara h 1 (~64 kDa) and some Ara h 1 fragments at ~49 and 85 kDa on gels. The roasted peanut extracts were very streaky and showed bands with a higher volume at around 59 and 80 kDa, compared to raw peanut extracts. There seems to be variation in previous publications regarding the presence of specific protein bands on SDS-gels extracted from raw and roasted peanuts. In accordance with the results in this chapter, Kopper et al. observed the presence of the monomeric an trimeric form of Ara h 1 on SDS-gels with raw peanut extract, while all high molecular weight proteins, such as Ara h 1 were lost during the roasting process (178˚C 20 min roasting, extraction with TE buffer (pH 8.3)) (Kopper et al.). Contrary, other publications show that the monomeric form of Ara h 1 is present, while the trimeric form of Ara h 1 is absent in raw peanut extracts, while both conformations are present to higher or lesser extent in roasted peanuts (Maleki et al. 2001, Maleki et al. 2000b, Mondoulet et al.). Beyer et al. observed the same but did not show raw peanut extract on the gels (Beyer et al. 2001). The varying observations are likely to be due to the differences in the roasting process, extraction buffer differences, which might influence the stability and conformation of Ara h 1, the sensitivity of stains on the gels, handling of the samples and possibly differences between varieties (Mondoulet et al. 2005). Nevertheless, the loss of protein bands (including the monomeric band of Ara h 1), the appearance of other protein bands and the streakyness of the roasted protein extracts on SDS-PAGE, was often reported previously. Accoring to Maleki et al. the roasted extracts appear as smears on SDS-PAGE rather than well-defined bands. The Ara h 1 monomer seems to become slightly smaller in size or disappears into larger molecular weight smears during roasting, while Ara h 2 remains visible as two distinct bands for a longer period of time during roasting. The increased streakyness of Ara h 1 was concluded to be the result of cross-linking and non-cross-linking of adducts as a result if the Maillard-reaction during roasting and the degradation of proteins during the heating process (Maleki et al. 2000a, Maleki, 2001). Furthermore it was shown that Ara h 1 from roasted peanuts has higher IgE binding (Maleki et al. 2001), which might be the result of a higher accessibility of epitopes and might also explain the high Ara h 1 detection in the ELISA. This shows that some Ara h 1 fractions, including the monomeric form were fragmented during the roasting process. It is likely that a higher state of fragmentation causes a higher detection rate of Ara h 1 in the ELISA kit (see above). This is substantiated by the fact that the detected Ara h 1 is twice as high as in all other performed extractions. On the other hand Mondoulet et al., who used commercially roasted peanuts, stated that the immunoreactivity was not statistically significant between roasted and raw peanut extracts (Mondoulet et al. 2005). However, the

proteins were treated with urea, which might have caused conformational differences in Ara h 1 similar to differences by roasting, in raw peanuts. The lower amount of Ara h 2 observed in this chapter in the roasted peanut extracts might be due to a lower extraction efficiency or, due to the loss of sulfhydryl groups and hence its conformation as a result of the Maillard reaction, which therefore might have a similar effect on Ara h 2 as DTT (Maleki et al. 2003, Traverso et al.). This effect would denature the Ara h 2 molecule and lower the accessibility of IgE epitopes for the primary antibody in the Ara h 2 ELISA kit. On the other hand Mondoulet et al. showed that in the case of Ara h 2, inhibition studies show no difference in immunoreactivity between Ara h 2 from raw or boiled peanut extract and a higher immunoreactivity after roasting. This might be the case if the loss of sulfhydryl groups would only be partial, in which case the conformation of Ara h 2 would remain in a mostly native (Maleki et al. 2003), keeping the epitopes intact. If the immunoreactiviity of Ara h 2 remains the same after roasting, it might also mean that the ELISA is correct and less Ara h 2 was extracted from roasted peanuts.

These observations show that thermal processing of peanuts can decrease the solubility of some proteins and may alter the allergenicity of a protein depending on the chemical properties of the allergens, but also the processing conditions. Consequently, the detection and quantification of allergens using ELISA assays can be affected after processing, through chemical modifications, the loss of conformational and gain of linear epitopes. Importantly, the effect of the food matrix on the conformation and detection of allergens is unknown (Wal 2003).  The grade of the structural changes can therefore not be predicted accurately.  In order to have reliable relative measures in regards of the ELISA detection, it was concluded that the best way to compare peanuts from different growing conditions (in the following chapters) is with raw peanuts.

### 3.9.5.  *Using the standard protocol no proteins can be extracted from seed coats*

Employing the widely used procedures in extracting peanut proteins, including homogenisation of the peanut material and extraction of proteins with various buffers, did not result in any detectable protein from the seeds coats. Harsher methods resulted in very small, almost undetectable levels of proteins from the seed coats. This shows that the widely used extraction procedures for peanut seeds do not extract any or only extremely low amounts of protein from seed coats. Therefore seed coats should not be used for peanut protein extractions in order to have more accurate peanut flour measurements. In most publications it

is not mentioned whether the peanut kernels were used with seed coats or if they were peeled before the peanut protein extraction; e.g. (Koppelman et al. 2001, Kottapalli et al. 2008, Krause et al. 2010), while sometimes it is mentioned that the peanut kernels were peeled (Blanc et al. , Mondoulet et al. 2005). Kang et al. investigated the temporal and spatial expression of peanut allergens Ara h 1, 2 and 3 in developing peanut seeds, and found that the allergens are tissue specific, and were not present in leaves, flowers, or roots, but in observed in seeds, particularly both embryonic axes and cotyledons; however, the seeds coats were not tested (Kang et al. 2007). The only study that tested the protein content in peanut skins for cattle consumption used samples, which were mixed with processed peanuts, broken nuts and, sometimes, nuts that may have been rejected during the preparation of peanuts for human consumption (Ahmed and Young 1982) and are thus not representative. Therefore it is not clear whether the seed coats should be used for extraction or not. Generally the results in this chapter confirm that the abundance of proteins in the seed coats is extremely low, especially compared to the vast amount of peanut proteins (around ~25%) in the actual peanut kernel and can therefore be neglected in allergen research.

### 3.9.6. *Variation of crude protein and Ara h 1 and 2 content in small peanut pools*

Although the Ara h 1 content varied slightly between the small peanut pools, a statistical difference could not be verified and, given that no significant differences in protein and Ara h 2 content were observed, it was concluded that small peanut pools of 10 peanuts are sufficient to test peanuts from different conditions in the following chapters. In most publications, no mention is made of the number of peanut kernels used for the production of peanut homogenate before weighing it in for the peanut extraction; e.g. (Boldt et al. 2005, Cong et al. 2007, Koppelman et al. 2003, Poms et al. , Schmidt et al. 2009). Nevertheless, Kottapalli et al. states that six peanuts per peanut cultivar were used to find differences in protein expression. Differences that might arise due to the variation among these small peanut pools were not evaluated and differences in protein expression between the cultivars were attributed as real differences, including differences in the abundance of fragments of allergen Ara h 3 (Kottapalli et al. 2008).

The slight (statistically insignificant) variances in Ara h 1 found here are probably due to the susceptibility of the Ara h 1 ELISA assay to conformational changes in the Ara h 1 molecule, which might occur at different rates in the replicates. It also might be possible that the amount of Ara h 3 (which was not tested) varies in the samples, such as detected by Kottapalli et al.

(Kottapalli, 2008). However, these differences might be due to extensive post-translational modification of this allergen (Liang et al. 2006) and are probably unavoidable between replicates, even if more peanuts were used. In any case, the results in this chapter show that pools of 10 peanuts show no significant variances in crude protein, Ara h 1 and 2, and are therefore sufficient for the experiments reported in the following chapters.

### 3.9.7.   *The most efficient extraction method for crude protein, Ara h 1 and Ara h 2*

The most efficient, convenient and non-denaturing extraction method for crude protein (and Ara h 1 and 2) was achieved when extracting hexane-defatted peanut flour with 20 mM Tris (pH 8.5) for 30 min at room temperature (21˚C). This method resulted on average in the solubilisation of 541 – 623 mg crude protein per g of defatted peanut flour. Given that peanuts contain around 50% lipid (www.pca.com.au) and the peanut flour used was defatted, the total protein content in the peanut kernels measured in this chapter was therefore 27 – 31%.  While longer extraction times and lower or higher temperatures did not influence the crude protein extraction efficiency, buffer composition and pH value did have an influence.


Generally there is no consensus in describing the crude protein extraction yield across the literature. It is often impossible to compare yields because only protein concentrations or percentages are given, rather than absolute values based on the peanut material. Nevertheless, from some publications the extraction yield could be calculated and it was observed that that the protein extraction efficiency differs vastly in the literature, due to variation in peanut protein extraction methods. For instance, Mondoulet et al. extracted approximately 14% crude protein from 1 g of raw peanut material by defatting with ether, extracting overnight at 4˚C, and resuspending the pellet after centrifugation with urea (Mondoulet et al. 2005). Chassiagne et al. extracted around 21% protein in two sequential steps: 1. Extraction with TBS buffer (pH 7.4); 2. Extraction with 20:80 ethanol/water mixture (Chassaigne et al. 2007). Koppelman et al. extracted 26% protein with Tris (pH 7.2) for 2 h (Koppelman et al. 2004). These values refer to the amount of soluble protein after extraction, which are dependent on the extraction method and do not consider proteins that might not have been solubilised. Koppelman et al. measured the protein content in ground (but not defatted) peanut material using the Kieldahl method, which measures indirectly all protein present (via N content and a peanut specific conversion factor), including insoluble protein. It was found that runner peanut kernels (such as Walter) contain 24 – 28% protein (Koppelman et al. 2001). The extraction yield of 27 – 31% crude protein from ground peanuts in this chapter is therefore around 100%. The yield

value obtained might exceed 100%, due to variation in the measurement or the protein content of the variety Walter. The high extraction efficiency using Tris buffer is further substantiated by the fact that the additions of SDS, DTT and/or urea, which are used to denature and solubilise proteins, were not able to increase this efficiency. In any case it is very likely that the majority of proteins could be solubilised and extracted with the method specified in this thesis chapter.

The most efficient extraction method for either crude protein and Ara h 1 and 2 was achieved when extracting hexane-defatted peanut flour with 20 mM Tris (pH 8.5) for 30 min at 21˚C. This method resulted on average in 10.7 – 16.5 mg Ara h 1 (1.8 – 2.8% of total protein) and 38.6 – 43.9 mg Ara h 2 (6.6 – 7.5% of total protein) per g of defatted peanut flour. Given that peanuts contain around 50% lipids (www.pca.com.au) and the used peanut flour was defatted, this means that 0.5 – 0.8% Ara h 1 and 1.9 – 2.2% Ara h 2 were present in the peanut kernels.

The Ara h 1 extraction was more susceptible to the extraction protocol, including temperature and time, than was Ara h 2. The extraction efficiency was increased when the extraction was performed for 30 min at 21˚C, rather than 1 h at 40˚C. The most efficient extraction method for either crude protein and Ara h 1 and 2 was achieved when extracting hexane-defatted peanut flour with 20 mM Tris (pH 8.5) for 30 min at 21˚C. This method resulted on average in 10.7 – 16.5 mg Ara h 1 (1.8 – 2.8% of total protein) and 38.6 – 43.9 mg Ara h 2 (6.6 – 7.5% of total protein) per g of defatted peanut flour. Given that peanuts contain around 50% lipids (www.pca.com.au) and the used peanut flour was defatted, this means that 0.5 – 0.8% Ara h 1 and 1.9 – 2.2% Ara h 2 were present in the peanut kernels.

The concentrations of Ara h 1 were lower in samples analysed in this chapter compared to previously published measurements (Koppelman et al. 2001, Krause et al. 2010) and the extraction yield of Ara h 1 seemed to be very susceptible to the extraction protocol. Krause at al. detected 32% Ara h 1 in crude protein extract and concluded, given that the protein content of a peanut is on average 25% (Koppelman et al. 2001) and based on the assumption that the amount of extracted crude protein was 100%, that 7.8% Ara h 1 is present in a standard peanut kernel (Krause et al. 2010). However, Krause et al. used ammonium bicarbonate (pH 8.0) as extraction buffer, which was shown in this chapter to have a relatively poor crude protein extraction efficiency: approximately 50% less efficient than Tris (pH 8.5), despite being efficient for Ara h 1 extraction. This means that if all Ara h 1 was extracted, around

15.6 Ara h 1 was present in the peanut kernel. Accordingly, Koppelman et al. (2001) state that Ara h 1 is 13 − 16% of total protein. However, these often-cited values derive from of 1D-gels, where an "Ara h 1 band" was identified, by running purified Ara h 1 as a standard and quantifying using densitometry measurements. Considering that most bands in a 1D-gel of a complex crude extract contains multiple proteins, it is likely that the Ara h 1 content as described in Koppelman et al. is too high (a measurement of multiple proteins with the same molecular weight). However, it was observed on 2D-gels in this chapter that the volume of Ara h 1 was very high and only a few small spots had similar molecular weights, substantiating Koppelman et al.'s densitometry measurements. It is therefore not within consistence that the amount of Ara h 1 accounts for only 1.8 −2.8% of crude protein in this chapter, although being highly abundant on the gel. The Ara h 1 ELISA results in this and the following chapters have to be regarded with caution, as they depend on the conformation of the Ara h 1 molecules in the samples and standards in the ELISA kits, which can be influenced by different storage conditions, the number of freezing/thawing cycles, the identity of the extraction buffer and the protocols used, by both the manufacturer of the allergens standards and the consumer.

The amount of Ara h 2 extracted from peanut flour in this chapter (6.6 − 7.5% of total protein) is consistent with the measurements of Koppelman et al., who found 6.2 − 7.7% Ara h 2 in the total protein, although just densitometry measurements of 1D-gels were used after extraction with 20 mM Tris (pH 8.2) (Koppelman et al. 2001). Chen at al. detected 4% Ara h 2 by a competitive ELISA (Chen et al. 2011), from peanut flour defatted with ether and extracted with TBS (pH 7.4) and EDTS-free protease inhibitors overnight at 4°C. Importantly, in contrast to total protein, the amount of certain allergens, such as Ara h 1 and 2, can only be determined when they are solubilised, which means their abundance in the peanut kernels is just an estimate and depends on the current most efficient extraction method. The absolute value of Ara h 1 and 2 in the peanut kernels is therefore unknown, although extraction efficiencies can be discussed.

## *3.10. Conclusion*

Different centrifugation times and temperatures did not influence the crude protein and Ara h 1 and 2 extraction efficiency. Ara h 1 extraction was susceptible to extraction time. The defatting reagent n-hexane resulted in significantly more crude protein and Ara h 1 and 2, compared to diethyl ether. Seed coats should not be used for extractions, because literally no protein is extracted using standard methods. Peanut pools of 10 peanuts can be used for comparison of different peanut batches. The most efficient and convenient extraction methods for crude protein, Ara h 1 and 2, that is compatible with all employed subsequent experiments is: extraction of n-hexane defatted peanut flour (without seeds coats) with 20mM Tris for 30 min at 21˚C. This method was used for the peanut samples in the following chapters of this thesis.

# Chapter 4

## Impact of elevated atmospheric $CO_2$ during plant growth on the allergen content of peanuts

### *4.1. Summary*

To test if their allergenicity is affected by projected global $CO_2$ concentrations, peanuts were grown in ambient and elevated atmospheric $CO_2$ concentrations in greenhouses. The growth conditions in the greenhouses were plotted over time, the development of the peanut plants was documented in photographs at various stages and the plant performance in the different treatments compared. Using the most suitable method from chapter 3, the peanut proteins were extracted and measurements made of crude protein and Ara h 1 and 2 abundance using ELISA kits. 1- and 2D-gel electrophoresis as well as 2D-DIGE were used to compare the expression of most individual proteins and allergens. Due to environmental differences between replicate greenhouses, no conclusions on the impact of elevated $CO_2$ on peanut allergenicity could be drawn. Although the conditions in the greenhouses varied between the greenhouses and had partly influenced plant performance, none of the allergens was completely absent or found at unusually high abundance in any of the samples, indicating that the environmental conditions did not influence the allergenicity of the peanuts.

## *4.2. Results*

### *4.2.1. First trial: set-up and outcomes of growing peanut plants in greenhouses with ambient and elevated $CO_2$*

In order to identify the difficulties that might arise during the relative long period of peanut growth (4 – 6 months) in the greenhouses, a preliminary trial was performed. Four similar greenhouses were allocated for growing peanuts. These greenhouses were temperature controlled (night / day: ~20˚C / ~30˚C) and two of them were fitted with gas cylinders connected to $CO_2$ supply systems and monitoring devices. These apparatus provided a constant atmospheric $CO_2$ concentration of around ~700 ppm during the daytime only as is standard practice for $CO_2$-enrichment experiments (Vu 2005) which is when photosynthesis and almost the entire associated $CO_2$ uptake occur. However, in the trial run no functioning independent data loggers to measure temperature and $CO_2$ concentration had been fitted; thus the system could be monitored only by inspecting the approximate temperature on a daily basis, and estimating the $CO_2$ concentration (by the technical staff in the greenhouse facilities) using data loggers that were not calibrated. After 28 days, a few days before the first flowers appeared on the plants, it was discovered that, due to a technical failure, the concentration of $CO_2$ in one of the greenhouses assigned to elevated $CO_2$ had been only ~400 ppm since the start of the trial. The value was adjusted from then on to ~700 ppm $CO_2$. In addition it was observed that Greenhouse 2 had been exposed each day to the direct sun all afternoon, while the other greenhouses had been shaded (4.2.2.1; Figure 4.1; Greenhouse 2).

Eight 30 cm-diameter pots per greenhouse were fitted with newspaper and filled with 14 l commercially available loamy soil (Greenlife native mix, Australia Native Lansdcapes Pty Ltd) mixed with 1 part to 2 parts of river sand. Runner peanut seeds (variety "Walter") were provided by the Peanut Company of Australia and five of them planted in each pot approximately 4 cm apart and 4 cm deep and covered with soil. The peanut seeds were watered with 1 l of water per pot each day for 14 days, then every second day for a further 14 days. Under the presumption that the peanut plants in each pot were competing for space and nutrients, the one tallest, greenest and most vigorous plant in each pot was left to grow, while the remaining, weaker plants were discarded 10 days after planting (DAP). Following the advice of the Peanut Company of Australia, 10 g of Osmocote (Scotts Australia Pty Ltd) was added to each pot to provide slow-release fertiliser for the peanut plants.

After 14 days some of the peanut plants started becoming mouldy on the base of the stems and small insects could be found in the pots. Therefore a fungicide was sprayed onto the plants (Yates Lime Sulphur Spray Fungicide) according to the manufacturer's instructions. At 74 DAP some of the plants had started to become yellow and weak, which was thought to be due to nitrogen deficiency. Therefore nitrogen fertiliser (urea) was applied to all plants. Moreover, mites could be detected on the leaves around this time, especially on the plants that had been infested with flies previously. Therefore lime sulphur (Yates Lime Sulphur Spray) was applied to the infested plants again. During the remaining period of plant growth most plants became mouldy on the base of the stem and were infested with flies and mites. Despite all efforts, only approximately 20% of the plants survived the trial experiment. After a few new shoots had started to grow in each greenhouse, indicating that mature peanut seeds were present in the soil, the peanuts were harvested (at 176 DAP). Surprisingly, after withholding watering for 4 days prior to harvesting, pulling out the peanut plants revealed that the lower half of the soil remained drenched with water while the upper half of the soil was dried out. This was due to the high evaporation rate in the greenhouses, which was caused by the rapid air-circulation and indicated a very low drainage capacity of the soil, which was too loamy. This effect was further enhanced by the small layer of newspapers on the bottom of the pots, and was clearly the reason for the mouldy bases of the stems and roots and the overall weakness of the peanut plants. A few plants were still able to produce mature peanut seeds, of which most appeared to be bleached or mouldy on the outside; however, most of the peanut seeds were immature.

The peanut kernels obtained from the trial experiment were not used for any analyses in this thesis but the trial gave an excellent opportunity to improve conditions for subsequent greenhouse experiments including: (1) conforming the solar radiation in Greenhouse 2 to that of the other greenhouses and ensuring better monitoring of most environmental conditions; (2) providing a soil composition with higher drainage capacity and better-draining pots; (3) increasing the sample size; (4) ensuring careful watering and monitoring of moisture in the lower part of the pots; (5) providing earlier application of liquid nitrogen fertilizer, and (6) developing a more sophisticated way to harvest for an increased yield of mature seeds.

The experimental procedure, addressing all mentioned improvements, is described in detail in the methods part of this thesis.

