Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 105 (2007) 1671–1681
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Investigation on sequential extraction of peanut allergens for subsequent analysis by ELISA and 2D gel electrophoresis

Hubert Chassaigne *, Marcel Brohée, Jørgen V. Nørgaard, Arjon J. van Hengel

Food Safety and Quality Unit, Institute for Reference Materials and Measurements, European Commission – DG Joint Research Centre, Retieseweg 111, B-2440 Geel, Belgium

Received 30 June 2006; received in revised form 20 April 2007; accepted 20 April 2007

Abstract

A two-step sequential extraction method of peanut proteins was proposed with the aim to investigate the protein composition and allergen content of peanut samples. The extraction procedure reported is fully compatible with subsequent analysis by enzyme-linked immunosorbent assays (ELISA) as well as 2D gel electrophoresis (2D PAGE). This sequential extraction method was used to study three different peanut varieties and three different types of food processing. Peanuts were analysed for total protein content and the extraction efficiency of raw and processed peanuts was determined. The total protein content of the three peanut varieties was found to be comparable, but their extraction efficiency varies. The peanut extracts were characterised by employing three different ELISA test kits specific to either the allergens Ara h 1 or Ara h 2, or to soluble peanut proteins. The content of both Ara h 1 and Ara h 2 differed in the raw peanut extracts of the three varieties. However, thermal processing resulted in much larger changes in detectability. Blanching significantly increases the detectability of Ara h 2, whereas Ara h 1 detection remains almost unchanged. After roasting a clear decrease of detectability was observed for both Ara h 1 and Ara h 2, although the effect is more severe for Ara h 1. 2D PAGE was employed to compare the protein profiles and abundances of peanut extracts. Statistically relevant differences were observed for the two different protein fractions obtained by using the described method, showing the relevance of this two-step sequential extraction method.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: Food allergy; Peanut proteins; Sequential extraction; Sample preparation; Enzyme-linked immunosorbent assay (ELISA); Two-dimensional gel electrophoresis (2D PAGE)

1. Introduction

Around 1–2% of the total population, and up to 8% of children suffer from some type of food allergy (Helm & Burks, 2000; Jansen et al., 1994; Mills, Jenkins, Alcocer, & Shewry, 2004; Ortolani, Ispano, Scibilia, & Pastorello, 2001; Woods et al., 2002). Although a large number of foods are known to be able to cause allergic reactions, a subset of only eight types of allergenic foods are responsible for causing more than 90% of all food allergies in the United States (Bush & Hefle, 1996). US legislation reflects the importance of those eight allergenic foods by means of a mandatory labelling of food products (108th Congress, 2nd Session (2004) S741, An Act to Amend the Federal Food, Drug, and Cosmetic Act, Washington, DC). The European Commission has widened this measure to protect allergenic consumers, by issuing Directives 2000/13/EC and 2003/89/EC which have now resulted in a mandatory labelling of 12 major allergenic foods in all its Member States. The majority of these 12 allergenic foods are of plant origin and are often consumed as seeds.

Within the allergenic food, protein components are usually the trigger of allergenic reactions (Mills, Madsen, Shewry, & Wichers, 2003), therefore it is of utmost importance to have specific and sensitive methods designed to detect allergens at low concentration and to control allergen-free products (Shin et al., 1998). Such methods are based on the

* Corresponding author. Fax: +32 14571787.
E-mail address: hubert.chassaigne@ec.europa.eu (H. Chassaigne).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.04.055

detection of either proteins or DNA fragments that indicate the presence of allergens or allergenic foods (Kiening et al., 2005; Poms, Klein, & Anklam, 2004).

Peanut is a highly allergenic plant food that is consumed as seeds. Peanut kernels contain a vast amount of proteins, amongst which there are at least eight allergens. The three major peanut allergens that are recognised by the vast majority of peanut allergic individuals and that have been studied extensively are Ara h 1, a vicilin-like protein (Dunwell, 1998; Pomes et al., 2005), Ara h 2, a conglutin-homologue protein (Maleki et al., 2000; Mills et al., 2003) and Ara h 3/Ara h 4, glycinin proteins (Piersma, Gaspari, Helfe, & Koppelman, 2005). These proteins have a high abundance in peanut which is likely to facilitate their detection. Peanut contains around 29% protein and the major allergen Ara h 1 accounts for approximately 20% of the total protein content with Ara h 2 accounting for ~10% (van Hengel, Anklam, Taylor, & Hefle, 2007).

The properties of peanut proteins are affected by heat treatments, and it is well known that submission of food to thermal processes causes protein denaturation or alteration, which often results in a decreased solubility. This hampers their extraction from food matrices (Mondoulet, Paty, & Dumare, 2005). Any analytical method based on the detection of proteins derived from allergenic food depends fully on an efficient, reliable and reproducible extraction of such proteins.

The aim of this work is to propose an improved method for protein extraction from peanuts. We report an advanced protocol for sequential protein extraction which takes into account the different properties of peanut proteins and their evolution as a result of food processing. Proteins from peanut samples need to be extracted efficiently and without degradation to ensure, as much as possible, that an accurate representation of the proteins in raw and processed material is obtained.

The sequential extraction method described here is fully compatible with immunological detection methods (Blais, Gaudreault, & Phillippe, 2003; Kiening et al., 2005; Yeung & Collins, 1996) and proteomic analysis by means of 2D PAGE. The two-step mild extraction method has been used to compare the extraction efficiency from three different varieties of raw peanuts and the effect of three different degrees of processing (blanching, mild roasting and strong roasting).

We have applied the complementary ELISA and 2D PAGE methods for the detection of a number of peanut proteins with a direct relevance to the allergenic potential of peanut. The composition of the peanut extracts has been investigated by employing ELISA test kits specific to Ara h 1, Ara h 2 or to soluble peanut proteins. The relative intensity of the ELISA signal obtained for the different protein extracts has been calculated for comparative purposes. Furthermore the extracts have been analysed by 2D PAGE to compare their representative protein maps, to establish respective protein abundances between extracts and to investigate the allergen content.

2. Materials and methods

2.1. Materials

2.1.1. Peanut samples

Three different peanut varieties (*Arachis hypogaea*) were obtained from different producers: Chinese peanut Red Skin (Unilever, The Netherlands), American peanut Medium Virginia (Golden Peanuts, USA) and American peanut Jumbo Runner (Golden Peanuts, USA).

The blanching method used by the manufacturer (Golden Peanuts, USA) was based on dry air impact. Processed peanut samples were produced in two steps: blanching (100 °C for 30 min) and dry roasting in hot air at 140 °C for 12 min (mild roasting) or for 20 min (strong roasting).

2.1.2. Chemicals

All chemicals used for sample preparation were obtained from VVW International (West Chester, PA, USA) or Sigma–Aldrich (St. Louis, MO, USA) and were at least analytical reagent grade. Water from a milli-Q water system (Millipore, Bedford, MA, USA) was used throughout. PlusOne chemicals for gel electrophoresis were obtained from GE Healthcare (Uppsala, Sweden).

