

Proteomics-Based Approach To Detect and Identify Major Allergens in Processed Peanuts by Capillary LC-Q-TOF (MS/MS)

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An MS-based method, combining reversed-phase capillary liquid chromatography (capillary LC) with quadrupole time-of-flight tandem mass spectrometry (nano-ESI Q-TOF MS/MS), was developed with the aim of identifying a set of peptides that can function as markers for peanut allergens. Emphasis was given to the identification of the three major peanut allergens Ara h 1, Ara h 2, and Ara h 3, because these proteins are considered to represent >30% of the total protein content of peanut and are directly relevant for the allergenic potential of this food. The analytical data obtained were used to perform databank searching in combination with de novo sequencing and led to the identification of a multitude of sequence tags for all three peanut allergens. Food processing such as roasting of peanuts is known to affect the stability of proteins and was shown to influence the detection of allergen sequence tags. The analysis of raw and roasted peanuts allowed the identification of five peanut-specific sequence tags that can function as markers of the specific allergenic proteins. For Ara h 1, two peptide markers were proposed, namely, VLEENAGGEQEER (m/z 786.88, charge 2+) and DLAFPGSGEQVEK (m/z 688.85, charge 2+), whereas for Ara h 2 only one peptide, RQQWELQGDR (m/z 439.23, charge 3+), was found to satisfy the required conditions. For Ara h 3, the two specific peptides, SPDIYNPQAGSLK (m/z 695.35, charge 2+) and SQSENFYVAFK (m/z 724.84, charge 2+), were selected. Other peptides have been proposed as indicative for food processing.

KEYWORDS: Peanut allergens; food processing; tryptic peptides; capillary high-performance liquid chromatography (capillary LC); nanoelectrospray quadrupole time-of-flight tandem mass spectrometry (nano-ESI Q-TOF MS/MS)

INTRODUCTION

Food allergens are a significant worldwide public health issue. Around 1–2% of the total population, and up to 8% of children, suffer from some type of food allergy (1–3). Peanuts are the most allergenic food known and are capable of triggering severe adverse reactions in sensitized individuals (4). The prevalence of peanut allergy has been estimated to be around 1.1% in the United States (5) and 0.5% in the United Kingdom (6). Human allergic reactions have been linked to several different proteins within the peanut. These allergenic proteins are characterized as seed storage proteins with molecular masses ranging from 10 to 70 kDa (7).

Currently, the only effective measure to prevent allergic reactions from occurring is the avoidance of the allergen-containing food. For peanut allergic individuals, total avoidance is often difficult because peanuts are increasingly being used in the diet as a valuable protein source and are present in a variety of processed foods. Over the recent years legislation has been issued aimed at a better safeguarding of the health of

allergic consumers. Within the European Union, Directives 2000/13/EC (8) and its amendment 2003/89/EC (9) regulate the labeling of foodstuffs and require a mandatory declaration of the presence of 12 major allergenic foods including peanut. For food producers and regulatory agencies specific and sensitive methods are urgently needed to detect allergens at the trace level and to control for allergen-free products.

Currently there are a variety of methods available that are used to determine the presence of allergenic ingredients in food products. Those methods often do not target a specific allergenic protein, but rather a marker indicative for the presence of the offending food (e.g., trace of peanuts) (10). In practice, proteins or DNA is targeted for this purpose. Protein-based methods utilize immunological techniques that employ antibodies raised against proteins specific for the allergenic food. Commercially available techniques of this type are lateral flow devices or dipsticks that are used for rapid screening and enzyme-linked immunosorbent assays (ELISA) that allow semiquantitative analyses. DNA-based methods are based on the amplification of DNA fragments specific for the allergenic food, by means of the Polymerase Chain Reaction (PCR). Among those, ELISA is probably the method that is used most commonly by the food industry and official food control agencies for the detection of