## *4.2.2. Experimental conditions*

### 4.2.2.1.    Greenhouse set-up

Based on the experiences gained from the trial (4.2.1), peanut plants were grown in a more sophisticated way in four temperature-controlled greenhouses, which were located on a rooftop at Macquarie University (Figure 4.1). Greenhouses 1 and 3 did not have any CO$_2$ supply and stayed at ambient CO$_2$ concentrations throughout the experiment. In both greenhouses with elevated CO$_2$ (Greenhouses 2 and 4) a CO$_2$ gas cylinder was fitted with a timer, controller and valve, which maintained the CO$_2$ concentration at around 700 ppm during the daytime. Notably, Greenhouses 1 and 2 were enclosed and had separate entries, while the replicate Greenhouses 3 and 4 were slightly bigger, had one external entrance and were connected via a door. The fluctuation of CO$_2$ from the elevated to the ambient greenhouse was kept to a minimum by rubber sealing in the doorframe and by closing the door immediately after entering and placing a cloth tube filled with sand in front of the door, which kept the CO$_2$ from flowing into Greenhouse 3
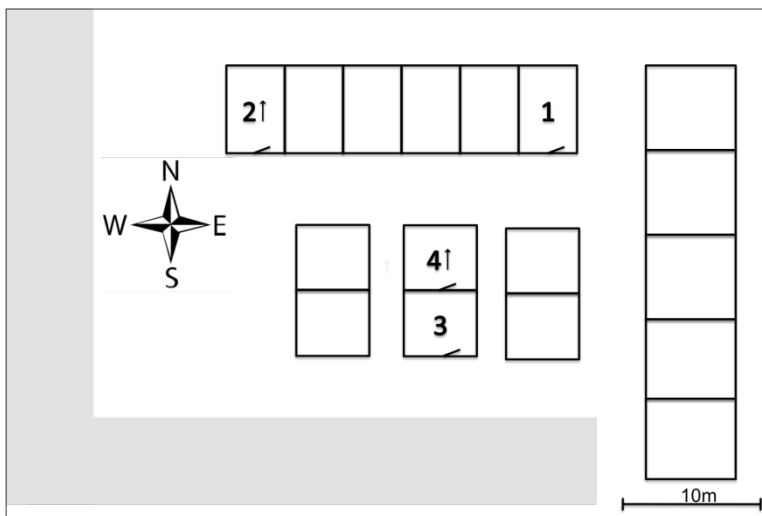


**Figure 4.1 Position and orientation of the greenhouses in this experiment**

The greenhouse facilities were located on a rooftop at Macquarie University in Sydney. Each of the black boxes represents one greenhouse. The four greenhouses allocated for this experiment are numbered. Arrows (↑) represent greenhouses with elevated atmospheric CO$_2$, while the other greenhouses have ambient CO$_2$. The entrances are marked in these greenhouses, showing that Greenhouses 1 and 2 are individual greenhouses, whereas Greenhouse 4 could be entered only through Greenhouse 3. The compass point allows an indication of the direction of the sun (note Sydney's latitude is 33" south and the sun appears to travel north during the course of the day). The grey boxes represent adjacent buildings (not taller than the greenhouses).

### 4.2.2.2.    CO$_2$ concentrations in the greenhouses

Because the aim of this experiment was to test the influence of elevated CO$_2$ during plant growth on the content of allergens in peanuts, the CO$_2$ concentration was the crucial condition in the experiment, and the corresponding data collected from Greenhouses 1 and 2 were

analysed using Matlab software (The Mathworks Inc., 2009). The data for each greenhouse were plotted and checked for any obvious errors. Some data points, as recorded by the data loggers, represented values of zero $CO_2$ (always around the time when the data were retrieved from the loggers) and were filtered from the data (using the "nan" function on Matlab) before calculating any subsequent functions. Greenhouse 2 contained around 325 ppm more $CO_2$ during the day compared to Greenhouse 1, which had 366 ppm $CO_2$, while the night-time values were very similar (Table 4.1). Furthermore, the average $CO_2$ concentration in the ambient-$CO_2$ Greenhouse 1 was approximately 50 ppm lower during the day compared to the night. Although Greenhouses 3 and 4 did not contain $CO_2$ data loggers, it was observed that the $CO_2$ concentration was around 400 – 410 ppm in greenhouse 3 and around 700 ppm in Greenhouse 4 during daytime throughout the experiment. The daily data for Greenhouses 2 and 4 (elevated $CO_2$) were therefore very similar, while the ambient-$CO_2$ Greenhouse 3 had approximately 50 ppm more $CO_2$ on average compared to greenhouse 1 during daytime. This was probably due to small flow of $CO_2$ from Greenhouse 4 to Greenhouse 3 through the door (Figure 4.1). With the existing data it cannot be calculated if this difference is statistically significant or not. According to technical staff working in the greenhouse the fluctuation is expected to be around 20 – 25 ppm.

**Table 4.1 Average atmospheric $CO_2$ concentration in the greenhouses during the experiment**

Averages and standard deviations are given for atmospheric $CO_2$ concentration during day and night.

| Greenhouse | Day $CO_2$ [ppm] | Night $CO_2$ [ppm] |
|---|---|---|
| 1 | 366 ± 41 | 410 ± 32 |
| 2 | 689 ± 35 | 443 ± 45 |
| 3 | ~400–410* | – |
| 4 | ~700* | – |

\* estimated value, due to random observations

To illustrate the fluctuation of $CO_2$ in Greenhouses 1 and 2, the measured values and their weekly running averages were plotted over time (Figure 4.2). The crosses in the figure represent individual measurements, which have been divided into day measurements from 06:00 to 18:00 h (red) and night measurements from 18:00 to 06:00 h (blue), while the line represents the weekly running averages of the day and night values. For Greenhouse 1 (with ambient $CO_2$) the day and night curves lie very close together, verifying that the $CO_2$ concentration was raised during the night by only 50 ppm on average, when the plants do not photosynthesize (and no $CO_2$ is taken from the atmosphere). In Greenhouse 2 (with elevated $CO_2$) the day and night values lie further apart, indicating that the elevation of $CO_2$ to about 700 ppm was relatively constant throughout the experiments. The red and blue crosses

between the weekly averages were measured around 06:00 or 18:00 h and indicate a decay in the $CO_2$ concentration, when turning the $CO_2$ supply on and off during that time.
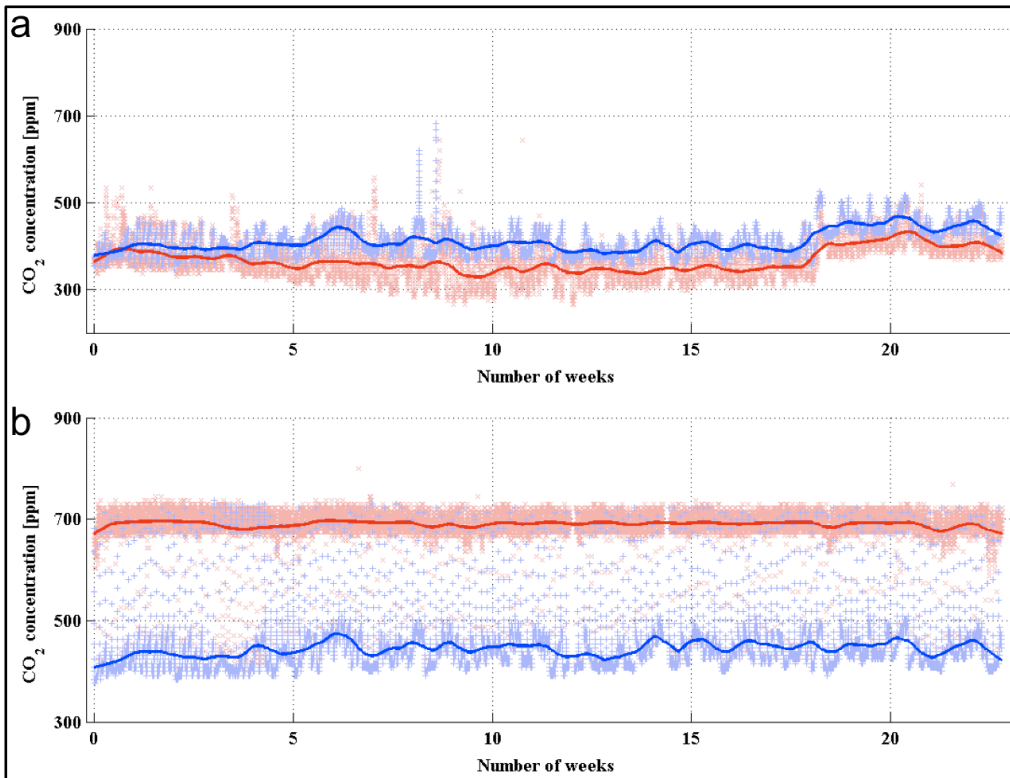


**Figure 4.2 Atmospheric $CO_2$ concentration in greenhouses 1 and 2 during the experiment**

(a) $CO_2$ concentration in Greenhouse 1; (b) $CO_2$ concentration in Greenhouse 2. Marks represent $CO_2$ concentrations measured during the day (red) and night (blue). Lines represent a time series of weekly averaged $CO_2$ data for day (red) and night (blue). The plots were produced using Matlab software (The Mathworks Inc., 2009). Number of weeks refers to the timecourse of the experiment in the greenhouses from 3 DAP (when the plants were moved into their designated greenhouses) to the last day of harvesting.

It was concluded that it is very likely that the greenhouses with elevated $CO_2$ (2 and 4) had similar $CO_2$ concentrations during the day, whereas ambient greenhouses (1 and 3) had slightly different average $CO_2$ concentrations. Greenhouse 3 had approximately 50 ppm more $CO_2$ during the day but, due to a lack of data, it is not clear if this is statistically significant. These conclusions were based on regular $CO_2$ measurements in Greenhouses 1 and 2 and random observations in Greenhouses 3 and 4 during daytime.

### 4.2.2.3. Environmental conditions other than $CO_2$ concentration

To be able to ascribe any effects on allergen composition of the peanut seeds to $CO_2$ concentration, it was crucial to test whether other environmental conditions, including light intensity, relative humidity and day and night temperatures, were comparable in all four greenhouses throughout the experiment.

**Table 4.2 Average light intensity, relative humidity and temperatures in the greenhouses during the experiment**

The light and humidity measurements were taken every 3 min for the entire time of the experiment. The temperatures were taken every 5 min. Averages and standard deviations are given.

| Greenhouse | Light intensity [lx] | Relative humidity [%] | Day temperature [°C] | Night temperature [°C] |
|---|---|---|---|---|
| 1 | 9.1e+03 ± 2.0e+04 | 67.6 ± 15.9 | 27.0 ± 2.5 | 20.2 ± 0.9 |
| 2 | 7.6e+03 ± 1.7e+04 | 56.1 ± 15.2 | 27.3 ± 2.2 | 19.6 ± 1.5 |
| 3 | 8.9e+03 ± 1.8e+04 | 63.3 ± 15.4 | 27.6 ± 1.9 | 20.1 ± 1.3 |
| 4 | 1.7e+04 ± 3.4e+04 | 69.7 ± 14.8 | 28.5 ± 1.9 | 21.2 ± 1.5 |

On first sight, the data suggest that the light intensity values in Greenhouses 1 – 3 were relatively similar, but higher in greenhouse 4 (Table 4.2). The relative humidity and day and night temperature appeared to be similar between all greenhouses (Table 4.2). However, these values do not illustrate the range of conditions to which the peanut plants were exposed over time and do not reflect whether there were any critical events (e.g. a heat event) in any of the greenhouses that might have affected the plants.

To determine the conditions to which the plants were exposed over time and identify any critical events, graphical time-series were generated by plotting weekly running averages of all measured values over time and comparing them to weekly running averages of average values among the greenhouses (Figure 4.3, left panel). The quantification of differences between the conditions in the greenhouses were facilitated further by plotting anomalies, which were obtained by subtracting the average values from the data points in the individual greenhouses (Figure 4.3, right panel).

The most important finding was that plants in Greenhouse 4 (red line) were exposed to more sunlight during the entire period of the experiment compared to plants in the other greenhouses. The residuals in the anomalies plot (Figure 4.3b) show that plants in Greenhouse 4 were exposed to 10,000 –30,000 lx greater light intensity than plants in the other greenhouses in the first 10 weeks of the experiment and 10,000 – 20,000 lx greater light intensity for the remaining time. It was also shown that plants in greenhouse 1 (green line) were exposed to 20,000 lx less than in the other greenhouses in the second week of the experiment. In week 5 the light intensity in Greenhouse 1 was 5000 lx above average, while Greenhouses 2 and 3 received 10000 – 150000 lx less than average. Greenhouses 1 and 2 received similar light intensities throughout the experiment. When plotting all light intensity measurements (including night measurements below 400 lx) the same result was obtained, except that the weekly averages were lower (as expected; not shown). Overall the data show

that the light intensities in the greenhouses were generally not constant between the treatments over time and that Greenhouse 4 received more light throughout the experiment than any of the other greenhouses.
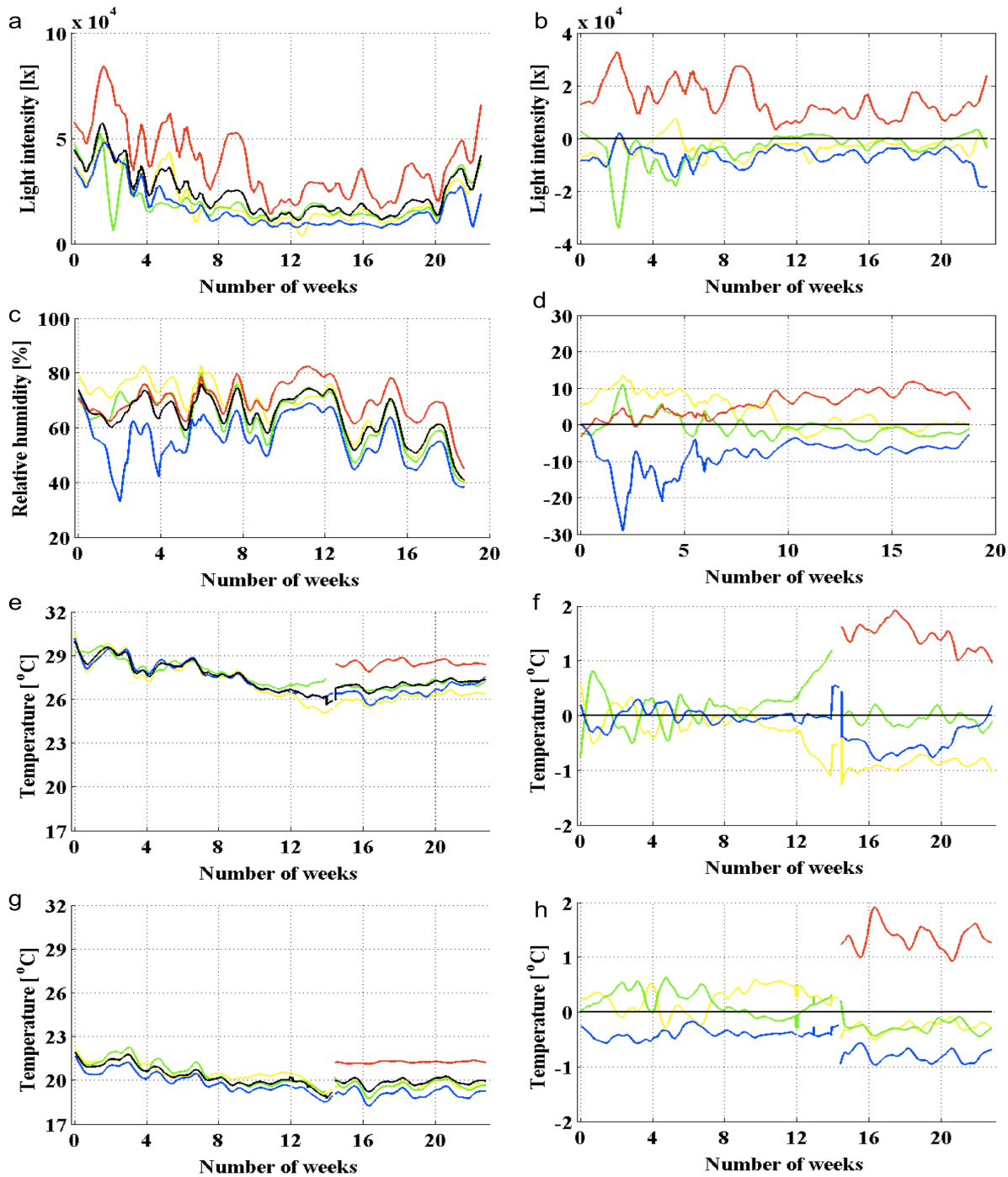


**Figure 4.3 Time series, averages and anomalies of environmental conditions in Greenhouses 1–4 during the experiment**

Weekly averaged time-series and anomalies for: (a,b) light intensity during day (values >400 lx); (c,d) total relative humidity; (e,f) temperatures during day (06:00–18:00); (g,h) temperatures during night (18:00–06:00); The relative humidity time series runs until the first day of harvesting at 133 DAP; The temperature data in Greenhouse 4 were recorded only from 101 DAP. The graphics were generated using Matlab (The Mathworks Inc., 2009). Number of weeks refers to the time-course of the experiment in the greenhouses from 3 DAP (when the plants were moved into their designated greenhouses) to the last day of harvesting 159 DAP. (yellow) Greenhouse 1; (blue) Greenhouse 2; (green) Greenhouse 3; (red) Greenhouse 4; (black) average between Greenhouse 1–4.

The plots showing relative humidity (Figure 4.3c,d) only cover the experiment until the first day of harvesting (133 DAP), because all following data contained errors due to equipment failure (recorded values for relative humidity were >10000%). Furthermore, some relative humidity measurements in greenhouse 1 were clearly in error (frequent sudden single drops in relative humidity to exactly 16.2%) in the first month of the experiment and were also filtered from the data. Markedly, the humidity in Greenhouse 2 dropped twice in the first four weeks to 20–30% below average, in contrast to all other greenhouses (Figure 4.3c,d). Greenhouse 1 appeared to have a higher relative humidity in the first seven weeks but came close to the average for the remaining time. In contrast, relative humidity in Greenhouse 4 appeared to increase gradually over time and was around 8–10% higher compared to the other greenhouses in the last 10 weeks. The relative humidity in Greenhouses 1 and 2 was very similar for most of the experiment.

The day temperatures in Greenhouses 1–3 were very close to average during night and day for the first few months but differed in the last few months (Figure 4.3e,f). The temperature of Greenhouse 1 dropped markedly to 1˚C below average during this time. However the average temperature was higher because data for Greenhouse 4 were available only for the last 2 months of the experiment and were constantly 1–2˚C higher than average. The night temperatures were very close to average throughout the experiment in Greenhouses 1–3 but Greenhouse 4 was constantly 1–2˚C warmer (Figure 4.3g,h).

Importantly, one extreme heat event was observed in the second week of the experiment in Greenhouse 4, which was not recorded in the data. The air-conditioning failed on a very hot day, so that the temperature rose to over 60˚C for almost all the day (10 DAP), and over 59˚C for several hours on the next day. The adjacent Greenhouse 3 was only slightly affected by this event, being around 1˚C warmer than Greenhouses 1 and 2 at this time, which could also be due to natural variation and was therefore not significantly influenced by the heat event in Greenhouse 4.

In summary, the environmental conditions other than CO$_2$ concentration were different between the greenhouses. In particular, Greenhouse 4 was exposed to higher light intensities throughout the experiment an extreme heat event in the first 2 weeks of plant growth (10 – 11 DAP).

### 4.2.3. Analysis of plant growth and peanut proteins/allergens

#### 4.2.3.1.    Observation of plants in the greenhouses

The peanut plants were observed throughout the experiment to ensure their vigour, to be able to react quickly to any irregularities and to monitor any differences in plant development between the greenhouses. The different stages of plant growth were recorded in each greenhouse by taking photographs with a digital camera (Figure 4.4). The first shoots were visible in all pots 3 DAP (Figure 4.4a), whereupon the pots were moved immediately to their respective greenhouses. At 10 DAP all plants in all greenhouses were around 10 cm tall and appeared to be healthy (Figure 4.4b). Based on the presumption that the peanut plants in each pot were competing for space and nutrients and to ensure that the sample size was as large as possible, only the two tallest, greenest and most vigorous plants per pot were left to grow at this stage.

A heat event (4.4.2) burned one to three leaves on almost every plant in Greenhouse 4 at 10 DAP (Figure 4.4c) and these heat-induced symptoms became worse over the coming weeks. Some of the plants dried out completely and died. The backup plants, which were placed in each greenhouse at the beginning of the experiment, were moved from Greenhouse 2 (the replicate elevated $CO_2$ greenhouse) to Greenhouse 4 and were transplanted into bigger pots. While most of the plants in greenhouse 4 recovered from the incident over the coming weeks, the backup plants did not cope well with the transplantation process and appeared to be very weak. They were therefore not used in the following experiments reported in this chapter.

The plants in all greenhouses (except for the transplanted backup plants) produced their first flowers around 32 DAP (Figure 4.4d). While new flowers appeared throughout the experimental period, they always withered after around 3 d after self-pollination, as this occurs naturally (Figure 4.4e). The pegs (Figure 4.4f) developed quickly and grew into the soil, where the seeds started to mature. While all plants looked mostly healthy (Figure 4.4g,h), the plants from Greenhouse 4 were still were losing stems at 59 DAP, most likely as a result of the early heat event. It was observed that they nevertheless grew large and had sturdier stems than plants from the other greenhouses.

**Figure 4.4 Peanut plants in the greenhouses**

Photographs were taken throughout the peanut plant growth period 07.03–13.08.2010. (a) 5 DAP: germinating peanuts;  (b) 10 DAP: most vigorous peanut plant was selected; (c) 11 DAP: a heat event burned outer leaves on the plants; (d) 32 DAP: first flowers appeared; (e) 35 DAP: first flowers were self-pollinated and withered; (f) 45 DAP: pegs are growing towards soil; undeveloped peanut at the end of peg visible; (g) 59 DAP: unfolding growing leaves; (g) 59 DAP: plants in the greenhouses 1, 2 and 3; (i) 59 DAP: the heat event still affecting the plants from greenhouse 4; (j) 160 DAP: last day of harvesting peanuts; peanut plant with pods; (k) 160 DAP: peanut pods; (l) 160 DAP: new shoots growing in the pots (from seeds that have not been harvested) 2–3 days after they were mature.

In order to maximise yield the peanut pods were harvested on three different dates: 133, 145 and 159 DAP (Figure 4.4g,h). A gentle harvesting technique was used so as not to disturb the plants, thus allowing additional mature seeds to form. Mature peanut seeds that were accidently left in the soil during harvesting germinated within 2–3 days in the pots (Figure 4.4i).