2.1.3. ELISA test kits

Three commercially available test kits targeting either a specific peanut allergen (Ara h 1 or Ara h 2) or total soluble protein were used:

- biokits peanut assay kit (Tepnel Biosystems, UK), targeting Ara h 1;
- peanut residue ELISA kit (Elisa Systems, Australia), targeting Ara h 2;
- ridascreen peanut ELISA kit (R-Biopharm, Germany), targeting total soluble peanut protein.

All ELISA test kits were used according to the manufacturers instructions, by following the enclosed protocols.

2.1.4. 2D gel electrophoresis system

The first dimension isoelectric focusing (IEF) separations were carried out on immobilised pH gradients (IPG) gel strips (gel dimensions: 70 × 3.0 × 0.5 mm and 240 × 3.0 × 0.5 mm, linear pH range: 3–10, polyacrylamide gel matrix) with an Ettan IPGphor unit (GE Healthcare, Uppsala, Sweden). Separation in the second dimension (SDS-PAGE) was carried out with an Ettan DALT 12 electrophoresis unit (Peltier cooling unit) with pre-cast Ettan DALT gels (260 × 200 × 1.0 mm, homogeneous 12.5, i.e., in the separating gel: $T = 12.5\%$, $C = 3\%$; separation range 10–100 kDa) and SDS-buffers (Laemmli buffers).

2.1.5. Image analysis and protein spots detection

2D Polyacrylamide gels were silver-stained using a Hoefer Autostainer system (GE Healthcare, Uppsala, Sweden).

After staining, the gels were scanned using an Image Scanner system (GE Healthcare) and the image treatment was performed using the Image Master 2D Platinium software 5.0.

2.2. Methods

2.2.1. Total nitrogen/protein determination in peanuts

Total nitrogen determination in different peanut samples was achieved applying the Dumas combustion method using the Elementar Vario MAX CN analyser (Elementar GmbH, Hanau, Germany). The measurements were performed on 10 independent samples (mass determined accurately) of each of the three peanut varieties selected for this study. The protein factor (5.41) known for peanut allows the evaluation of the protein concentration (Holland et al., 1991).

2.2.2. Sequential extraction and sample preparation

The different peanut varieties were ground under liquid nitrogen in order to obtain a fine and homogeneous powder. The extraction scheme was designed to separate protein into two fractions soluble in TBS buffer and ethanol/water mixture, respectively. Extraction was done in two steps: (1) TBS buffer (20 mM Tris + 150 mM NaCl) pH 7.4 and (2) ethanol/water mixture, 20:80 (Fig. 1). Ten milliliter buffer was added to ca. 500 mg of ground peanuts and the solution was mixed by vortexing. The samples were put in an ultrasonic bath for 20 min (for cell disruption), on ice (4 °C) for protease inhibition. The same procedure was repeated for the second extraction step (using ethanol/water mixture, 20:80) (Fig. 1). The tubes were centrifuged at 16,000*g* and the supernatant was pipetted carefully through the lipid layer without disturbing the pellet.

The proteins were precipitated to remove the remaining interferents (detergents, salts, peptides) and to concentrate proteins before analysis. Precipitation solution (trichloroacetic acid (TCA)/acetone solution) (600 µl) was added to 100 µl of protein sample in microcentrifuge tubes, mixed well by vortexing and incubated on ice (4 °C) for 15 min. The tubes were then centrifuged at 16,000*g* for 5 min and the supernatant was removed. Milli-Q water (25 µl) was added to disperse the pellet. One milliliter ice-cold acetone (−20 °C) was added to wash the pellet. The tubes were agitated by vortexing until the pellet was fully dispersed and incubated at −30 °C for one night. The tubes were then centrifuged at 16,000*g* for 5 min and the supernatant was discarded. The proteins were re-suspended (i) in a copper-containing solution (alkaline pH) for total protein determination, (ii) in the isoelectric focusing rehydration solution prior to 2D PAGE or (iii) in the original extraction buffer (TBS buffer or ethanol/water mixture) and further diluted in the different ELISA buffers for ELISA measurements.

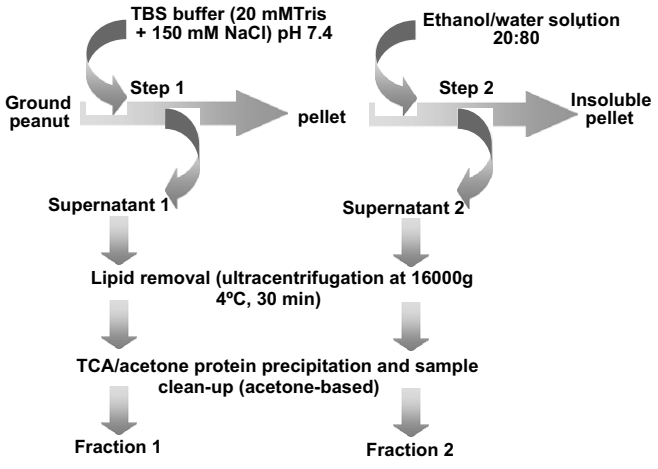

Fig. 1. Two-step sequential extraction procedure and sample preparation – experimental workflow.

2.2.3. Protein quantification of peanut extracts

A calibration curve with a bovine serum albumin (BSA) standard was established in the range 0–50 µg protein. Six standard solutions were prepared (0, 10, 20, 30, 40 and 50 µg). Two different methods for protein quantification were employed:

(1) The 2D quantification kit from GE Healthcare (Uppsala, Sweden) based on binding of copper ions to proteins re-suspended in solution. For this Eppendorf tubes containing 1–50 µl of the protein extracts to be assayed were prepared in duplicate. Precipitant (TCA) (500 µl) and co-precipitant (acetone) (500 µl) were added to each tube before mixing. The tubes were centrifuged at 16,000*g* for 5 min and the supernatant was completely removed. Copper solution (100 µl) and milli-Q water (400 µl) were added and the samples were vortexed briefly. One milliliter of working colour reagent was added to each tube. The samples were incubated at room temperature for 15–20 min and the absorbance of each sample and standard was read at 480 nm.

(2) The Smith copper/bicinchoninic assay based on reduction of copper ions (Smith et al., 1985). For this 1 ml of working reagent (mixture 50:1, Bicinchoninic acid (BCA) solution: 4% cupric sulfate) was added to the protein pellets. The samples were then incubated at 60 °C for 15 min and the tubes were allowed to cool to room temperature. The absorbance of all reactions was then recorded at 562 nm within 10 min.

