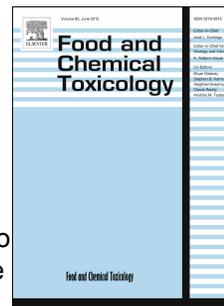


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**Peanut digestome: identification of digestion resistant IgE binding peptides**

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*Abbreviation:* Ambic, ammonium bicarbonate; BBM, brush border member enzymes; DTT, dithiothreitol; IAA, iodoacetamide; TAME, *p*-toluene-sulfonyl-L-arginine methyl ester ; TCA, trichloroacetic; TFA, trifluoroacetic ; SSF, Simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.



Antigenic  
characterization

Proteomic  
characterization

peanut digesta

## 1. Introduction

Peanut allergy is one of the most widespread and severe IgE-mediated food allergies, with an estimated prevalence of 1% in children and 0.6% in adults within the general population of developed countries (Ben-Shoshan et al., 2010; Sicherer and Wood 2013). The complex allergome of peanut consists of several type I protein allergens triggering immune responses with different symptoms and prognosis, depending on characteristics of the offending protein (Vereda et al. 2011, Lauer et al. 2009, Nicolaou and Custovic 2011, Mittag et al. 2004). Ara h 1, Ara h 2, and Ara h 3 are major allergens associated with primary sensitization to peanut (Mueller et al. 2014). Ara h 1 is a 63.5-kDa vicilin-type (7S) seed storage protein, representative of the cupin superfamily, which naturally occurs as up to 600-700 kDa non-covalent aggregates (van Boxtel et al, 2006). Ara h 3 is a glycinin-like protein (11S) consisting of a 60-kDa polypeptide post-translationally cleaved in acid and basic subunits which remain linked each other by a disulphide bond, similarly to the 11S plant seed storage protein signature (Boldt et al. 2005). Ara h 2 belongs to the conglutinin (2S albumin) superfamily and their folding resembles that of  $\alpha$ -amylase/trypsin inhibitors from cereal kernels (Mueller et al. 2011). Ara h 2 comprises two isoforms, namely Ara h 2.01 (17-kDa) and Ara h 2.02 (19-kDa). Ara h 2.02 includes an insertion of 12 extra amino acid residues, constituting an additional IgE-binding epitope and is a more effective IgE cross-linker than Ara h 2.01 (Chatel et al 2003). Ara h 6 is a 14.5-kDa 2S albumin co-member, sharing 59 % sequence identity, secondary and tertiary structure homology as well as immune cross-reactivity patterns with Ara h 2 (Koppelman et al, 2005; Lehmann et al. 2006). World Health Organization and International Union of Immunologic Societies Subcommittee (WHO/IUIS) catalogued several additional minor peanut allergens ([www.allergen.org](http://www.allergen.org)), including Ara h 5 (profilin), Ara h 8 (Bet v 1 birch pollen homologue), Ara h 9 or lipid transfer protein (LTP), the latter being a relevant peanut allergen especially in the Mediterranean area (Krause et al. 2009). Ara h 10 (oleosin 1) and Ara h 11 (oroleosins) are not usually associated with severe allergic reactions (Zhuang and Dreskin 2013).

Apart from geographical differences in the sensitization profiles (Sicherer and Wood 2013), conglutinin Ara h 2 and Ara h 6 have been proven as the most harmful for peanut allergic subjects, in term of basophil activation, IgE-binding properties and skin prick test (Burks et al. 1991, Blanc et al. 2009). Both Ara h 2 and Ara h 6 are tightly coiled, heat-stable and resistant to gastrointestinal digestion (Suhr et al. 2004), which are structural features shared by a large number of common food allergens (Astwood et al. 1996). Early studies showed that digestion of Ara h 2 and Ara h 6 by pepsin and/or chymotrysin produce large stable fragments with unmodified immunological potential (Apostolovic et al. 2016). On the contrary, peanut allergen Ara h 1 and Ara h 3 have been described as highly susceptible to proteases (Koppelman et al. 2010). Nevertheless, peptides resulting from gastro-duodenal digestion of Ara h 1 retain T cell stimulatory, sensitizing capability and IgE-binding properties (Eiwegger et al. 2006, Bøgh et al. 2009), probably due to the formation of exceptionally stable non-covalent peptide aggregates (Bøgh et al. 2012).

Susceptibility of peanut food allergens to proteolysis has been typically assayed by using single purified proteins (Bøgh and Madsen 2016), due to the drawbacks of analyzing the heterogeneous digestome of peanut as a whole food. Such an approach could suffer from scarce relevance, since the protein aggregation state, the interaction of allergens with non-protein components naturally occurring in whole foodstuff (e.g. polysaccharide, lipid), presence of protease inhibitors and the protein-protein interactions affect the accessibility of proteases to allergens, thereby contributing to the bioaccessibility and hence to the bioavailability of allergenic determinants (epitopes) (Teuber 2002). Nowadays, the advances in “omic” sciences (*i.e.*, proteomics, metabolomics, lipidomics) have enabled the assessment of the food digestome as well as the identification of stable allergens and the monitoring of IgE-binding epitopes sequentially released upon digestion of complex matrices (Picariello et al. 2011, Picariello et al. 2013).

Another relevant aspect, barely addressed so far, is the analysis of peptide fragments arising from the proteolysis process. In fact, the majority of the studies aimed at assessing the digestion stability of allergens only monitored the degradation of allergens by SDS-PAGE and Western Blot,

neglecting the release of immunologically active proteolytic peptides, which escape the electrophoretic detection. Mapping the peanut resistant peptides harbouring IgE epitopes might improve the knowledge about the allergenic determinants and the pathogenic mechanism, paving the way to new immunotherapeutic approaches (Bannon et al. 2001, Li. et al 2003). These considerations prompted us to simulate the digestion of whole raw peanuts using an *in vitro* multicompartmental static digestion model with physiological relevance (Minekus et al., 2014), which includes the oral, gastric, duodenal and intestinal phases. IgE-binding (poly)peptides resulting from digestion were characterized by integrated proteomic/peptidomic and immunochemical assays.

## 2. Materials and Methods

### 2.1 Chemicals

Raw peanuts (Virginia variety) were provided by Besana (Milano, Italy). HPLC-grade solvents were from Merck (Whitehouse Station, NJ, USA). Pepsin, trypsin, chymotrypsin, dithiothreitol (DTT), iodoacetamide (IAA), Tris-HCl, urea, guanidine chloride, ammonium bicarbonate (Ambic), phospholipids, trichloroacetic (TCA), trifluoroacetic (TFA), *p*-toluene-sulfonyl-L-arginine methyl ester (TAME) and the modified Lowry assay kit were purchased from Sigma-Aldrich (St Louis, MO, USA). Egg lecithin was from Lipid Products (Redhill, UK). Reagents for electrophoresis analysis were from Bio-Rad (Milan, Italy). Brush border membrane (BBM) vesicles were purified from porcine jejunum according to Cheeseman and O'Neill, 2006, as previously detailed (Picariello et al. 2015). The aminopeptidase activity was determined by colorimetric assay using *p*-nitroaniline as the substrate, while the total activity of BBM peptidases was assayed by HPLC using angiotensin I as a substrate (Picariello et al. 2015).