In summary, the peanut plants developed similarly in all greenhouses and, except for the heat event in Greenhouse 4, no major event threatened the plants. A number of mature peanuts could be harvested from each plant in the greenhouses and used for analysis as described in the following paragraphs. The peanut plants from all four greenhouses were used for the analysis of plant performance and the harvested kernels for the content of protein and Ara h 1 and 2. However, because of the heat stress and relatively higher light intensity to which the plants from Greenhouse 4 were exposed, samples from this greenhouse were *post hoc* excluded in the comparison of the effects of ambient $CO_2$ on the allergen content of peanuts.

### 4.2.3.2.        Peanut samples acquired from the greenhouses

Based on the presumption that the peanut plants in each pot were competing for space and nutrients and only the dominant plant would give representative results, only the taller and more productive plant of the two plants in each pot was used for analyses described in the following paragraphs. Although peanuts from all four greenhouses were used to test the effect on plant performance, *post hoc* only the data from Greenhouse 1, 2 and 3 were used to draw conclusions regarding the effects of elevated $CO_2$ on peanut plants in the following chapters. This was a result of the distinct light intensity conditions and the heat event that had stressed the peanut plants in Greenhouse 4. Extracts from Greenhouse 4 were therefore excluded from experiments involving 2D gels, Western blots and 2D-DIGE.

### 4.2.3.3.        Effects of growth conditions on size and yield of plants

Experiments were conducted to test whether the conditions in all four greenhouses had an effect on the performance of the peanut plants. However, only the data from Greenhouse 1, 2 and 3 were used to draw conclusions regarding the effects of elevated $CO_2$ on peanut plants. Box-plots and means and 95% confidence intervals were plotted along with a two-way ANOVA, showing significant differences or similarities in plant performance between the treatments (Figure 4.5). The yield, dry weight of shoots, and weight of pods and seeds were acquired after harvesting. Root weights were not measured because of the very fine structure of the roots, which made the roots difficult to handle.
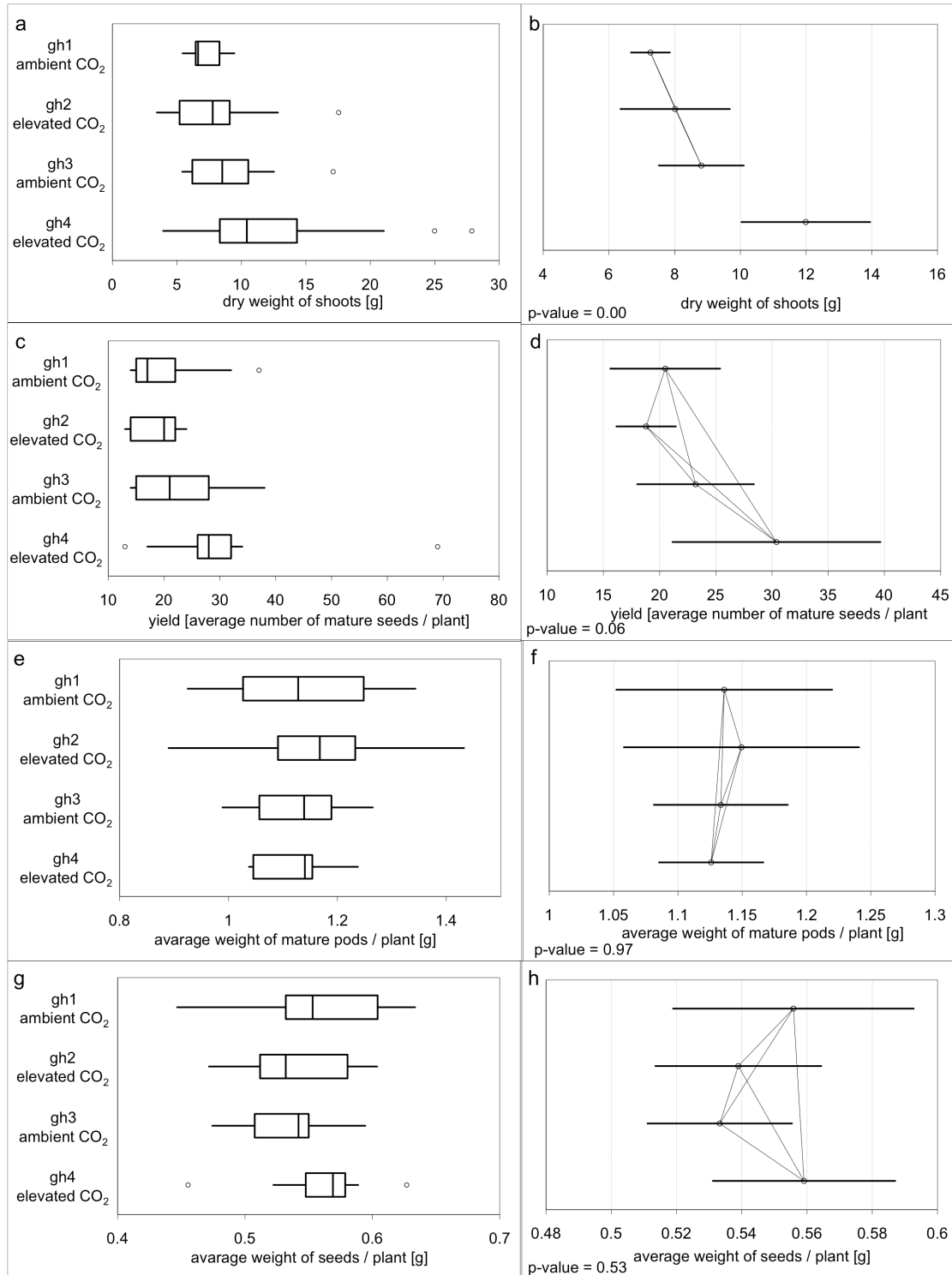
**Figure 4.5 Yield and average weight of shoots, pods and seeds of peanuts grown in greenhouses with ambient and elevated $CO_2$ concentration**

Box-plots and means with 95% confidence intervals. (a,b) Dry weight of the peanut shoots; (c,d) Yield as average number of mature seeds per plant; (e,f) Average weight of mature peanut pods per plant; (g,h) Average weight of seeds per plant. A line between two means represents a significant similarity, while the absence of a line between means represents a significant difference between these means. The p-values are given for comparison of all data groups with one-way-ANOVA; in all figures n = 10 plants per treatment.

The dry weight data for the 10 peanut shoots per greenhouse showed that there were highly significant differences in plant growth between the greenhouses (Figure 4.5a,b). While the dry weight of most peanut shoots from Greenhouses 1–3 was 6–9 g, more than 50% of the plants in Greenhouse 4 had a dry weight of more than 10 g. Comparing the means and 95% confidence intervals, and according to the one-way-ANOVA (analysis of variance), Greenhouses 1–3 appeared to produce shoots with similar dry weights. The dry weights of shoots from Greenhouse 4 were significantly higher than those from Greenhouses 1 and 3 but similar to those from Greenhouse 2, with a p-value just above 0.05 (0.0572). The shoots from plants in Greenhouse 4 also appeared to be sturdier compared to shoots from the other greenhouses, with thicker stems, but less leaves. It was concluded that the distinct growth conditions in Greenhouse 4 had an influence on the weight of the shoots, while no effect of $CO_2$ (in Greenhouses 1–3) could be detected.

Peanut yield was determined by counting the number of mature seeds per plant, which were identified by size, reddish colour and dry texture of the seed coat. In Greenhouses 1 and 2 more than 50% of plants contained 15–22 mature seeds per plant (Figure 4.5c,d), while 50% of plants in greenhouse 3 had a larger number of mature seeds (20–27) per plant on average. Nevertheless, the plotted means for all samples were statistically similar (Figure 4.5d). An effect of elevated $CO_2$ on the yield of peanut could therefore not be identified. Although most of the plants in Greenhouse 4 contained more mature seeds than the plants from the other greenhouses (26–31), the difference was not significant.

The average weights of mature peanut pods and peanut seeds were similar in all treatments and did not show any correlation with the conditions in the greenhouses (Figure 4.5e,f and g,h).

Overall, the peanut plant performance was most affected by the environmental conditions in Greenhouse 4, which was exposed to higher levels of sunlight during the entire period of plant growth, as well as a heat event that put the plants under stress in the second week of the experiment. The effect was only significant for the dry weight of the plant shoots. The $CO_2$ concentration in the greenhouses did not seem to have any effect on shoot dry weight, yield, pod weight and seed weight.

**4.2.3.4.**      **Total protein concentration and Ara h 1 and 2 content of peanuts grown in greenhouses with ambient and elevated CO$_2$ concentrations**

In order to extract peanut proteins and allergens according to the findings in Chapter 3 (3.9.7), eight peanut plants were randomly chosen (out of 10 per greenhouse). Ten peanuts per plant were pooled (Chapter 3.8.6.) and the seed coat removed (3.8.5) before defatting the raw peanuts (3.8.4) with hexane (3.8.3) and extracting once (3.3.3) with 20 mM Tris (pH 8.5) (3.3.1) for 30 min at 21˚C (3.8.2.). The amount of crude protein and Ara h 1 and 2 were measured with the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and commercial Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.). To illustrate the results and determine differences in the measured protein and allergen concentrations from peanuts grown in different greenhouses, box-plots and means with 95% confidence intervals were plotted and one-way ANOVA applied.

There were significant differences ($p < 0.01$) in the amount of extracted protein from the peanuts (protein content expressed as mg of protein per g of peanut flour) harvested from Greenhouses 1–4 (Figure 4.6a,b). The protein extracts obtained from plants grown in Greenhouse 2 contained on average the highest amount of protein per g peanut flour (768 ± 14 mg), while protein extracts obtained from plants grown in Greenhouse 4 (the replicate greenhouse with elevated CO$_2$) contained on average the lowest amount of protein per g of peanut flour (683 ± 12 mg). This further supports the finding that the distinct light conditions and/or the heat event (4.2.2.3) in Greenhouse 4 had a significant impact on the plants in this greenhouse. Greenhouse 1 appeared to have the highest spread of values ranging from around 670 to 800 mg protein extracted per g peanut flour. Although the protein content per g of peanut flour of peanuts from Greenhouses 1 and 2 was similar when all greenhouses were compared with each other (4.6b), the protein content was significantly higher ($p < 0.02$) in plants grown in elevated CO$_2$ when the data from the two ambient-CO$_2$ greenhouses (1 and 3) were combined and compared to the elevated CO$_2$ Greenhouse 2 (Figure 4.6c,d). 128-while 768 mg (± 14 mg) was the average in extracts from plants from elevated CO$_2$ samples.

The amount of Ara h 1 in the protein extracts was significantly different ($p < 0.01$) between the greenhouses (Figure 4.6e,f). Extracts from Greenhouse 2 (elevated CO$_2$) had a significantly lower Ara h 1 content than extracts from Greenhouses 3 and 4 and the highest variance of Ara h 1 content (~3–14 mg Ara h 1 per g of peanut flour), while the amount was lower but statistically similar to extracts from the ambient-CO$_2$ greenhouse 1. Extracts from Greenhouse 4 contained on average the highest amount of Ara h 1 (~13–17 mg), which was

statistically higher than extracts from Greenhouses 1 and 2. When comparing the Ara h 1 content of peanuts from ambient and elevated $CO_2$ (without Greenhouses 4), the greenhouses with ambient $CO_2$ had significantly higher Ara h 1 content (Figure 4.6g,h), indicating a possible $CO_2$ effect. The percentage of Ara h 1 in the crude protein reflects very similar findings to those for Ara h 1 content.

The amount of Ara h 2 in the protein extracts was significantly different ($p < 0.01$) between the greenhouse treatments (Figure 4.6m,n). Extracts from Greenhouse 2 had the highest spread of Ara h 2 values (~15–83 mg Ara h 2 per g of peanut flour) with most esxtracts containing the lowest measured Ara h 2 content. Extracts from Greenhouse 4 contained on average the highest amount of Ara h 2 (~68–89 mg). Although the Ara h 2 content in extracts from Greenhouse 4 was similar to that from greenhouse 3, the other greenhouses had significantly less Ara h 1 and 2. When comparing the ambient $CO_2$ Greenhouses 1 and 3 with the elevated $CO_2$ Greenhouse 2, the Ara h 2 content was statistically similar and the amount of Ara h 2 was not correlated with the $CO_2$ concentration (Figure 4.6o,p).

As a result of having the lowest amount of crude protein per g peanut flour but the highest amount of Ara h 2, Greenhouse 4 had the highest percentage Ara h 2 of crude protein (Figure 4.6qr). When determining the corresponding percentages for Greenhouses 1–3 it emerged that the Ara h 2 percentage was significantly lower in the greenhouses with elevated $CO_2$ compared to the ambient-$CO_2$ greenhouses. This is because the amount of crude protein was on average significantly lower in the ambient-$CO_2$ greenhouses, while the Ara h 2 content was similar.
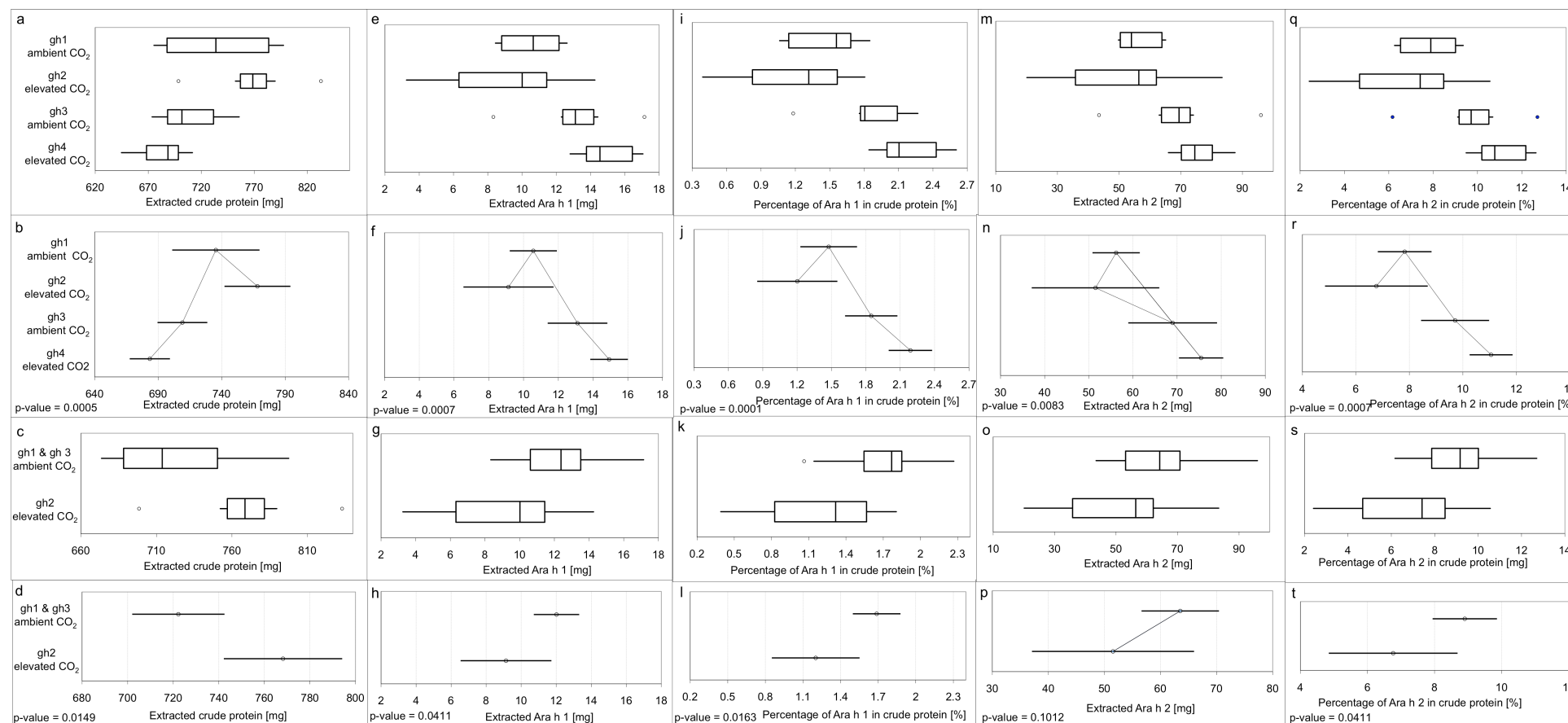
**Figure 4.6 Crude protein and Ara h 1 and 2 content of peanut seeds from plants grown in greenhouses with ambient and elevated $CO_2$**

Box-plots and means with 95% confidence intervals: (a,b) Amount of extracted crude protein per g of peanut flour in all four greenhouses and (c,d) Greenhouses 1–3, ambient vs. elevated $CO_2$; (e,f) Amount of extracted Ara h 1 in all four greenhouses and (g,h) Greenhouses 1–3, ambient vs. elevated $CO_2$; (i,j) Percentage of extracted Ara h 1 of extracted crude protein in all four greenhouses and (k,l) Greenhouses 1–3, ambient vs. elevated $CO_2$; (m,n) Amount of extracted Ara h 2 in all four greenhouses and (o,p) greenhouses 1–3, ambient vs. elevated $CO_2$; (q,r) Percentage of extracted Ara h 2 of extracted crude protein in all four greenhouses and (s,t) Greenhouses 1–3, ambient vs. elevated $CO_2$. Each extraction derived from a pool of 10 seeds per plant and was performed in triplicate. The amount of crude protein and Ara h 1 and 2 was measured in triplicate with the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and commercial Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.). All measurements are based on mg per g of peanut flour. gh = greenhouse

Remarkably, although crude protein had been extracted and measured in exactly the same way, all extracts from greenhouse-grown peanuts, including from plants grown in ambient $CO_2$, contained on average significantly more protein (20.5%) per g of peanut flour (722 ± 40 mg) than the parental seeds (the seeds were used for sawing peanuts in the greenhouses) that were used throughout Chapter 3 (3.9.6; 574 ± 28 mg on average for all pooled samples).

These data show that the distinct conditions in Greenhouse 4 resulted in a significantly lower amount of crude protein in the peanut seeds and significantly higher Ara h 1 and 2 amount and percentage of crude protein, compared to all other greenhouses. This further supports the decision to remove data corresponding to extracts from Greenhouse 4 *post-hoc* from the subsequent experiments involving 2D gels, Western blots and 2D-DIGE. Furthermore, peanut seeds from elevated $CO_2$ contain on average significantly more crude protein but significantly less Ara h 1 and a significantly lower percentage of Ara h 2, even though the total amount of Ara h 2 was not significantly different compared to peanut seeds grown in ambient $CO_2$.

### 4.2.3.5.     1D-, 2D gels and 2D-DIGE

After having tested the effect of growth conditions on the amount of extracted crude protein and Ara h 1 and 2, qualitative differences in other proteins between extracts from greenhouses with ambient and elevated $CO_2$ were examined using 1- and 2D-gel electrophoresis (Invitrogen, Carlsbad, CA). Because 1D-gel electrophoresis is inexpensive and relatively simple to perform, protein extracts from all greenhouses were applied. However, in order to focus on the effect of $CO_2$ on the proteins and allergens in the peanuts seeds, protein extracts from Greenhouse 4 were omitted from more expensive and complex experiments such as 2D-gels, 2D-DIGE and Western blots.

Protein patterns for all extracts subjected to 1D-gel electrophoresis from Greenhouses 1–4 looked very similar (Figure 4.7). The pattern was very similar to that obtained for the 20 mM Tris (pH 8.5) extracts analysed in Chapter 3, with the same major and minor bands being present. A minor protein band at 58 kDa was absent in almost 40% of the extracts made but this was the case equally for all greenhouses conditions. Neither the different $CO_2$ concentrations in the greenhouses, nor the distinct conditions in Greenhouse 4, had an influence on the 1D-gel electrophoresis pattern of the peanut protein extracts.
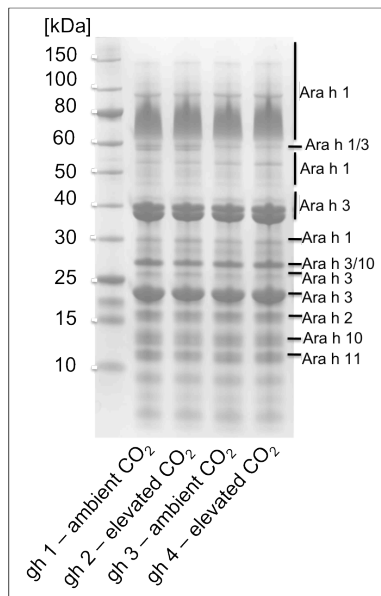
**Figure 4.7 1D-gel electrophoresis of crude protein extracts from peanuts grown in Greenhouses 1–4**
An equal volume of extract was run on each lane of the gel. Gel electrophoresis was performed with all eight extracts per greenhouse and each extraction was derived from a pool of 10 seeds per plant. Only a single run representing one sample from each greenhouse is shown. The protein identities derive from mass spectrometry experiments; details are in Appendix 2.

2D-gel electrophoresis was performed to obtain a higher resolution of individual proteins in the extracts from the greenhouse treatments. Due to a limited budget, only a subset of samples could be used. These consisted of four biological replicates of samples from ambient and elevated $CO_2$ concentrations; i.e. four plants grown in ambient $CO_2$ and four plants grown in elevated $CO_2$ were randomly selected, 10 mature seeds per plant were pooled and used for one peanut protein extraction. The samples from Greenhouse 4 that were exposed to distinct conditions compared to the other greenhouses, such as heat-stress and elevated light conditions (see 4.4.2), were *post-hoc* omitted from the experiments.