2.2.4. Characterisation of protein extracts by 2D PAGE

The protein pellets obtained after TCA/acetone precipitation and sample clean-up for the extracts in TBS buffer and ethanol/water mixture were solubilised in 450 µl isoelectric focusing rehydration solution (8 M urea, 0.5% [(cholamido-propyl)-dimethylammonio]-propane sulfonate (CHAPS), 0.5% IPG buffer, 0.002% bromophenol blue). Subsequently 24 cm immobilised pH gradients (IPG) gel strips with a linear pH range (3–10) were rehydrated for 12 h at 20 °C using 450 µl rehydration solution. Isoelectric focusing was performed for 53,500 Vh with the initial

voltage limited to 500 V for 1 h, and then stepped up to 1000 V for 1 h and finally to 8000 V for 6.5 h. An accurate amount of 100 μg of protein was loaded on each individual gel.

After isoelectric focusing, the IPG gel strip was prepared for transfer to the second dimension by soaking, with gentle agitation, for 15 min in an equilibration solution (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and dithiothreitol (DTT)). The equilibrated IPG gel strip was embedded at the top of the SDS-PAGE gel in molten 1% (w/v) agarose in cathodic electrode buffer. A set of molecular mass markers was applied to paper IEF sample application pieces (MW range 14–94 kDa). A tris–glycine buffer pH 8.3 was used for the second dimension (SDS-PAGE). Six gels (homogeneous 12.5, 260 × 200 × 1 mm) for each extraction step (TBS and ethanol/water extracts) were run at 5 W per gel for 15 min (25 W), then 20 W per gel for 5 h (100 W) with the peltier cooling system at 25 °C. Gels were post-stained with silver according to the protocol of Shevchenko, Wilm, Vorm, and Mann (1996). After staining, the gels were scanned using the image scanner system and image analysis was performed using Image Master 2D Platinium software 5.0 (GE Healthcare).

3. Results and discussion

3.1. Determination of peanut protein content

In order to be able to determine the efficiency of peanut protein extraction it was necessary to first determine the total protein content of the peanuts. This can be determined by elemental analysers based on the Dumas method (Kirsten, 1983) that permit the determination of N/protein in various plant materials, e.g., peanuts, from samples ranging from milligrams to multi-grams. The Dumas combustion method is most accurate and represents an attractive alternative to the classical Kjeldahl Method, which requires a long and fastidious sample digestion procedure prior to analysis (Lee, Nguyen, & Littlefield, 1996; Wiles, Gray, & Kissling, 1998; Yeomans & Bremner, 1991).

Results from 10 independent measurements were obtained for each of the three peanut varieties selected for this study. Measurements were based on the complete combustion of ca. 500 mg of ground raw peanut samples for which the masses were determined accurately. The total nitrogen content (in %, w:w) for the peanut varieties Medium Virginia, Jumbo Runner, and Red Skin is given in Table 1. As expected, all three varieties have a comparable protein content (Table 1), which lies around 22%.

3.2. Investigation on a two-step mild extraction method for peanut protein

The efficiency of protein and allergen extraction is clearly dependant on the composition of the extraction

Table 1
N/protein determination for three different peanut varieties using the Dumas analyser and protein quantification in different extracts using two assays

Peanut variety	American peanut								Chinese peanut							
	Medium Virginia			Jumbo Runner					Red Skin							
Treatment	Raw			Raw	Blanched				Raw			Mild roasting			Strong roasting	
Nitrogen content (%, w:w)	4.11 ± 0.03			3.95 ± 0.07	–				4.06 ± 0.04			–			–	
Protein content[d] (%, w:w)	22.23 ± 0.14			21.35 ± 0.38	–				21.99 ± 0.22			–			–	
Extract	1[a]	2[b]	Total[c]	1	2	Total	1	2	Total	1	2	Total	1	2	Total	1	2	Total
Protein recovery[e] (% of protein in raw peanut)	19.0 ± 0.9	8.4 ± 0.6	27.4 ± 0.7	15.4 ± 1.0	10.8 ± 1.6	26.2 ± 1.0	12.7 ± 0.3	12.9 ± 1.4	25.6 ± 0.9	20.6 ± 0.1	16.1 ± 1.5	36.7 ± 0.9	5.1 ± 0.3	1.5 ± 0.2	6.5 ± 0.3	3.3 ± 0.2	1.1 ± 0.2	4.4 ± 0.2
Protein recovery[f] (% of protein in raw peanut)	16.9 ± 0.7	10.7 ± 2.0	27.6 ± 1.3	13.4 ± 0.6	14.3 ± 1.5	27.7 ± 1.0	13.4 ± 0.5	12.7 ± 2.3	26.1 ± 1.1	20.3 ± 0.5	15.5 ± 2.5	35.9 ± 1.4	5.2 ± 0.5	1.4 ± 0.3	6.6 ± 0.4	3.5 ± 0.3	1.2 ± 0.2	4.7 ± 0.5

[a] Extract in TBS buffer.
[b] Extract in ethanol/water mixture, 20:80.
[c] (1 + 2).
[d] Calculated using the protein factor for raw peanut (5.41).
[e] Copper-binding assay and spectrophotometric detection at 480 nm.
[f] Copper reduction assay and spectrophotometric detection at 562 nm.

buffer. This is confirmed by Poms, Klein, et al. (2004), Poms, Capelletti, and Anklam (2004) and Westphal, Pereira, Raybourne, and Williams (2004), who have used different buffers such as phosphate-buffered saline (PBS), hepes-buffered saline (HBS), carbonate and tris buffers with different pHs and concentrations for the extraction of peanut, soybean and sesame. However, such approaches do not provide information on the protein fractionation according to different solubilities. A two-step sequential extraction method, as depicted in Fig. 1, was proposed for this study in order to separate proteins into two fractions of different solubility. This simple fractionation strategy is likely to lead to an improved detection of different peanut proteins of different solubility and of low-abundance proteins by 2D gel electrophoresis. The choice of TBS buffer at pH 7.4 for extraction was based on previous work (Poms, Capelletti, et al., 2004). Non-denaturant buffers like this have shown to give high extraction efficiencies and can be used to solubilise proteins in order to be compatible with ELISA test kits (Poms, Capelletti, et al., 2004). A lower pH will only solubilise a subset of peanut proteins since most peanut proteins have isoelectric points between 3 and 6.5 (Koppelman et al., 2001).

Higher extraction efficiencies might be achieved by addition of surfactants and reducing agents to the extraction solvent (Watanabe et al., 2005). However, the use of such extraction buffers requires detection methods that are not affected by the buffer components and affects the integrity of the proteins thereby hampering proteomic analysis.

In this study a sequential extraction method was employed using a mixture of ethanol and water for the second extraction step. The choice of this buffer is based on previous studies (Breiteneder & Radauer, 2004; Breiteneder & Mills, 2005). According to Breiteneder and Radauer (2004) plant proteins can be classified into families and superfamilies on the basis of their structural and physicochemical properties. The prolamin superfamily contains the alcohol-soluble storage protein in plants. This superfamily includes three different groups of proteins including 2S storage albumins such as the peanut Ara h 2 (conglutin-homologue protein).