### 2.2 Sera of peanut allergic patients

Sera were collected from peanut allergic subjects (N=8, 80% male), all from Regione Campania (Southern Italy), according to the ethical requirements. The local Ethics Committee approved the study. The allergy symptoms ranged from urticaria to angioedema and anaphylaxis. The clinical features of the allergic individuals enrolled in this study are reported in Table S1. Diagnosis of IgE-mediated allergy to peanut was confirmed by skin prick test (SPT) and oral food challenges. Either a SPT peanut extract or fresh peanut (prick-by-prick) was applied to the patients' volar forearm. Tests were performed using a 1-mm single peak lancet (ALK, Copenhagen, Denmark), with histamine dihydrochloride (10 mg/mL) and isotonic saline solution (0.9% NaCl) as the positive and negative controls, respectively. Reactions were recorded based on the largest diameter (in millimeters) of the wheal and flare at 15 min. A SPT result was considered "positive" if the wheal was 3 mm or larger, without a reaction to the negative control.

The total serum IgE was quantified with the ImmunoCAP system (Phadia, Uppsala, Sweden). All the serum samples were stored at  $-20\text{ }^{\circ}\text{C}$  before being used. Any sensitization was regarded as positive when the total IgE was greater than 0.35 kUA/L.

### *2.3 In vitro gastroduodenal-BBM digestion of whole peanuts*

*In vitro* oral-gastro-duodenal digestion of peanuts was performed in triplicate, according to Minekus et al. 2014. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the harmonized conditions. All digestion steps were carried out in a shaking incubator at  $37\text{ }^{\circ}\text{C}$  and 170 rpm. For the oral phase, peanuts were grossly minced using a coffee grinder and 100 mg of the resulting coarse powder was suspended in 207  $\mu\text{L}$  of SSF (included of 1500 U/mL of human salivary amylase) and incubated for 2 min. Subsequently, oral digesta were mixed with 320  $\mu\text{L}$  SGF containing 8  $\mu\text{L}$  of previously sonicated phospholipids (10 mg/mL). The pH was adjusted to 2.7 and 40  $\mu\text{L}$  of porcine pepsin (3000 U/mg) at a concentration of 12 mg/mL was added. Sample was incubated for 2 h at  $37\text{ }^{\circ}\text{C}$ . Pepsin hydrolysis was stopped by raising the pH to 7.0 with 1 M sodium bicarbonate. The duodenal digestion was carried out 2 h at  $37^{\circ}\text{C}$  after incorporating 640  $\mu\text{L}$  of SIF, bile salts (16 mg), porcine pancreatic lipase (1 mg), trypsin (0.7 mg, 100 U/mg as TAME activity),  $\alpha$ -chymotrypsin (0.3 mg, 40 U/mg) and pancreatic  $\alpha$ -amylase (1.1 mg, 10 U/mL). A final step of intestinal digestion was performed with of BBM (13  $\mu\text{U}/\mu\text{L}$ , final concentration) after adjusting pH to 7.2 with 1.0 M sodium bicarbonate. After 4 h at  $37^{\circ}\text{C}$ , peptidases were inactivated by immersion in boiling water for 5 min. Digesta were then filtered by Millex GV 0.22  $\mu\text{m}$  (Millipore, Billerica, MA, USA) and lyophilized.

### *2.4 Purification of peanut digesta samples*

After digestion, sample was defatted through two 10 min extraction with diethyl ether under magnetic stirring, followed by centrifugation at 10,000g (10 min). Large-sized polypeptides were precipitated with TCA up to a final concentration of 30% (w/v). After centrifugation, pellet was

four-fold washed with 1 mL of cold acetone, to remove residual TCA. The protein pellet was resuspended in 50 mM Tris-HCl, pH 7.0 and fractionated with Econo-pack 10-DG size exclusion chromatography (SEC) pre-packed columns (Bio-Rad), using 50 mM Tris as the eluent. Effluents were collected in 1 mL fractions and polypeptides monitored by the UV-absorbance at 280 nm (Ultrospec 160 2100 pro, Amersham Biosciences, Milan, Italy). Proteins (>6kDa) and peptide (MW<6 kDa) fractions were separately pooled. The low-sized peptide fraction was further desalted using Sep-Pak C18 pre-packed cartridges (Waters, Milford, MA, USA) washed with aqueous 0.1% TFA (v/v) and eluted with 70% ACN (v/v)/0.1% TFA (v/v). Proteins and peptides were concentrated in a speed-vac and finally lyophilized.

### 2.5 SDS-PAGE Analysis

Digested protein fraction >6 kDa and urea-extracted proteins were loaded onto a precast 12% polyacrylamide gel (Bio-Rad) either under reduction or non-reduction conditions for SDS-PAGE analysis. The whole protein fraction of peanuts, extracted according to Koppelman et al. 2016, was used as a reference to monitor the proteolysis. Proteins were visualized with blue silver (G250) staining. The gel was imaged with a scanner and processed using the LABScan software 3.00 (Amersham Bioscience).

For proteomic analysis, protein bands were manually excised, destained with acetonitrile/25mM Ambic (1/1, v/v) and dried under vacuum after dehydration in acetonitrile. Gel pieces were rehydrated with 20  $\mu$ L of a 12 ng/ $\mu$ L trypsin solution in 50 mM Ambic for 45 min on an ice-cold bath. Afterward, the excess of trypsin solution was discarded and the protein bands were incubated overnight at 37°C. The tryptic peptides were two-fold extracted in 40  $\mu$ L of 50% acetonitrile containing 2.5% (v/v) formic acid and dried using a speed-vac.

### 2.6 Western blotting analysis

IgE binding peanut proteins were detected by immunoblot analysis, performed using a pool of sera from N=8 children allergic to peanuts, as the source of specific IgE. SDS-PAGE resolved proteins were electroblotted onto nitrocellulose membrane using a Trans-blot cell (Bio-Rad) at 120 V for 60 min. The membrane was blocked for 1 h at room temperature with 5% (w/v) bovine serum albumin (Sigma-Aldrich) in a Tris-buffered saline solution with 0.05% Tween 20 (TBS-T) and incubated overnight at 4 °C with the pooled sera diluted 1:50 in TBS-T. After extensive washing (3 x 10 min with TBS-T), the membrane was incubated 1 h with monoclonal peroxidase-conjugated anti-human IgE antibody developed in goat (Sigma, cod. A9667), diluted 1:10000 with TBS-T. The membrane was rinsed with TBS-T (3 ×10 min) and finally with TBS (1 ×10 min) before development. Chemiluminescence reagents (ECL Plus WB reagent, GE Healthcare. Milan Italy) and X-ray film (Kodak, Chalons/Saône, France) were used to visualize the immunoreactive protein bands at various exposure times (0.5-5 min range).