After adding 25 µg of crude protein extract per sample into IPG buffer containing detergent (Chaps), denaturants (urea and thiourea) and a reducing agent (DTT), the protein samples were absorbed into IPG strips (pH 3–10 non-linear) and focussed up to 100,000 kVh with a maximum of 5,000 V. The second dimension was run on 12% Bis-Tris gels (12% Criterion XT Bis-Tris precast gels, Bio-Rad Laboratories Inc) and analysed using the Progenesis Same Spot software (Nonlinear Dynamics Ltd.).
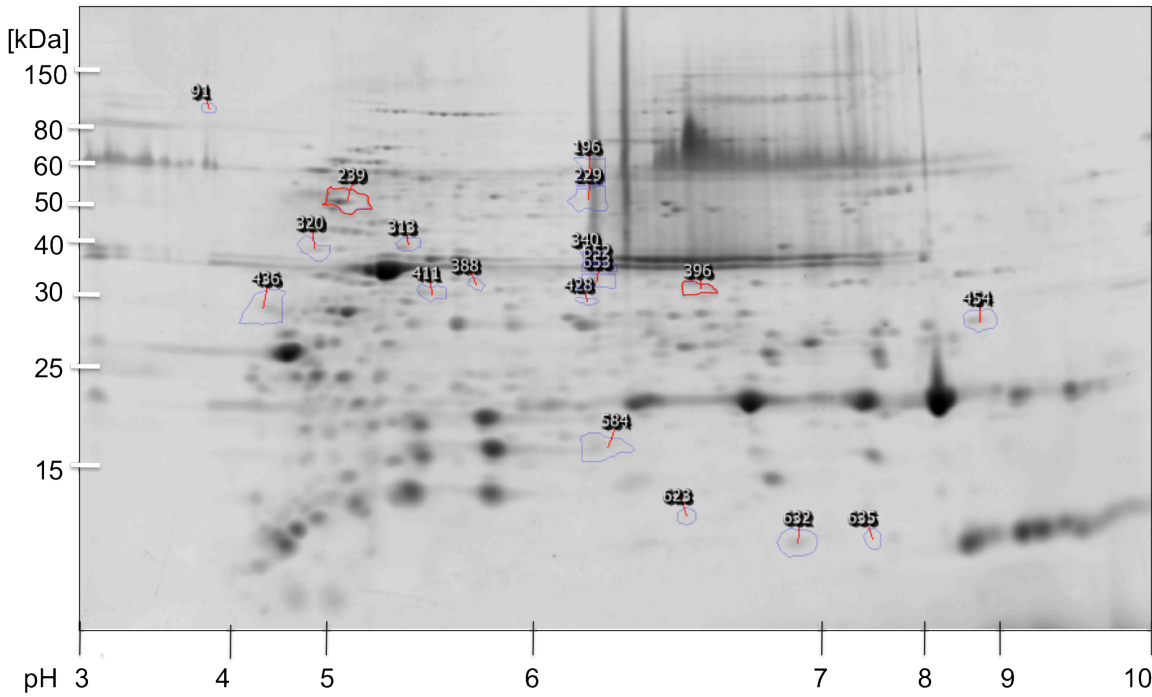
**Figure 4.8 Protein spots identified with significantly different spot volumes in crude protein extracts from peanuts grown in ambient and elevated $CO_2$ concentrations on 2D-gels**

2D-gels were performed with four crude protein extracts obtained from peanut seeds of four plants grown in ambient $CO_2$ (Greenhouses 1 and 3) and with four crude protein extracts from four plants grown in elevated $CO_2$ (Greenhouse 2). IPG strips (pH 3–10 non-linear, Bio-Rad Laboratories Inc.) were rehydrated with 25 µg protein and focussed up to 100,000 kVh with a maximum of 5,000 on a Protean IEF cell (Bio-Rad Laboratories Inc.). The second dimension was run on 12% Bis-Tris gels (12% Criterion XT Bis-Tris precast gels, Bio-Rad Laboratories Inc). The 2D gels were stained with Sypro Ruby and scanned using a Typhoon FLA 9000 laser scanner (GE Healthcare, General Electric Company, 2011), before analysing using the Progenesis Same Spot software (Nonlinear Dynamics Ltd.). The reference gel is shown and displays all protein spots that differed significantly in volume (p < 0.05) between crude proteins samples obtained from plants grown in ambient and elevated $CO_2$. Red marked spots were used for identification of proteins with mass spectrometry (Table 4.3; details and further not marked protein identities are listed in Appendix 2).

Nineteen protein spots were found to differ significantly in volume between the $CO_2$ treatments, ranging from 10–100 kDa and with putative pI values of 3.8 – 8.9 (Figure 4.8 and Table 4.3). The protein spots with significantly different volume were generally small or faint spots and none of the major protein spots were affected. Only two protein spots were identified using mass spectrometry and revealed to be Ara h 1 and 3.

**Table 4.3 Significantly different spots as detected on 2D-gels of peanut extracts from greenhouses with ambient CO₂ and elevated CO₂**

Identity, significance, magnitude and averaged normalised volumes of significantly different spots as detected on 2D-gels of protein extracts from peanuts grown in greenhouses with ambient (Greenhouses 1 and 3) an elevated atmospheric CO₂ (Greenhouse 2). Only two spots (239 and 396; labelled red in Figure 4.8) were employed in mass spectrometry (detailed pictures and graphics showing the normalised volumes for each spot can be found in Appendix 3). Spot number, p-values and averaged normalised volumes of protein spots were acquired using Progenesis software (Nonlinear Dynamics Ltd.). The molecular weight and pI-values were estimated from the 2D-gels. The identification of proteins was obtained with an in-house peanut allergen database and NCBInr as marked (full list and details in Appendix 2).

| Spot no. | Identification MS/MS | | | | Anova [p] | Fold[b] | MW [kDa] | pI | Average normalised Volumes | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | NCBI acc. no. | Isoallergen or protein | Fragment (full length) [kDa] | MS spot no.[a] | | | | | ambient CO₂ | elevated CO₂ |
| 313 | | – | | | 0.004 | 1.2 | 40 | 5.4 | 2.5e+005 | 3.0e+005 |
| 584 | | – | | | 0.005 | 1.3 | 18 | 6.25 | 6.3e+005 | 4.9e+005 |
| 340 | | – | | | 0.006 | 3.9 | 38 | 6.25 | 1.6e+006 | 4.1e+005 |
| 652 | | – | | | 0.006 | 2.0 | 37 | 6.25 | 2.4e+006 | 1.2e+006 |
| 239 | gi\|9864777 | Ara h 3 | Yes (60) | 33 | 0.008 | 1.5 | 51 | 5.1 | 1.8e+006 | 1.2e+006 |
| 320 | | – | | | 0.015 | 1.6 | 39 | 4.9 | 2.9e+005 | 1.8e+005 |
| 632 | | – | | | 0.018 | 2.1 | 10 | 6.9 | 3.1e+005 | 6.6e+005 |
| 653 | | – | | | 0.021 | 1.7 | 34 | 6.2 | 6.4e+005 | 3.8e+005 |
| 396 | gi\|1168391 | Ara h 1 | Yes (71) | 73 | 0.024 | 1.7 | 31 | 6.6 | 1.8e+005 | 3.1e+005 |
| 411 | | – | | | 0.025 | 1.4 | 30 | 5.5 | 2.9e+005 | 2.0e+005 |
| 229 | | – | | | 0.026 | 3.7 | 52 | 6.2 | 2.6e+006 | 7.0e+005 |
| 623 | | – | | | 0.026 | 1.4 | 12 | 6.5 | 5.5e+004 | 3.9e+004 |
| 196 | | – | | | 0.030 | 2.7 | 59 | 6.2 | 2.9e+006 | 1.0e+006 |
| 91 | | – | | | 0.032 | 1.6 | 100 | 3.8 | 2.0e+004 | 1.3e+004 |
| 635 | | – | | | 0.033 | 1.8 | 10 | 7.4 | 3.9e+004 | 7.1e+004 |
| 428 | | – | | | 0.035 | 1.8 | 29 | 6.2 | 7.3e+004 | 4.0e+004 |
| 436 | | – | | | 0.038 | 1.3 | 28 | 4.4 | 9.0e+005 | 6.7e+005 |
| 388 | | – | | | 0.044 | 1.6 | 31 | 5.8 | 7.8e+004 | 1.3e+005 |
| 454 | | – | | | 0.049 | 1.2 | 28 | 8.9 | 3.1e+005 | 3.8e+005 |

[a] Spot number in mass spectrometry table (Appendix 2)
[b] Fold difference in mean abundance of protein spots

All of the averaged normalised volumes of the protein spots of both treatments were persistently high. No protein spot was highly abundant in one sample set but not in the other. This shows that the same set of proteins were present in peanuts from ambient and elevated CO₂. However, it was not clear whether the differences in spot volumes of the protein spots were due to different protein concentrations in the protein extracts or due to gel-to-gel variation.

To overcome gel-to-gel variation that might have occurred and further validate the results, 2D-DIGE (two-dimensional difference gel electrophoresis) was performed, where two protein samples and one internal standard are labelled with different fluorescent dyes (CyDye2, CyDye3 and CyDye5) before running them in equal concentrations on the same 2D-gel under the usual experimental conditions. For this all eight samples from Greenhouse 2 (elevated CO$_2$) and eight random samples from Greenhouse 1 and 3 (ambient CO$_2$) were used. It was taken into account that one Cy-Dye might stain the proteins more than the other and lead to misleading results, so each gel had alternate Cy-Dye combinations: on the first gel, CyDye3 was used to stain the elevated sample and CyDye5 the ambient sample, while on the second gel the staining was *vice-versa*, etc.. The results of the 2D-DIGE experiments were also analysed using the Progenesis Same Spot software (Nonlinear Dynamics, Ltd.). Surprisingly only three significantly differing protein spots volumes between high and ambient CO$_2$ extracts could be located (Figure 4.9).
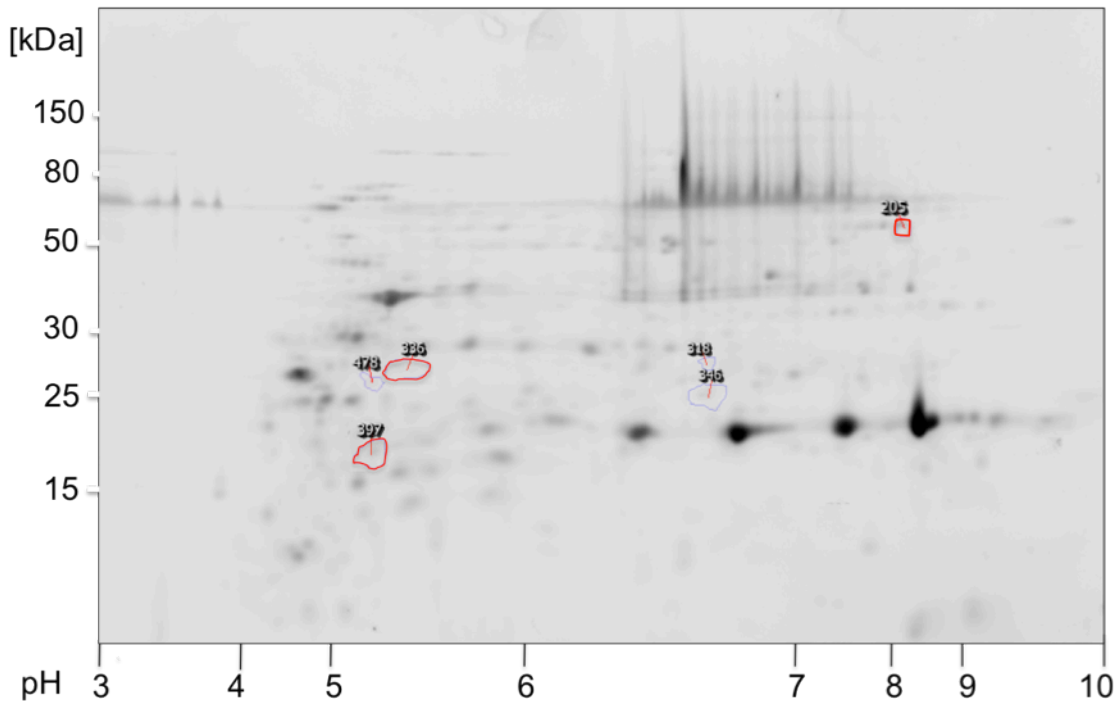


**Figure 4.9 2D-DIGE gel with protein extracts from peanuts grown in greenhouses in ambient and elevated CO$_2$**

2D-DIGE gels were performed with eight crude protein extracts obtained from peanut seeds of eight plants grown in ambient CO$_2$ (Greenhouses 1 and 3) and with eight crude protein extracts from eight plants grown in elevated CO$_2$ (Greenhouse 2). After staining the proteins extracted from seeds from different growth conditions either with Cy3 or Cy5, an equal mixture of all sixteen protein extracts was mixed and stained with Cy2. Equal amounts of two protein extracts and the mixed standard, adding up to 25 µg in total, were applied to IPG strips (pH 3–10 non-linear, Bio-Rad Laboratories Inc.) and focussed up to 100,000 kVh with a maximum of 5,000 on a Protean IEF cell (Bio-Rad Laboratories Inc.). The second dimension was run on 12% Bis-Tris gels (12% Criterion XT Bis-Tris precast gels, Bio-Rad Laboratories Inc). The 2D gels were scanned using a Typhoon FLA 9000 laser scanner (GE Healthcare, General Electric Company, 2011) and analysed using the Progenesis Same Spot software (Nonlinear Dynamics Ltd.). The reference gel is shown and displays all protein spots that differed significantly in volume (p < 0.05) between crude proteins samples obtained from plants grown in ambient and elevated CO$_2$. Red marked spots were used for identification of proteins with mass spectrometry (Table 4.4; details and further not marked protein identities are listed in Appendix 2).

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**Table 4.4 Significantly different spots volumes as detected on 2D-DIGE of peanut extracts from greenhouses with ambient $CO_2$ and elevated $CO_2$**

Identity, significance, magnitude and averaged normalised volumes of significantly different spots as detected on 2D-DIGE of protein extracts from peanuts grown in greenhouses with ambient (Greenhouses 1 and 3) and elevated atmospheric $CO_2$ (Greenhouse 2). Only two spots (397 and 336; labelled red in Figure 4.9) were subjected to mass spectrometry (detailed pictures and graphics showing the normalised volumes for each spot can be found in Appendix 4). Spot number, p-values and averaged normalised volumes of protein spots were acquired using Progenesis software (Nonlinear Dynamics Ltd.). The molecular weight and pI-values were estimated from the 2D-gels. The identification of proteins was obtained with an in-house peanut allergen database and NCBInr as marked (full list in Appendix 2 and 3).

| Spot no. | NCBI acc. no. | Isoallergen or protein | Fragment (full length) [kDa] | MS spot no. [a] | Anova [p] | Fold [b] | MW [kDa] | pI | Average normalised Volumes ambient $CO_2$ | elevated $CO_2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 205 | gi\|1345681 | Catalase[c] | No (57) | 141 | 0.000 | 1.3 | 57 | 8.3 | 1.00 | 1.25 |
| 478 | – | | | | 0.009 | 1.3 | 27 | 5.3 | 0.83 | 1.01 |
| 397 | No match | | | 60 | 0.015 | 1.2 | 19 | 5.3 | 0.98 | 1.17 |
| 318 | – | | | | 0.026 | 1.2 | 28 | 6.7 | 0.93 | 1.10 |
| 346 | – | | | | 0.032 | 1.1 | 25 | 6.7 | 1.05 | 1.14 |
| 336 | gi\|118776570 | Ara h 3 | Yes (60) | 65 | 0.040 | 1.1 | 28 | 5.4 | 1.04 | 1.15 |

[a] Spot number in mass spectrometry table (Appendix 2)
[b] Fold difference in mean abundance of protein spots
[c] Results from NCBInr database; protein spot 172 (64) was not found in peanuts, but a range of other plants

The significantly different protein spots detected on the 2D-DIGE gels (Figure 4.9) were not found on the conventional 2D-gels (Figure 4.8). Because of its reduced gel-to gel variation, it is likely that the significantly different protein spots in the 2D-DIGE are more accurate compared to the conventional 2D gels. Since merely the volumes of the protein spots detected on 2D-DIGE were different (as opposed to a lack of protein spots in peanuts from one treatment; see averaged normalised volumes in Table 4.4, and individual protein spots in Appendix 4), it was concluded that only six spots had slightly but significantly different average volumes and all protein spots were present. Thus, in general, there were no qualitative differences in allergen abundance (present vs. absent proteins or allergens) peanut extracts from ambient and elevated greenhouses.

It was therefore concluded that the significant differences in Ara h 1 and 2 abundance, as found using ELISA assays (Chapter 4.5.3), were too small to be detected on the 2D-gels and 2D-DIGE.

#### 4.2.3.6. Western blotting

The recognition of allergens in protein extracts from peanuts grown in ambient and elevated $CO_2$ (Greenhouses 1–3) by IgE antibodies in the serum of a peanut allergic patient (Appendix 2) was tested by performing Western blotting after 2D-gel electrophoresis. The limited amount of available serum allowed only two biological replicates to be run for ambient and elevated $CO_2$ samples. The Western blots (Figure 4.10) displayed the same protein spots that had been observed in the Western blots with Tris-extracted samples previously (Chapter 3.3.4.). Notably, both Western blots performed with protein extracts from ambient $CO_2$ greenhouses showed one intense and 11 additional faint spots compared to elevated $CO_2$ samples and other Tris samples from previous chapters. This observation was also made in one sample in the next chapter with peanut samples from Childers (Chapter 5.3.5.) and surprisingly was found to be consistent in the duplicates. It could not be clarified whether the majority faint protein spots were actual signals on the blots or increased background. None of the corresponding protein spots in the 2D-DIGE was found to show a positive signal on the Western blots.
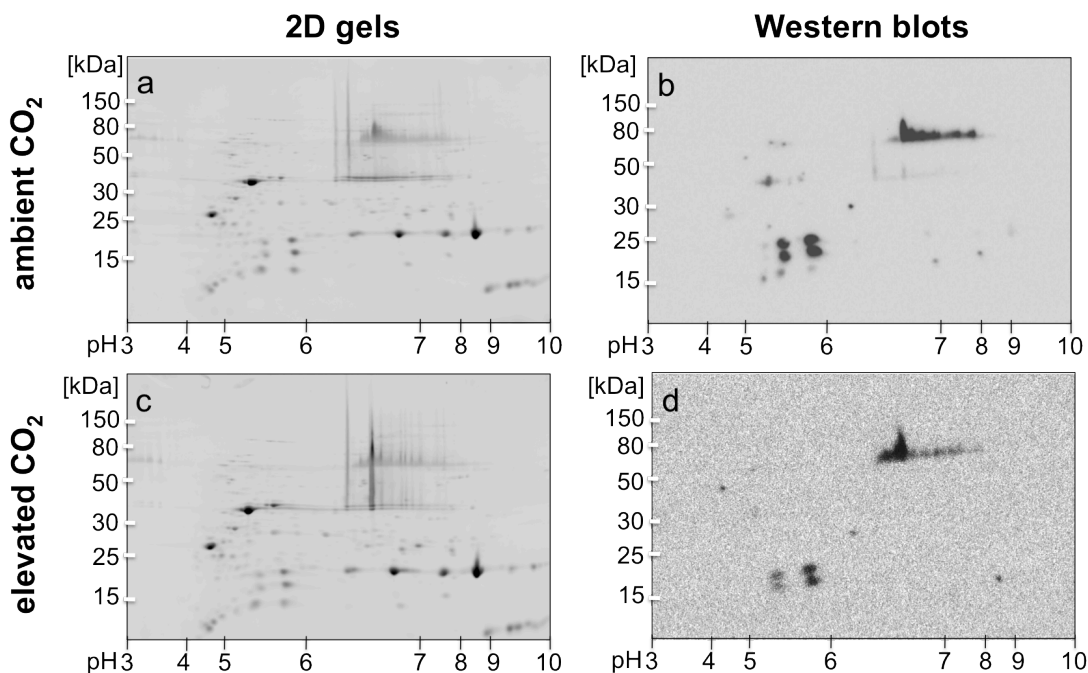


**Figure 4.10 Protein pattern on 2D-gels and Western blots of peanuts grown in ambient and elevated $CO_2$**

(a,b) Protein pattern of peanuts grown in ambient $CO_2$; the assays were done in duplicate (one sample each for greenhouse 1 and 3) (c,d) Protein pattern of peanuts in elevated $CO_2$ (duplicates from Greenhouse 2) 2D-gels and subsequent Western blots were performed with two crude protein extracts obtained from peanut seeds of two plants grown in ambient $CO_2$ (Greenhouses 1 and 3) and with two crude protein extracts from two plants grown in elevated $CO_2$ (greenhouse 2). IPG strips (pH 3–10 non-linear, Bio-Rad Laboratories Inc.) were rehydrated with 25 µg protein and focussed up to 100,000 kVh with a maximum of 5,000 on a Protean IEF cell (Bio-Rad Laboratories Inc.), run on 12% Bis-Tris gels (12% Criterion XT Bis-Tris precast gels, Bio-Rad Laboratories Inc) and blotted onto a nitrocellulose membrane (Bio-Rad Laboratories Inc.). The Western blots were exposed to serum of a peanut allergic patient with IgE against Ara h 1, 2 and 3 (to a lower extent) stained and secondary antibody containig horseradish peroxisase and developed using the ImmunStar HRP Chemiluminescence kit (Bio-Rad Laboratories Inc.). The blots were scanned using a G box (Synoptics Ltd.). The identities of most protein spots are listed in Table 4.5 and Appendix 2.