Efficient extraction was facilitated by mechanical destruction of cells and cell walls by means of ultrasonic treatment. Extraction and sample preparation was performed at low temperatures (4 °C) to inhibit protease activities. Another key point of the sample preparation concerns the elimination of major interferents, namely lipids and polyphenols. The extraction of lipids in a solvent (*n*-heptane or *n*-hexane) leads to an important lost of the most hydrophobic proteins. In this method lipid extraction was replaced by ultracentrifugation which is likely to reduce this loss (Granier, 1988). This step is of major importance for peanut extracts because of the high lipid content (ca. 46%). Precipitation techniques rapidly separate proteins from phenolic compounds (Englard & Seifter, 1990). Protein purification and concentration was achieved by TCA/acetone precipitation followed by re-solubilisation (i) in the isoelectric focusing rehydration buffer prior to 2D PAGE or (ii) in the original extraction buffer (TBS buffer or ethanol/water mixture) and further diluted in the different ELISA buffers for ELISA measurements.

3.3. Extraction efficiency of the sequential extraction method

Proteins were extracted from all three peanut varieties as well as from blanched and roasted peanuts using the two-step sequential extraction method. The protein concentration of the extracts was determined using two parallel approaches. First a method based on binding of copper ions to proteins was used and the results of these analyses were then confirmed using the Smith method based on the reduction of copper. Standard curves were generated by plotting the absorbance of the standards against the quantity of protein and used to determine the protein concentration of the samples. Each data point is based on four independent extractions for each variety and treatment and five replicates per measurement. The protein recovery is calculated as the percentage of the protein content in raw material as previously determined by the Dumas method (%, w:w).

The results in Table 1 show that for raw peanuts the first extraction step (extraction in TBS buffer) solubilises around 15–21% of the total peanut protein. For the peanuts Red Skin and Jumbo Runner the second extraction step (extraction with ethanol/water, 20:80) adds another 70–78% of proteins of extract 1 to the total extract. In the case of Medium Virginia this value was found to be between 44% and 63% depending on the quantification method (copper-reduction or copper-binding method). The total extraction efficiency obtained with the sequential extraction method was found to be similar (25–26%) for the two peanut varieties Medium Virginia and Jumbo Runner, but a little higher in the case of the variety Red Skin (36%) especially due to a higher recovery for extract 2. These first results clearly show the importance of the second extraction step that adds a significant amount of protein to the total peanut extract. Further investigations aim at determining whether this second extract is bringing valuable information on the protein composition and more specifically on allergen content of peanut samples.

The validity of using the second extraction buffer was assessed by comparing the data of the sequential extraction (Table 1) with two successive extractions using the only TBS buffer (pH 7.4). Total protein quantification as well as 2D PAGE experiments have been performed in order to establish the respective protein content (protein quantification using in both extracts using the 2D quantification kit) and protein maps after two successive extractions using only TBS buffer. Results have shown that extract 2 of raw peanuts represents only ~10% of extract 1 when using two consecutive extractions with the same buffer compared to an average of ~65% when the two different extraction buffers are employed. Furthermore 2D gels are virtually

identical for both TBS extracts when loading the same protein amount on 2D gels (data not shown).

Previously, an analysis of total protein content in peanut and protein concentrations in peanut extracts have been reported by Koppelman et al. (2001). However the protein content of both ground peanut and peanut extracts had been determined by total nitrogen measurement using the Kjeldahl method. The precision of this analytical method is lower (SD of 3.0 expressed in % w:w of total protein in peanut) compared to the one obtained in our work with the copper-binding method (SD 0.1–1.6). The recovery of extraction obtained by Koppelman et al. (2001) using Tris–HCl buffer was in the range of 18–26 ± 3% depending on the variety, whereas the ultrasonic-assisted sequential extraction as described in this study results in recoveries in the range of 25–36% depending on the peanut variety.

Two peanut varieties Jumbo Runner and Red Skin were chosen as examples to study the influence of thermal treatment on the amount of peanut protein extracted. This is of major importance since the extractability of peanut proteins, that are the causative agents for allergenic reactions, is reduced in peanuts destined for human consumption that have generally undergone heat treatments like roasting.

The results in Table 1 show that in the case of the selected peanut variety Jumbo Runner the blanching process does not significantly affect the protein recovery, since the total protein amount before and after blanching give similar results for both extracts 1 and 2. Roasting has however an immediate effect on protein extractability (Kopper et al., 2005; Westphal et al., 2004), especially for the second extraction step (Table 1). For mild roasted Red Skin peanuts, the extraction efficiency is reduced by 75% and 91% for extracts 1 and 2, respectively. After strong roasting, this reduction increases further to 82% and 95% (Table 1). This suggests that the extraction of hydrophilic proteins (extracted in the first step) are less affected by the thermal treatment than the more hydrophobic proteins extracted during the second step.

3.4. Ara h 1, Ara h 2 and soluble protein detection by ELISA

Three commercially available ELISA test kits that specifically detect Ara h 1, Ara h 2, or soluble peanut protein were employed to compare the peanut extracts. Since all extracts that were analysed with the ELISA test kits were obtained using the sequential extraction method and not with the extraction buffer supplied with the ELISA test kit the values in Table 2 are only given for comparative purposes. Each measurement was made in triplicate for each of the peanut extracts and the average values were calculated and normalised for the total extract of raw peanut. The results of the total extracts of processed peanut are then expressed as percentage of the signal obtained for the corresponding raw material. Table 2 lists the pea-

Table 2
Immunochemical detection for different peanut extracts using three different ELISA test kits specific to Ara h 1, Ara h 2 and to the total soluble proteins

Peanut variety	American peanut									Chinese peanut Red skin								
	Medium Virginia			Jumbo Runner						Red Skin								
Treatment	Raw			Raw			Blanched			Raw			Mild roasting			Strong roasting		
Extract	1[a]	2[b]	Total[c]	1	2	Total	1	2	Total	1	2	Total	1	2	Total	1	2	Total
ELISA A (as % of signal of raw peanut)	59.3 ± 0.8	40.7 ± 0.8	100 ± 2	53.4 ± 0.4	46.6 ± 0.7	100 ± 1	42.2 ± 0.5	49.3 ± 0.6	91.5 ± 0.5	50.9 ± 0.6	49.1 ± 0.8	100 ± 1	11.0 ± 0.1	3.98 ± 0.04	15.0 ± 0.3	5.96 ± 0.06	2.05 ± 0.03	8.01 ± 0.04
ELISA B (as % of signal of raw peanut)	62.7 ± 0.9	37.3 ± 0.3	100 ± 1	79.7 ± 0.7	20.3 ± 0.2	100 ± 2	79.8 ± 0.9	32.2 ± 0.6	112 ± 1	55.8 ± 0.9	44.2 ± 0.6	100 ± 2	55.8 ± 0.9	3.20 ± 0.02	59.0 ± 0.9	43.4 ± 0.1	3.83 ± 0.09	47.2 ± 0.4
ELISA C (as % of signal of raw peanut)	78.6 ± 0.6	21.4 ± 0.8	100 ± 1	65.1 ± 0.3	34.9 ± 0.1	100 ± 1	26.5 ± 0.7	14.5 ± 0.3	41.0 ± 0.6	72.3 ± 0.8	27.7 ± 0.5	100 ± 2	7.50 ± 0.04	1.50 ± 0.02	9.01 ± 0.09	1.63 ± 0.01	0.62 ± 0.01	2.25 ± 0.02

ELISA A: Kit targeting Ara h 1.
ELISA B: Kit targeting Ara h 2.
ELISA C: Kit targeting total soluble peanut protein.
[a] Extract in TBS buffer.
[b] Extract in ethanol/water mixture, 20:80.
[c] (1 + 2).

nut content (in %) of the three peanut varieties as determined by using three different ELISA test kits.

