University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

Faculty Publications in Food Science and Technology

Food Science and Technology Department

2016

Allergenicity attributes of different peanut market types

Stef J. Koppelman
University of Nebraska-Lincoln, stefkoppelman@zonnet.nl

Shyamali Jayasena
University of Nebraska-Lincoln, sjayasena2@unl.edu

Dion Luykx
HAL Allergy, Leiden, The Netherlands

Erik Schepens
HAL Allergy, Leiden, The Netherlands

Danijela Apostolovic
HAL Allergy, Leiden, The Netherlands

See next page for additional authors

Follow this and additional works at: http://digitalcommons.unl.edu/foodsciefacpub

Part of the Dietetics and Clinical Nutrition Commons, and the Food Science Commons

Authors

Stef J. Koppelman, Shyamali Jayasena, Dion Luykx, Erik Schepens, Danijela Apostolovic, Govardus A.H. de Jong, Thomas G. Isleib, Julie A. Nordlee, Joseph L. Baumert, Steve L. Taylor, Hsiaopo Cheng, and Sohelia Maleki
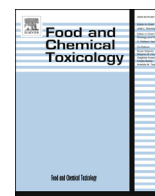
Allergenicity attributes of different peanut market types

Stef J. Koppelman [a, *], Shyamali Jayasena [a], Dion Luykx [b], Erik Schepens [b], Danijela Apostolovic [b, 1], Govardus A.H. de Jong [c], Thomas G. Isleib [d], Julie Nordlee [a], Joe Baumert [a], Steve L. Taylor [a], Hsiaopo Cheng [e], Soheila Maleki [e]

[a] Food Allergy Research and Resource Program, Department of Food Science & Technology, University of Nebraska, 279 Food Innovation Center, Lincoln, NE 68588-6207, USA
[b] HAL Allergy, J.H. Oortweg 15-17, 2333 CH, Leiden, The Netherlands
[c] TNO, Utrechtseweg 48, 3704 HE, Zeist, The Netherlands
[d] North Carolina State University, 210 Method Road, Greenhouse Unit 3, Raleigh, NC 27695-7629, USA
[e] US Dept. of Agriculture, Agricultural Research Service, Food Allergy Research, 1100 Robert E. Lee blvd., New Orleans, LA 70124, USA

ARTICLE INFO

ABSTRACT

Four different market classes of peanut (Runner, Virginia Spanish, and Valencia) are commonly consumed in Western countries, but for some consumers peanuts are a main cause of food-induced anaphylaxis. Limited information is available on the comparative allergenicity of these distinct market classes. The aim of this study was to compare allergenicity attributes of different peanut cultivars.

The protein content and protein profiles were highly comparable for all tested cultivars. All cultivar samples contained the major allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6, as assessed by SDS-PAGE and RP-HPLC, although some minor differences in major allergen content were found between samples. All samples were reactive in commercial ELISAs for detection and quantification of peanut protein. IgE-binding potency differed between samples with a maximum factor of 2, indicating a highly comparable allergenicity.

Based on our observations, we conclude that peanuts from the main market types consumed in Western countries are highly comparable in their allergenicity attributes, indicating that safety considerations with regard to peanut allergy are not dependent on the peanut cultivar in question.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Peanut (Arachis hypogaea L) is a seed crop legume that is widely used for human food purposes because of its high nutrition value (Oerise et al., 1974) and sensory attributes. The overall annual production of peanut (including Runner, Virginia, Spanish, and Valencia) in the U.S. in 2014 was 2.4 million tons (Anonymous, USDA NASS report, 2015) harvested from 1.4 million acres. The primarily grown species of peanut include two subspecies: hypogaea (Virginia market type) and fastigiata, the latter divided into two varieties fastigiata vulgaris (Spanish market type) and fastigiata fastigiata (Valencia market type). The Runner market type is a hybrid of fastigiata and hypogaea subspecies (Krapovicakas, 1969) and accounts for the majority (78.7%) of the U.S. peanut production. The Virginia market type accounts for approximately 19.9% of the U.S. peanut production. The Runner type is used primarily for the manufacture of peanut butter, and the large-kernelled Virginia type is marketed mainly as snack peanut and in-the-shell peanut products. The Spanish and Valencia market types are commercially less important, representing a combined 1.4% of the overall U.S. peanut production. The Spanish type, with rounder and smaller kernels, is used for snack peanuts, peanut butter and confections while the longer podded Valencia type, containing three to five kernels in each shell, is marketed mostly in the shell for roasting and boiling (American Peanut Council website, 2015).

Peanuts are widely known as potent allergens and count together with tree nuts for the majority of anaphylactic reactions to food (Sicherer and Sampson, 2007). Approximately 0.6% of adults and 1–2% of children/infants in the U.S. are affected by peanut allergy (Dyer et al., 2015; Sicherer et al., 2010). Unfortunately there is

no treatment currently available to cure peanut allergy and therefore, peanut-allergic patients must avoid consuming peanut. Several experimental immunotherapies such as oral immunotherapy (Anagnostou et al., 2014; Varshney et al., 2011), sublingual immunotherapy (Burks et al., 2015; Fleischer et al., 2013), and epicutaneous immunotherapy (Sampson et al., 2015) show promising results for desensitizing peanut-allergic patients, although complete tolerance to peanut using these approaches appears to occur in only a limited number of patients. A new approach using modified peanut allergens (Bencharitiwong et al., 2015) for subcutaneous immunotherapy is also currently being investigated (ClinicalTrials.gov identifier NCT02163018). The active compound for such therapies is essentially based on peanut proteins that can redirect the immune system. The dosage of peanut protein given in these therapies is controlled for the efficacy and safety of treatment, but it is not known if the source of peanut protein plays a role too.

To help peanut allergic consumers adhere to their peanut avoidance diets, the food industry has invested significant resources to ensure clear labeling of peanut-containing products and has also developed allergen control best practices to prevent peanut cross-contact in food products produced on shared equipment or in shared processing facilities. The validation of the effectiveness of cleaning protocols can be monitored using immunoassays to detecting peanut residue on equipment surfaces and quantifying peanut residue in food samples. Such immunoassays have different sensitivities for specific peanut allergens (Jayasena et al., 2015), i.e. some detect mainly Ara h3, and others detect mainly Ara h2 and Ara h2. Because it is not know if different peanut market types contain different levels of these peanut allergens., it is not known if certain peanut market types are under- or overestimated with such assays.

On occasion, pre-packaged food products have been shown to contain undeclared peanut residue at varying concentrations (Remington et al., 2013). Studies to quantify the risk that undeclared peanut residue poses to peanut allergic consumers who may eat such products rely on population threshold distributions modeled from peanut allergic individuals who have undergone a low-dose peanut challenge using various market types of peanuts (Taylor et al., 2010, 2015). It is not known if different peanut market types have different potencies thereby affecting both individual and population-based thresholds for peanut.

Some studies have investigated differences in the allergenic properties of various peanut types. It was shown that the four main market types (Runner, Virginia, Spanish, and Valencia) had comparable contents of the major allergens, Ara h 1 and Ara h 2 (Koppelman et al., 2001). Although the analytical tools used in that study may have been adequate in that era, quantitation of the major allergens would nowadays require more sophisticated analytical techniques. Also, since the time of that study other major peanut allergens have been identified that should also be taken into account. A more recent study compared different cultivation conditions on the allergen composition of Spanish peanuts (Walczyk et al., 2013) while Kottapalli et al. (Kottapalli et al., 2008) used 2D electrophoresis and proteomics to compare the protein profiles of the four market types commonly grown in the U.S. (Runner, Virginia, Spanish, and Valencia). The authors conclude that Valencia and Runner market types do not contain Ara h 3; however, the 2D gels indicate spots at the approximate position of Ara h 3 that were not identified. Due to complex post-translational processing of Ara h 3 (Piersma et al., 2005), it may migrate in 1D and 2D gel electrophoresis conditions at positions deviating from what is expected, possibly explaining why the authors concluded that Ara h 3 is absent in Valencia and Runner peanuts (Kottapalli et al., 2008). Another proteomics study made a detailed analysis of the proteins present in two different peanut types, i.e. Virginia and an Indonesian type named Kacang Asin or Bali peanut (Schmidt et al., 2009). Over 100 protein spots from 2D electrophoresis were identified, and it was shown that the level of Ara h 1 was substantially lower in the Kacang Asin peanut (Schmidt et al., 2009). Other peanut allergens were present in both peanut types in comparable amounts, but the analytical techniques used were only semi-quantitative, i.e. intensity if mass spectrometry signals (Schmidt et al., 2009).