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peanut allergens in food products. Commercially available ELISA test kits are used to measure specific peanut allergens (Ara h 1 and Ara h 2) or total soluble peanut protein. The typical limit of detection (LOD) that can be achieved with these kits was found to be in the range of 1–5 ppm (11). A major drawback of immunological methods as described above is the fact that the epitopes that are detected are usually not known and cross-reactivity with matrix components can result in false positive results. In contrast to this, the detection of DNA markers benefits from having a well-defined target analyte, and the use of a combination of database analysis and experimental specificity testing could lower the chance of obtaining false positive results due to matrix components. However, the allergenicity of a food is caused by its proteins and not its DNA; therefore, the presence of peanut DNA in a food product does not guarantee the presence of allergens and vice versa (e.g., when purified peanut proteins are used as an ingredient).

Given the limitations of the methods described above, it is clear that a confirmatory method is needed to provide an unambiguous identification of (allergenic) proteins. A mass spectrometry-based proteomic approach, which combines the separation and identification of individual proteins, can provide a solution for this. The technique has already been employed with a focus on the identification of allergens (12), but it holds potential to study allergen detection in food products (7) and allergen stability. Despite the need for costly specialized equipment, this method can provide an unambiguous identification of allergenic proteins present in a food product (10), which is a major advantage compared to methods based on immunological techniques or DNA analysis.

We have applied MS-based proteomics for the detection of a number of peanut proteins that have a direct relevance to the allergenic potential of peanut. The three major peanut allergens that are recognized by the vast majority of peanut allergic individuals and have been studied extensively are Ara h 1 (vicilin-like protein) (13), Ara h 2 (conglutin-homologue protein) (14), and Ara h 3/Ara h 4 (glycinin proteins) (15). These proteins have a high abundance in peanut, which is likely to facilitate their detection. Peanut contains around 29% protein, and the major allergen Ara h 1 accounts for approximately 20% of the total protein content. Another ~10% is Ara h 2, whereas Ara h 3/Ara h 4 are also abundant allergens.

Ara h 1 is known to be a 63–68 kDa glycoprotein (16); its levels do not vary significantly among varieties or among peanuts grown in different geographical regions (17). The protein is well characterized in both biochemical and genetic terms (18–20). Ara h 2, another major allergen, was purified from peanut and migrates as a doublet in SDS-PAGE around 17–20 kDa (21) and consists of two isoforms (22). The allergen Ara h 3 has recently been purified from peanuts. It contains an acid and a basic subunit (15, 23) that remain covalently linked by an intermolecular disulfide bridge (15, 24). Recently it was shown that Ara h 3 and Ara h 3/4 are isoallergens (25, 26).

Prior to the development of MS-based methods for the detection of peanut allergens in food, it is necessary to establish which allergen sequence tags derived from Ara h 1, Ara h 2, and Ara h 3/Ara h 4 can be detected and what protein coverage can be achieved. In addition to this, the effect of food processing has to be taken into account. Prior to consumption, peanuts usually undergo roasting, and a variety of roasting conditions are used by the food industry. Processing steps such as heating, for example, dry roasting, are known to have a high impact on the protein stability and structure (26). The effect of processing on the MS detection of peanut allergen sequence tags needs

therefore to be investigated. Finally, there is a general agreement that the detection limits for food allergens need to be somewhere between 1 and 100 mg of allergenic protein per kilogram food product (11, 28); therefore, the LOD of the MS detection needs to be investigated, and the sequence tags that can still be detected close to the LOD need to be identified.

In this study we have used raw and mild- and strong-roasted peanut samples that were analyzed with a combination of capillary LC and Q-TOF tandem mass spectrometry for both the separation and identification of specific highly stable peptides. The identification of allergen sequence tags, the effect of roasting on their detection, and the minimal amounts of protein required for an unambiguous identification of the allergens have been determined. In addition to this, the data obtained by MS analysis were evaluated for their use to trace the processing history of peanuts based on the detection of peptides that are specific either for raw or roasted peanut.