### 2.7 LC-high resolution (HR)-MS/MS analysis

Mass spectrometry analysis was performed using a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), online coupled with an Ultimate 3000 ultra-high performance liquid chromatography instrument (Thermo Scientific). Samples were resuspended in 0.1% (v/v) formic acid solution, loaded through a 5 mm long, 300 µm i.d. pre-column (LC Packings, USA) and separated by an EASY-Spray™ PepMap C18 column (2 µm, 15 cm x 75 µm) 3 µm particles, 100 Å pore size (Thermo Scientific). Eluent A was 0.1% formic acid (v/v) in Milli-Q water; eluent B was 0.1% formic acid (v/v) in acetonitrile. The column was equilibrated at 5% B. Peptides were separated applying a 4–40% gradient of B over 60 min. The flow rate was 300 nL/min. The mass spectrometer operated in data-dependent mode and all MS1 spectra were acquired in the positive ionization mode with an  $m/z$  scan range of 350 to 1600. Up to 10 most intense ions in MS1 were selected for fragmentation in MS/MS mode. A resolving power of 70,000 full width at half maximum (FWHM), an automatic gain control (AGC) target of  $1 \times 10^6$  ions and a maximum ion

injection time (IT) of 256 ms were set to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM. In order to prevent repeated fragmentation of the most abundant ions, a dynamic exclusion of 10s was applied. Ions with one or more than six charges were excluded. Spectra were elaborated using the Xcalibur Software 3.1 version (Thermo Scientific). Mass spectra were elaborated using the Proteome Discoverer 2.1 software (Thermo Scientific), restricting the search to *Arachis Hypogea* database extracted from the NCBI (downloaded on March 2017).

Database searching parameters for identification of SDS-PAGE protein bands were the following: Met oxidation and pyroglutamic for N-terminus Gln as variable protein modifications; carboxymethylcysteine as a constant modification; a mass tolerance value of 10 ppm for precursor ion and 0.01 Da for MS/MS fragments; trypsin as the proteolytic enzyme; missed cleavage up to 2. Database searching parameters for identification peptides in digests were the same described above, except for no modification of cysteine residues included and no proteolytic enzyme selected.

The false discovery rate and protein probabilities were calculated by Target Decoy PSM Validator working between 0.01 and 0.05 for strict and relaxed searches, respectively. Proteins were considered confidently identified based on at least four sequenced peptides.

### 2.8 RP-HPLC and DOT-BLOT analysis

Peanut digests were fractionated using a HP1100 modular system (Agilent, Palo Alto, CA, USA) equipped with a RP-HPLC a C18 (5 $\mu$ , 4.6 mm i.d., 300A, 250 mm) reverse-phase column (Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL/min. Solvent A was 0.1% TFA (v/v) in water; solvent B was 0.1% TFA in acetonitrile. Separation of the peptides was effected with a 5-70% linear gradient of solvent B over 90 min, following 5 min of isocratic elution at 5% B. The column effluents were monitored at  $\lambda=214$  and 280 nm using a diode-array detector.

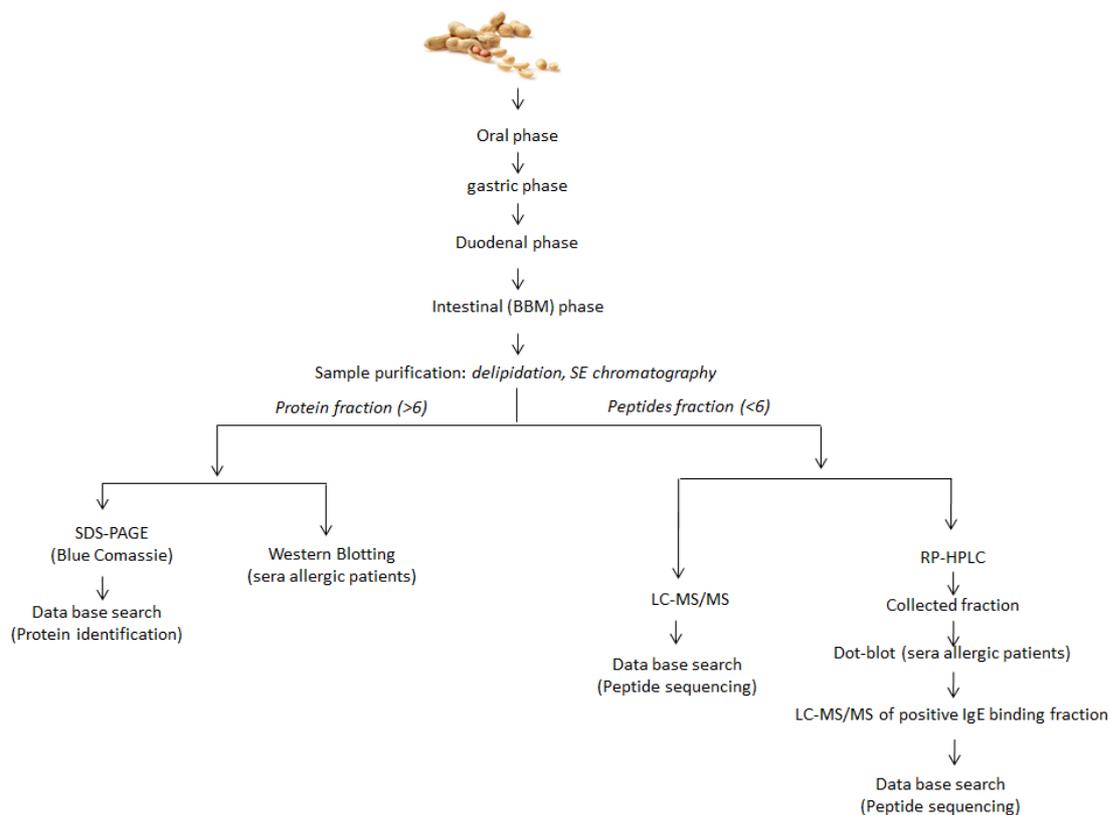
For dot-blot assay, the manually collected HPLC fractions were spotted onto a 0.22- $\mu\text{m}$  trans-blot nitrocellulose membrane (Bio-Rad) and developed using the same protocol as the Western blot assay. The whole protein extract was used as the positive control.

### 3. Results

#### 3.1 Gastrointestinal digestion of whole peanut

The stability of IgE binding proteins following gastrointestinal digestion of whole raw peanut, was determined by immunochemical and proteomic analysis, according to the workflow diagram shown in Figure 1.

A widespread commercial variety of peanuts (Virginia cv) was used in this study (Koppelman et al. 2016). Whole peanuts were digested according to the harmonized *in vitro* static digestion model (Minekus et al. 2014), based on the following phases: oral phase through mastication, gastric and duodenal phases with a sequential addition of digestive enzymes in physiological concentration ranges. The model was finally integrated with pig intestinal BBM mimicking the jejunal phase of peptide degradation. After *in vitro* digestion, samples were defatted and protein and peptide fractions were separated by SEC.



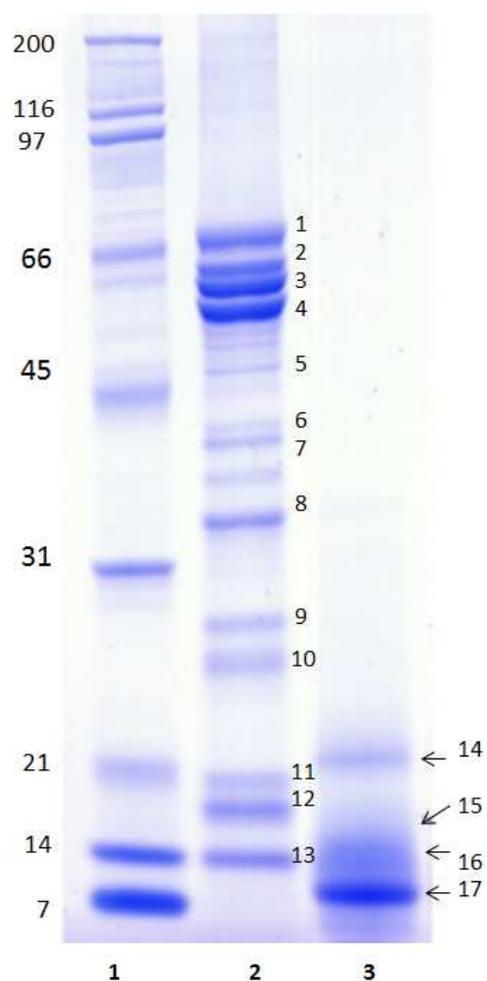
**Fig. 1:** Schematic workflow of the experimental approach employed (immunoassay and MS-based analysis) for the study and characterization of proteolytic digesta resulting from *in vitro* simulated digestion of whole peanuts.