This study quantitatively compares the allergenicity of the four main peanut market types (Runner, Virginia, Spanish, and Valencia). We have determined the protein content and protein profiles, and have applied different immunoassays to compare antigenic and allergenic potency. Furthermore, we have applied a reversed-phase HPLC method to quantify the allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6. This knowledge will serve in the development of well-characterized peanut immunotherapy materials for peanut allergy, and will also support risk-assessment and food safety programs for the food industry.

2. Material and methods

2.1. Reference peanut allergens and peanut kernel samples

The purified peanut allergens, Ara h 1, Ara h 2, Ara h 3 and Ara h 6, were obtained from lyophilized stock preparations made as described earlier (de Jong et al., 1998; Koppelman et al., 2003, 2005). Virginia peanuts were obtained from the North Carolina State University Department of Crop Science (Raleigh, NC), Runner and Spanish peanuts from the USDA-ARS National Peanut Research laboratory (Dawson, GA), and Valencia peanut from the New Mexico State University Agricultural Science Center (Clovis, NM). All peanut samples were used raw. Table 1 provides an overview of the peanut cultivar samples. Peanuts were shelled and initially stored according to the guidelines for cold storage of peanuts (American Peanut Council, 2006) for several months. The peanut kernels were later repackaged and stored at −20 °C. The nitrogen content of the intact peanut kernels was determined by the combustion method using a LECO FP-428 nitrogen analyzer at 950 °C combustion temperature (LECO Corp., St. Joseph, MI). Conversion to protein was done by multiplying the nitrogen value with 5.46 (Jones, 1931).

2.2. Preparation of extracts

10 to 15 g of peanut kernels was manually ground with a mortar and pestle until a fine, homogeneous paste was obtained. Three different extracts were prepared. The first series of extracts was prepared by mixing 2 g of ground peanut with 20 mL of extraction buffer (0.01 M Ammonium-bicarbonate, pH 7.9) in a 50 mL Falcon tube. Tubes were vortexed and placed in a rotator device (10 rpm) overnight at 2−8 °C. Tubes were centrifuged at 4500 rpm (2830 × g) for 45 min at 4 °C, and an aliquot of the middle layer was collected and transferred into 15 mL tubes and centrifuged again (4500 rpm; 2830 × g) for 45 min at 4 °C. Again, an aliquot of the middle layer was collected and transferred to several 1.5 mL tubes and centrifuged at 14,000 rpm (10,000 × g) for 20 min at room temperature (RT). Clarified solutions were collected from the middle portion of each microcentrifuge tube; these were pooled per sample, aliquoted in small volumes and stored at −80 °C until further use. Where transportation was required, samples were shipped frozen. The soluble protein concentration in the pooled extracts was determined by Bradford analysis (Sigma−Aldrich, USA) using a bovine serum albumin standard (Sigma−Aldrich) and a UV/Vis spectrophotometer (PerkinElmer, USA). This first series of extracts is referred to as aqueous extracts and was used for protein

Table 1
Protein and major allergen content of peanut samples.

Sample			Total protein			Major allergen content			
No.	Description	Market type	In kernel	Extracted	Yield of extraction (%)	Ara h 1 %	Ara h 2 %	Ara h 3 %	Ara h 6 %
			Leco analysis	Bradford analysis					
			(% W/W)	(mg/ml)					
1	Exp-27-1516	Runner	27.6	21.5	78%	12.1	4.4	77.7	3.8
2	GA06G	Runner	25.3	20.7	82%	15.4	5.8	82.5	4.2
3	Tiftguard	Runner	26.8	19.9	74%	16.1	6.3	71.4	3.7
4	Flo7	Runner	26.4	21.7	82%	11.7	4.5	78.0	4.4
5	Florunner 4/14	Runner	29.3	17.8	61%	17.8	5.5	75.9	2.5
6	Olin Spanish	Spanish	28.9	23.6	82%	15.3	5.7	83.5	4.8
7	Valencia C	Valencia	29.7	24.3	82%	14.6	6.5	80.1	4.8
8	Valencia A	Valencia	26.3	20.5	78%	17.1	4.5	77.9	4.1
9	H&W 102	Valencia	26.7	17.8	67%	14.1	3.5	69.9	5.2
10	NC9	Virginia	21.6	14.5	67%	20.9	8.0	58.0	9.7
11	NC10C	Virginia	25.6	15.6	61%	22.0	7.3	82.7	5.7
12	NC-V11	Virginia	24.7	15.9	64%	22.1	6.6	57.7	6.3
13	NC 12C	Virginia	20.7	18.1	87%	16.4	7.2	62.2	7.6
14	Gregory	Virginia	24.3	17.7	73%	19.1	6.8	61.9	6.9
15	Perry	Virginia	22.2	17.7	79%	14.5	6.1	62.3	8.0
16	Phillips	Virginia	21.4	15.8	74%	23.7	8.0	62.8	7.8
17	Brantley	Virginia	21.8	18.3	84%	16.9	5.7	65.9	7.8
18	Bailey	Virginia	24.7	14.9	60%	21.4	7.8	68.7	6.0
19	Sugg	Virginia	25.0	19.8	79%	15.3	7.0	69.9	6.4
20	Champs	Virginia	24.0	19.5	81%	15.2	6.6	64.0	5.3

concentration determination, SDS-PAGE analysis, ELISA, and RP-HPLC analysis. The second series of extracts was made by mixing 20 mg of ground peanut with 0.5 mL of non-reducing Laemmli buffer (8% glycerol, 4% SDS, and 0.01% bromphenol blue in 0.066 M Tris HCl, pH 6.8) in the absence of a reducing agent. Suspensions were mixed and then centrifuged at 1400 rpm (100 × g) for 20 min at RT and subsequently at 70 °C for 20 min. Supernatants were collected and stored at −20 °C until use. The same procedure was used to prepare the third series of extracts, except that the Laemmli buffer contained 1% β-mercapto-ethanol as a reducing agent. The second and third series of extracts were used for SDS-PAGE analysis only, and are referred to as SDS extracts.

2.3. SDS-PAGE analysis

SDS-PAGE analysis was performed with 12% Bis-Tris gels (XCell SureLock Mini-Cell; Invitrogen, USA) according to the instructions of the manufacturer. Aqueous extracts were diluted to 2 mg/mL and then mixed (1:1 V/V) with 2 × concentrated Laemmli buffer, either in the presence or absence of 2% β-mercapto-ethanol. SDS extracts were diluted 10-fold in Laemmli buffer, either in the presence or absence of 1% β-mercapto-ethanol, resulting in a load of 6 μg/lane. Molecular weight marker was from ThermoFisher Scientific, product reference Mark 12, catalogue number LC5677, Following electrophoresis, gels were incubated for 1 h in 12% TCA as a fixation step, and proteins were subsequently visualized by SimplyBlue SafeStain (Invitrogen, USA) according to the instructions of the manufacturer.