For the raw peanuts, the results obtained for extract 1 using the Ara h 1 specific ELISA are similar for all three peanut varieties and ranges from 50% to 60% of the value of the total extract. Table 2 shows that the extraction efficiency of Ara h 1 was almost doubled using the sequential extraction method when compared to a single extraction with a mild extraction buffer (e.g., TBS buffer). The data obtained with the Ara h 2 specific ELISA show that the value in extract 1 is more variable and ranged from 55% to 80%. This indicates that Ara h 2 is mostly detected in extract 1 and that the amount of ethanol/water extractable Ara h 2 varies by a factor 2 in the different peanut varieties tested. For the third assay, which employs an ELISA specific to soluble peanut protein, the peanut content of extract 2 represents 21–35% of that of the total extract depending on the peanut variety. Therefore, the sequential extraction method used here achieved an increase in detectability, the extend of which depends on peanut variety and on the type of ELISA kit employed.

In addition to the values for the raw peanuts Table 2 also lists the results of the processed peanuts. The recovery of peanut content is given for the extracts obtained from processed peanut and expressed in % of signal of the raw peanut extract using the same assay. After blanching, the value of Jumbo Runner, as determined with the Ara h 1 specific ELISA, was found to be slightly lower than that of the total raw peanut extract (91.5%). In contrast to this, the value for Ara h 2 showed an increase where 112% of the value of raw peanut was found in extracts of blanched peanuts. Interestingly, after blanching the value for Ara h 2 remained stable in extract 1, while for extract 2 this value increased to 158% of that of the raw peanut of the selected peanut variety Jumbo Runner. It remains however unclear whether this would apply to other peanut varieties as well.

The variety Red Skin was used to study the effect of roasting on the determination of peanut content (Table 2). The values obtained with the Ara h 1 specific ELISA, were found to be 15% and 8% of that of the total raw peanut extract after mild roasting and strong roasting, respectively. This decrease is less pronounced for extract 1 compared to extract 2 both after mild and strong roasting. The values obtained with the Ara h 2 ELISA kit, were found to be 59% and 47% of that of the total raw peanut extract after mild roasting and strong roasting, respectively. Surprisingly the Ara h 2 content was found to be quite stable for extract 1, whereas in fraction 2 the value of Ara h 2 showed a clear decrease after roasting. The heat stability of Ara h 2 and to a lesser extend that of Ara h 1 compare favourably to that of other soluble peanut proteins since after roasting the largest decrease was observed when the ELISA targeting soluble protein was employed (Table 2).

The effect of industrial processing on the detectability of peanut proteins like Ara h 1 and Ara h 2 is complex as it may involve significant transformations in the 3D structure of the proteins and the possible glycation of proteins and peptides during heat treatment. The denaturation of the proteins also has an important effect on their solubility and on their detectability by ELISA since antibodies are often specifically binding the native protein forms. The detectability of Ara h 1 after blanching remains almost unchanged whereas the detectability of Ara h 2 in extract 2 seems to increase significantly indicating that modification of the protein properties as a result of blanching increase the solubility of Ara h 2. The results obtained after mild and strong roasting of the peanuts show an important decrease of the detectability of Ara h 1 which is in line with the decrease in total protein recovery. The detection of Ara h 2 in extract 2 follows the same pattern. But, in contrast to this, roasting was found to affect the extractability of this allergen in extract 1 to a much lesser extent. These results also support the previously reported good thermal stability of Ara h 2 (Gruber, Becker, & Hofmann, 2005). The influence of the Maillard reaction on the allergenicity and detectability of the protein has been studied using a commercial ELISA test kit specific to Ara h 2 (Gruber et al., 2005), which has shown that thermal treatment of recombinant Ara h 2 in the presence of carbohydrates induces a strong increase in detectability. A recent study shows an association between advanced glycation end products (AGE) occurring in peanut protein and increased IgE binding of roasted peanut (Chung & Champagne, 2001).

3.5. Characterisation of protein extracts by 2D PAGE

The characteristics of individual peanut proteins and in particular their solubility determine their extractability in the two extraction buffers used in this study. A comparative analysis of extract 1 and extract 2 was performed after protein separation using 2D PAGE, which is a powerful tool for the analysis of the complex peanut protein mixtures. The suitability of this technique to characterise individual proteins lies in its enormous resolving power (Garfin, 2003). The aim of establishing 2D protein maps was to support this idea of having a two-step sequential extraction to extract different individual proteins. 2D PAGE and subsequent image analysis of the 2D protein maps allows to highlight the most significant differences in protein abundance and to perform statistical treatment on the large number of data generated.

The analysis of 2D gels in which protein extracts from raw peanuts were separated was carried out using the Image Master software (GE Healthcare). The gel images were sorted in two classes, classes 1 and 2 corresponding to the extracts 1 and 2, respectively (Fig. 2). In order to allow a statistical analysis six gels were analysed for each of the two classes. The spot detection mode used aimed to identify a maximum number of protein spots, but requires a good compromise between the sensitivity and the specificity of the spot detection algorithm. For classes 1 and 2, respectively 255 and 212 spots were detected (Fig. 2a and b).