MATERIALS AND METHODS

Materials. Raw and roasted peanut samples (*Arachis hypogaea*) of the variety Red Skin were obtained from Unilever (The Netherlands). Peanut processing was done by the supplier following standard industrial processing technology. Before roasting, the peanuts were blanched to remove the skins. Roasting was performed in a continuous roaster, with an oven temperature set at 140 °C. Roasting times varied depending on desired characteristics. Mild-roasted (12 min roasting time) and strong-roasted peanuts (20 min roasting time) were obtained and used in this study.

Chemicals. All chemicals used for sample preparation were obtained from VVW International (West Chester, PA) and were of at least analytical reagent grade. Water from a Milli-Q water system (Millipore, Bedford, MA) was used throughout. RapiGest was obtained from Waters (Manchester, U.K.) and sequencing grade trypsin from Merck (Darmstadt, Germany). SupraPure formic acid and HPLC grade acetonitrile for mass spectrometry were from Sigma-Aldrich (St. Louis, MO).

Mild Extraction and Protein Quantification. The peanut samples were ground under liquid nitrogen to obtain a fine and homogeneous powder. The complete two-step sequential extraction and sample preparation have been described previously (29). Extraction was essentially performed in two steps (at 4 °C) using (1) TBS buffer (20 mM Tris + 150 mM NaCl), pH 7.4, and (2) an ethanol/water mixture, 20:80.

Protein quantification of peanut extracts was performed using two different assays, the Smith copper/bicinchoninic method (30) and the 2D quantification kit of GE Healthcare (Uppsala, Sweden). For mild-roasted Red Skin peanuts, the extraction efficiency was reduced by 75 and 91% for extracts 1 and 2, respectively. After strong-roasting, this reduction increases further to 82 and 95% for extracts 1 and 2, respectively (29). Extracts 1 and 2 were combined for analysis using the MS-based method, taking the ratio of concentrations of extracts 1:2 as a mixing ratio.

Trypsin Digestion of Peanut Protein. Trypsin digestion was performed as follows: 500 μ L of trypsin solution of 0.1 μ g/ μ L was prepared and placed on ice before use. One milligram of RapiGest (Waters) was resuspended into 500 μ L of 50 mM ammonium bicarbonate (pH 8.6) to give a 0.2% solution (w/v). The precipitated protein pellet [ca. 100 μ g of protein determined by using a 2D quantification kit (GE Healthcare)] was dissolved in 50 μ L of the 0.2% RapidGest solution and vortexed. Five microliters of 500 mM dithiothreitol (DTT) solution was added for protein reduction (final concentration = 50 mM), and the sample was heated at 60 °C overnight. The sample was then cooled to room temperature, 10 μ L of 100 mM iodoacetamide solution was added for protein alkylation, and the sample was placed in the dark for 30 min (room temperature).

Tryptic digestion was achieved by adding 40 μ L of trypsin solution to the protein tube (1:50, protease/protein ratio), and the solution was gently flicked to mix. The sample was incubated at 37 °C for 2 h at

pH 8.6. Ten microliters of 500 mM HCl was added to the digested protein sample (final concentration = 43.5 mM) for enzyme precipitation. The sample was incubated at 37 °C for 45 min before centrifugation at 16100g for 10 min. The supernatant was transferred to another microcentrifuge tube and stored at -30 °C before capillary LC Q-TOF (MS/MS) analysis.

Capillary High-Performance Liquid Chromatography (Capillary LC). Capillary LC-MS/MS analyses were performed on a capillary flow-liquid chromatography system (CapLC, Waters). A precolumn Accurate microflow splitting system (LC Packings, Sunnyvale, CA) was used. The binary CapLC pump was operated at a flow rate of 10 μ L/min and a flow rate of 1 μ L/min was obtained after a 1:10 split. An auxiliary pump for sample injection was operated at a flow rate of 15 μ L/min.