2.4. Reverse-phase HPLC

RP-HPLC experiments were performed on an Agilent 1200 system operated by ChemStation software. UV detection was performed at 215 nm. Aqueous peanut extracts' were analyzed on an XBridge Phenyl column (2.1 mm × 150 mm, 3.5 μm particle size, Waters Chromatography). The column was protected by an Xbridge Phenyl guard cartridge (2.1 mm × 10 mm, 3.5 μm particle size, Waters Chromatography). The mobile phase solvent A consisted of 0.1% TFA (V/V) whereas the mobile phase solvent B contained

acetonitrile with 0.085% TFA(V/V) The column was equilibrated with 90% solvent A and 10% solvent B. The column was run at 50 °C with a flow rate of 0.5 mL/min. After loading the sample (10× diluted, 5 μL injected or 150× diluted, 10 μL injected), the column was eluted with a gradient of increasing percentage of solvent B using the following increments: 10% solvent B for 5 min, from 10 to 20% solvent B in 5 min, from 20 to 30% solvent B in 20 min, from 30 to 40% solvent B in 50 min. Subsequently, the column was stripped with 80% solvent B and re-equilibrated with 10% solvent B. Quantification of the major peanut allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 in the different peanut extract samples was performed by using external calibration curves of the purified native Ara h 1, Ara h 2, Ara h 3 and Ara h 6 (25–400 ug/mL, 5 μL injected, based on A_{280} measurements and the extinction coefficients of the different allergens), respectively. The amounts of the different major allergens (in percentages) were calculated in relation to the total protein content of the aqueous peanut extracts. Measurements were performed in duplicate and the percentages were averaged.

2.5. Peanut ELISA by commercial test kits

Two commercial ELISA test kits were used in this study: the Neogen Veratox® for peanut allergen (Lansing, MI USA) and the Morinaga Institute of Biological Science, Inc. Peanut ELISA kit (Japan). Aqueous extracts were tested (in duplicate) in a broad series of dilutions (ranging from 0.0125 to 0.1 μg/mL prepared in the extraction buffers provided by the respective kits), essentially following the instructions of the kit manufacturers. Plates were read at the wavelength specified by each kit using a microtiter plate reader (BioTek Instruments Inc. USA).

2.6. IgE-binding by ELISA

A serum pool was composed of sera from 8 U.S. patients with peanut allergy (patient's characteristics are summarized in Table 2). Specific IgE was analyzed by ImmunoCap and ImmunoCap ISAC (ThermoFisher, USA). All individual sera had IgE to Ara h 1, Ara h 2, Ara h 3, and Ara h 6, and five of these sera had additional IgE to Ara h 8 as well. A serum pool was constructed by mixing equal volumes

Table 2
Patient characteristics and serology.

Subject#	Sex/Age (yr)	Case history[a]	Other food allergy[b]	Other atopy	SPT[c] Peanut	IgE (ISU)[d] Ara h 1	Ara h 2	Ara h3	Ara h 6	Ara h 8	Ara h 9	Peanut	IgE (kUa/L)[e] Peanut
1	F/13.9	RA, P, U, AE, W, E, P(H)	a	yes	9	51.7	64.5	27.1	76	5.15	0	224.38	53
2	M/35	SZ, P(UR), L, R, N, AP, LS, OAS	e, s, tn, pe, bn, ln	yes	35	60.4	31.3	15.1	64.5	2.05	0	141.38	575
3	F/24	P, L, W, C, N, DY, OAS	s, tn, ml, ap, st	yes	20	50.2	24	14.5	50.2	2.48	0	378.36	72.5
4	M/19	W, TS, P	a, h	no	12	96.7	98.5	58.2	124.3	0.67	0	173.27	787
5	F/32	AS, NC, DY	s, tn, m, f	yes	ND	39.6	22.3	3.42	8.43	35.4	0	n.d.	30.1
6	M/35	L, AE, U, AP	s, l, tn, c, f	no	15	57.7	18.4	5.71	21.3	0	0	n.d.	85.5
7	M/35	LS, U, DY, E, OT	unknown	unknown	ND	33.4	30.2	29.6	15	0	0	n.d.	74.3
8	M/32	DY,U, AE,	s, pe, ln, c, cs	unknown	ND	10.6	9.26	0.83	10.7	0	0	n.d.	15.4
Pool	n.a.	n.a.	n.a.	n.a.	n.a.	53.4	39.3	15.2	76.5	4	0	251	n.d.

n.a.: Not applicable; n.d. Not determined.

[a] AE: angioedema; AP: abdominal pain; AS: asthma; C: cough; DY: dypsnea; E: emesis; L: laryngeal edema; LS: lip swelling; N: nausea; NC: nasal congestion; OAS: oral allergy symptoms; OT: odd taste; P (H), (UR):pruritis (hand), (upper respiratory); R: rhinorrhea; RA: rash; SZ: sneeze; TS: tongue swelling; U: urticaria; W: wheeze.

[b] a: almond; ap: apple; bn: bean; c: crustacea; cs: cashew; e: egg; f: fish; h: hazelnut; l: legumes; ln: lentil; m: milk; ml: melon; pe: pea; s: soy; st: strawberry; tn: tree nut.

[c] Skin prick test; diameter of wheal in milimeters.

[d] Peanut IgE by ISAC (ISU).

[e] PN ICAP: Specific IgE to peanut (ImmunoCAP value for serum IgE) expressed in kUa/L.

of individual serum. Peanut-specific and individual peanut allergen-specific IgE levels in this pool were confirmed (Table 2). Serum from both an atopic patient with no peanut allergy and a non-atopic patient were included as negative controls.

IgE-ELISA was performed essentially as described earlier[17] with some modifications. Maxisorp microtiter plates (Nunc Maxisorp flat bottom immune plates, Rochester, NY) were coated with 100 μL per well of 5 μg/mL of peanut extract #1 (sample 1 in Table 1) prepared in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$; pH 9.6), and incubated overnight at 4 °C. Plates were washed with 0.01 M phosphate buffered saline containing 0.05% (V/V) Tween 20 (PBS-T, pH 7.4) and subsequently blocked with PBS-T containing 1% BSA. For each peanut cultivar extract (#1 to 20), a series of dilutions ranging from 0 to 2000 μg/mL was prepared in blocking buffer. Each of these dilutions was mixed 1:1 with the pooled sera that had already been pre-diluted 15 fold in blocking buffer and incubated at 37 °C for 2 h. The pre-incubated serum + inhibitor mixtures were then transferred to the coated ELISA plates and incubated for 2 h at 37 °C. The plates were washed with PBS-T and incubated for 1 h at 37 °C with 100 μL/well of mouse anti-human IgE conjugated with HRP diluted 1:15,000 in blocking buffer. Plates were again washed with PBS-T and 100 μL per well of TMB substrate was added and incubated in the dark for 15 min. The reaction was then stopped by adding 100 μL per well of 1N HCl. The absorbance of each well was measured at 450 nm using a microtiter plate reader (BioTek Instruments Inc. USA). Assays were performed in triplicate. Concentrations of peanut protein required for 50% inhibition (IC_{50}) of IgE-binding were calculated as described previously (Koppelman et al., 2001).

3. Results and discussion

3.1. Protein content of peanut kernels and aqueous extracts

The intact peanut kernels contained proteins levels ranging from 20.7 to 29.7% (relative to total weight) with a mean protein content for all samples of 25.2 ± 2.7% (Table 1), which is in line with known average values of protein content in peanut (Oerise et al., 1974). The differences we see in protein content between the samples can be explained by both genetic and environmental factors, in particular the variety cultured and climate and weather conditions (Cobb and Johnson, 1973). In aqueous extracts with the protein content determined by Bradford analysis (Table 1), the levels of soluble peanut protein were somewhat lower than

predicted based on the 25% protein content of intact peanut kernels (60–81% of predicted). The samples extracted 1:10 (w/v) should have contained a theoretical concentration of 25 mg/mL if all of the protein in the kernel was solubilized and subsequently extracted. Sathe et al. also found that extractable protein for peanut as determined by the Bradford method was somewhat lower that the theoretically expected value based on the nitrogen content of peanut kernels (Sathe et al., 2009). This can be due to incomplete extraction (Leco analysis measures the nitrogen content in the solid material of peanut, independent on extractability or solubility of different proteins), or because the two methods are calibrated by different means.