**Table 4.5 Identities and intensities of peanut proteins spots detected in Western blots with peanuts grown in greenhouses with ambient and elevated $CO_2$**

The proteins matching the spot pattern in the Western blots in Figure 4.10 were subjected to mass spectrometry (Appendix 2). The spot number corresponds to the labelling in Figure 3.8 (Chapter 3.3.2.3). The identity and intensity of the protein spots is listed below, (+) indicating high intensity and (–) a low intensity of the protein spot. If the intensity is not marked, the protein spot was absent in the respective Western blot (Figure 4.10).

| MS spot no [a] | Identification MS/MS | | Intensity of protein spot | |
|---|---|---|---|---|
| | Isoallergen or protein | Fragment | Ambient $CO_2$ | Elevated $Co_2$ |
| 128 – 139 | Ara h 1 | Yes | +++ | +++ |
| 37 | Ara h 1 and 3 | Yes | +++ | +++ |
| 38 | Ara h 3 | Yes | +++ | +++ |
| 45 | Ara h 3 | Yes | +++ | + |
| 42 | Ara h 3 | Yes | | |
| 32 | Ara h 3 | Yes | + | |
| 72 | Ara h 3 | Yes | | |
| 30 | Ara h 3 | Yes | + | |
| 28 | Ara h 3 | Yes | + | + |
| 29 | Ara h 3 | Yes | | |
| 91 | Ara h 6 | Yes | + | |
| 25 | Ara h 6 and 8 | Yes | + | |
| 36 | Ara h 5 [b] | No | + | |
| 50 | Ara h 7 precursor [b] | No | + | |
| 60 | Ara h 7 precursor [b] | No | + | |
| 56 | Ara h 7 precursor [b] | No | | |
| 55 | Ara h 9 [b] | Yes | + | |
| 26 | Thioedoxin fold [c] | No | +++ | +++ |
| 27 | *No match* | | +++ | +++ |
| 51 | *No match* | | + | |
| 98 | *No match* | | | |
| 86 | *No match* | | | |
| 116 | *No match* | | | |
| 117 | *No match* | | | |
| 107 | *No match* | | | |
| A | - | | + | |
| B | - | | + | |
| C | - | | + | |
| D | - | | | |
| E | - | | | |
| F | - | | | |

[a] Spot number in mass spectrometry table (Appendix 2)
[b] Not significant, no other match
[c] Result from NCBInr database; no match in in-house peanut allergen database

## *4.3.    Discussion*

### *4.3.1.    The peanut plants in the four greenhouses were exposed to different conditions for part of the growth period*

Although the greenhouse conditions were relatively similar on average over the whole experiment, the high-resolution plot of the conditions over time shows that the plants were at times exposed to variations between the greenhouses. In the literature it is not common to talk about the precision of the conditions in such detail and usually only the average value and standard deviations of conditions, such as temperatures are mentioned (Singer et al. 2005, Wayne et al. 2002, Ziska L. H. et al. 2007). Importantly, these averages do not show if the conditions were different over short periods of time, although such periods might still influence the development of the plants. Therefore it is valuable to make the effort to show the conditions the plants were actually exposed to at all stages of development, as has been done in the plots in this chapter (Figure 4.3).

Generally, the differing environmental conditions in Greenhouse 4, such as the inconsistencies of temperature (including the heat-event) and the consistently distinct light conditions, led to the *post-hoc* exclusion of this greenhouse from the analysis of the effects of CO$_2$ on plant performance and peanut allergen abundance. Recordings suggested that Greenhouse 3 had a few days of much lower light intensity than the other greenhouses but this is most likely due to shadowing of the sensor by the growing plants during this time and was therefore ignored. With aid of the plots, it could be determined that the only difference between the greenhouses was the low humidity in the elevated-CO$_2$ Greenhouse 2 during the first five weeks, which might have influenced the development of the plants as discussed below (4.6.3).

### *4.3.2.    Chamber, greenhouse and FACE experiments*

Conditions in greenhouses are partly weather-dependent but are likely to differ from an entirely realistic growth experiment on the field, including the confinement of root space, available soil nutrients, radiation exchange (walls can interfere with incident radiation, especially solar UV-B, but also general solar irradiance level and thermal energy exchange), wind patterns and other climatic conditions (Aldrich and White 1969 2008). This creates an altered microclimate inside the greenhouses compared to field conditions. Additionally the fumigation system can induce a chamber effect, by forming CO$_2$ gradients (Ainsworth E. A. et al. 2008), although this was avoided in the experiments reported here by using an effective ventilation system. In conclusion, the investigation of growth of plants in greenhouses but

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with realistic field - environment interactions is impossible (Curtis and Wang 1998). Moreover, the root-zone volume is a very important factor (Bindi et al. 2001, Taub et al. 2008) in assessing a clear response to $CO_2$, air pollutants, or any other treatment (Ainsworth E. A. et al. 2008, Arp 1991, Thomas R. B. and Strain 1991). As chamber experiments usually involve rooting of plants in pots, rather than in the ground, root volume effects, such as a lower yield in many crops, should be taken into consideration. In using greenhouses, however, important variables, such as temperature, nutrient availability, amount of precipitation and humidity can be controlled to accommodate the plants' needs or answer an experimental question. Greenhouses are very convenient to work with, as they are large enough for fairly large sample sizes and relatively big plants. By allowing work inside the greenhouse, they provide an easy handling/working space and are particularly valuable as a setting to identify mechanisms of plant responses at the molecular, biochemical and physiological scales (Ainsworth E. A. et al. 2008). Furthermore, they are very cost effective.

In contrast to greenhouses, growth chambers are totally controlled environments and artificial atmosphere and light intensity has to be supplied constantly. Therefore, for a successful experiment, highly reliable equipment is critical to provide comparable conditions between chambers, without being dependent on the orientation to the sun. However, growth chambers are very limited in space and only small plants or seedlings are suitable for these experiments. They were therefore not suitable for growing peanuts, as the plants (and therefore the pots required) are relatively big.

FACE (free-air $CO_2$-Experiments) currently provide the most realistic experimental system to test the effects of future $CO_2$ conditions. To date they are the only way to investigate plant-environment interactions under realistic field conditions and elevated $CO_2$ (Curtis and Wang 1998). They simulate future $CO_2$ conditions in an open-air environment, without perturbing the soil–plant–atmosphere continuum and without limiting growing space, or altering microclimate, precipitation or pest/pathogen access. The experimental plots of these facilities are typically larger than those in greenhouse experiments and allow more information to be obtained on plant systems throughout co-operative integrated field experiments (Ainsworth E. A. et al. 2008, Pinter et al.). On the other hand, the costs of establishing and maintaining a FACE experiment, while depending largely on the size and the required $CO_2$ level for the experiment, are usually very high and reach easily the million barrier (Raison et al. 2007). FACE is therefore not feasible for most studies, including the one reported in this thesis.

In conclusion, greenhouse experiments were in the best choice for this study, due to their cost effectiveness and relatively good space requirements.

All biochemical methods used in experiments reported in this chapter to test the allergen content are discussed in the general discussion (Chapter 6). General difficulties associated with testing the allergenicity of crops with lowered abundance of allergens are discussed in a book chapter recently accepted for publication, which is attached in Appendix 5.

### 4.3.3. *Performance of peanut plants grown in ambient and elevated $CO_2$ was similar (Greenhouses 1–3)*

Of the two peanut plants per pot, one plant was usually much bigger, stronger and had a higher seed yield. This plant was therefore more competitive for above-ground resources, such as light, and below-ground resources, including water and at least 20 essential mineral nutrients that differ in molecular size, valence, oxidation state and mobility within the soil (Casper and Jackson 1997, Wilson 1988) in this confined space. There is little evidence to justify the common assumption that adding environmental resources (e.g. fertilisation) reduces competitive effects, and it has been shown that the competitive imbalance is often even greater at higher resource levels (Wilson 1988). Therefore the bigger plant from each pot represented most closely a plant grown in the field and was chosen for analysis in this study.

The peanut plants did not show any differences in performance, such as dry weight of shoots, seed yield, seed weight and pod weight between ambient and elevated $CO_2$ conditions (when comparing data from Greenhouses 1 – 3). It has been shown in previous studies that under favourable conditions the elevation of atmospheric $CO_2$ from 400 to 700 or 800 ppm has positive effects on peanut plant growth, including foliage dry weight, dry weight of pods and seed yield (Mortley D. G. et al. 1997, Stanciel et al. 2000). For the experiments conducted here, there are a few factors and that might have affected the usual $CO_2$ response on yield and plant growth and caused the relatively poor plant performance observed. Precise monitoring over time showed that Greenhouse 2 (elevated $CO_2$) had much lower humidity in the first 5 weeks of growth. This is likely to have negatively affected the development of gynophores and hence the seed yield. It has been shown in previous studies that foliage and pod dry weights, total seed yield, and seed maturity of peanut plants were significantly lower at 50% compared to 85% relative humidity. Furthermore, flowering occurred 3 days later, and fewer flowers and gynophores were present under lower relative humidity (Lee et al. 1972, Mortley D.G. et al. 2000). Additionally it was shown that gynophores grew more rapidly at 95%

compared to 50% relative humidity, which was thought to be due to lowered ethylene production (Lee et al. 1972).

Another factor affecting the $CO_2$ response might have been the confinement of the peanut plants in pots, which is likely to have influenced seed yield. Although the peanut plants were grown in big pots (14 l) following the recommendations of PCA, this confinement restricted the root-zone volume, which is an important factor affected plant performance (Bindi et al. 2001, Taub et al. 2008), including assessment of responses to $CO_2$ (Ainsworth E. A. et al. 2008, Arp 1991, Thomas R. B. and Strain 1991). This is particularly the case of peanuts, because the gynophores penetrate into the ground so that the peanut seeds can develop in the soil. In the experiments conducted here it was observed repeatedly that gynophores were growing outside the pots as the plants were "reaching out" for additional space beyond the edges of the pots. This might have prevented some peanut seeds from developing and maturing and caused the greenhouse with elevated $CO_2$ to have a similar yield to the greenhouses with ambient $CO_2$. Furthermore, the replicate set-up of the greenhouses was not ideal (4.6.1) and Greenhouse 3 (ambient $CO_2$) had 50 ppm more $CO_2$ on average than Greenhouse 1 during the day. The slightly higher concentration of in $CO_2$ might have influenced the plant growth positively, which is substantiated by the fact that plants from this greenhouse had on average slightly higher shoot dry weight and yields.

It is likely that a combination of the listed factors diminished the influence of elevated $CO_2$ on the peanut plants. The fact that the expected effects of elevated $CO_2$ on plant performance, which serve as an indicator for a successful $CO_2$ experiment, were diminished by a combination of several factors, raises the following question: Were the results obtained on the allergenicity of peanuts affected by the differing conditions in the greenhouses, such as decreased humidity in greenhouse 2, or were they are due to the elevated atmospheric concentration of $CO_2$?

### 4.3.4. *Plants from Greenhouse 4 had significantly higher shoot dry weights*

Plants in Greenhouse 4 had significantly higher shoot dry weight compared to plants from Greenhouses 1 – 3. The seed yield was similar to the other greenhouses. The heat event and distinct light conditions, as well as the elevated $CO_2$, could have influenced plant performance. It has been reported that short-term (1–6 days) exposure to temperatures above optimum cause significant yield loss in peanut, primarily due to the reduced proportion of flowers that produce pegs and pods (Vara Prasad P. V. et al. 1999, Vara Prasad Pagadala V. et

al. 2000). These authors showed that the number of pegs and pods was affected significantly only when the peanuts were exposed to > 33°C for 6 days. The maximum temperature tested (48°C) did not lead to any significant effects on yield when the plants were exposed for only 2 days (Vara Prasad Pagadala V. et al. 2000). Nevertheless, it was observed that the number of pegs and pods was significantly affected by higher temperatures and exposure time and that the greatest sensitivity of the peanut plants to hot days occurred in the period beginning 6 d before and 15 d after flowering (Vara Prasad Pagadala V. et al. 2000), which supports the earlier finding that peanuts are more sensitive to stress in the reproductive phase than the vegetative phase (Cox 1979, Ketring 1984).

Long-term exposure to an elevated temperature of 35°C resulted in a smaller seed yield (Ketring 1984) temperature reproductive and vegetative development peanuts. Interestingly, Prasad et al. showed that the combined effect of long-term elevated temperatures (up to 44°C) and elevated CO$_2$ concentrations (700 ppm), resulted in very similar seed set numbers, but higher plant weights (shoot and root) (Vara Prasad P. V. et al. 2003). In the experiments reported in this thesis, the similar seed yield in ambient and elevated CO$_2$ and elevated shoot weight of peanut plants from Greenhouse 4 is therefore probably a result of the short-term exposure to elevated temperature of around 60°C for 2 days just before flowering and the elevated CO$_2$ concentration. It is unclear to what extent the elevated light intensity might have played a role but it may be that the temperature was slightly elevated throughout the experiment due to higher radiation. The temperature data for the last few weeks in Greenhouse 4 support this.

### 4.3.5. Greenhouse conditions and CO$_2$ concentration had effects on the crude protein content of peanuts

The crude protein concentration was higher in peanuts grown in elevated CO$_2$ compared to ambient CO$_2$ (when comparing Greenhouse 1 – 3) but the relative abundance of individual proteins was the same. Greenhouse 4 had significantly less protein than its replicate Greenhouse 2. Earlier studies showed that elevated CO$_2$ treatments do not affect the oil and protein content of peanut (Burkey et al. 2007) and soybean seeds (Heagle et al. 1998, Thomas J.M.G. et al. 2003). Taub et al. showed in a meta-analysis with 228 major food crops, that species other than peanut showed consistently lower protein concentrations in elevated CO$_2$ (540–958 ppm) compared with ambient CO$_2$ (315 –400 ppm). This includes allergenic plants, such as wheat and soybean, but although soybean had a significant lower protein content in elevated CO$_2$, the difference was only 1.4% and the magnitude was dependent on the

experimental setting (Taub et al. 2008). It has been demonstrated in some studies that environmental conditions can significantly influence the seed protein content in some legumes (Burstin et al. 2011, Frimpong et al. 2009, Oluwatosin 1997, Saxena et al. 2002). However, nitrogen supply is the most important factor affecting protein content and composition (Triboi et al. 2000). The observed variations were therefore largely due to the soil type and its moisture and nutrient level. Environmental effects that were associated with nitrogen nutrition, such as drought, soil density, root diseases and pests, might also influence seed protein content (Burstin et al. 2011, Burstin et al. 2007, Lawn and Rebetzke 2006, Matthews and Arthur 1985, Oluwatosin 1997). The fact that the protein concentrations reported in this chapter were significantly higher (rather than similar or lower, as expected) for peanuts from the greenhouse with elevated $CO_2$ might be due to the combined influence of other factors. Because the fertilisation, including the addition of nitrogen and watering was the same in all the greenhouses, it is unlikely that variations in these parameters would account for the difference in protein concentration. It is rather the result of the low humidity in the first five months in Greenhouse 2 (elevated $CO_2$) or the slightly increased $CO_2$ concentration in Greenhouse 3. However, since there are no publications available on the combined effects of humidity and $CO_2$ on peanut plants, it is difficult to know whether these parameters are likely to be responsible.

The low crude protein concentration in Greenhouse 4 is also very likely due to a combined effect of elevated $CO_2$, the heat event and the high light intensity in this greenhouse.

It is impossible to state with certainty that the increased protein concentration in peanuts from plants grown under elevated $CO_2$ is indeed due (partly or wholly) to the elevated $CO_2$ and no conclusions can be drawn without testing the impact on the environmental conditions individually.

### 4.3.6.  *Greenhouse conditions had a small but statistically significant effect on the Ara h 1 and 2 content of the peanut plants*

The amount of Ara h 1 and 2 was lower in peanuts grown in elevated $CO_2$, than in ambient $CO_2$ (when comparing Greenhouses 1 – 3). This effect was significant for the absolute abundance of Ara h 1, as well as the percentage of Ara h 2 in the crude protein. Greenhouse 4 had significantly more protein than its replicate greenhouse 2.

These effects were too small to be detectable on 2D-DIGE and no other altered abundance of allergens could be detected. Although it could not be clarified if the differences observed on Western blots were due to increased background, the observed differences on Western blots seemed to suggest that the IgE binding might change on 2D-gels in different plant growth conditions. Earlier studies that examined the effects of elevated CO$_2$ on the allergenicity of plants mostly gave attention to the differences in amount of allergenic tissues, such as the amount of pollen (Wayne et al. 2002) or the time of pollination (Menzel 2000). Up until now, there has been only one study that examined the direct effect of elevated CO$_2$ on the allergen content in the plant per se: Singer et al. was able to show in ELISA assays, that ragweed (*Ambrosia artemisiifolia*) shows a 1.8 - fold higher amount of the major ragweed allergen Amb a 1 in pollen grown at current compared to pre-industrial CO$_2$ and 1.6- fold more Amb a 1 at future atmospheric CO$_2$ concentrations (700 ppm CO$_2$), compared to current conditions, although total pollen remained unchanged (Singer et al. 2005). This shows that the amount of allergen might be expected to change with elevated CO$_2$ in plants generally. However, the attempt to analyse the allergen content of peanuts in elevated CO$_2$ in this chapter is the first of its kind for allergenic food plants.

Due to the irregularities in environmental conditions in the greenhouses it is impossible to draw conclusions on how the CO$_2$ influences the abundance of allergens Ara h 1 and 2. The results are rather a combined effect of a few environmental factors mentioned in the paragraphs above.

### 4.3.7. *Other factors that might influence the allergenicity of peanuts*

When discussing the impact of a future CO$_2$ environment on the allergen quantity of peanuts and other food crops it is important to take in account other factors that will change with higher CO$_2$. In a future CO$_2$ environment, soils play an important role in plant performance, as their CO$_2$ concentration will increase along with the atmosphere. Long-term elevated CO$_2$ has major impacts on soil pH and increases its capacity to hold nutrients, like organic carbon and total nitrogen and promotes net mineral-N mineralisation. These findings may affect how plants respond to an elevated CO$_2$ atmosphere and therefore alter the potential of allergenic plants (Aalberse 2000). Extreme weather events, like heat waves, cyclones and precipitation events, may alter plant performance and allergenicity as well, by influencing the expression pattern of certain proteins, which might be increased/decreased due to a stress response. Peanuts are often grown in semi-arid regions, where the maximum temperatures reach > 40˚C for short periods of time. Furthermore, the global average surface temperature is projected to

rise by another 1.8 – 4.0 °C by 2100 (IPCC 2007). As mentioned previously, this might influence the performance of some plants, including peanuts (Vara Prasad P. V. et al. 2003) and might result in altered concentrations of allergens. However, crop-growing regions might move, so that the temperatures stay at an optimum. Another important factor might be the future atmospheric composition. It has been shown, for instance, that rising concentrations of air pollutants, such as ozone, also influence the effect of elevated CO$_2$ on crop growth and yield, and *vice versa* (Allen 1990, Booker and Fiscus 2005, Booker et al. , Long Stephen P et al. 2005 2007). The impact of these parameters on the abundance and allergenicity of allergens is unknown.

Importantly, this study concentrates on the "botanical view" of allergenicity, by addressing allergen content. The actual allergic potential is dependent on the immune system of allergic individuals, which is influenced by a whole range of unpredictable factors, such as eating habits, pollution, life style, changes in exposure to allergens (e.g. allergen defeating cleaning products) etc.. It is important, nevertheless, to determine possible changes in allergen exposure in the future to obtain a comprehensive view so that estimates of risk to patients can be made. Although this does not ensure that the allergenicity of peanuts can be lowered, it is a worthwhile attempt to understand the mechanisms behind allergy.

## *4.4.    Conclusions*

Unfortunately, no conclusion can be drawn on the impact of CO$_2$ on the allergenicity of peanuts from the present study. It can be seen, however, that environmental conditions do have an impact on the plant growth of peanuts and that diverse conditions, such as reduced humidity and elevated CO$_2$, can interfere with each other. Overall, the CO$_2$ effect combined with other factors did not lead to an over-expression or lack of particular proteins, including allergens, in the peanuts, and therefore did not result in a different allergenicity of allergic peanuts.

# Chapter 5

# Protein and Ara h 1 and 2 content of peanuts grown in different regions in Australia

## 5.1.  Summary

In order to develop new breeding strategies allowing production of peanuts with lower allergenicity, the relative concentrations of peanut allergens from the selected commercial peanut variety "Walter" grown in different climate regions in Australia were compared. The outcome has both scientific and potentially commercial value and is particularly important for PCA (a partner for the research reported in this chapter) as peanut allergy is the principle health concern associated with consumers of the company's products.

## 5.2.  Peanut samples

To test the hypothesis that the abundance of major peanut allergens is different from plants grown in various peanut-growing regions in Australia, which encountered differences in climate variables, runner peanuts (variety "Walter") were acquired from PCA and analysed. The samples came from a variety evaluation trial performed by PCA from November 2009 to March 2010 in three peanut-growing regions in Queensland, Australia: two regions in South Queensland, one close to Kingaroy and the other around 250 km north-east of Kingaroy, close to Bundaberg, and a third region in North Queensland close to Kairi, around 1500 km north of Bundaberg (Figure 5.1a). In Kingaroy two sites were set up at the Redvale and Taabinga Research Stations. In Bundaberg one site was located at the Bundaberg Research Station and another site at Russo Farms close to Childers, around 50 km south-west of Bundaberg. In Kairi only one site was set up at the Kairi Research Station (Figure 5.1b). To obtain triplicate samples for each of the five locations, three plots were set up at each site in a randomised block design, each of which was approximately 5 m long by 2 rows and contained approximately 150 plants in total. The peanuts from each plot were pooled by PCA and a subsample was allocated for the study reported in this chapter.