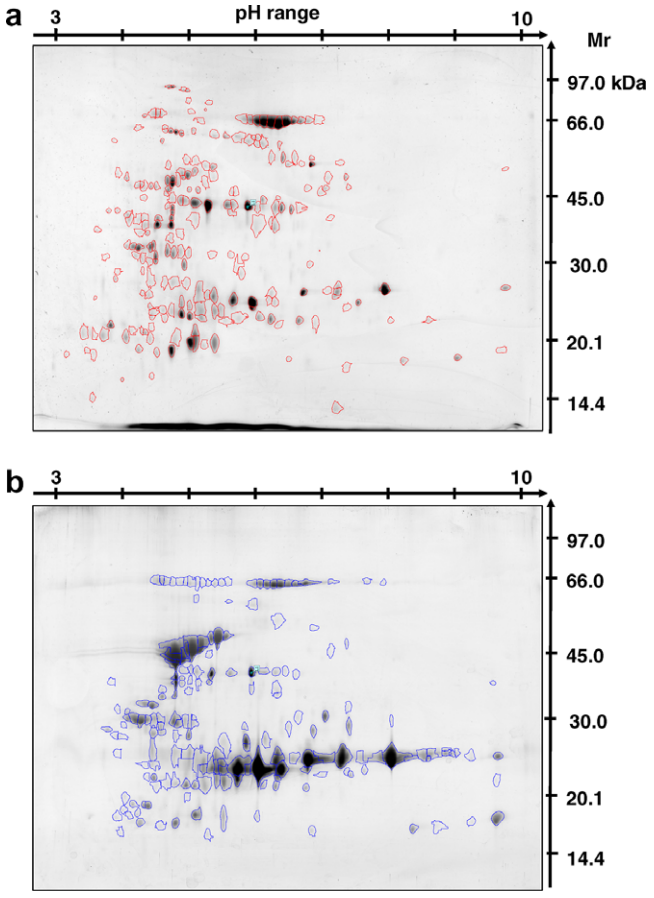

Fig. 2. 2D gels of peanut extracts after silver staining obtained from protein extract of raw peanut of the variety Red Skin: (a) proteins in extract 1 and (b) proteins in extract 2.

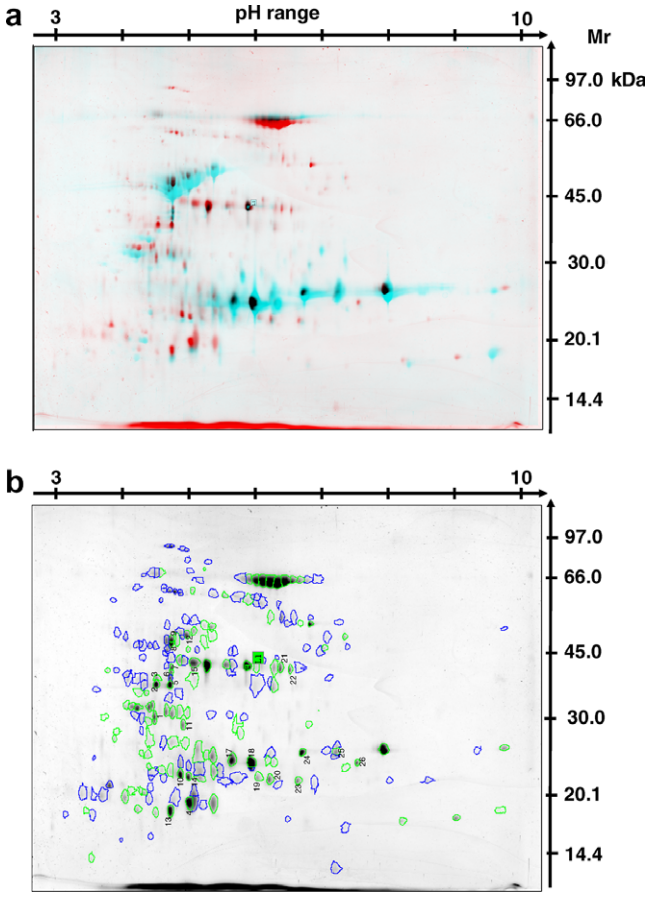

Fig. 3. 2D gel matching: (a) overlay of the protein maps in a transparent mode: red map corresponds to the proteins in extract 1 and blue map to the proteins in extract 2 and (b) matched spots appear in green and unmatched in blue, spot numbers corresponding to the spot list generated after statistical treatment. (For the interpretation of colour in this figure legend, the reader is referred to the Web version of this article.)

Using a transparent colour overlay allows visualisation of similarities and differences between the two protein extracts as shown in Fig. 3a. In this mode, the gels from classes 1 and 2 are presented in red and blue, respectively. Red spots represent proteins present in extract 1 and blue spots in extract 2. After spot matching, a total number of 138 spots were found to be common in the two extracts, therefore the percentage of matching was 62%. In Fig. 3b matched spots are shown in green whereas blue spots correspond to the unmatched spots. In this study alignment of the gels was achieved using a landmark named L1 (Fig. 3b). The gel report consists of a list of paired spot IDs (Table 3). The volume of each spot is calculated as the volume above the spot border situated at 75% of the spot height and the total volume of all spots in the gel is established. After normalisation, 130 spots were validated for data analysis and statistical treatment.

Analysis of the spot values between the two classes (1 and 2) allows the analysis of variation in protein abundance between the two protein extracts. The Kolmogorov test was used to determine if the two data sets (obtained for class 1 and 2) differed significantly. The empirical distribution for the spot values of each individual group (the same spot protein present in all gels) in the two classes (1 and 2) is established and a factor is defined as the maximum distance between the empirical distribution function of the two samples (working on the vol.% of the list of matched spots). If this factor K is greater than a particular decision limit ($K \geqslant 1$), a statistically significant difference between the samples exists. A total number of 30 spots was reduced to 26 spots after filtration (4 spots present a large dispersion within the same class) of which 16 proteins were found to be relatively more abundant in extract 1 (1/2 in the range 2.7–7.8) and 10 relatively more abundant in extract 2 (1/2 in the range 0.1–0.8) (Table 3 and spot ID shown in Fig. 3b).

The apparent molecular mass of the selected proteins was determined by co-electrophoresis of protein markers (Table 3). The pI values were determined according to the scale of the linear immobilised pH gradient strips (Table 3). Ara h 1, the major peanut allergen is known to be a 63–68 kDa glycoprotein (Burks et al., 1991). In our work, eight matched spots on the same line of mass (ca. 66 kDa) are detected in this corresponding area of the gel (Fig. 3b). However, no significant difference in abundance between the two extracts 1 (class 1) and 2 (class 2) was

Table 3
Report on 2D gel analysis (spot matching and normalisation of data) and statistical treatment

Pair number	Molecular mass (kDa)	Isoelectric point (pI)	% Volume class 1 (mean value)	% Volume class 2 (mean value)	Volume ratio 1/2
1	30–35	4.4	0.45	0.10	4.50
2	35–40	4.5	1.10	0.16	6.78
3	40–45	4.5	0.56	0.12	4.66
4	19–20	5.0	1.83	0.46	3.96
5	35–40	4.7	1.18	0.18	6.49
6	40–45	4.7	0.26	0.15	1.83
7	40–45	4.7	0.80	1.02	0.79
8	45–50	4.7	0.30	0.40	0.73
9	45–50	4.7	0.58	0.35	1.63
10	23–25	4.9	1.20	0.35	3.47
11	28–30	4.9	0.51	0.27	1.88
12	50–55	5.0	0.63	3.75	0.17
13	18–19	4.8	2.89	0.64	4.54
14	23–25	5.0	0.80	0.22	3.68
15	40–45	5.1	1.72	0.17	9.85
16	40–45	5.3	0.27	2.27	0.12
17	24–26	5.7	2.01	5.83	0.35
18	24–26	5.9	4.48	11.70	0.38
19	22–24	6.0	0.37	0.13	2.76
20	22–24	6.2	4.00	0.51	7.79
21	40–45	6.4	0.05	0.08	0.60
22	40–45	6.5	0.34	0.05	7.04
23	22–24	6.7	0.42	0.13	3.11
24	26–28	6.7	1.22	5.20	0.23
25	26–28	7.2	0.52	6.06	0.09
26	24–25	7.6	0.35	0.07	4.85