3.2. Protein profile

Fig. 1 shows the protein profiles of the aqueous peanut extracts both under non-reducing and reducing conditions. Purified reference peanut allergens shown in the first four lanes were used to assign the bands. Ara h 1, peanut vicilin (7S globulin), migrates at both non-reducing and reducing conditions at approximately 63 kDa. Ara h 2 and Ara h 6, peanut conglutin (2S albumin), migrate at approximately 15/17 and 13 kDa, respectively, under non-reducing conditions and at 17/19 and 15 kDa under reducing conditions due to unfolding of the tight protein core (Apostolovic et al., 2013). Ara h 3, peanut legumin (11 S), consists of an N-terminal and C-terminal chain that are held together by disulfide bridges (Piersma et al., 2005). The N-terminal chain is extensively post-translationally processed, leading to various molecular weights and Ara h3, under non-reducing conditions, migrates as various bands. Upon reduction, the two chains are dissociated resulting in 14, 42/45 kDa (N-terminal, acidic chain) and 25 kDa (C-terminal, basic chain) bands (Piersma et al., 2005). Under non-reducing conditions, Ara h 1 and Ara h 3 co-migrate, while Ara h 2 and Ara h 6 are well separated from Ara h 3 (basic 25 kDa chain). Under reducing conditions, Ara h 1 and Ara h 3 are well separated, at the costs of lower resolution of Ara h 2, Ara h 6, and the 25 kDa basic chain of Ara h 3. With both electrophoretic conditions in place, each individual allergen can be assigned more reliably. Fig. 1 clearly shows that all of the peanut extracts contained Ara h 1, Ara h 2, Ara h 3 and Ara h 6. Qualitatively, the intensities of these allergens are similar between different peanut extracts, although only semi-quantitative statements can be made based on the SDS-PAGE data. Under reducing conditions, the N-terminal chain of Ara h 3 appears differently among various cultivar samples. The majority (16/20) of
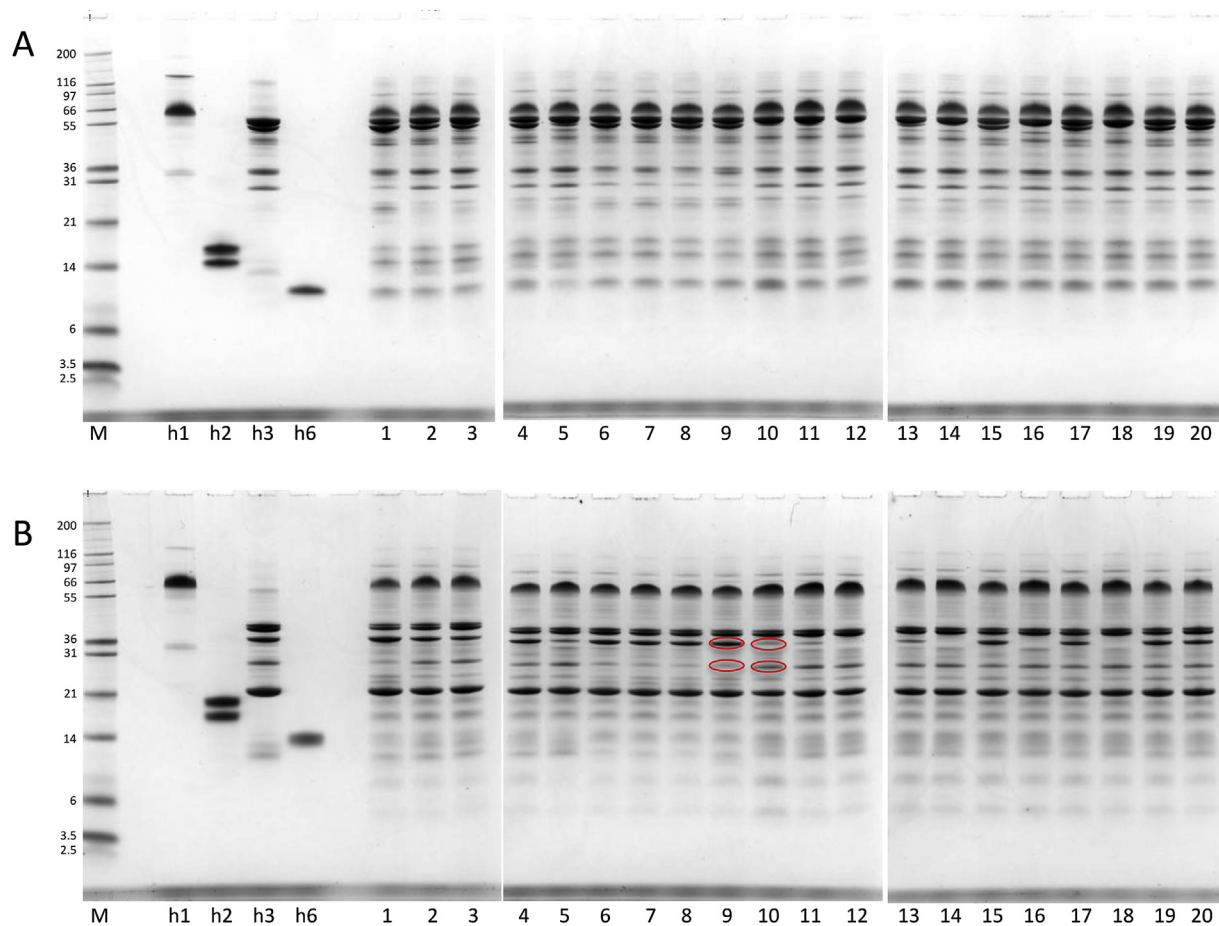
Fig. 1. Protein profiles of aqueous extracts of peanut samples. Panel A: Non-reducing conditions. Panel B: Reducing conditions. M: Marker proteins (indicated in the left margin in kDa); h1: Ara h 1 (1 μg/lane); h2: Ara h 2 (2 μg/lane); h3: Ara h 3 (5 μg/lane); h6: Ara h 6 (1 μg/lane). Numbers 1 to 20 refer to sample numbers as described in Table 1; 6 μg/lane was loaded.

samples have a triplet at 40–45 kDa. Some samples (no. 12, 14, 16, and 18) lack the lowest band of this triplet. Furthermore, with some samples (no. 5, 11, and 13) the intensity of this band is lower. For the samples that lack this band or have this band at low intensity, the intensity of a band at approximately 30 kDa is higher (indicated in Fig. 1B for samples 9 and 10 as examples). This band is also present in purified Ara h 3 and was earlier shown to be a post-translationally processed part of the N-terminal chain of Ara h 3 (Piersma et al., 2005). There seems to be a correlation between the lower intensity (or absence) of the lower band of the 42–45 kDa triplet with the intensity of the 30 kDa band, however not for all samples. In particular sample 4, in which both the lower band of the 42–45 kDa triplet as well as the 30 kDa band can be observed. Apparently in peanut kernels, Ara h 3 can be found at different stages of post-translational processing. This phenomenon is not observed for the C-terminal chain of Ara h 3, or Ara h 1, Ara h 2, and Ara h 6 (Fig. 1).

An SDS-containing buffer was used to prepare extracts of ground peanut in order to increase the efficiency of extracting lipophilic and amphiphilic proteins such as peanut oleosins (apparent molecular weights between 14 and 17 kDa (Schwager et al., 2015)). Schwager et al. demonstrated extraction of lipophilic and amphiphilic proteins with a similar SDS-containing buffer (Schwager et al., 2015). The protein profiles of the extracts obtained with SDS-containing buffer (Fig. 2) show similar proteins bands to those of the aqueous extracts (Fig. 1). A minor difference

may be the faint band at approximately 150 kDa that can be observed in the profiles of extracts made with the SDS-containing buffer. This band is trimeric Ara h1 with different solubility (Maleki et al., 2000), and appears to be soluble in the presence of SDS. The trimeric form of Ara h1 can also be observed as minor band in the protein profile of purified Ara h1, in line with literature (Maleki et al., 2000). For both extraction conditions we see bands at the 14–17 kDa molecular weight area. Some of these bands are from Ara h2, Ara h3, and Ara h6, but some other bands with low intensity are unassigned and may be oleosins. Having no further data available, we can only speculate on this. Overall the aqueous extraction was efficient in extraction of similar proteins from the ground peanut kernels compared to extraction of peanut protein with the SDS-containing buffer. The optimized pH (8.2) and the long duration of extraction may have contributed to this observation.