**Figure 5.1 Commercial peanut-growing regions and sites from which the peanut seeds for analysis in this chapter were obtained**

The peanut plants were grown at the Redvale Research Station and Taabinga Research Station in the peanut growing region Kingaroy; in the Bundaberg growing region at Russo Farms close to Childers and at the Bundaberg Research Station; and in the Kairi Research Station. Three plots of peanut plants were grown in each site by PCA and a subset of samples from these locations was provided for the analyses in this chapter. The maps were generated using Google Maps (http://maps.google.com/).

## *5.3. Results*

### *5.3.1. Climatic conditions in the peanut-growing regions*

In order to obtain data on the climatic conditions of the various peanut-growing regions, the closest weather stations to the peanut field sites were identified. One weather station in each of the three regions (Kingaroy, Bundaberg and Kairi) was identified and the monthly maximum and minimum temperatures, total monthly rainfall and monthly average solar exposure obtained from the Bureau of Meteorology, Australia (www.bom.gov.au) and plotted using Excel (Microsoft Office, 2008). The monthly averages of the conditions measured in each station as well as the overall averages were plotted against time (Figure 5.2, left panel). Furthermore the anomalies were plotted so that the individual averages can be compared easier with the overall average of the conditions (Figure 5.2, right panel).

For the growing period (November 2009 to March 2010), the average daily maximum temperature in the Kingaroy decreased from 32˚C in November, when it was 2˚C above average, to 26˚C in March, when it was 1˚C below average (Figure 5.2a,b). The corresponding temperature in Bundaberg was almost steady with only a slight increase from 29 to 31˚C between November and January, after which it decreased to 28˚C in March. The temperature in Bundaberg showed a steady increase when compared with the overall averages between all regions, being below average at the beginning of the growing season and above average at the end. The temperature in Kairi fluctuated between 27 and 30˚C throughout the growth season and was up to 2˚C below average until January. Overall, the average daily maximum temperature was up to 4˚C different between the three regions.

In Kingaroy the daily minimum temperature was between 15 to 19˚C, which was 1.0 to 2.3˚C below average (Figure 5.2c,d). Kingaroy had the highest difference between daily maximum and minimum temperatures. In Bundaberg the daily minimum temperature was 19.5 to 23˚C, which was 2 to 3˚C above average, and this site had the lowest difference between daily maximum and minimum temperatures. In Kairi the daily minimum temperatures were on average 16 to 20˚C, which is close to the average of all three regions. There was a slight increase in daily minimum temperature in all regions from November to March.
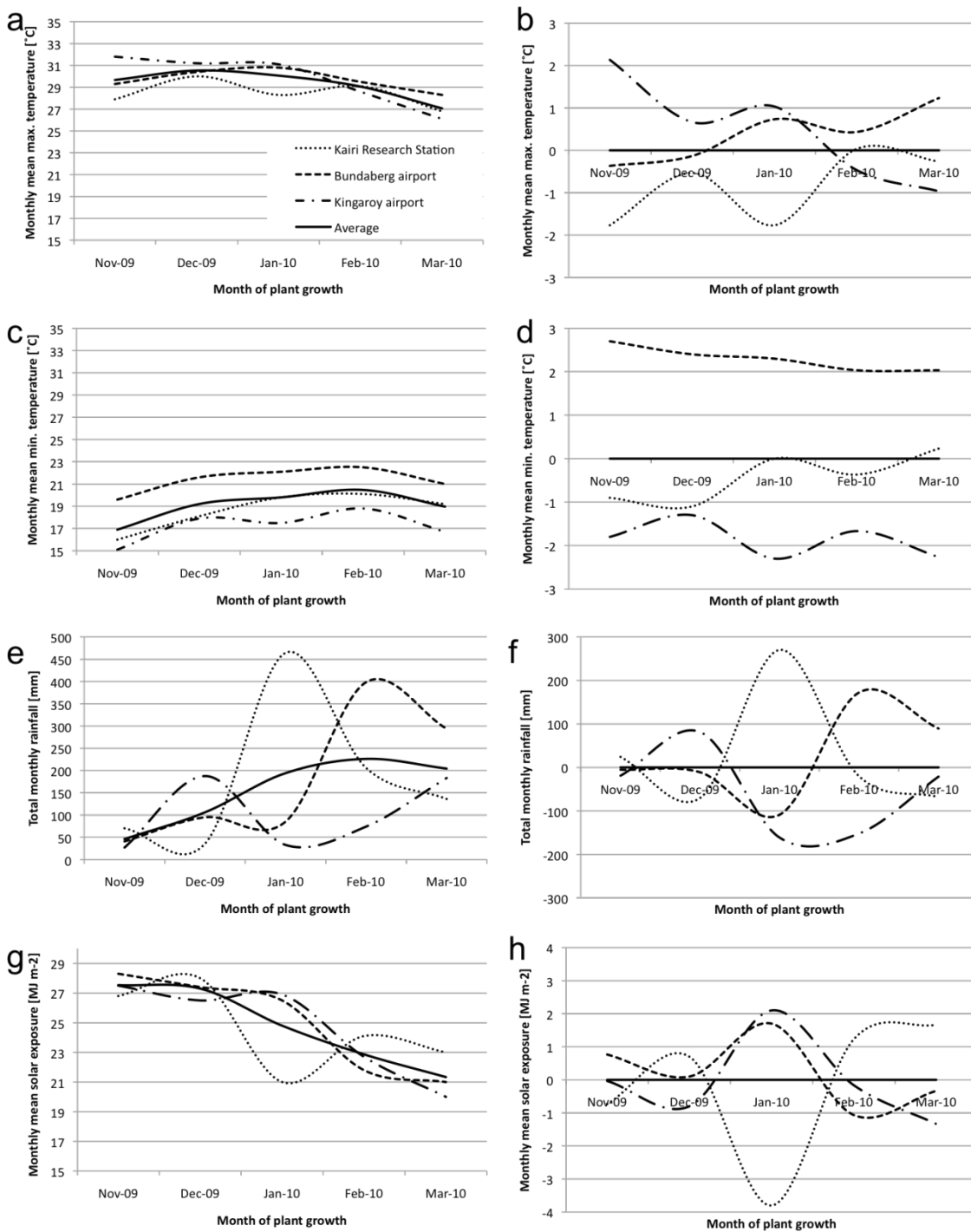
**Figure 5.2 Climatic conditions during the growth period (November 2009 to March 2010) of peanut plants grown in the regions Kingaroy, Bundaberg and Kairi**

Average monthly maximum temperature, rainfall, solar exposure, temperature and humidity recorded at weather stations closest to Kingaroy, Bundaberg and Kairi. (a,b) Average and anomalies of maximum daily temperature; (c,d) Average and anomalies of minimum daily temperature; (e,f) Total monthly rainfall and anomalies of rainfall; (g,h) Mean solar exposure and anomalies. The graphics were generated with Excel (Microsoft Office, 2008) using monthly averages of daily measurements obtained from the Bureau of Meterology, Australia (www.bom.gov.au).

The rainfall differed most between the peanut growing regions (Figure 5.2e,f). In November the total monthly rainfall was low in all three regions. In December total rainfall in Kingaroy was 190 mm, which was around 90 mm above average, but the rainfall then declined and remained below average in the next three months. In Bundaberg the rainfall was between 50 and 100 mm from November until March but then increased to 400 mm in February (~180 mm above average) and 300 mm (~90 mm above average) in March. Kairi was the driest region in December but the wettest in January, with 465 mm of rain, which was 270 mm above average. In February and March the rainfall in Kairi was 200 and 150 mm, respectively, values close to the average rainfall between the regions.

Solar exposure in Kingaroy and Bundaberg stayed between 26 and 28 MJ*m$^2$ from November until January and then decreased gradually to around 20–21 MJ*m$^2$ in March (Figure 5.2g,h). The solar exposure in Kairi was similar in November and December but 4 MJ*m$^2$ lower on average in January, while it was around 2 MJ*m$^2$ above average in Kingaroy and Bundaberg.

In summary, Kingaroy had the largest differences between daily maximum and minimum temperature, less rainfall than the other regions in January and February and a decrease in solar exposure from January to March. Bundaberg had the smallest differences between daily maximum and minimum temperature, the highest rainfall in February and March and a very similar solar exposure to Kingaroy. Kairi had the lowest maximum temperatures between November and January but the minimum temperatures were close to average. In January it rained more than in any other region throughout the plant growth season. At the same time the solar exposure in Kairi was much below average for that region. Other conditions, such as availability of nutrients (e.g. the peanuts were inoculated) and soil composition, were kept to an optimum for the peanuts as they would be if peanuts were cultivated by farmers in the regions.

The three peanut-growing regions lie relatively close to each other and despite the observed differences in environmental conditions, as expected, the plants in all regions were exposed to rather favourable conditions for peanut farming.

### 5.3.2.  *Plant performance*

Because the peanut plants examined in this chapter were grown by PCA, differences in plant growth and yield could not be determined, and only the weights of seeds were compared

between the treatments. For that, weights of 10 peanuts from each of the samples (triplicates per sites) were measured. One-way ANOVA was applied and the results were illustrated with box-plots and means with 95% confidence intervals (Figure 5.3).
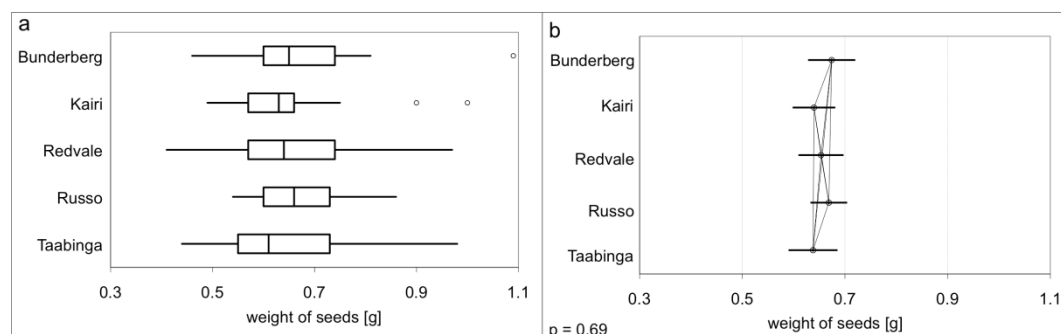


**Figure 5.3 Weight of peanut seeds from five locations in Australia**

The weights of peanut seeds from Redvale, Taabinga, Childers, Bundaberg and Kairi were compared using (a) Box plots and (b) means with 95% confidence intervals. A line between two means represents a significant similarity, while the absence of a line between means represents a significant difference between these means. The p-values are given for comparison of all data groups with one-way-ANOVA; In all panels n = 10 plants per replicate and three replicates per treatment. The peanuts were grown and provided by PCA.

The mean weight of the peanut seeds was between 0.6 and 0.7 g. Weights of the peanuts from the five treatments did not show any significant differences ($p > 0.5$). As expected, the peanut seeds from all locations had similar weights and appearance exactly as described on the PCA website (www.pca.com.au) and do not show any unusual characteristics, showing that none of the peanut regions had a effect on the appearance of the peanuts, which further indicates that the plants were grown under optimal conditions.

### 5.3.3.  *Total protein concentration and Ara h 1 and 2 content of peanuts grown in different locations in Australia*

In order to extract peanut proteins and allergens according to the findings in Chapter 3, 10 peanuts per replicate (with three replicates per location) were pooled were pooled (Chapter 3.8.6.) and the seed coat removed (3.8.5) before defatting the raw peanuts (3.8.4) with hexane (3.8.3) and extracting once (3.3.3) with 20 mM Tris (pH 8.5) (3.3.1) for 30 min at 21°C (3.8.2.). The levels of crude protein and Ara h 1 and 2 were measured with the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and commercial Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.) and expressed as mg per g flour. To illustrate the results and determine differences in the measured protein and allergen concentrations from peanuts grown in different locations, box-plots and means with 95% confidence intervals were plotted and one-way ANOVA applied.

Protein extracts of peanut plants grown in Redvale, Taabinga, Bundaberg and Kairi had a similar spread of protein contents (Figure 5.4a). While peanuts from Redvale, Taabinga and Bundaberg contained approximately 540 – 620 mg protein per g flour, Kairi contained the lowest protein content measured (498 – 598 mg). Protein extracts from peanut plants grown in Childers contained on average the highest amount of protein and the smallest variance with approximately 640 – 660 mg protein per g of peanut flour. Overall, the statistical analysis showed that peanuts from the five locations had different content of crude protein ($p < 0.05$) per g of peanut flour. However, when the means and 95% confidence intervals were compared (Figure 5.4b), only protein extracts from Kairi (545 ± 51 mg) and Childers (650 ± 9 mg) were significantly different to each other and both were statistically similar to protein extracts derived from plants grown in Redvale (577 ± 34 mg), Taabinga (621 ± 45 mg) and Bundaberg (573 ± 27 mg). This result should be interpreted with caution, as there is a significant heterogeneity of variance of the protein samples from Childers. The fact that peanuts from Bundaberg, which is very close to Childers and has very similar conditions, have a very similar protein content as peanuts grown in Kairi, substantiates the assumption that also the peanuts from Childers similar protein contents and the differences are due to the variance caused by the small sample size.

As shown with commercial ELISA kits (Indoor Biotechnologies Inc.), the Ara h 1 content in the peanut samples from all five locations was statistically very similar and on average between 12.9 and 13.8 mg per g of peanut flour ($p > 0.9$; Figure 5.4c,d). The amount of Ara h 2 was also statistically similar ($p > 0.5$), with values between 35.4 and 46.6 mg per g peanut flour (Figure 5.4e,f).

The peanut protein samples from plants grown in Redvale, Bundaberg and Kairi contained a similar amount of protein ($p > 0.05$) compared to the seeds used throughout Chapter 3 (pooled samples had 574 ± 28 mg protein; Chapter 3.8.6), which were obtained from PCA in Kingaroy in early 2010. The protein content of peanuts from Childers was on average 76 mg per g flour higher ($p < 0.01$) compared to the peanuts from Chapter 3, while peanuts from Taabinga were around 47 mg higher ($p < 0.05$). The peanuts grown in ambient-$CO_2$ greenhouses as reported in Chapter 4 (4.2.3.4) contained 722 ± 40 mg per g flour, which was 129 mg more than the average protein content of peanuts in this chapter.
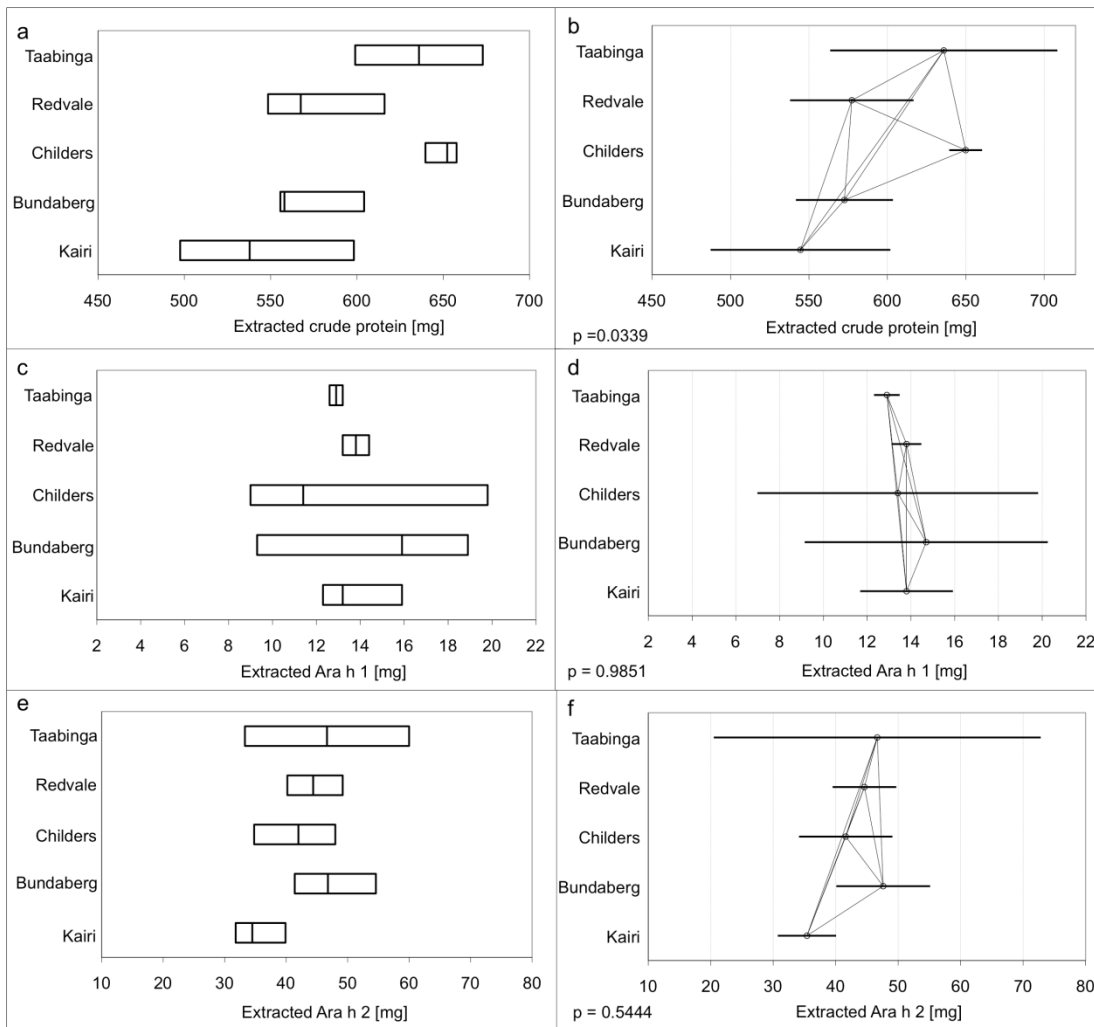
**Figure 5.4 Crude protein and Ara h 1 and 2 quantity of peanuts grown in different locations in Australia**

Box plots and means with 95% confidence intervals for protein extracts derived from five locations in Australia: (a,b) Amount of extracted crude protein; (c,d) Amount of extracted Ara h 1; (e,f) Amount of extracted Ara h 2. Each extraction derived from a pool of 10 seeds per sample and three biological replicates were used for each location. The amount of crude protein and Ara h 1 and 2 was measured in triplicate with the 2D Quant kit (Amersham Biosciences-GE Healthcare, Uppsala, Sweden) and commercial Ara h 1 and 2 ELISA kits (Indoor Biotechnologies Inc.). All measurements are based on mg per g of peanut flour.

The Ara h 1 content was statistically similar (p > 0.05) when the samples from the five locations and the samples from the ambient greenhouses and the samples in Chapter 3 (pooled samples; Chapter 3.9.6.) were compared.

The Ara h 2 content of peanuts from the five locations was very similar to that of the peanuts used in Chapter 3 (pooled samples; Chapter 3.9.6.). The Ara h 2 content was significantly higher in peanuts from Kingaroy, Taabinga, Childers and Kairi compared to the content in peanuts from the ambient greenhouses in Chapter 4 (4.2.3.4.). Peanuts from Bundaberg appeared to have more Ara h 2 than the peanuts from the ambient greenhouses, but the difference was not significant.

### 5.3.4. Comparison of 1- and 2D gels and 2D-DIGE

After having tested the amount of extracted crude protein and Ara h 1 and 2 in peanuts from plants grown at five different locations, qualitative differences in other proteins between extracts were examined using 1- and 2D-gel electrophoresis (Invitrogen, Carlsbad, CA).

Protein patterns for all extracts subjected to 1D gel electrophoresis (Figure 5.5) from all locations appeared very similar. The pattern was very similar to that obtained for extracts made using 20 mM Tris (pH 8.5) analysed in Chapters 3 (Figure 3.5.) and 4 (4.7.), with the same major and minor bands being present. Therefore it was concluded that none of the locations had an influence on the band pattern of the peanut protein extracts at the 1D-gel resolution.
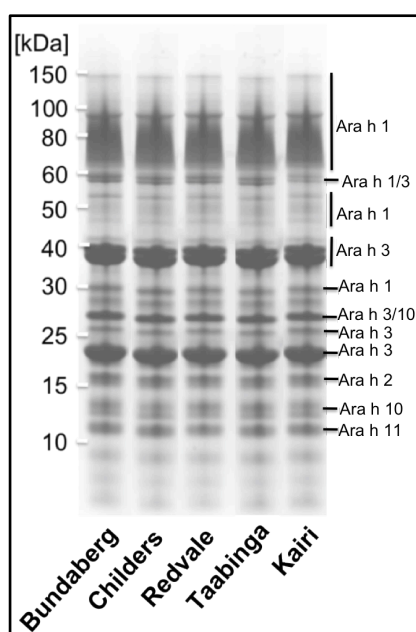


**Figure 5.5 1D-gel electrophoresis of extracts of one peanut variety grown in different locations in Australia**

The same volume of extract was run on each lane of the gel. Gel electrophoresis was performed with all three biological replicates per location and each extraction was derived from a pool of 10 seeds per plant. Only one of the biological replicates is shown in the figure. The protein identities derive from mass spectrometry experiments; details are in Appendix 2.