### 3.2 SDS-PAGE analysis digesta sample

The disappearance of peanut proteins after simulated digestion was monitored by SDS-PAGE (Fig. 2), compared with whole protein extract as the reference control of undigested proteins. Individual electrophoretic bands were identified by peptide sequencing using LC-MS/MS analysis, as reported in Table 1. Ara h 1 was identified at approximately 68.2 kDa (band 1) and as less abundant isoforms at 58.2 (band 2) and 33.4 kDa (band 8). Ara h 3 migrated in three different bands at 58.2 54.9 and 52.4 kDa (band 2, 3, and 4 respectively), close to Ara h 1. Low-abundance bands identified as Ara h 3 were detected between 52.4 and 17.2 kDa (bands 5-12). The high heterogeneity observed for Ara h 3 is a consequence of multiple post-translational proteolytic events, involving the N-terminal of the acidic subunit, which produces isoforms of various molecular size (Piersma et al. 2005). A

similar processing has already been described for 11S glycinin storage proteins from other plant sources (Dickinson et al. 1989). The Ara h 2 migrated as a doublet at 17.8 (band 11) and 17.2 kDa (band 12) which were assigned to the Ara h 2.01 and Ara h 2.02 isoforms, respectively, according to migration (Koopelman et al. 2010). The Ara h 6 was identified as a well resolved band at 16.3 kDa (band 13). Minor allergens Ara h 7, Ara h 8 and Ara h 10 were also detected in band 12.

As expected, after gastrointestinal digestion of whole peanuts, the electrophoretic protein pattern radically changed, since large-sized proteins were no longer detectable, while the gel exhibited multiple polypeptide fragments at MW estimated between 7 and 23 kDa (Fig. 1). MS/MS-based analysis revealed the presence of Ara h 3 fragments in all these bands (bands 14-17), indicating that large domains of Ara h 3 survived proteolytic degradation. Ara h 6 fragments were detected in band 16 and 17, whilst practically intact residual Ara h 2 was revealed exclusively in band 15. No signal assigned to Ara h1 allergen was detected in the gel, confirming its susceptibility to digestion leading to low MW peptide products (Koopelman et al. 2010).



**Fig. 2: SDS-PAGE comparison of undigested and digested peanut proteins under non-reducing conditions.** Lane 1, molecular marker. Lane 2, whole peanut protein extract (urea extract). Lane 3, protein extract obtained from *in vitro* simulated gastrointestinal digestion of whole peanuts.

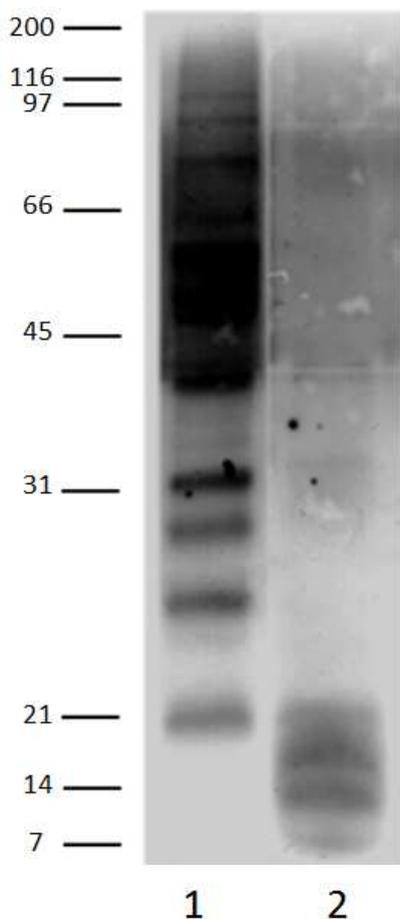
**Table 1:** Identification of protein bands from SDS-PAGE through LC-HR-MS/MS of in gel produced tryptic peptides (Fig. 2).

	Band <sup>a</sup>	Accession	Description	Allergen name <sup>b</sup>	Coverage <sup>c</sup>	Peptides <sup>d</sup>	aa <sup>e</sup>	MW [kDa] <sup>f</sup>	calc. pI <sup>f</sup>	Score Sequest HT <sup>g</sup>	Peptides SEQUEST HT <sup>h</sup>
Before digestion	1	N1NG13	Seed storage protein Ara h1	Ara h 1	65	64	626	71,302	7,06	689,59	64
	2	Q8LKN1	Allergen Arah3/Arah4	Ara h 3	73	32	538	61,7	5,72	489,49	32
		N1NG13	Seed storage protein Ara h1	Ara h 1	59	43	626	71,302	7,06	266,06	43
	3	Q647H3	Arachin Ahy-2	Ara h 3	56	31	537	61,494	5,73	499,05	31
	4	Q647H3	Arachin Ahy-2	Ara h 3	55	25	537	61,494	5,73	182,64	25
	5	Q647H3	Arachin Ahy-2	Ara h 3	50	23	537	61,494	5,73	205,92	23
	6	Q8LKN1	Allergen Arah3/Arah4	Ara h 3	47	18	538	61,7	5,72	116,78	18
	7	Q9FZ11	Gly1	Ara h 3	58	27	529	60,412	5,64	225,42	27
	8	A1DZF0	Arachin 6	Ara h 3	48	24	529	60,339	5,54	336,71	24
		N1NG13	Seed storage protein Ara h1	Ara h 1	43	26	626	71,302	7,06	137,80	26
	9	A1DZF0	Arachin 6	Ara h3	33	18	529	60,339	5,54	94,68	18
	10	A1DZF0	Arachin 6	Ara h 3	59	32	529	60,339	5,54	356,90	32
	11	Q6PSU2	Conglutin-7	Ara h 2	40	5	172	20,102	6,34	31,61	5
Q647G5		Oleosin 1	Ara h 10	27	3	169	17,741	9,58	15,39	3	
12	A1DZF0	Arachin 6	Ara h 3	33	15	529	60,339	5,54	77,368	15	
	Q6PSU2	Conglutin-7	Ara h 2	36	5	172	20,102	6,34	41,520	5	
	Q647G5	Oleosin 1	Ara h 10	36	6	169	17,741	9,58	23,170	6	
	B0YIU5	Ara h 8 allergen isoform	Ara h 8	38	3	153	16,402	5,2	16,86	3	
	B4XID4	Ara h 7 allergen	Ara h 7	26	3	164	19,326	7,9	9,83	3	
13	A5Z1R0	Ara h 6	Ara h 6	61	9	145	16,909	6,52	60,31	9	
After digestion	14	Q9FZ11	Gly1	Ara h 3	32	12	529	60,412	5,64	33,78	12
	15	Q5I6T2	Arachin Ahy-4	Ara h 3	32	14	531	60,699	5,48	56,624	14
		52001227	2S protein	Ara h 2	46	11	179	20,837	7,36	52,84	11
	16	Q9FZ11	Gly1	Ara h 3	34	15	529	60,412	5,64	64,41	15
		A5Z1R0	Ara h 6	Ara h 6	57	11	145	16,909	6,52	62,38	11
	17	Q9FZ11	Gly1	Ara h 3	18	8	529	60,412	5,64	42,81	8
A5Z1R0		Ara h 6	Ara h 6	52	8	145	16,909	6,52	29,11	8	