3.3. Quantification of major allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6 by RP-HPLC

The RP-HPLC method allowed the identification and quantification of the major peanut allergens (Ara h 1, Ara h 2, Ara h 3 and Ara h 6) in extracts from several commercially important peanut market types. These allergens appear as separate peaks in the chromatograms of the samples (Fig. 3), and exhibit the same elution characteristics of the reference proteins Ara h 1, Ara h 2, Ara
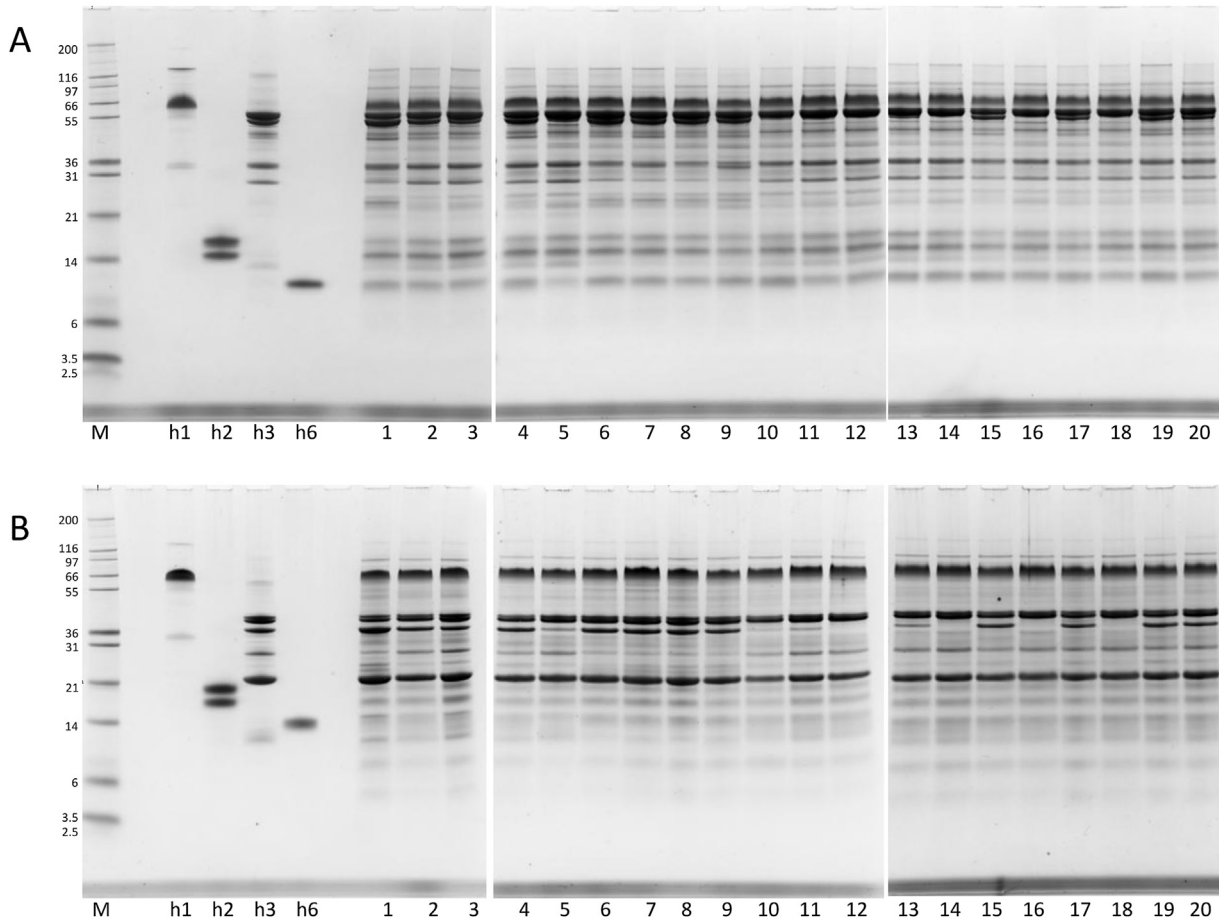
Fig. 2. Protein profiles of SDS extracts of peanut samples. Panel A: Non-reducing conditions. Panel B: Reducing conditions. M: Marker proteins (indicated in the left margin in kDa); h1: Ara h 1 (1 μg/lane); h2: Ara h 2 (2 μg/lane); h3: Ara h 3 (5 μg/lane); h6: Ara h 6 (1 μg/lane). Numbers 1 to 20 refer to sample numbers as described in Table 1; a theoretical amount (based on the assumption that peanut contains 25% protein) of 8 μg/lane was loaded.
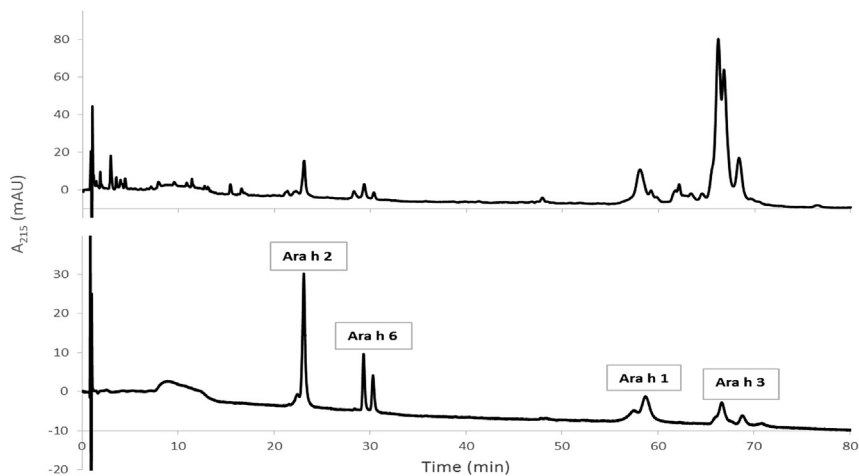

Fig. 3. RP-HPLC chromatograms of an aqueous peanut extract and the purified peanut major allergens. X-axis: Elution time in minutes; Y-axis: Absorbance at 215 nm (mAU). Upper panel: Aqueous peanut extract of sample 1. Lower panel: mix of equal amounts (200 μg/mL, 5 μL injected) of purified peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6 (indicated in text boxes).

h 3 and Ara h 6. Table 1 summarizes the amount of each allergen present in the extracts and expressed as a percentage of the total protein in the extracts. These values are abased on comparison of the surface areas of the relevant peaks with those of known amounts of reference proteins. Based on our quantitative analysis, we estimate that Ara h 1 ranges from 11.7 to 23.7%, with a mean (±SD) of 17.1 (±3.4) %. Ara h 2 ranges from 3.5 to 8.0% with a mean (±SD) of 6.2 (±1.3) %. These values are in line with earlier work

showing that Ara h 1 and Ara h 2 in similar peanut market types ranged from 12 to 16% (Ara h 1) and from 5.9 to 9.3%, (Ara h 2) (Koppelman et al., 2001). Ara h 3 is clearly the most abundant protein in all samples, with an estimated range from 57.7 to 83.5% with a mean ($\pm$SD) of 70.6 ($\pm$8.6) %. No previously published data quantifying the Ara h 3 content of peanuts is available; however, the large quantity of Ara h 3 in peanut is nevertheless expected since the bands of Ara h 3 dominate the peanut protein profile as shown in this study (Figs. 1 and 2) and many other publications (Hefle et al., 1995; Koppelman et al., 2003; Fu and Maks, 2013; Zhuang and Dreskin, 2013). There are also no data on the amount of Ara h 6 in peanut. Based on our analysis, we estimate that Ara h 6 content ranges from 2.5 to 9.7% with a mean ($\pm$SD) of 5.8 ($\pm$1.8) %. As expected based on the protein profiles (Fig. 1), all four allergens were present in all tested samples. The ranges of major allergen content are not substantially different between different market types, or between varieties within a single cultivar (Runner and Virginia). The minor differences we see may be explained by the genetic background of the peanut and climate and weather conditions. These factors are known to influence the protein content of peanut kernels and may as well affect the expression of individual proteins. However, at this stage we have no data to support this hypothesis.