2D-gel electrophoresis was performed to obtain a higher resolution of individual proteins in the protein extracts from the greenhouse treatments. Due to a limited budget, only a subset of samples could be used. These consisted of three biological replicates of samples from three locations: Redvale, Childers and Kairi. After adding 25 μg of protein extract per sample into IPG buffer containing detergent (Chaps), denaturants (urea and thiourea) and a reducing agent (DTT), the protein samples were absorbed into IPG strips (pH 3–10 non-linear) and focussed up to 100,000 kVh with a maximum of 5,000 V. After reduction and alkylation of the proteins

on the strips, the second dimension was run on 12% Bis-Tris gels (12% Criterion XT Bis-Tris precast gels, Bio-Rad Laboratories Inc) and analysed using the Progenesis Same Spot software (Nonlinear Dynamics Ltd.).

Of the 422 protein spots analysed in total on 2D-gels, 45 spots were found to differ significantly in volume between protein extracts from peanuts grown in Redvale, Childers and Kairi. These spots corresponded to 10–100 kDa with putative pI values of 4–9 (Figure 5.6; significantly different spots are marked). The protein spots with significantly different volumes were distributed throughout the gel and included both major and minor protein spots.
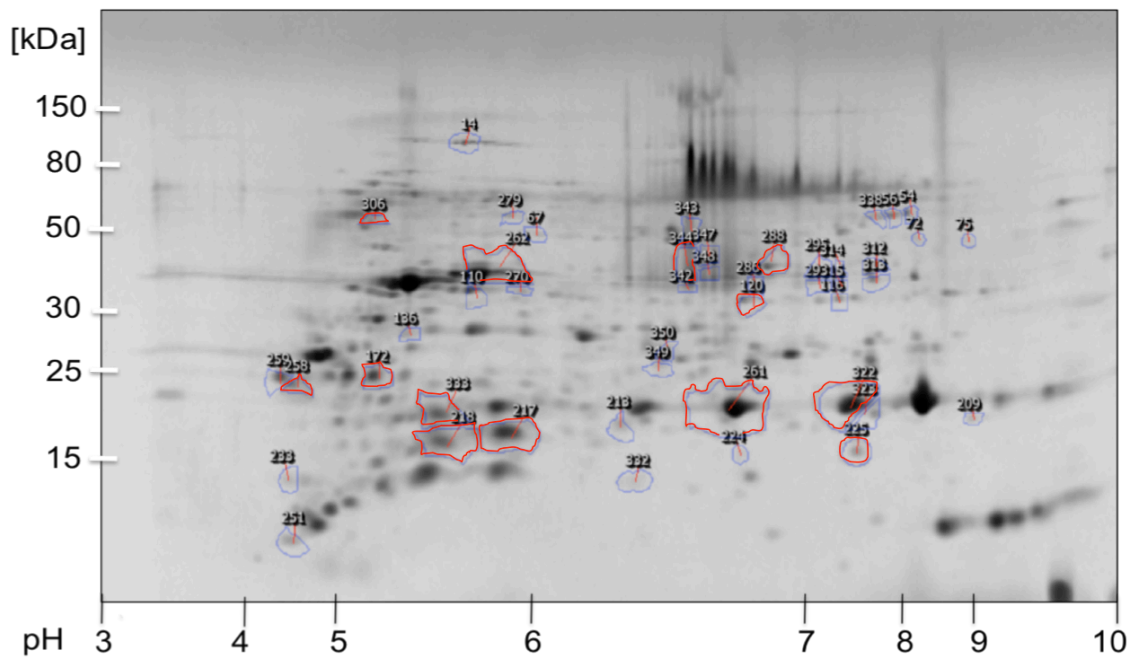


**Figure 5.6 2D-gel of crude protein extracts from peanuts grown in Redvale, Childers and Kairi**

Protein spots with significantly different spot volumes (p < 0.05) between crude proteins samples from three locations in Australia are highlighted on the displayed 2D-reference gel. 2D-gels were performed with three biological replicates of crude protein extracts obtained from peanut seeds from three location is Australia: Redvale, Childers and Kairi. IPG strips (pH 3–10 non-linear, Bio-Rad Laboratories Inc.) were rehydrated with 25 µg protein and focussed up to 100,000 kVh with a maximum of 5,000 on a Protean IEF cell (Bio-Rad Laboratories Inc.). The second dimension was run on 12% Bis-Tris gels (12% Criterion XT Bis-Tris precast gels, Bio-Rad Laboratories Inc). The 2D-gels were stained with Sypro Ruby and scanned using a Typhoon FLA 9000 laser scanner (GE Healthcare, General Electric Company, 2011) before analysis using the Progenesis Same Spot software (Nonlinear Dynamics Ltd.). The reference gel is shown and displays all protein spots that differed significantly in volume (p < 0.05) between crude proteins samples obtained from plants grown at the three locations. Red marked spots were used for identification of proteins with mass spectrometry (4.3; details and further not marked protein identities are listed in Appendix 2).

**Table 5.1 Protein spots with significantly different spot volumes of crude protein extracts of peanuts from Redvale, Childers and Kairi as detected on 2D-gels**

Only the 13 spots identified in mass spectrometry (highlighted red in Figure 5.6.) are listed here (a detailed list of all protein spots with significantly different spot volumes, detailed pictures and graphics showing the normalised volumes can be found in Appendix 4). Spot number, p-values and averaged normalised volumes of protein spots were acquired using Progenesis software (Nonlinear Dynamics Ltd.). The molecular weight and pI-values were estimated from the 2D-gels. The identification of proteins was performed with reference to an in-house peanut allergen database and NCBInr as marked (full list and details are in Appendix 2).

| Spot no. | Identification MS/MS | | | MS spot no. a) | Anova [p] | Fold b) | MW [kDa] | pI | Average normalised volumes | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | NCBI acc. no. | Isoallergen or other protein | Fragment (full length) [kDa] | | | | | | Redvale | Childers | Kairi |
| 262 | gi\|57669861 | Ara h 3 | Yes (60) | 32 | 0.001 | 2.1 | 38 | 5.6 | 8616 | 1.8e+004 | 1.2e+004 |
| 333 | No match | | | 27 | 0.003 | 1.3 | 19 | 5.5 | 2730 | 3577 | 2684 |
| 261 | gi\|37789212 | Ara h 3 | Yes (61) | 40 | 0.003 | 1.4 | 21 | 6.7 | 1.6e+004 | 2.0e+004 | 1.4e+004 |
| 258 | gi\|9864777 | Ara h 3 | Yes (60) | 97 | 0.009 | 2.2 | 23 | 4.3 | 305.6 | 234.4 | 511.1 |
| 218 | gi\|115187464 | thioredoxin fold c) | No | 26 | 0.010 | 1.3 | 17 | 5.5 | 3084 | 4137 | 3484 |
| 344 | gi\|21314465 | Ara h 3 | Yes (62) | 153 | 0.012 | 4.8 | 42 | 6.5 | 8110 | 4277 | 1686 |
| 288 | No match | | | 57 | 0.019 | 1.9 | 42 | 6.9 | 1604 | 943.8 | 859.6 |
| 120 | gi\|1168391 | Ara h 1 | Yes (71) | 73 | 0.021 | 1.4 | 34 | 6.8 | 277.3 | 352.2 | 390.3 |
| 217 | gi\|37789212 | Ara h 3 | Yes (61) | 37 | 0.030 | 1.3 | 18 | 5.8 | 6532 | 7034 | 5521 |
| | gi\|1168391 | Ara h 1 | Yes (71) | | | | | | | | |
| 306 | gi\|9864777 | Ara h 3 | Yes (60) | 35 | 0.031 | 1.6 | 53 | 5.1 | 514.7 | 526.7 | 329.3 |
| | gi\|1168390 | Ara h 1 | Yes (70) | | | | | | | | |
| 322 | gi\|9864777 | Ara h 3 | Yes (60) | 41 | 0.034 | 1.5 | 21 | 7.4 | 9274 | 1.3e+004 | 8584 |
| 225 | No match | | | 56 | 0.035 | 1.5 | 17 | 7.5 | 616.4 | 473.5 | 398.3 |
| 172 | gi\|1708792 | Galactose-binding lectin c) | Yes (29) | 64 | 0.045 | 1.4 | 25 | 5.2 | 1429.6 | 1115.9 | 1507.1 |

a) Spot number in mass spectrometry table (Appendix 2)
b) Fold difference in mean abundance of protein spots
c) Results from NCBInr database; protein spot 172 (64) was not found in peanuts, but for green plants (*Viridiplantae*)

Only the 13 protein spots that were identified using mass spectrometry are listed in Table 5.1 (a detailed list of all protein spots with significantly different spot volumes, detailed pictures and graphics showing the normalised volumes can be found in Appendix 4). Seven of the 13 protein spots identified were Ara h 3 and three were Ara h 1 (two protein spots were a mixture of both allergens). All of the averaged normalised volumes of the proteins spots from samples from Redvale, Childers and Kairi showed that spots differed only in volume (rather than presence/absence) and none of the protein spots were highly abundant in one sample set but not in the other. This shows that all proteins visualized were present in peanuts from all five locations.

It was not clear whether the differences in spot volumes of the protein spots were due to different protein concentrations in the protein extracts or due to gel-to-gel variation, which may have been high because only three biological replicates were used for each location. To overcome gel-to-gel variation that might have occurred and further substantiate the results, 2D-DIGE was performed, where two protein samples and one internal standard are labelled with different fluorescent dyes (Cy2, Cy3 and Cy5) before running them in equal concentrations on the same 2D-gel under the usual conditions. Because two samples were applied on one gel, three biological replicates from each of the five locations were used. This was in contrast to the conventional 2D-gels, where only three replicates from three locations were used. It was taken into account that one Cy-Dye might stain the proteins more than the other and lead to misleading results, so each gel had alternate Cy-Dye combinations. Because the mixed internal standard contained all proteins in equal amounts, and was applied to each gel, a relative quantitative comparison between samples on different gels was possible.
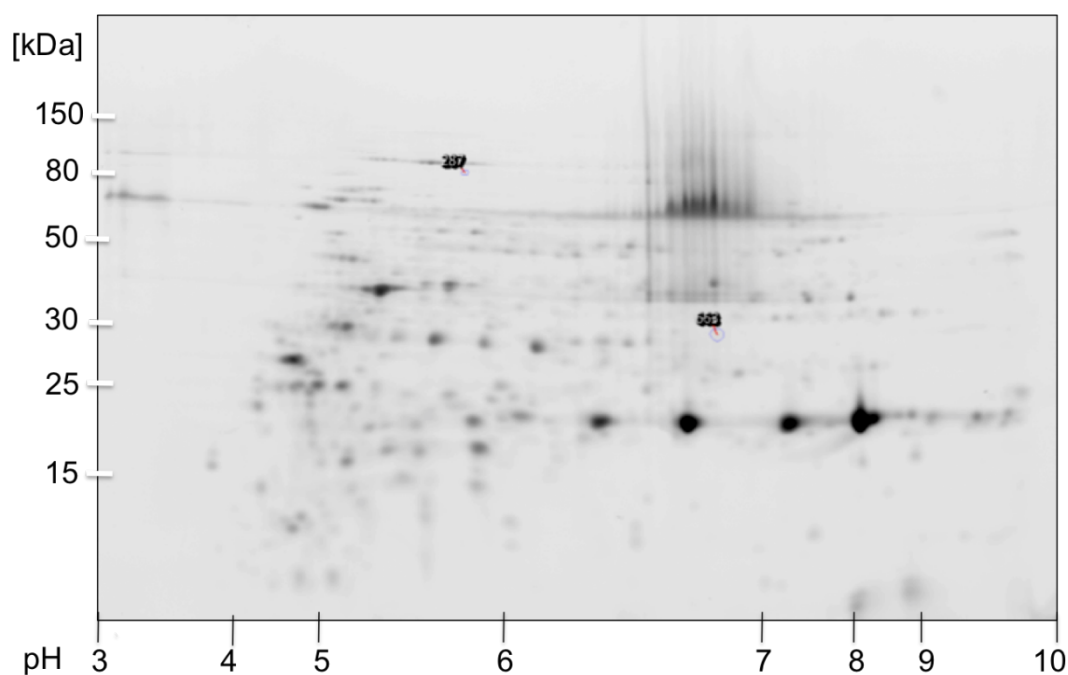


**Figure 5.7 2D-DIGE of crude protein extracts from peanuts grown in five locations in Australia**

Protein spots with significantly different spot volumes (p < 0.05) between crude proteins samples from five locations in Australia are highlighted on the displayed 2D-DIGE-reference gel. 2D-DIGE was performed with three biological replicates of crude protein extracts obtained from peanut seeds from five location is Australia: Redvale, Taabinga, Childers, Bundaberg and Kairi. After staining the proteins extracted from seeds from the different locations either with Cy3 or Cy5, an equal mixture of all 15 protein extracts was mixed and stained with Cy2. Equal amounts of two protein extracts and the mixed standard, adding up to 25 µg in total, were applied to IPG strips (pH 3–10 non-linear, Bio-Rad Laboratories Inc.) and focussed up to 100,000 kVh with a maximum of 5,000 on a Protean IEF cell (Bio-Rad Laboratories Inc.). The second dimension was run on 12% Bis-Tris gels (12% Criterion XT Bis-Tris precast gels, Bio-Rad Laboratories Inc). The 2D gels were scanned using a Typhoon FLA 9000 laser scanner (GE Healthcare, General Electric Company, 2011) and analysed using the Progenesis Same Spot software (Nonlinear Dynamics Ltd.). Most not marked protein identities are listed in Appendix 2).

**Table 5.2 Protein spots with significantly different spot volumes of crude protein extracts of peanuts from five different locations in Australia as detected on 2D-DIGE**

Spot number, p-values and averaged normalised volumes of protein spots were acquired with Progenesis software (Nonlinear Dynamics Ltd.). The molecular weight and pI-values were estimated from the 2D-gels.

| Spot no. | Anova [p] | Fold [a] | MW [kDa] | pI | Average normalised volumes | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Taabinga | Redvale | Childers | Bundaberg | Kairi |
| 287 | 0.016 | 1.5 | 81 | 5.8 | 0.658 | 0.972 | 0.748 | 0.990 | 0.910 |
| 663 | 0.041 | 1.4 | 29 | 6.9 | 1.135 | 0.962 | 0.831 | 1.099 | 1.113 |

[a] Fold difference in mean abundance of protein spots

The results of the 2D-DIGE experiments were analysed using the Progenesis Same Spot software (Nonlinear Dynamics, Ltd.). Surprisingly, only two protein spots with significantly different spot volumes between peanut protein extracts from the different locations were found (Figure 5.7, Table 5.2). These spots were minor and could not be identified with mass spectrometry.

The two protein spots with significantly different spot volumes detected on the 2D-DIGE gels (Figure 5.7) were not found on the conventional 2D-gels (Figure 5.6). Importantly, all five locations were compared in the 2D-DIGE experiments but only three locations with the conventional 2D-gels. Because the gel-to gel variation is reduced in 2D-DIGE experiments (where two samples and one mixed standard are run on the same gel) it is likely that identification of the significantly different protein spots in the 2D-DIGE was more reliable than those identified with the conventional 2D-gels. The different spot volumes that were detected using 2D-gels were therefore thought to be due to gel-to gel-variation. Furthermore, since only the volumes of the protein spots detected on 2D-DIGE were different (as opposed to the lack of specific protein spots in peanuts from one or more locations), it was concluded that there were no detectable qualitative differences in peanut extracts from the five locations and all proteins visualized as spots were present in all samples, with only two very small spots having slightly different average volumes.

In conclusion, peanuts grown in three peanut-growing regions in Australia have the same relative concentrations of individual proteins including major allergens. These findings were substantiated by the Ara h 1 and 2 ELISA results (5.4.1), which showed no difference between Ara h 1 and 2 content in peanut seeds from the five locations.

### 5.3.5.  *Western blots*

The recognition of antigens in protein extracts from peanuts grown in the different locations (Figure 5.8) by antibodies in a serum of a peanut-allergic patient (Appendix 2) was tested by

performing Western blotting after 2D-gel electrophoresis. The limited amount of available serum allowed only two biological replicates to be run for three locations: Redvale, Childers and Kairi. The Western blots (Figure 5.8) displayed the same protein spots that had been observed in the Western blots with Tris-extracted samples previously (Chapter 3.3.4.) except that two additional spots were detected. The Western blots made with protein extracts from Kairi, Redvale and Childers had the same intense spot patterns but protein extracts from Childers and Kairi resulted in fainter protein spots. It could not be clarified whether the majority of the faint protein spots were actual signals on the blots or merely the result of increased background.
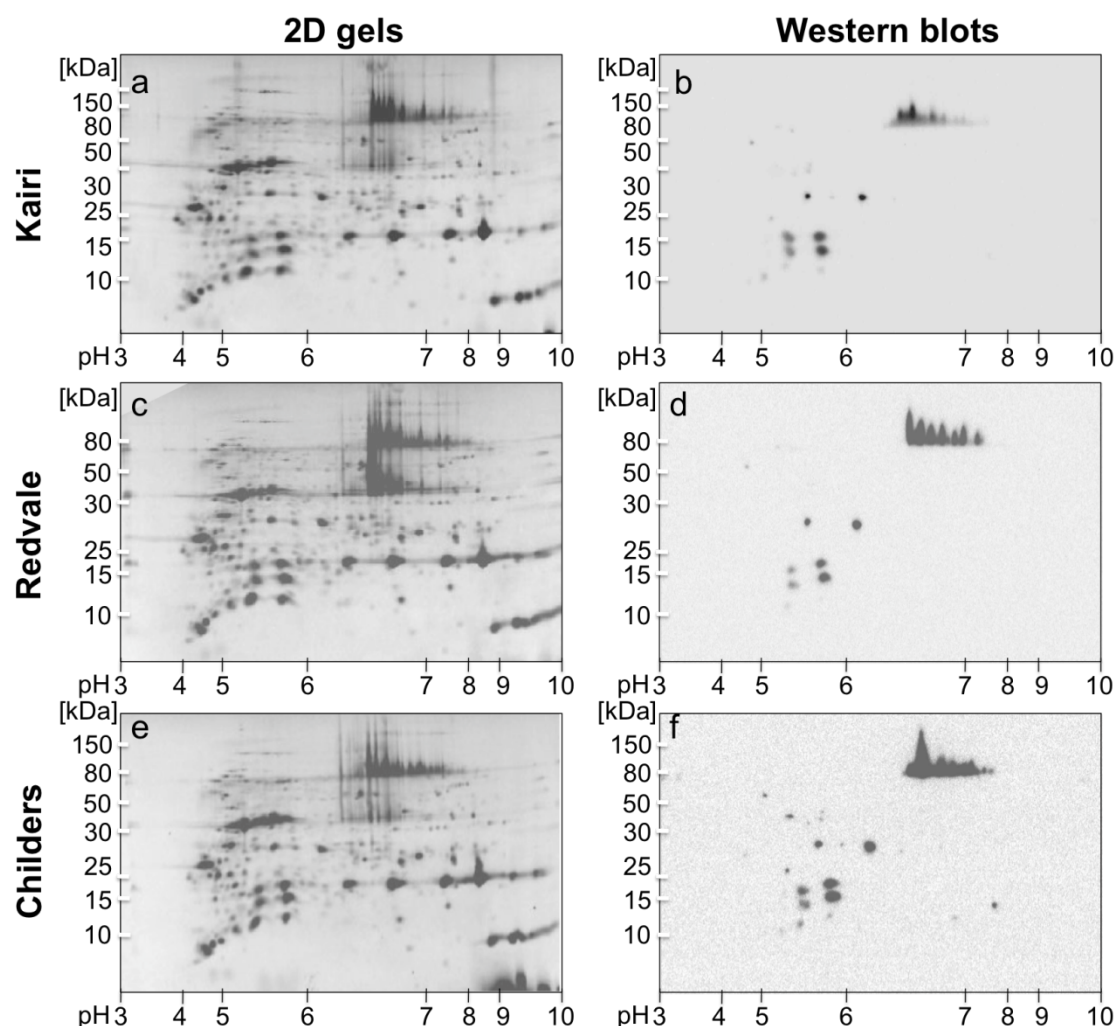


**Figure 5.8 Protein patterns on 2D-gel electrophoresis and Western blots of peanuts grown in different locations in Australia**

2D-gels and Western blots of crude protein extracts from Walter peanuts grown in different locations in Queensland, Australia (a,b) Kairi, (c,d) Redvale and (e,f) Russo. IPG strips (pH 3–10 non-linear, Bio-Rad Laboratories Inc.) were rehydrated with 25 µg protein and focussed up to 100,000 kVh with a maximum of 5,000 on a Protean IEF cell (Bio-Rad Laboratories Inc.), run on 12% Bis-Tris gels (12% Criterion XT Bis-Tris precast gels, Bio-Rad Laboratories Inc) and blotted onto a nitrocellulose membrane (Bio-Rad Laboratories Inc.). The Western blots were exposed to serum of a peanut-allergic patient, incubated with a secondary antibody containing horseradish peroxidase and developed using the ImmunStar HRP Chemilumisnescence kit (Bio-Rad Laboratories Inc.). The blots were scanned using a G Box (Synoptics Ltd.). Only one Western blot per treatment is shown because the duplicates were very similar. The identities of most protein spots are listed in Table 5.3 and Appendix 2.