Experimental conditions optimized for capillary LC were as follows: peptide mixtures from the trypsin digest of 100 μ g of protein (starting material) were injected by the autosampler and loaded at a flow rate of 15 μ L/min (using the auxiliary pump) on a LC Packings C₁₈ capillary cartridge (300 μ m i.d., 150 mm length, 5 μ m particle size) for desalting and preconcentration for 5 min. A postinjection switch valve was activated, and a binary gradient was generated using the binary pump. Peptides were eluted from the trap column with increasing organic phase (acetonitrile), refocused, and separated on a Grace Vydac C₁₈ High-loading capacity capillary column (150 μ m i.d., 150 mm length, 3 μ m particle size, 190 Å porosity) (flow rate = 1.0 μ L/min). The eluents used were (A) 0.2% HCOOH in Milli-Q H₂O/CH₃CN, 95:5, and (B) 0.2% HCOOH in Milli-Q H₂O/CH₃CN, 5:95. The linear gradient used to achieve the separation was as follows: 0–5 min, 95% A; 5–115 min, 5–100% B; 115–135 min, 100% B; 135–140 min, 0–95% A. The column was re-equilibrated for 10 min with 95% A prior to the next injection.

Nanoelectrospray Q-TOF Tandem Mass Spectrometry (NanoESI Q-TOF MS/MS). Nanoelectrospray experiments were conducted on a quadrupole time-of-flight mass spectrometer (Q-TOF Ultima Global, Waters, Manchester, U.K.) equipped with a nanoelectrospray Z spray source. Waters NanoEase emitters were used for the nanoflow sprayer (360 μ m o.d., tip 90 μ m o.d. $\times$ 20 μ m i.d., length = 7 cm). A zero dead volume microunion with PEEK fused silica microfittings was used to connect the outlet of the capillary column directly to the nanoflow sprayer.

The operating conditions of the Q-TOF mass spectrometer were as follows: capillary voltage, 3.2 kV; sample cone voltage, 100 V; source temperature, 80 °C. The instrument was operated in the positive ion mode. Time-of-flight (TOF) was performed in a continuous extraction mode. In the positive linear mode (V mode), an accelerating voltage of 9.10 kV was used for the TOF, and the microchannel plates (MCP) value of the detector was set to 2200 V. The TOF analyzer was calibrated in MS/MS mode on a daily basis using Glu¹-Fibrinopeptide B (Sigma-Aldrich) in the range m/z 70–1500.

Full scans (MS mode) were performed over the m/z range 200–1500 with a scan time of 15 μ s and an interscan time of 0.1 s. For peptide fragmentation the instrument was operated in “MS survey” mode (MS/MS), which means that a fragmentation of ions is achieved when a minimum intensity (specified value) is detected (up to three channels available for a simultaneous fragmentation of the three most abundant ions). The quadrupole mass filter was set with low mass (LM) and high mass (HM) resolution settings of 10 and 10. The collision energy was varied between 5 and 55 V according to the mass and charge state of the respective peptides. MS to MS/MS switch criteria were as follows: threshold, 10 counts/s; charge states, +2, +3, +4. The MS/MS to MS switch occurs when the TIC is falling below 5 counts/s or after a defined time of 10 s.

The fragment ion spectra obtained from the “MS survey” mode were processed using Mass Lynx version 4.0 (Waters), a software program that converts MS/MS raw data to peak lists for database searching. Then, the MS/MS spectra of each peptide were processed by the Mass Lynx add-on Maximum Entropy 3 (MaxEnt 3) and converted into a file format suitable for further data analysis. The algorithm of MaxEnt 3 deconvolutes charge state and isotopic information in a continuum spectrum to generate a centroid spectrum containing only monoisotopic singly charged peaks.