A clinical study applying skin-prick tests demonstrated that peanut allergens Ara h 2 and Ara h 6 are 100- to 1000-fold more potent than Ara h 1 and Ara h 3 (Peeters et al., 2007), and it is therefore important to closely evaluate Ara h 2 and Ara h 6 specifically. Even though these allergens are less abundant (around 6% each, together approximately 12% of the total protein in peanut) compared to Ara h 1 and Ara h 3 (about 17% and 71%, respectively; Table 1), they may dominate the allergenic potential of peanut protein. The observation that levels of both Ara h 2 and Ara h 6 differ a maximum of about 2- to 3-fold between samples suggests a minimal impact on the overall difference in allergenicity among peanut market types, cultivars or varieties.

3.4. Reactivity in commercial kits for peanut residue detection and quantification

When the peanut extracts were evaluated by two commercially available peanut ELISA kits (Neogen Veratox® for Peanut Allergen kit and the Morinaga Institute of Biological Science, Inc. Peanut ELISA kit) at a normalized protein concentration of 1 mg/mL, the Neogen kit overestimated the peanut protein content (about 4-fold compared to the expected value) and the Morinaga kit underestimated the content (mean of 60% of the expected value). A similar observation has been reported by Jayasena et al. with these two kits with the NIST (National Institute of Standards and Technology, Gaithersburg, MD) standard reference material 2387 peanut butter and is most likely due to the calibration of the kits for which the kit manufacturers supply their own independent standard in absence of international standard materials (Jayasena et al., 2015). The ranges of reactivity of the samples are from 2.8 to 4.5 mg/mL (mean $\pm$ SD = 4.0 $\pm$ 0.5) for the Neogen kit, and 0.44 to 0.92 (mean $\pm$ SD = 0.6 $\pm$ 0.1) mg/ml for the Morinaga kit (Table 3).

Jayasena et al. (2015) showed that these two kits have distinctly different specificities for the major allergens. The Neogen kit detects predominantly Ara h 3 with about a 30-fold lower reactivity to Ara h 1, while Ara h 2 and Ara h 6 are minimally recognized. The Morinaga kit recognizes Ara h 2 and Ara h 6, but had very low recognition of Ara h 1 and Ara h 3 (both about 1000-fold less reactive; Jayasena et al., 2015). A comparison was made between the peanut protein content of the samples assayed with the Morinaga kit and the sum of Ara h 2 and Ara h 6 as determined by RP-HPLC. Although all samples are relatively close to each other, there

is a positive correlation between the two parameters ($R^2 = 0.895$). An inverse correlation was found between the reactivity in the Morinaga kit and the Ara h 3 content as determined by RP-HPLC ($R^2 = 0.63$). Reactivity in the Neogen kit was not related to content of any of the peanut allergens specifically, possibly because this ELISA recognizes two different types of peanut allergens (Ara h 3 and to a lesser extent Ara h 1).

3.5. Reactivity in IgE-binding assay

To compare the IgE-binding potency of the different peanut samples, the aqueous extracts were tested in a competitive inhibition IgE-ELISA using a pool of sera from peanut-allergic patients as a source of IgE. Because such ELISAs have no standard or reference material, sample #1 was used as the basis to compare all other samples. IgE-binding to peanut protein from sample #1 was inhibited with sample #1 itself (positive control) and all other samples, and the concentration needed to inhibit 50% of this signal was calculated (IC_{50}). Table 3 shows the IC_{50} values which ranged from 0.20 to 0.41 μg/mL. Attempts to make allergen extracts or allergens hypo-allergenic, for example by chemical modification or biotechnological modification, aim to reduce the IgE-binding at least 100-fold, preferably more (Apostolovic et al., 2013; Ibarrola et al., 2004; Swoboda et al., 2007; van Bilsen et al., 2013; Versteeg et al., 2011). The narrow range of IC_{50} values reported here (only a 2-fold difference) indicates that the peanut samples have a highly comparable IgE-binding potency. This small difference in IgE inhibition between the most and least potent sample is in line with the weak positive correlation between IgE-binding potency and the content of Ara h 1, Ara h 2 and Ara h 6, and a (weak) inverse relation between IgE-binding potency and content of Ara h 3 ($R^2 = 0.42, 0.34, 0.21,$ and 0.11 for Ara h 1, Ara h 2, Ara h 6 and Ara h 3, respectively). Correlation coefficients are most likely low due to the narrow range in which the parameters vary (2- to 3-fold). Together this may suggest that Ara h 3 is not as relevant of an IgE-binding protein, as Ara h 1, Ara h 2 and Ara h 6, the peanut allergens of primary concern. This is in line with the current understanding of the relevance of individual peanut allergens (Chen et al., 2011; Ballmer-Weber et al., 2015).

3.6. Consequences for allergenicity

We have compared the allergenicity attributes of 20 peanut varieties, i.e., protein content and profile, major allergen content, IgG- and IgE-binding potencies. These attributes are highly comparable for all samples. The peanut varieties included in this study are most relevant for human consumption, both in the U.S. and in other Western countries. Currently in U.S. peanut production, GA06G (sample no. 2) is the dominant Runner type peanut produced, whereas Bailey (sample no. 18) is the most commonly produced Virginia type. Together, Runner and Virginia peanuts make over 98% of the U.S. production with Runner at 78.7% and Virginia at 19.9%. Some exotic, naturally occurring, peanut varieties that have clearly different allergenicity attributes have been described in literature (Krause et al., 2010; Ramos et al., 2009; Schmidt et al., 2009), but these peanut types are not commonly consumed in Western countries. Using genetic modification, attempts were made to develop new varieties of peanut that are low in certain allergens (Chandran et al., 2015; Chu et al., 2008; Knoll et al., 2011; Riascos et al., 2010). None of these peanuts are commercially available or used by the food industry in the U.S. or other Western countries.

While we have compared important parameters that are prerequisites for allergenicity, we have not compared allergenicity *in vivo*. This would require clinical investigations, studies in

Table 3
Reactivity of peanut samples in various ELISAs.

Sample No.	Reactivity in peanut ELISA		IgE-binding
	Relative to kit standard (= 1)		IC50 (µg/mL)
	Neogen	Morinaga	
1	4.27	0.50	0.41
2	4.05	0.60	0.33
3	4.41	0.61	0.35
4	2.75	0.61	0.29
5	3.67	0.47	0.27
6	3.88	0.61	0.27
7	4.14	0.63	0.23
8	3.36	0.48	0.25
9	4.22	0.44	0.31
10	3.61	0.92	0.23
11	4.17	0.66	0.20
12	3.30	0.63	0.22
13	4.17	0.71	0.25
14	4.32	0.76	0.20
15	4.46	0.71	0.29
16	4.11	0.85	0.21
17	4.53	0.69	0.29
18	4.35	0.78	0.27
19	3.88	0.64	0.24
20	3.36	0.57	0.24

The values for the two commercial kits (Neogen and Morinaga) represent the total peanut protein as detected by each respective kit in 1 mg/mL of peanut extracts.

validated animal models, or in cell-based ex-vivo methods, all of which are beyond the scope of the current study. Nevertheless, a clinical study carried out by Peeters et al. has shown that Ara h 2 and Ara h 6 are the most potent peanut allergens (Peeters et al., 2007), and our observation that the levels of these particular allergens do not differ substantially between the peanut types suggests that the in vivo allergenicity of the peanut types will also be comparable.