**Table 5.3 Identities and intensities of peanut proteins spots detected in Western blots with peanuts grown in Kairi, Redvale and Childers**

The proteins matching the spot pattern in the Western blots in Figure 5.8 were subjected to mass spectrometry (Appendix 2). The spot number corresponds to the labelling in Figure 3.8 (Chapter 3.3.2.3). The identity and intensity of the protein spots are listed below: (+++), high intensity; (+) low intensity. No marking under Intensity indicates that the protein spot was absent in the respective Western blot (Figure 5.8).

| Spot no. | Identification MS/MS | | | Intensity of protein spot | | |
|---|---|---|---|---|---|---|
| | Isoallergen or protein | Fragment | MS spot no [a] | Kairi | Redvale | Childers |
| 1 | Ara h 1 | Yes | 128-139 | +++ | +++ | +++ |
| 25 | Ara h 1 and 3 | Yes | 37 | +++ | +++ | +++ |
| 22 | Ara h 3 | Yes | 38 | +++ | +++ | +++ |
| 14 | Ara h 3 | Yes | 45 | +++ | +++ | +++ |
| 7 | Ara h 3 | Yes | 32 | + | | + |
| 15 | Ara h 3 | Yes | 72 | | | |
| 5 | Ara h 3 | Yes | 30 | + | | + |
| 9 | Ara h 3 | Yes | 28 | | | |
| 10 | Ara h 3 | Yes | 29 | | | |
| 26 | Ara h 6 | Yes | 91 | + | | |
| 27 | Ara h 6 and 8 | Yes | 25 | + | + | + |
| 28 | Ara h 5 [b] | No | 36 | + | | + |
| 3 | Ara h 7 precursor [b] | No | 50 | | | |
| 20 | Ara h 7 precursor [b] | No | 60 | | | |
| 30 | Ara h 7 precursor [b] | No | 56 | | | + |
| 29 | Ara h 9 [b] | Yes | 55 | | | + |
| 24 | Thioedoxin fold [c] | No | 26 | +++ | +++ | +++ |
| 21 | *No match* | | 27 | +++ | +++ | +++ |
| 12 | *No match* | Yes | 49 | +++ | +++ | +++ |
| 2 | *No match* | | 51 | | | + |
| 11 | *No match* | | 98 | | | |
| 13 | *No match* | | 86 | + | | + |
| 18 | *No match* | | 116 | | | |
| 19 | *No match* | | 117 | | | |
| 16 | *No match* | | 107 | | | |
| 17 | *No match* | | 75 | + | | + |
| A | - | | | + | | |
| B | - | | | | | |
| C | - | | | + | | + |
| D | - | | | | | |
| E | - | | | | | |
| F | - | | | | | |

[a] Spot number in mass spectrometry table (Appendix 2)
[b] Not significant, no other match
[c] Result from NCBInr database; no match in in-house peanut allergen database

Overall, despite a constant variance of faint protein spots, there seemed to be no qualitative difference in recognition of antigens in Western blots among protein extracts from peanuts grown in the different locations.

## *5.4. Discussion*

Commercial peanuts (runner peanuts, variety "Walter"), grown in three peanut-growing regions in Australia (Kingaroy, Childers/Bundaberg and Kairi) had the same relative concentrations of individual crude proteins, including Ara h 1 and 2. 2D-DIGE showed that there was no difference in individual protein expression between the regions, including a range of allergens that were identified via mass spectrometry. Western blotting with peanut allergic serum highlighted the same major spots. Taken together, these data mean that the peanuts of the variety "Walter" from the three peanut-growing regions in Australia are most likely to have the same allergenic properties and are not suitable for commercialising a peanut with lowered allergen content by PCA.

It has been demonstrated in some studies that environmental conditions can significantly influence the seed protein content in some legumes (Burstin et al. 2011, Frimpong et al. 2009, Oluwatosin 1997, Saxena et al. 2002). However, nitrogen supply is the most important factor affecting protein content and composition (Triboi et al. 2000). Therefore, conditions that are associated with nitrogen nutrition, such as droughts, soil density, root diseases and pests might also influence seed protein content (Burstin et al. 2011, Burstin et al. 2007, Lawn and Rebetzke 2006, Matthews and Arthur 1985, Oluwatosin 1997).

There are differences in the environmental conditions the peanut plants presented in this chapter were exposed to in the three regions. However, it can be assumed that they were optimal for growing peanuts, because these regions are used to farm and commercialise them. The nutrition of the peanuts, including nitrogen, was furthermore controlled, meaning that it were kept at an optimum, such as in usual farming conditions. Therefore the relative concentration of the individual proteins is likely to be very similar . However, with emerging concerns regarding the peanut allergens, it is valuable to verify this theory and test allergen abundance of peanuts grown in different regions.

In contrast to the allergen composition of different peanut varieties (Koppelman et al. 2001, Kottapalli et al. 2008, Krause et al. 2010, Schmidt et al. 2009), little information is available about the allergenic characterisation of peanuts grown in distinct peanut-growing regions with different environmental conditions. To date only one publication from 2001 (Koppelman et al. 2001), compared the amount of allergens Ara h 1 and 2 in peanuts, including a runner variety (comparable to the "Walter" variety used here) grown in two regions: USA and Argentina.

The peanuts contained similar amounts of crude protein and allergens Ara h 1 and 2 and the results are consistent with the data presented in this chapter. However, Koppelman et al. (2001) used methods that have since been outdated; for example, densitometry measurements of 1D-gels were performed to quantify the allergens Ara h 1 and 2, and none of the protein bands was identified as such, but classified by running a purified sample in a neighbouring lane of the gel, raising questions about whether all existing isoforms were present. Furthermore, only pooled serum was used for IgE binding studies to test the allergenicity of the samples (Koppelman et al. 2001). Given that each band in 1D-gels is likely to contain a mixture of proteins, this identification method is sub-optimal and the quantification method imprecise. The IgE binding studies of Koppelman et al. (2001) did not distinguish between Ara h 1 and 2 and other allergens, which might have contributed to the IgE binding. Rather, the authors could give only a broad overall estimation of allergenicity, without being able to identify which allergens were responsible. In this thesis chapter, more sophisticated methods, some of which have been developed in recent years, were used to obtain more powerful data that substantiated Koppelman's results. These data are discussed in the general discussion (Chapter 6).

The extractable crude protein content of raw peanut was measured and evaluated in this study using the most efficient and viable extraction method (20 mM Tris, pH 8.5, 30 min, 21°C) described in Chapter 3 of this thesis. Thus, the quantity and quality of the protein content and Ara h 1 and 2 factions and the protein pattern on Western blots were based on the extraction and the influence of the buffer on the proteins and epitopes in the peanuts (see General discussion, Chapter 6). Allergens that were not sufficiently extracted were not investigated and might have different quantities in the peanut from the different regions. However, the high extraction efficiency of Tris for the major allergens and the small differences in environmental parameters between the peanut-growing regions mean this it is not likely that major allergens are affected.

Another limitation of this study, due a lack of resources, is the small number of replicates in some of the experiments, such as the measurement of crude protein or the Western blots, which resulted in heterogeneity of variance between the samples. However, in order to be statistically valid, each experiment was done with at least three biological replicates and more technical replicates if applicable, except for the Western blots, which were done only in duplicates due to availability of serum. In order to confirm that the statistical difference in protein concentration between Childers and Kairi is only a result of this heterogeneity and the

peanut protein concentrations are actually similar, more technical replicates are necessary. The fact that the protein content of peanuts from Bundaberg, which is very near to Childers, was statistically similar to the protein content from Kairi and that the 2D-DIGE experiments did not show major differences it is nonetheless most likely that the total protein content of peanuts from Kairi and Childers are similar. Furthermore, the Western blots were performed with only one patient's serum (and a non-peanut allergic serum as a negative control), which means that only subsets of the epitopes present were recognised. The Western blots were performed with previously denaturated proteins, which might have lost some epitopes. This cannot be avoided when using this powerful but limited method of analysis. Nevertheless, this study shows effectively that the allergens are similar in the peanuts from the different treatments and that there should be no difference in allergic reaction for patients from peanuts from the three different peanut-growing regions.

The methods used in this chapter to test the allergen content are discussed in the general discussion (Chapter 6). General difficulties associated with testing the allergenicity of crops with lowered amount of allergens is discussed in a recently accepted book chapter, which is attached as Appendix 5.

## *5.5.* *Conclusions*

Despite a relatively small number of replicates, it was effectively shown that the extractable proteins from peanut plants grown in three regions in Australia are similar and provide the same set of allergens, and therefore a similar thread to peanut allergenic patients.

# Chapter 6

# General discussion

## *6.1.* *Protein content differed between peanuts grown in greenhouses and peanuts grown in the field*

The amount of crude protein was measured using the 2D Quant kit, which has been used for peanut extracts previously in the literature (Kim et al. 2011). The amount of protein and allergens was expressed as protein as per g of peanut flour, as in other published work (Poms et al. 2004). This form makes it easy to compare the quantity of proteins from other studies, rather than giving a concentration, which is dependent on the respective base material and buffer volume. The extraction of peanut proteins from n-hexane defatted peanut flour with 20 mM Tris (pH 8.5) for 30 min at 21°C resulted in the solubilisation of 541 – 623 mg crude protein per g of defatted peanut flour as reported in chapter 3. The average protein content of extracts from plants grown in the greenhouses under ambient $CO_2$, as reported in Chapter 4, was significantly higher (722 ± 10 mg protein per g peanut flour) than those in Chapter 3. This is likely due to the different growth conditions in the field compared to greenhouses. Differences in crude protein content are most likely due to different levels of available nitrogen, which is the most important factor affecting protein content and composition (Triboi et al. 2000).

Factors influencing the nitrogen availability could include the soil type and its moisture and nutrient level (Burstin et al. 2011, Burstin et al. 2007, Lawn and Rebetzke 2006, Matthews and Arthur 1985, Oluwatosin 1997). A major difference in nitrogen supply between field and greenhouse conditions is the symbiosis with rhizobia, which fix nitrogen after becoming established inside root nodules of legumes. In the greenhouses artificial nitrogen fertiliser was used instead. In Chapter 5 peanuts from Redvale, Taabinga and Bundaberg contained approximately 540 – 620 mg protein per g flour, a very similar amount to the protein extraction in Chapter 3. Kairi contained a slightly lower amount of protein (498 – 598 mg), but the differences are not significant. Protein extracts from peanut plants grown in Childers contained on average the highest amount of protein and the smallest variance with approximately 640 – 660 mg protein per g of peanut flour. The small variance might be due to the small number of replicates. It could also be that the soil quality in Childers was different with different amounts of available nitrogen. Importantly, the peanuts used throughout this

thesis were from the same variety "Walter", which is a "hi-oleic" variety of peanuts (www.pca.com.au). The high content of oleic fatty acid does not affect on peanut allergenicity and high-oleic peanuts show the same risk of allergy as normal peanuts (Chung Si-Yin et al. 2002).

## 6.2.   Comparison of ELISA results

The quantification of allergens Ara h 1 and 2 was done by using commercial sandwich ELISA kits employing monoclonal antibodies (Indoor Biotechnologies Inc.). Despite the lack of global standardised reference allergens (van Ree R. et al. 2008) and the dependence of the ELISA outcome on the degree of processing and extraction method of the allergens (Chapter 3), these ELISA kits have been commonly used throughout more recent literature e.g. (Dodo et al. 2008, Pomes et al. 2003, Yu et al. 2011) and found to be excellent tools to measure the Ara h 1 and 2 content in food products.

The pooled peanuts in the field-grown peanuts (Chapter 3) resulted on average in 10.7 – 16.5 mg Ara h 1 and 38.6 – 43.9 mg Ara h 2 per g of defatted peanut flour. While the amount of Ara h 1 in peanuts grown in ambient $CO_2$ in the greenhouses was very similar (13 ± 3.9 mg Ara h 1 per g peanut flour), around a third more Ara h 2 was detected (64 ± 13 mg Ara h 2 per g peanut flour). The addition of nutrients can increase the trypsin inhibitor in a dose-dependent manner, while total soluble protein content remains unaffected (Cipollini and Bergelson 2001). This relationship and the increased amount of crude protein in the peanuts grown in greenhouses, shows that it is likely that more nutrients were available for the plants in the greenhouses compared to the field. The Ara h 1 concentration in plants from Chapter 5 (13 –14 mg per g of peanut flour) was very similar to those in Chapter 3. This is also the case for the Ara h 2 content in peanuts from Chapter 5 (35-47 mg per g of peanut flour). The peanut kernels from Chapter 3 were grown in a similar way in the field to the peanuts in Chapter 5, and thus the results were expected.

## 6.3.   Comparison of 1D- and 2D-Gels and 2D DIGE

All 1D-gels prepared with crude protein from raw peanuts using Tris were extremely similar in all cases throughout the thesis. The 2D-gels also showed mostly similar patterns. It was, however, observed that the intensity of the Ara h 1 isoforms (mass spectrometry label 128– 139) differed in the replicates. This might be due to different abundance of Ara h 1 isoforms or differences in glycosylation of Ara h 1 fragments, which would affect the various pI values. The use of technical and biological replicates was financially unfeasible in this study.

By doing technical replicates, the gel-to gel variation can be assessed; however, the data could be significantly skewed and could result in inaccurate conclusions. Instead biological replicates were chosen, which allow the determination of variations within a treatment.

The protein profile and relative concentrations of proteins extracted from peanuts grown in the three greenhouses and three regions in Australia were visualised in high-resolution 2D-DIGE experiments. This technique allows reliable relative quantification of multiple proteins in a single gel electrophoresis experiment, where gel-to-gel variation is removed by the incorporation of an internal standard (Marouga et al. 2005). This approach has been used successfully in the recent years when comparing relative concentrations of peanut allergens (Chassaigne et al. 2009, Kottapalli et al. 2008, Schmidt et al. 2009).

## *6.4.    Comparison of Western blots*

The Western blot approach does not compare with the analysis of the allergenicity of peanuts by *in-vitro* histamine release from sensitized human or humanized basophils (Goodman et al. 2008), which requires large volumes of serum not accessible in this project, or the gold standard the double-blind, placebo-controlled, food challenge DBPCFC (Peeters et al. 2007), This was not feasible in this study because of the high risk for allergic patients. Additionally, it is unfortunate that only one serum was available for testing the IgE recognition of peanut extracts on Western blots. Nevertheless, it was still possible to compare the variation of a small subset of allergenic protein fragments between the treatments on Western blots, including the major allergens Ara h 1, 2 and 3. Possible compensatory effects which might have occurred between treatments; e.g. if less Ara h 1 but more Ara h 3 would be expressed in the samples (Krause et al. 2010) would likely be detected. While IgE binding studies with unfractionated protein extracts reveal the overall allergenicity of a protein extract in a powerful way, Western blots have the advantage that they can allow the identification of specific proteins affected in the extracts. However, this is the case after the proteins are denatured with SDS, urea, etc. Western blotting is still a very powerful method in allergen research commonly used to find protein spots that bind to antibodies in serum from peanut allergic patients (Chassaigne et al. 2009, Schmidt et al. 2009, Schmitt et al. 2010).

The 2D Western blots prepared with 20 mM Tris extracts in Chapter 3, as well as all Western blots in Chapter 4 and 5 have been prepared the same way. However, the recognised protein spot patterns vary substantially between the chapters. Some proteins spots are visible on each gel, such as spots 26, 27, 37, 38 (which might contain Ara h 2 in addition to Ara h 1, 3 and

non-allergenic proteins; see discussion in Chapter 3.4), as well as spot 45 (Ara h 3) and spots 128 – 129 (Ara h 1). Other protein spots seem to vary in intensity (42, Ara h 3), while some appear seemingly random between treatments. With the number of replicates it is impossible to assign these changes to the treatment of the samples and it was suspected these spots might represent increased background. It is suspected that the protein spots display different epitopes in the corresponding protein spots. The differences in the protein spot pattern are either due to differential expression of the proteins, varying stages of posttranslational modifications on the gel, which means that some Ara h 3 fragments have not been split yet or likely due to unavoidable variations in the procedure. These might include a variation is blotting efficiency, which might have occurred unnoticed (the Ponceau stain used to test the successful blotting of proteins had a low sensitivity and did not show all small spots), differences when applying Chemiluminescence reagent or in the detection. Nonetheless, it is possible to conclude with high confidence that the protein spots that are present on all the Western blots contain epitopes that are recognised by the IgE in the patients' serum. Importantly, the Western blots performed with TBS in Chapter 3 are much more intense and the duplicates are very similar, indicating at least a higher likelihood of an increased detection rate in TBS extracts on Western blots. To validate if the different spot patterns are due to differential expression of allergens in the peanut seeds, more technical and biological replicates of Western blots should be performed. It would be valuable to use a range of patient's sera to assess the spectrum of IgE recognition to obtain a more comprehensive insight into the impacts of the peanut protein extraction buffer to Western blotting.

## 6.5.  *Influence of extraction protocol on results in Chapters 4 and 5*

Only the extractable protein content of raw peanut was measured and evaluated in Chapter 4 and 5 using the most efficient and viable extraction method (20 mM Tris, pH 8.5, 30 min, 21˚C) found in Chapter 3 of this thesis. Thus, the quantity and quality of protein and Ara h 1 and 2 fractions and the pattern on Western blots are based on the extraction and the influence of the buffer on the proteins and epitopes in the peanuts. Allergens that have not been sufficiently extracted were not investigated and might have been overseen. Additionally, it was shown that extraction patterns of proteins can differ between buffers. There is a possibility that use of a single buffer may have obscured differences which may have been detected had buffers other than Tris been used. However, the compatibility of 20 mM Tris with all subsequent experiments and its extremely high extraction efficiency make it a very good buffer for a comprehensive overview of the effects of growth conditions to major allergens in peanuts.

# General conclusions

The most interesting findings in this thesis were that buffer composition and pH value, as well as extraction protocol, including the choice of defatting reagents, strongly influence the extraction efficiency of peanut crude proteins and major allergens. Despite some variance between the Western blot results in the chapters, the detection and outcome of subsequent methods of analysis, such as SDS-PAGE, ELISA and Western blots, were strongly affected by the extraction buffer and protocol.

Depending on the scientific question asked, reagents should be selected with care and results interpreted considering possible structural effects. Extraction buffers and protocols and their effects and influence on the properties of allergens should be empirically investigated and made publicly available. Nevertheless, a standardisation in the extraction of both peanut protein samples and standards such as in ELISA kits is essential for quantifying and analysing allergenicity reliably and reproducibly. Standardisation in expressing the allergen quantity should also be attained, to be able to compare results between publications (e.g. ng allergen per g hexane-defatted peanut flour or ng allergen per peanut). Due to extremely low or undetectable protein quantities, seed coats should be omitted from normal peanut protein extractions, to obtain a better accuracy in weighing in peanut flour.

Although an impact of $CO_2$ on the allergenicity of peanut plants could not be demonstrated conclusively, it was observed that some environmental conditions, such as extreme heat events, could cause differences in allergen expression. Furthermore, it was observed that some environmental effects on peanuts, such as relative humidity and elevated $CO_2$, might interfere with each other.

The amount of crude protein and major allergens was very similar in peanuts from the three commercial growing regions in Australia, indicating a very similar allergenicity.

# Future directions

This thesis substantially contributes towards standardisation of the measurement of peanut allergens by demonstrating the critical importance of extraction protocols, both in preparing extracts of samples and in the provision of standards for immunoassays. The lack of previous recognition of the dependence of extraction on the conditions used, has led to significant disagreement about allergen content in the literature. In future, either different conditions will need to be used for optimal measurement of different allergens, or the different efficiencies of extraction for each allergen for a common and most optimal buffer will need to be established. To fully establish this, will be necessary to evaluate further extraction times and temperatures to this thesis, to be able to evaluate the impact on crude protein and individual allergens. Extraction methods that do not employ buffers, such as phenol extraction (Kottapalli et al. 2008), should also be included. It will also be necessary to evaluate these extracts by a variety of assay systems (as performed in this thesis) as these too can differ in their capacity to detect differences. This includes more technical and biological replicates of Western blots to validate the different spot patterns observed in 1D western blots. To overcome the high variance in 2D western blots, methods of detection other than chemiluminescence should be tested, such as by radiological methods which might be more reproducible and quantitative. It would also be necessary to use a wider range of patient's sera to assess the spectrum of IgE recognition, as both other allergens and other epitopes of different structure and stability on individual allergens may be recognised. In some cases, such as for Ara h 1 it would also be valuable to study the extraction efficiency in more detail, e.g. shorter frequencies of extraction times. As defatting is likely to be an essential part of the extraction process, it would also be interesting to study the effect of the different defatting reagents, as well as studying non-defatted peanut extraction in more detail to determine whether other allergenic components are lost in the treatments. Additionally, the effects of the extraction protocol on changes to the glycosylation of allergens and their subsequent IgE binding capacity, should be evaluated and included. This is essential for identifying the exact involvement of protein glycosylation in peanut allergy and needed to characterise the molecular mechanisms responsible for triggering allergenic reactions towards peanut exposure.

It is important to stress that results from this thesis showed that peanut allergen detection assays such as ELISAs (e.g. for Ara h 1) are highly context-dependent and the exact extraction protocol of samples and standards crucial for a reliable quantification. The development of new ELISA assays for either Ara h 1 other peanut allergens should therefore use extraction protocols for maximum allergen extraction efficiency. Furthermore, antibody pairs used in ELISAs (i.e. Sandwich-ELISAs) should utilise epitopes, which are more stable under different extraction conditions. This is essential when testing, e.g. hypo-allergenic peanuts for reduced allergencity. Part of this work has been done in this thesis already and it would be very valuable to continue this work for understanding and effectively quantifying allergenicity in the future.

Finally, the impact of climate change in particular elevated $CO_2$, is likely influenced by other unforeseeable climatic factors, which can in combination influence the allergen content of peanuts. These changes are however only small and will probably not affect the overall allergenicity of peanuts on a larger scale. Because peanuts are commercialised crops, it would be more interesting to test the influence of nitrogen supply on the expression of the major peanut allergens. This might include different amounts of nitrogen fertilizer, but also differences between external nitrogen supply and the endogenous supply of nitrogen supplied via nitrogen fixation by symbiotic Rhizobia bacteria. Both strategies are common when growing peanuts commercially.

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