<sup>a</sup> Protein band from SDS-PAGE (Fig. 2); <sup>b</sup> allergen name according to WHO/IUIS database ([www.allergen.org](http://www.allergen.org)); <sup>c</sup> sequence coverage (%); <sup>d</sup> number of peptides identified; <sup>e</sup> number of amino acids (AA), <sup>f</sup> theoretical Mr and pI values; <sup>g</sup> sum of the scores of the individual peptides from the Sequest HT search; <sup>h</sup> number of distinct peptide sequences in a protein group from the SEQUEST HT search.

### 3.3 Western blotting of digested sample

The IgE-reactivity of digested and undigested peanut proteins was assayed by immunoblotting using pooled sera from eight pediatric patients diagnosed with food allergy to peanut (**Fig. 3**). Before digestion, almost all the major peanut proteins appeared as IgE-reactive, mainly due to the individual heterogeneity in the recognition patterns of the allergic subjects. Consistently with SDS-PAGE, native allergens were no longer detectable in gastrointestinal digests, while neo-formed low MW bands were immunoreactive. The immunoreactive band at 23.0 kDa was identified from the corresponding Coomassie stained electrophoretic gel (Fig. 1, Table 1) as Ara h 3 fragments. The IgE-binding fragments in the immunoreactive bands between 20 and 7 kDa were not univocally assigned, due to co-migration of fragments arising from Ara h 2, Ara h 3 and Ara h 6.



**Fig. 3: Western Blotting analysis.** Undigested (lane 1) and digested (lane 2) peanut extracts immunostained using specific IgE from pooled sera of (N=8) pediatric allergic subjects.

### 3.4 LC-MS/MS analysis of digesta sample

The peptide fraction of digested samples collected after SEC separation (see Figure 1) was analyzed by LC-HR-MS/MS to identify the released peptides resistant to digestion. Table 2 lists the parent proteins identified through MS/MS based peptide sequencing. Details about peptide sequences are reported in Table S2. Figure 4 highlights the sequence of peptides identified in the digesta belonging to Ara h 1, Ara h 3, Ara h 2 and Ara h 6 main isoforms. Notably, protein coverage data generated by peptide sequencing, might be not fully exhaustive, considering that database search is challenged by the non-tryptic nature of the peptides, the non predictable cleavage specificity as well as the extreme heterogeneity of the parent protein subset. Despite such drawbacks, LC-HR-MS/MS analysis revealed a heterogeneous mixtures of peptides (nearly 800 identified sequences) with molecular size up to 5000 kDa. The bulk of signals was assigned to fragments of Ara h 3 isoforms, which mapped 69% of the primary structure, indicating a significant stability of Ara h 3-derived peptides to gastrointestinal protease.

A lower number of peptides (MW between 400 and 2000 Da) matched with Ara h 1, showing a sequence coverage of 22%. Peptides arising from Ara h 2 and Ara h 6 digestion were detected as well. In particular, two and five peptides from Ara 6 and Ara h 2 were identified, respectively. Similarly, six peptides attributed to Ara h 8, occurred in the peptide mixture. Proteolytic fragments of actin, conarachin, 13-lipoxygenase were also detected, though with poor sequence coverage. Three peptides arising from the digestion of an oleosin allergen, namely Ara h 10, were identified as well. As regarding these hydrophobic proteins, we can not exclude that large oleosin-derived peptides remained embedded in the lipid matrix and were removed during defatting process of digesta.

**Ara h 1  
(N1NG13)**

MRGRVSLML LGIIVLIASV SATHAKSSPY QKKTENPCAQ RCLQSQQEP DLKQKACES RCTKLEYDPR CUYDFRGHTG TINQRSPPE RIRGRQFCIV DLDRQPRRE EGGRWGPAGP  
 REREPREDNR QREDNRRRS HQQPKIRPE GFCECEWCT PGSHVPEETS PNNPFYFSPR RFSTRYGNQN GRIRVLQRFD QSRQFQNLQ NHRIVQIEAK PNTIVLPRHA DADNIVIQQ  
 GQATIVVANG NNRKSFNLDE GHALRIPSGF ISYILNRHDN QNLRVAKISM FVNTPGQFED FFPASSRDQS SYLQGFSRNT LEAAFNAEFN EIRVLLEN ACCGQEERQQ RRVSTRSSEN  
 NEGVIKVKSK EHVEELTKHA KSVSKIGSEE EQDITNPINL RECEPDLSEN FGLLFEVKPD KKNPQLQDLD MMLTCVEIKE GALMLPHFNS KAVIIVWVK GTGNLELVAV REEQQRGRR  
 EEEDEDEEE EGSNREVRRY TARKEGDVDF IMPAAHPVAI NASSELHLIG FGINAENNR IFLAGQDMV IDQIEQARD LAFPGSGQV ENLIRNQKES HFVSARFQQ SQSPSSPEKE  
SPEKEDQEE NQGGKGPLLS ILKAFN

**Ara h 3  
(Q516T2)**

MAKLELSFC FCFLVLGASS ISFRQQPEEN ACFFRINA RFDNRISSE CVIETWPN QEFECAGVAL SRLVLRNAL RRFVSNAQ EIIFIQQSGRY EGLIFPGCPS TYEEPAQQR  
 RSQSQRPPRR LQEDQSQQQ QDSHQVHRF DEGDIAVPT GVAFWLNDH DIIVAVSLT DTNNNDQLD QFPAPFLAG NHEQELRY QSSQSRRS LPYSPYSPQS QPRQEREFS  
 PRGQHSRRER ACQEEENEGG NIFSGFPEF LEQAFQVDG QIVQNLGEN ESEEGAVT VAGGLRILSH DRKGADEE EYDEBEYED EDRRRGRGS RGRNGIET ICTASVKNI  
GRNPSFDIYN EQAGSLKTN DINLLILRM GLSAEYGNLY RNALVPHYN INAHSIIYAL RGRAHVQVD SNGNRVDEE LQEGHVLVVE QNFVAGKSC SINFYVAFK IDSRSIANL  
AGENSVIDNI PEEVVANSY LQREQARQ MINNPKFFV SPSQSPAV A

**Ara h 2  
(Q6PSU2)**

DHLENTHSI TITTIMAKLI ILVALALELL AAHASARQQW ELQGDRRQDS QLERNLRPC EQHLMQKIQR DEDSYGRDP SPSQDFYSPS QDPRRDPYS PSPYDRRAG SSQHQEROCN  
ELNEFENNQ CMCEALQQIM ENQSDRLQGR QQEQQFKREL RNLEQQCGLR APQRGLEVE SGGRRY

**Ara h 6  
(A1DZE9)**

MAKSTILVAL LALVIVAHAS AMRRERGRQG DSSSCERQVD KVNLPCEQH IMQRMGEQE QVDSYDIRST RSSDQQQRC DELNEMENTQ RMGEALQQK MENQCCKLQD REMVEQFKRK  
 LMDLAQQCNF RAPPKCDLIV NGRRC

**Fig. 4:** Primary sequences of the major peanut allergens Ara h 1, Ara h 3, Ara h 2 and Ara h 6. The highlighted domains correspond to peptides arising from simulated digestion of whole peanut, identified by LC-HR-MS/MS. The underlined sequences belonging to Ara h 1 and Ara h 3 correspond to predicted IgE-binding regions. The boxed sequences are Ara h 3 IgE-binding peptides as assessed by Dot-blot and LC-HR-MS/MS.