Taken together, we believe it is fair to say that there are no important differences in allergenicity attributes between peanuts types commonly consumed in Western countries. This has implications for several disciplines in food science and food allergy research. First, because the protein content and extractable protein is highly comparable for all samples, no major differences are expected for the analysis of samples from food products based on different peanuts which is an important finding for the food industry that must design appropriate allergen control plans to manage peanut cross-contact in shared facilities. This is illustrated by the results of the two commercial ELISA's for the detection of peanut. Such assays will provide results independent of the background of the peanut.

Second, this information will allow the food industry and regulators to make sound risk assessment and risk management decisions without the need to determine which peanut variety or varieties are used in a finished food product. Many have assumed that the allergenic characteristics (content of the major allergens and potency) of the common peanut market types, cultivars and varieties used in food products do not different significantly; however, there was no published data to systematically document this until now.

Third, several initiatives are ongoing aiming to develop immunotherapy for peanut allergy. Such therapy is based on peanut protein that is administered to patients via different routes. Not all studies published so far have disclosed the market type of peanuts that are used, but all have used U.S. peanuts obtained from commercial sources. More importantly, the clinical benefit of these studies is evaluated by oral food challenges using defined amounts of peanut protein. Knowing that the different peanut types are very similar in allergenicity attributes allows comparing food challenge results from different studies and this facilitates the evaluation of clinical benefits of the various peanut-immunotherapy studies.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2016.02.016

References

Anagnostou, K., Islam, S., King, Y., Foley, L., Pasea, L., Bond, S., Palmer, C., Deighton, J., Ewan, P., Clark, A., 2014. Assessing the efficacy of oral immunotherapy for the desensitisation of peanut allergy in children (STOP II): a phase 2 randomised controlled trial. Lancet 383, 1297–1304.

Anonymous, January 2015. United states department of agriculture, national agricultural statistics service. Crop Prod. 2014 Summ. ISSN: 1057-7823

Apostolovic, D., Luykx, D., Warmenhoven, H., Verbart, D., Stanic-Vucinic, D., de Jong, G.A., Velickovic, T.C., Koppelman, S.J., 2013. Reduction and alkylation of peanut allergen isoforms Ara h 2 and Ara h 6; characterization of intermediate- and end products. Biochim. Biophys. Acta 1834, 2832–2842.

Ballmer-Weber, B.K., Lidholm, J., Fernández-Rivas, M., Seneviratne, S., Hanschmann, K.M., Vogel, L., Bures, P., Fritsche, P., Summers, C., Knulst, A.C., Le, T.M., Reig, I., Papadopoulos, N.G., Sinaniotis, A., Belohlavkova, S., Popov, T., Kralimarkova, T., de Blay, F., Purohit, A., Clausen, M., Jedrzejczak-Czechowcz, M., Kowalski, M.L., Asero, R., Dubakiene, R., Barreales, L., Mills, E.N., van Ree, R., Vieths, S., 2015. IgE recognition patterns in peanut allergy are age dependent: perspectives of the EuroPrevall study. Allergy 70, 391–407.

Bencharitiwong, R., van der Kleij, H.P., Koppelman, S.J., Nowak-Węgrzyn, A., 2015. Effect of chemical modifications on allergenic potency of peanut proteins. Allergy Asthma Proc. 36, 185–189.

van Bilsen, J.H., Knippels, L.M., Penninks, A.H., Nieuwenhuizen, W.F., De Jongh, H.H., Koppelman, S.J., 2013. The protein structure determines the sensitizing capacity of Brazil nut 2S albumin (Ber e1) in a rat food allergy model. Clin. Transl. Allergy 3, 36.

Burks, A.W., Wood, R.A., Jones, S.M., Sicherer, S.H., Fleischer, D.M., Scurlock, A.M., Vickery, B.P., Liu, A.H., Henning, A.K., Lindblad, R., Dawson, P., Plaut, M., Sampson, H.A., 2015. Consortium of food allergy research. Sublingual immunotherapy for peanut allergy: long-term follow-up of a randomized multicenter trial. J. Allergy Clin. Immunol. 135, 1240–1248.

Chandran, M., Chu, Y., Maleki, S.J., Ozias-Akins, P., 2015. Stability of transgene expression in reduced allergen peanut (Arachis hypogaea L.) across multiple generations and at different soil sulfur levels. J. Agric. Food Chem. 63, 1788–1797.

Chen, X., Zhuang, Y., Wang, Q., Moutsoglou, D., Ruiz, G., Yen, S.E., Dreskin, S.C., 2011. Analysis of the effector activity of Ara h 2 and Ara h 6 by selective depletion from a crude peanut extract. J. Immunol. Methods. 372, 65–70.

Chu, Y., Faustinelli, P., Ramos, M.L., Hajduch, M., Stevenson, S., Thelen, J.J., Maleki, S.J., Cheng, H., Ozias-Akins, P., 2008. Reduction of IgE binding and nonpromotion of Aspergillus flavus fungal growth by simultaneously silencing Ara h 2 and Ara h 6 in peanut. J. Agric. Food Chem. 56, 11225–11233.

Cobb, W.Y., Johnson, B.R., 1973. Physicochemical properties of peanuts. In: Wilson, C.T. (Ed.), Peanuts: Culture and Uses. American Peanut Research and Education Association Inc., Stillwater, Oklahoma, USA.

Dyer, A.A., Rivkina, V., Perumal, D., Smeltzer, B.M., Smith, B.M., Gupta, R.S., 2015. Epidemiology of childhood peanut allergy. Allergy Asthma Proc. 36 (1), 58–64.

Fleischer, D.M., Burks, A.W., Vickery, B.P., Scurlock, A.M., Wood, R.A., Jones, S.M., Sicherer, S.H., Liu, A.H., Stablein, D., Henning, A.K., Mayer, L., Lindblad, R., Plaut, M., Sampson, H.A., 2013. Consortium of Food Allergy Research (CoFAR). Sublingual immunotherapy for peanut allergy: a randomized, double-blind, placebo-controlled multicenter trial. J. Allergy Clin. Immunol. 131, 119–127.

Fu, T.J., Maks, N., 2013. Impact of thermal processing on ELISA detection of peanut allergens. J. Agric. Food Chem. 61, 5649–5658.

Hefle, S.L., Helm, R.M., Burks, A.W., Bush, R.K., 1995. Comparison of commercial peanut skin test extracts. J. Allergy Clin. Immunol. 95, 837–842.

Ibarrola, I., Sanz, M.L., Gamboa, P.M., Mir, A., Benahmed, D., Ferrer, A., Arilla, M.C., Martínez, A., Asturias, J.A., 2004. Biological characterization of glutaraldehyde-modified Parietaria judaica pollen extracts. Clin. Exp. Allergy 34, 303–309.

Jayasena, S., Smits, M., Fiechter, D., de Jong, A., Nordlee, J., Baumert, J., Taylor, S.L., Pieters, R.H., Koppelman, S.J., 2015. Comparison of six commercial ELISA kits for their specificity and sensitivity in detecting different major peanut allergens. J. Agric. Food Chem. 63, 1849–1855.

Jones, D.B., 1931. Factors for converting percentages of nitrogen in foods and feeds into percentages of protein. U. S. Dep. Agric. Circ. 183, 1–22.

de Jong, E.C., van Zijverden, M., Spanhaak, S., Koppelman, S.J., Pellegrom, H., Penninks, A.H., 1998. Identification and partial characterization of multiple major allergens in peanut proteins. Clin. Exp. Allergy 28, 743—751.

Knoll, J.E., Ramos, M.L., Zeng, Y., Holbrook, C.C., Chow, M., Chen, S., Maleki, S., Bhattacharya, A., Ozias-Akins, P., 2011. TILLING for allergen reduction and improvement of quality traits in peanut (Arachis hypogaea L.). BMC Plant Biol. 11, 81.

Koppelman, S.J., Vlooswijk, R.A.A., Knippels, L.M.J., Hessing, M., Knol, E.F., van Reijsen, F.C., Bruijnzeel-Koomen, C.A.F.M., 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.