All volume spots value are expressed in pixels and normalised (%). Data for each matched spot generated using central tendency (mean value) and dispersion (standard deviation).
The chosen statistics are midrange (100%) and mean square deviation (MSD). Kolmogorov–Smirnov statistical test is used.

observed for the respective spots. Our results obtained with the Ara h 1 specific ELISA test also showed a similar amount of Ara h 1 detected in both extracts 1 and 2 of raw peanut. In Burks et al. (1992), a second major peanut allergen, Ara h 2, was first isolated by anion-exchange chromatography. Ara h 2 showed two large, closely migrating, IgE-specific bands in SDS-PAGE, with a mean molecular mass of ca. 17 kDa. 2D gel electrophoresis revealed the protein divided in four distinct spots at a mean molecular mass of 17 kDa and a mean pI of 5.2 (Burks et al., 1992). In our work, the corresponding mass lines of the gel shows several spots among which the four spots 10, 14, 13 and 4 (mean molecular mass of ca. 20 kDa and mean pI of ca. 5) were matched and were shown to have a higher abundance in fraction 1 (class 1) than in fraction 2 (class 2) (Table 3 and Fig. 3b). This result is also in accordance with the data obtained using the Ara h 2 specific ELISA, where Ara h 2 was shown to be present mostly in extract 1 of raw peanut.

These results show that the second extract contains proteins that are also present in extract 1 but that there are significant differences in abundance. The 26 proteins showing such differences in abundance (Table 3) represent only a subset of the actual differences in proteins extracted by the two different extraction buffers. Proteins that were not matched, for instance because they are detected only in one of the extracts, are not included in this list. This approach targeting individual peanut proteins is showing the relevance of the two-step sequential extraction method.

4. Conclusions

This study describes the development of a two-step mild sequential extraction procedure for peanut proteins that utilises non-denaturant conditions to be compatible with ELISA and 2D PAGE. The extraction efficiency was studied for peanuts of three varieties and found to be in a range between 25% and 36% of the total peanut protein content. Our results using the ELISA test kit specific to soluble peanut protein indicate that the extraction efficiency is higher than when a single extraction buffer (as supplied with commercial ELISA test kits for the detection of peanut) is employed. Therefore the sensitivity of such ELISA test kits can be improved when proteins are extracted using a sequential extraction procedure reported in this study. However, this protein fractionation has been developed mainly for an improved detection of low-abundant peanut proteins by using 2D PAGE and to target different allergens of different solubilities by using immunodetection techniques.

We also observed clear differences in extraction efficiency as well as in peanut content (as determined using the three different ELISA test kits) which were dependant on the variety of peanut. This kind of variability is a major concern for the quantification of peanut content in food

products. Food processing techniques are also known to affect the detectability and our study confirms that ELISA test kits give significant lower values for peanut content after heat treatment. Although this might be partially contributed to conformational changes of the epitopes recognised by the antibodies of such ELISA test kits, our results indicate that it is largely due to a reduction of the extraction efficiency. Such a reduction in extraction efficiency is different for individual peanut proteins as shown by the relative heat-stability of Ara h 2.

The influence of heat treatments on the detection of peanut in a cookie matrix was reported by Koch et al. (2003) who studied the detection of raw and roasted peanut in cookies by employing commercially available peanut ELISA test kits targeting soluble peanut protein, in a semi-quantitative fashion. The recovery rate for raw peanut was found to be between 106% and 136% for the cookies containing 500–2500 mg/kg raw peanut of the variety Jumbo Runner, while the recovery rates for roasted peanut (5 min at 153 °C) from the same variety were between 30.4% and 46.8%. It is apparent that (semi) quantification of peanut content in food products requires a standard material. The recent launch of IRMM-481, a peanut test material kit which contains five peanut varieties that underwent five different types of heat treatments is aimed to facilitate this.

2D PAGE analysis has clearly shown that the reported mild sequential extraction method solubilises two distinct sets of proteins, thereby increasing not only the amount of individual peanut proteins that can be extracted compared to a one-step method but also extending the total number of proteins extracted, when compared to using a single mild extraction buffer. The generation of 2D protein maps can assist in a better characterisation of peanut protein extracts or extracts of peanut-containing food products. This technique can also be employed to study the heat-stability of individual peanut proteins in processed peanut and to identify peanut specific marker proteins (e.g., protein spots from Ara h 1 or Ara h 2) for the analysis of food products.

Acknowledgement

The authors acknowledge Jozef Mast for the measurements of nitrogen/protein content using the Dumas analyser.

References

Blais, B. W., Gaudreault, M., & Phillippe, L. M. (2003). Multiplex enzyme immunoassay system for the simultaneous detection of multiple allergens in foods. *Food Control, 14*, 43–47.

Breiteneder, H., & Radauer, C. (2004). A classification of plant food allergens. *Journal of Allergy and Clinical Immunology, 113*, 821–830.

Breiteneder, H., & Mills, E. N. C. (2005). Molecular properties of food allergens. *Journal of Allergy and Clinical Immunology, 115*, 14–23.

Burks, A. W., Williams, L. W., Helm, R. M., Caunnaughton, C., Cockrell, G., & O'Brien, T. J. (1991). Identification of a major peanut allergen, Ara h 1, in patients with atopic dermatitis and positive peanut challenges. *Journal of Allergy and Clinical Immunology, 88*, 172–179.

Burks, A. W., Williams, L. W., Caunnaughton, C., Cockrell, G., O'Brien, T. J., & Helm, R. M. (1992). Identification and characterisation of a second major allergen, Ara h 2, with use of the sera of patients with atopic dermatisis and positive peanut challenge. *Journal of Allergy and Clinical Immunology, 90*, 962–969.

Bush, R. K., & Hefle, S. L. (1996). Food allergens. *Critical Reviews in Food Science and Nutrition, 36*(Supplement), S119–S163.

Chung, S. Y., & Champagne, E. (2001). Association of end-product adducts with increased IgE binding of roasted peanuts. *Journal of Agriculture and Food Chemistry, 49*, 3911–3916.

Dunwell, J. M. (1998). Cupins: A new superfamily of functionally-diverse proteins that include germins and plant seed storage proteins. *Biotechnology and Genetic Engineering Reviews, 15*, 1–32.