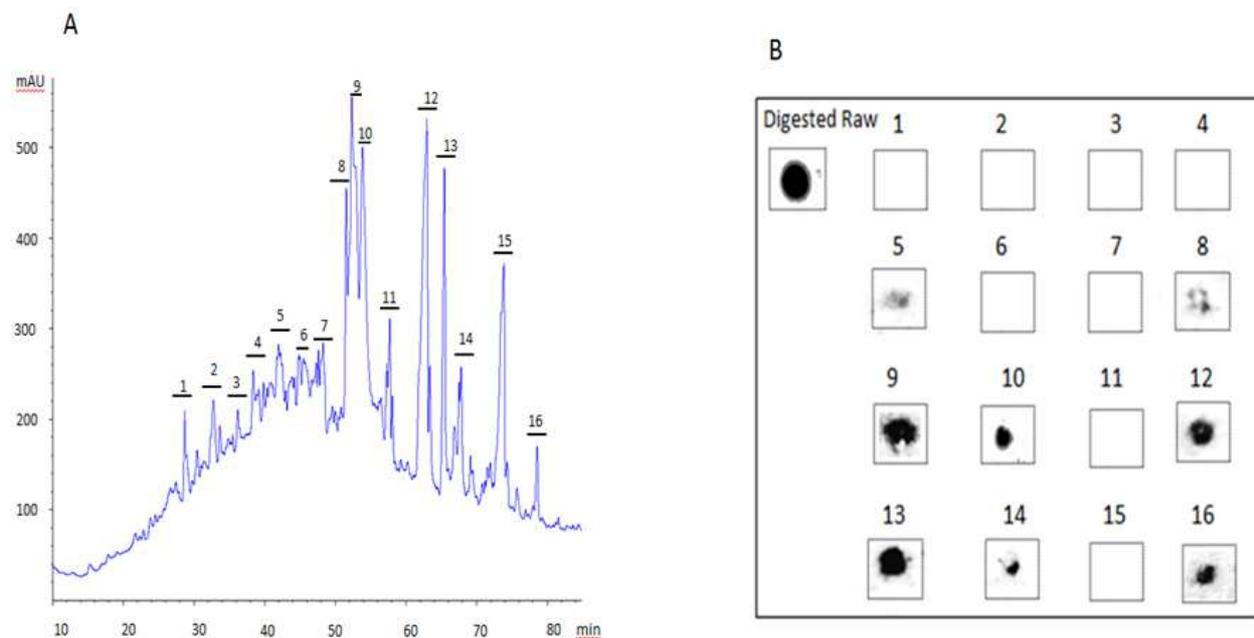
**Table 2:** Identification of parent peanut proteins through LC-HR-MS/MS sequencing of peptide digests

Accession	Description	Allergen name <sup>a</sup>	Coverage <sup>b</sup>	Peptides <sup>c</sup>	aa <sup>d</sup>	MW [kDa] <sup>e</sup>	calc. pI <sup>e</sup>	Score SEQUEST HT <sup>f</sup>	Peptides SEQUEST HT <sup>g</sup>
Q516T2	Arachin Ahy-4	Ara h 3	69	403	531	60,69	5,48	6531	403
Q9FZ11	Gly1	Ara h 3	69	392	529	60,41	5,64	6523	392
B5TYU1	arachin Arah3 isoform	Ara h 3	69	346	530	60,58	5,55	5560	346
A1DZF0	arachin 6	Ara h 3	69	356	529	60,33	5,54	5925	356
<u>Q0GM57</u>	iso-Ara h3	Ara h 3	69	260	512	58,22	5,59	3464	260
P02872	peanut agglutinin precursor	Ara h agglutinin	35	25	273	29,30	5,66	224	25
B0YIU5	Ara h 8 allergen isoform	Ara h 8	69	6	153	16,40	5,2	41	6
A0A0A1EUV7	actin		25	15	323	35,86	5,97	168	15
N1NG13	Seed storage protein Ara h1	Ara h 1	22	41	626	71,30	7,06	630	41
B3IXL2	Main allergen Ara h1	Ara h 1	22	35	614	70,24	6,86	554	35
Q647H1	Conarachin		20	28	662	75,88	5,4	232	28
Q647H2	Arachin Ahy-3	Ara h 3	18	16	484	54,53	5,59	130	16
Q6PSU2	seed storage protein SSP1	Ara h 2	16	5	187	21,77	6,39	56	5
Q647G5	oleosin 17.8	Ara h 10	13	3	169	17,74	9,58	8	3
Q647G9	Conglutin	Ara h 6	7	2	145	16,91	6,01	36	2
A1DZE9	Conglutin 8 hypogaea	Ara h 6	7	3	145	16,82	6,54	20	3
Q4JME6	13-lipoxygenase		3	5	863	97,41	5,53	28	5

<sup>a</sup> allergen name according to WHO/IUIS database ([www.allergen.org](http://www.allergen.org)); <sup>b</sup> sequence coverage (%); <sup>c</sup> number of peptides identified; <sup>d</sup> number of amino acids (AA), <sup>e</sup> theoretical MW and pI values; <sup>f</sup> sum of the scores of the individual peptides from the SEQUEST HT search; <sup>g</sup> number of distinct peptide sequences in a protein group from the SEQUEST HT search.

### 3.5 Characterization of IgE binding peptides

IgE binding capability of peptides (<6kDa) was assessed by HPLC fractionation and Dot-blot analysis (Fig. 5a and 5b). Six out of sixteen spotted fractions (panel B) tested positive to IgE. Fractions 9, 10, 12, 13 and 16 exhibited the most intense response, indicating the presence of peptide(s) harboring IgE epitope(s). Fraction 5, 8 and 14 were also positive, although at a weaker intensity. IgE positive-HPLC fractions were sequenced by LC-HR-MS/MS (Table 3). Details about MS/MS based peptide sequencing are given in Table S3. The reference linear IgE-binding epitopes are taken from previous studies (Rabjohn et al. 1999, Rougé et al. 2009, Jin et al. 2009, Shinmoto et al. 2010). Overall, IgE-binding peptides were from Ara h 3, releasing three specific domains that appear particularly resistant to proteolysis (Fig. 4, boxed regions). In the HPLC peaks 12 and 13, “chopped” forms of a single peptide deriving from the protein region 260-274 (referred to amino acid sequences of Q5I6T2) (Fig.4) harbored the epitope GNIFSGFTPEFLEQA (Rabjohn P. et al 1999, Jine et al 2009). The same epitope was also encrypted in peptides arising from Ara h 3 isoform (Q9FZ11), where an alanine is replaced by a glutamic acid residue (GNIFSGFTPEFLAQA) (Table 3). In contrast, the HPLC peaks 8, 9 and 10 contained peptides from Ara h 3 isoforms encrypting the IgE-binding epitope KNNNPFKFFVPP (Rougé et al. 2009). Partial sequences of a further Ara h 3 linear epitope (KKNIGRNRSPDIYNP), identified by Rouge et al 2009, eluted in the HPLC peaks 14 and 16. Finally, the N-terminal fragment of Ara h 3 harboring the IgE-binding epitope IETWNPNNQEFECAG (Rabjohn et al 1999) occurred in fraction 5. Noteworthy, exclusively Ara h 3 fragments were IgE-positive, whilst no immunoreactive peptide of Ara h 1 was identified.