Koppelman, S.J., Knol, E.F., Vlooswijk, R.A.A., Wensing, M., Knulst, A.C., Hefle, S.L., Gruppen, H., Piersma, S., 2003. Peanut allergen Ara h 3: isolation from peanuts and biochemical characterization. Allergy 58, 1144—1151.

Koppelman, S.J., de Jong, G.A., Laaper-Ertmann, M., Peeters, K.A.B.M., Knulst, A.C., Hefle, S.L., Knol, E.F., 2005. Purification and immunoglobulin E-binding properties of peanut allergen Ara h 6: evidence for cross-reactivity with Ara h 2. Clin. Exp. Allergy 35, 490—497.

Kottapalli, K.R., Payton, P., Rakwal, R., Agrawal, G.K., Shibato, J., Burow, M., Puppala, N., 2008. Proteomics analysis of mature seed of four peanut cultivars using two-dimensional gel electrophoresis reveals distinct differential expression of storage, anti-nutritional, and allergenic proteins. Plant Sci. 175, 321—329.

Krapovicakas, A., 1969. The origin, variability and spread of the groundnut (Arachis hypogaea). In: Ucko, P.J., Falk, I.S. (Eds.), Domestication and Exploitation of Plants and Animals. Gerald Duckworth Co., London, UK, p. 247.

Krause, S., Latendorf, T., Schmidt, H., Darcan-Nicolaisen, Y., Reese, G., Petersen, A., Janssen, O., Becker, W.M., 2010. Peanut varieties with reduced Ara h 1 content indicating no reduced allergenicity. Mol. Nutr. Food Res. 54, 381—387.

Maleki, S.J., Kopper, R.A., Shin, D.S., Park, C.W., Compadre, C.M., Sampson, H., Burks, A.W., Bannon, G.A., 2000. Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. J. Immunol. 164, 5844—5849.

Oerise, N.L., Lau, H.A., Ritchey, S.J., Murphy, E.W., 1974. Yield, proximate composition and mineral element content of three cultivars of raw and roasted peanuts. J. Food Sci. 39 (2), 264—266.

Peeters, K.A., Koppelman, S.J., van Hoffen, E., van der Tas, C.W., den Hartog-Jager, C.F., Penninks, A.H., Hefle, S.L., Bruijnzeel-Koomen, C.A., Knol, E.F., Knulst, A.C., 2007. Does skin prick test reactivity to purified allergens correlate with clinical severity of peanut allergy? Clin. Exp. Allergy. 37, 108—115.

Piersma, S.R., Gaspari, M., Hefle, S.L., Koppelman, S.J., 2005. Proteolytic processing of the peanut allergen Ara h 3. Mol. Nutr. Food Res. 49, 744—755.

Ramos, M.L., Huntley, J.J., Maleki, S.J., Ozias-Akins, P., 2009. Identification and characterization of a hypoallergenic ortholog of Ara h 2.01. Plant Mol. Biol. 69, 325—335.

Riascos, J.J., Weissinger, A.K., Weissinger, S.M., Burks, A.W., 2010. Hypoallergenic legume crops and food allergy: factors affecting feasibility and risk. J. Agric. Food Chem. 58, 20—27.

Remington, B.C., Baumert, J.L., Marx, D.B., Taylor, S.L., 2013. Quantitative risk assessment of foods containing peanut advisory labeling. Food Chem. Toxicol. 62, 179—187.

Sampson, H.A., Agbotounou, W.K., Thebault, C., Ruban, C., Martin, L., Yang, W.H., Sussman, G.L., Brown-Whitehorn, T.F., Nadeau, K.C., Cheema, A.S., Leonard, S.A., Pongracic, J.A., Sauvage-Delebarre, C., Assa'ad, A.H., De Blay, F., Bird, J.A., Tilles, S.A., Boralevi, F., Bourrier, T., Shreffler, W.G., Hebert, J., Green, T.D., Gerth Van Wijk, R., Knulst, A.C., Kanny, G., Kowalski, M.L., Schneider, L.C., Benhamou, P.H., Dupont, C., 2015. Epicutaneous Immunotherapy (EPIT) is effective and safe to treat Peanut Allergy: a multi-national double-blind placebo-controlled randomized phase IIb trial. J. Allergy Clin. Immunol. 135, AB390.

Sathe, S.K., Venkatachalam, M., Sharma, G.M., Kshirsagar, H.H., Teuber, S.S., Roux, K.H., 2009. Solubilization and electrophoretic characterization of select edible nut seed proteins. J. Agric. Food Chem. 57, 7846—7856.

Schmidt, H., Gelhaus, C., Latendorf, T., Nebendahl, M., Petersen, A., Krause, S., Leippe, M., Becker, W.M., Janssen, O., 2009. 2-D DIGE analysis of the proteome of extracts from peanut variants reveals striking differences in major allergen contents. Proteomics 9, 3507—3521.

Schwager, C., Kull, S., Krause, S., Schocker, F., Petersen, A., Becker, W.M., Jappe, U., 2015. Development of a novel strategy to isolate lipophilic allergens (oleosins) from peanuts. PLoS One 10 (4), e0123419.

Sicherer, S.H., Sampson, H.A., 2007. Peanut allergy: emerging concepts and approaches for an apparent epidemic. J. Allergy Clin. Immunol. 120, 491—503.

Sicherer, S.H., Muñoz-Furlong, A., Godbold, J.H., Sampson, H.A., 2010. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. J. Allergy Clin. Immunol. 125, 1322—1326.

Swoboda, I., Bugajska-Schretter, A., Linhart, B., Verdino, P., Keller, W., Schulmeister, U., Sperr, W.R., Valent, P., Peltre, G., Quirce, S., Douladiris, N., Papadopoulos, N.G., Valenta, R., Spitzauer, S., 2007. A recombinant hypoallergenic parvalbumin mutant for immunotherapy of IgE-mediated fish allergy. J. Immunol. 178, 6290—6296.

Taylor, S.L., Moneret-Vautrin, D.A., Crevel, R.W., Sheffield, D., Morisset, M., Dumont, P., Remington, B.C., Baumert, J.L., 2010. Threshold dose for peanut: risk characterization based upon diagnostic oral challenge of a series of 286 peanut-allergic individuals. Food Chem. Toxicol. 48, 814—819.

Taylor, S.L., Houben, G.F., Baumert, J.L., Crevel, R.R., Allen, K.J., Dubois, A.E., Knulst, A.C., Remington, B.C., Kruizinga, A.G., Blom, W.M., Brooke-Taylor, S., 2015. Understanding food allergen thresholds requires careful analysis of the available clinical data. J. Allergy Clin. Immunol. 135, 583—584.

Varshney, P., Jones, S.M., Scurlock, A.M., Perry, T.T., Kemper, A., Steele, P., Hiegel, A., Kamilaris, J., Carlisle, S., Yue, X., Kulis, M., Pons, L., Vickery, B., Burks, A.W., 2011. A randomized controlled study of peanut oral immunotherapy: clinical desensitization and modulation of the allergic response. J. Allergy Clin. Immunol. 127, 654—660.

Versteeg, S.A., Bulder, I., Himly, M., van Capel, T.M., van den Hout, R., Koppelman, S.J., de Jong, E.C., Ferreira, F., van Ree, R., 2011. Glutaraldehyde-modified recombinant Fel d 1: a hypoallergen with negligible biological activity but retained immunogenicity. World Allergy Organ J. 4, 113—119.

Walczyk, N.E., Smith, P.M., Tovey, E., Wright, G.C., Fleischfresser, D.B., Roberts, T.H., 2013. Analysis of crude protein and allergen abundance in peanuts (Arachis hypogaea cv. Walter) from three growing regions in Australia. J. Agric. Food Chem. 61, 3714—3725.

Zhuang, Y., Dreskin, S.C., 2013. Redefining the major peanut allergens. Immunol. Res. 55, 125—134.