Englard, S., & Seifter, S. (1990). Precipitation techniques. *Methods in Enzymology, 182*, 285–300.

Garfin, D. E. (2003). Two-dimensional gel electrophoresis: An overview. *Trends in Analytical Chemistry, 22*, 263–272.

Granier, F. (1988). Extraction of plant proteins for two-dimensional electrophoresis. *Electrophoresis, 9*, 712–718.

Gruber, P., Becker, W. M., & Hofmann, T. (2005). Influence of the Maillard reaction on the allergenicity of Ara h 2, a recombinant major allergen from peanut (*Arachis hypogaea*), its major epitopes, and peanut agglutinin. *Journal of Agriculture and Food Chemistry, 53*, 2289–2296.

Helm, R. M., & Burks, A. W. (2000). Mechanisms of food allergies. *Current Opinions in Immunology, 12*, 647–653.

Holland, B., Welch, A. A., Unwin, I. D., Buss, D. H., Paul, A. A., & Southgate, D. A. T. (1991). *The composition of foods* (5th ed.). Cambridge: Royal Society of Chemistry.

Jansen, J. J., Kardinaal, A. F. M., Huijber, G., Vleig-Boestra, B. J., Martens, B. P., & Ockhuizen, T. (1994). Prevalence of food allergy and intolerance in the Dutch adult population. *Journal of Allergy and Clinical Immunology, 93*, 446–456.

Kiening, M., Niessner, R., Drs, E., Baumgartner, S., Krska, R., Bremer, M., et al. (2005). Sandwich immunoassays for the determination of peanut and hazelnut traces in foods. *Journal of Agriculture and Food Chemistry, 53*(9), 3321–3327.

Kirsten, W. J. (1983). Rapid, automatic, high capacity Dumas determination of nitrogen. *Microchemical Journal, 28*, 529–547.

Koch, P., Schäppi, G. F., Poms, R. E., Wüthrich, B., Anklam, E., & Battaglia, R. (2003). Comparison of commercially available ELISA kits with human sera-based detection methods for peanut allergens in foods. *Food Additives and Contaminants, 20*, 797–803.

Koppelman, S. J., Vlooswijk, R. A. A., Knippels, L. M. J., Hessing, M., Knol, E. F., van Reijsen, F. C., et al. (2001). Quantification of major peanut allergens Ara h 1 and Ara h 2 in the peanut varieties Runner, Spanish, Virginia and Valencia, bred in different parts of the world. *Allergy, 56*, 132–137.

Kopper, R. A., Odum, N. J., Sen, M., Helm, R. M., Stanley, J. S., & Burks, A. W. (2005). Peanut protein allergens: The effect of roasting on solubility and allergenicity. *International Archives of Allergy and Immunology, 136*, 16–22.

Lee, D., Nguyen, L. D. V., & Littlefield, S. (1996). Comparison of methods for determination of nitrogen levels in soil, plant, and body tissues, and water. *Communications in Soil Science and Plant Analysis, 27*, 783–793.

Maleki, S. J., Kopper, R. A., Shin, D. S., Park, C. W., Compadre, C. M., Sampson, H., et al. (2000). Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *The Journal of Immunology, 164*, 5844–5849.

Mills, E. N. C., Madsen, C., Shewry, P. R., & Wichers, H. J. (2003). Food allergens of plant origin – Their molecular and evolutionary relationships. *Trends in Food Science and Technology, 14*, 145–156.

Mills, E. N. C., Jenkins, J. A., Alcocer, M. J. C., & Shewry, P. R. (2004). Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. *CRC Review in Food Science and Nutrition, 44*, 379–407.

Mondoulet, L., Paty, E., & Dumare, M. F. (2005). Influence of thermal processing on the allergenicity of peanut proteins. *Journal of Agriculture and Food Chemistry, 53*, 4547–4553.

Ortolani, C., Ispano, M., Scibilia, J., & Pastorello, E. A. (2001). Introducing chemists to food allergy. *Allergy, 56*, 5–8.

Piersma, S. R., Gaspari, M., Helfe, S. L., & Koppelman, S. J. (2005). Proteolytic processing of the peanut allergen Ara h 3. *Molecular Nutrition and Food Research, 49*, 744–755.

Pomes, A., Helm, R., Bannon, G., Burks, A., Tsay, A., & Chapman, M. (2005). Monitoring peanut allergen in food products by measuring Ara h 1. *Journal of Allergy and Clinical Immunology, 111*, 641–645.

Poms, R. E., Klein, C. L., & Anklam, E. (2004). Methods for allergen analysis in food: A review. *Food Additives and Contaminants, 21*, 1–31.

Poms, R. E., Capelletti, C., & Anklam, E. (2004). Effect of roasting history and buffer composition on peanut protein extraction efficiency. *Molecular Nutrition and Food Research, 48*, 459–464.

Shevchenko, A., Wilm, M., Vorm, O., & Mann, M. (1996). Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Analytical Chemistry, 68*, 850–858.

Shin, D. S., Compadre, C. M., Maleki, S. J., Kopper, R. A., Sampson, H., Huang, S. K., et al. (1998). Biochemical and structural analysis of the IgE binding sites on Ara h 1, an abundant and highly allergenic peanut protein. *Journal of Biological Chemistry, 273*, 13753–13759.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., et al. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry, 150*, 76–85.

van Hengel, A. J., Anklam, E., Taylor, S. L., & Hefle, S. L. (2007). Analysis of food allergens. Practical applications. In Y. Pico (Ed.), *Food toxicants analysis* (pp. 189–229). Amsterdam: Elsevier.

Watanabe, Y., Aburatani, K., Mizumura, T., Sakai, M., Muraoka, S., Mamegosi, S., et al. (2005). Novel ELISA for the detection of raw and processed egg using extraction buffer containing a surfactant and a reducing agent. *Journal of Immunological Methods, 300*, 115–123.

Westphal, C. D., Pereira, M. R., Raybourne, R. B., & Williams, K. M. (2004). Evaluation of extraction buffer using the current approach of detecting multiple allergenic and nonallergenic proteins in food. *Journal of AOAC International, 87*, 1458–1464.

Wiles, P. G., Gray, I. K., & Kissling, R. C. (1998). Routine analysis of proteins by Kjeldahl and Dumas methods: Review and interlaboratory study using dairy products. *Journal of AOAC International, 81*, 620–632.

Woods, R. K., Stoney, R. M., Raven, J., Walters, E. H., Abramson, M., & Thien, F. C. (2002). Reported adverse food reactions overestimate true food allergy in the community. *European Journal of Clinical Nutrition, 56*, 31–36.

Yeomans, J. C., & Bremner, J. M. (1991). Carbon and nitrogen analysis of soils by automated combustion techniques. *Communications in Soil Science and Plant Analysis, 22*, 843–850.

Yeung, J. M., & Collins, P. G. (1996). Enzyme immunoassay for determination of peanut proteins in food products. *Journal of AOAC International, 79*, 1411–1416.