**Fig. 5:** HPLC fractionation (A) and dot-blot analysis of individual fractions (B) of low MW peptides (<6kDa) arising from simulated digestion of whole raw peanut. IgE-immunoreactive peptides were identified by LC-HR-MS/MS.

**Table III:** Sequences harbouring IgE binding peptides, identified in IgE-immunoreactive HPLC fractions (Figure 5). Details about MS/MS based peptide sequencing are reported in Table S1.

Accession	Amino acid sequence	LC	MH <sup>+</sup> (Da)
Q647H3; Q9FZ11	LNAQRPDNRLESEGGYIETWNPNN	#5	2787.30979
Q8LKN1; Q647H4;Q61W G5; Q9SQH7; Q516T2.	LNAQRPDNRIESEGGYIETWNPNN	#5	2787.30979
Q647H4.	NRSPDIYNPQAGSLKTANELNLLILR	#8	2910.57749
Q9SQH7	QLKNNNPFKFFVPPFQQSPR --KNNNPFKFFVPPFQQSPR	#8	2416.25553 2192.13993
Q647H3; Q516T2	NRSPDIYNPQAGSLKTANDLNLIL- NRSPDIYNPQAGSLKTANELNLLILR	#9	2740.46281 2910.57957
Q647H3; Q516T2	QLKNNNPFKFF----- -----FKFFVPPFQQSP ----NNPFKFFVPP-----	#10	1379.71064 1468.76234 1206.63060
Q9SQH7; Q516T2	AGQEEENEENEGGNIFSGFTPEFLEQAFQVDDR---- ---EENEENEGGNIFSGFTPEFLEQAFQVDDR---- -----EGGNIFSGFTPEFLEQAFQVDDRQIVQ ----EENEENEGGNIFSGFTPEFLEQAFQVDDR---- -----ENEGGNIFSGFTPEFLEQAFQVDDR---- -----NEGNIFSGFTPEFLEQAFQVDDR---- -----EGGNIFSGFTPEFLEQAFQVDDR----	#12	3360.50791 3104.37456 3071.47702 2975.33585 2846.28862 2717.23686 2603.20561
Q8LKN1; Q647H3; Q647H4	AGQEENEGGNIFSGFTPEFLAQAFQVDDR---- AGQEENEGGNIFSGFTPEFLAQAF-----	#12	3301.50290 2688.22197
Q61WG5; Q9SQH7	QLKNNNPFKFFVPPFQQSPR QLKNNNPFKFFVPPF----	#12	2416.24857 1819.95452
Q516T2; Q9SQH7	---EENEENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGE- ----EENEENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGE- EENEENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLR--- EGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGEN EGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGE- EGGNIFSGFTPEFLEQAFQVDDRQIVQNLR--- ----EEDEENEGGNIFSGFTPEFLEQAFQVDDR----- ----EENEENEGGNIFSGFTPEFLEQAFQV----- EGGNIFSGFTPEFLEQAFQV-----	#13	4141.91421 4012.89492 3826.81826 3754.80979 3640.76406 3454.71054 2976.32573 2589.17803 2332.08745
Q8LKN1; Q647H3; Q647H4; Q9FZ11.	AGQEENEGGNIFSGFTPEFLAQAFQVDDRQIL----- ----EENEENEGGNIFSGFTPEFLAQAFQVDDRQIVQNLRGEN EENEENEGGNIFSGFTPEFLAQAFQVDDRQIVQNLRGE- ----EENEENEGGNIFSGFTPEFLAQAFQVDDRQIVQNLR--- EGGNIFSGFTPEFLAQAFQVDDRQILQ ----QENEENEGGNIFSGFTPEFLAQAF----- -----EGGNIFSGFTPEFLAQAF-----	#13	3655.73288 4068.92885 3954.88588 3768.84463 3027.48223 2286.03105 1931.92278
Q647H4.	KKNIGRNRSPDIYNPQAGSLKTANELNLLILRWL -KNIGRNRSPDIYNPQAGSLKTANELNLLILRWL --NIGRNRSPDIYNPQAGSLKTANDLNLILRWL ----RNRSPDIYNPQAGSLKTANDLNLILRWL -----NRSPDIYNPQAGSLKTANDLNLILRWL-----	#13	3906.18349 3778.09853 3635.97622 3351.82325 3195.73061
Q61WG5; Q647H4.	NRSPDIYNPQAGSLKTANELNLLILRWL NRSPDIYNPQAGSLKTANELNLLIL--- NRSPDIYNPQAGSLKTANELNL-----	#14	3209.73337 2754.48044 2415.22611
Q647H4; Q647H3; Q9FZ11; Q516T2	NRSPDIYNPQAGSLKTANELNLLILRWLGLSAEYGNLYR NRSPDIYNPQAGSLKTANDLNLILRWLGLSAEYGNLYR NRSPDIYNPQAGSLKTANDLNLILRWLGLSAEYGNLY- --SPDIYNPQAGSLKTANDLNLILRWLGLSAEYGNLYR NRSPDIYNPQAGSLKTANELNLLILRWLGLSAEYG--- NRSPDIYNPQAGSLKTANDLNLILRWLGL----- NRSPDIYNPQAGSLKTANELNLLILRWL----- NRSPDIYNPQAGSLKTANDLNLILRWL-----	#16	4433.33387 4419.32294 4263.21840 4149.17348 3887.04555 3365.84731 3209.74209 3195.71914

## Discussion

The mechanism by which dietary proteins sensitize and elicit an allergic reaction remains substantially unresolved. In particular it is still fervently debated whether gastrointestinal digestion stability could be an effective predictor of allergenicity. Anyway, it is widely accepted that many among the most common food allergens are digestion resistant proteins and probably induce sensitization at the level of the intestinal tract (Asero and Antonicelli 2011). Unquestionably, the digestion stability increases the probability that a food protein (or its derived peptide) can sensitize an individual, because in addition to the skin, respiratory and oral mucosa ones, also the intestinal route of sensitization becomes accessible. In the last years, the scientific community has paid remarkable attention to the evaluation of the stability of food allergens along the human digestive tract, also providing information at the molecular level about the proteolytic fraction resulting from the digestion process (Huby et al. 2000). In a perspective of molecular resolved diagnostic and therapeutic approaches, these insights contribute to the precise identification of epitopic determinants able to reach the intestinal mucosa in immunological active form, with relatively high likelihood to trigger immunological reactions in sensitised subjects. To this aim, more or less physiological-correspondent *in vitro* protocols have been developed to simulate human digestion. Early attempts evaluated the digestion stability of purified allergens by mimicking only the gastric phase of digestion (Astwood et al. 1996). Later on, digestion protocols were extended also to the pancreatic phase (Fu et al. 2002, Wickham et al. 2009). In the case of peanuts, nearly all model studies indicated that Ara h 1 and Ara h 3 are broken down into small peptide fragments within minutes, while Ara h 2 and Ara 6 remain almost unaffected by digestion (Koopelman et al. 2010). The digestion resistance might justify the predominant clinical relevance of Ara h 2 and Ara h 6 allergens (Flinterman et al, 2007). At the same time, these outcomes left partially unanswered the question why and how extremely proteolysis-labile proteins, such as as Ara h 1 and Ara h 3, could be associated with primary sensitization.

The digestion and the immunogenic potential of allergenic proteins in a more realistic context, also taking into consideration the allergen containing food matrix, have remained poorly investigated so far. In this paper, we aimed at investigating the stability of peanut allergens throughout the digestive process and at identifying the potential antigenic determinants surviving the digestion. However, compared to the existing works, which are for the most tailored to the assessment of allergen stability using standard purified proteins, we herein introduced a further complexity factor represented by the whole peanut matrix to reproduce more realistically what happens after consumption of peanuts. To this purpose, we applied the harmonized *in vitro* digestion procedure, developed for mimicking protein degradation of unfractionated foodstuff (Minekus et al. 2014). This digestion model was also integrated with an additional step with porcine jejunal BBM enzymes. The jejunal phase of peptide degradation is a fundamental step for assessing the intestinal stability of large protein fragments produced upstream, during the gastric and duodenal phases (Mamone et al. 2007, Picariello et al. 2016).

In line with previous studies (Apostolovic et al. 2016), the outcomes of the digestion assays confirmed the substantial stability of Ara h 2 and Ara h 6, since these latter produced protein fragments with MW only slightly lower than their parent proteins, as detected by SDS-PAGE. Consistently, LC-HR-MS/MS of the small MW peptides revealed that Ara h 2 and Ara h 6 released a number of peptides relatively low.

The most striking result of the current study was the additional identification of digestion stable Ara h 3 large sized fragments (7-21 kDa). Such a finding contrasts with most of the previous literature, which claimed the almost complete susceptibility of Ara h 3 to gastrointestinal proteases (Koppelman et al. 2010). The partial resistance of Ara h 3 to digestion was also confirmed by LC-HR-MS/MS analysis of peptide digests, that contained several peptides with MW between 800 and 5000 Da mapping the Ara h 3 isoforms for 69% overall. A reasonable explanation of this finding may be the “*masking effect*” by the peanut matrix, delaying or impairing the protein degradation and altering the pattern of the peptide fragments released by proteolysis. Indeed, the peanut body

includes a medium protein content and high level of lipids and polysaccharides, which may affect the proteolysis, sparing several immunological active polypeptides. It has been demonstrated that some type of polysaccharides decrease the digestibility of peanut allergens and increased the number of large-sized IgE-binding polypeptides (Mouencoucon et al. 2004). Similarly, reports assessing digestion stability of other food allergens (e.g.  $\beta$ -lactoglobulin, Act d 2 kiwi allergens,  $\beta$ -conglycinin) confirmed this trend (Mouencoucon et al. 2004, Polovic et al. 2007, De Angelis et al. 2017). The presence of lipids, like phospholipids naturally occurring in foods may greatly alter susceptibility of allergens to digestion, as well. For example, phosphatidylcholine hinders the enzymatic degradation of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin (Moreno et al. 2005, Mandalari et al. 2009).

In addition to proteins, we evaluated the IgE-binding properties of peptides resulting from simulated digestion. By using a combined approach based on HPLC, dot-blot and LC-HR-MS/MS, for the first time, to the best of our knowledge, it was evaluated the IgE-binding property of peptide fragments produced as a consequence of the physiological digestion. Interestingly, linear epitopes GNIFSGFTPEFLEQA and IETWNPNN encrypted in digestion-stable Ara h 3 domains were sequenced. Their epitope nature had previously been assessed using overlapping synthetic peptides (Rabjohn et al. 1999) and their conformation within native Ara h 3 protein was determined by crystallographic methods (Jin et al. 2009). Notably, it has been reported that the side chain of GNIFSGFTPEFLEQA and IETWNPNN was nearly completely buried in the folded native allergen, thereby being not exposed to the IgE capture (Jin et al. 2009). However, as suggested by the same authors and confirmed in current study, the Ara h 3 peptides harboring these epitopes became available for interaction with immune system effectors upon gastrointestinal release. These same authors also identified two additional linear Ara h 3 epitopes (DEDEYEYD and VTVRGGLRILSPDRK) that, on the contrary, are exposed on the surface of the allergen in the native folding and so immediately available for IgE-binding. Although peptides containing these epitopes occurred in the peanut digesta (Fig. 4, Table S1), none of them was IgE-immunoreactive in

our conditions. This apparent incongruence is likely due to a poor specificity of these epitopes for the specific IgE in the pool of pediatric sera used in the current study. To this purpose, it has to be underlined that IgE-based immunochemical assays for the identification of allergenic determinants are in general affected by a high degree of individual variability.

Unlike Ara h 3, Ara h 1 was completely degraded and no derived peptide showed immunoreactivity. LC-HR-MS/MS analysis of digesta revealed Ara h 1 peptides with MW lower than 2kDa, which mapped 22 % of the whole protein. Although some of the Ara h 1 sequenced peptides included already described epitopes (Fig. 4, Table S3), these were not reactive against serum IgE in our conditions. Bøgh et al. 2009 demonstrated that gastro-duodenal digests from Ara h 1, containing peptides of size less than 2 kDa, could induce degranulation response with a similar magnitude as intact Ara h 1. These small peptides were shown to be aggregated in large complexes, which was hypothesized to be the reason for their eliciting capacity (Bøgh et al. 2009).

## 5. Conclusion

Our results point out the importance to investigate the digestion process of whole food, instead of purified allergen proteins, clearly increasing the correspondence of the model systems with human physiology. Remarkably, at the moment a digestion model assuring the complete *in vitro-in vivo* correspondence is not available, taken into account the large range of factors affecting human digestion and the complexity of an ordinary meal. Notwithstanding this, a static *in vitro* multi-compartmental model, recently developed in the framework of the EU Infogest Cost Action with the precise aim to harmonize digestion conditions based on human physiology, has been applied for the evaluation of allergen stability of food matrices (Picariello et al. 2015, Mamone et al. 2015), providing physiologically consistent outcomes (Egger et al. 2016). Along with the advancement of omic sciences, it is plausible to assume that all products arising from food digestion can be “*simply*” characterized in order to define with high accuracy the metabolic fate of either toxic or bioactive

molecules. With regard to food allergens, improving the understanding of the spatio-temporal evolution of allergens in the gastrointestinal tract will facilitate the development of more sensitive and effective antibodies to detect food allergens, pushing to the edge the limit of allergen detection in complex matrices, and will support establishing threshold levels of sensitization/elicitation. In perspective, such an analysis could contribute to predict the allergenicity of proteins from novel and alternative foods.

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- A realistic picture of the potentially allergenic peanuts is provided.
- Large fragments of Ara h 3, survive to in vitro gastrointestinal digestion;
- Ara h 3 IgE binding epitopes are released during gastrointestinal digestion.
- Ara h 1 is completely degraded following in vitro gastrointestinal digestion.
- Peanut matrix slows the gastrointestinal digestion process of allergen proteins.