When specific peptides were selected for measuring their relative signal intensity, the “Multiple ion monitoring” mode was used (up to 32 channels available). “MS/MS scan” mode was run in parallel to confirm the identity of the measured ions (up to 32 channels for MS/MS fragmentation). The “MS survey scan” mode could obviously not be used for this purpose as the peaks in the ion chromatograms appear serrated because the MS system alternates between the MS and MS/MS modes as a means both to measure ion intensity and to generate a mass spectrum for protein sequencing. A list of m/z values was specified, and collision energies of 30 and 35 eV were set up for ions with charge states of +2 and +3, respectively.

Databank Search and Sequence Analysis. Peptide fragment ion lists, generated as described before, were analyzed by searching sequence databases with Protein Lynx Global Server 2.1 (Waters). For database searching, the mass spectrometry protein sequence database of Swiss-Prot/TremBL and a reduced database of known peanut allergens were used. From the Swiss-Prot/TremBL protein database two reported sequences of Ara h 1 and Ara h 2, translated from their respective nucleotide sequences, were retrieved. The amino acid sequences of four Ara h 3 entries were found in Swiss-Prot/TremBL; however, only two entries are shown for sequence alignment of the amino acid sequences to simplify the presentation of results. Accession numbers were as follows: allergen Ara h 1, clone P41B [precursor] (P43238) (31); allergen Ara h 1, clone P17B [precursor] (P43237) (18, 32); allergen Ara h 2 isoform [fragment] (Q7Y1C0) (33); allergen Ara h 3 glycinin [fragment] (O82580) (34); allergen Ara h 3/Ara h 4 (Q8LKN1) (35).

Auto de novo sequencing (Auto mode) with combined database searching was also performed using the Protein Lynx Global Server version 2.1. Spectra were searched with a peptide tolerance of 100 ppm and fragment mass tolerance of 0.1 Da. The enzyme entry was set for trypsin, and the maximum number of missed cleavages with trypsin was set to 3. A certain number of peptide modifications were considered (carbamidomethylated cysteine and oxidized methionine), and the variable modification mode was selected for searching.

The protein server used for this work has been designed to facilitate automated, unattended protein identification and characterization from MS/MS data sets. This was achieved by chaining automated data interpretation modules in a hierarchical, strategic manner within an “MS workflow” method editor. With this workflow, data were submitted to different modules, beginning with the smallest when the data set was large: (i) restricted database searching with MS/MS data, (ii) de novo sequencing, (iii) BLAST search to further characterize more proteins from the remaining data, and (iv) investigation of the presence of peptide modifications and homologous substitutions. Search results were validated when at least three consecutive measured fragment ions of a peptide matched theoretical b- or y-fragment ions of a known protein sequence tag. After databank searching and sequence analysis, each identified peptide was assigned a “peptide score” by a probability-based score algorithm, which is an indication for the reliability of the peptide identification (scale 1–100).

The method employs a discriminant function with classified “good” and “bad” spectra, producing a single quality peptide score. The acceptance of spectra starts from a peptide score of 25 at a significance level of $P < 0.05$ (interval of confidence of 95%) for the individual peptides. Only peptides fulfilling all conditions listed above are validated, whereas the low-score peptides (<25 at a significant statistical difference) are not considered for protein identification.

The peptide identification was validated by the accurate mass of peptides and peptide fragments. Three runs were made for each sample, and the parallel MS and MS/MS data acquisition was shown to be highly reproducible; only the peptides found in the three replicate runs were accepted as confident identifications.

Limits of Detection of the Method. Validated peptide ions specific for the three major allergens, Ara h 1, Ara h 2, and Ara h 3, detected in raw peanuts as well as in processed peanuts were selected. Detection limits (LOD) of the MS method were defined as a signal-to-noise ratio of 3:1 for the peptides ions measured in the MS mode (multiple ion monitoring of selected ions). LODs were evaluated by injecting three times the tryptic digests of three independent extracts of a peanut sample at different concentrations. The undiluted sample corresponded to an

amount of 1 μ g of protein loaded on the capillary LC column. Several dilutions of the same sample were made, and LODs were expressed as the amount of protein used as starting material for trypsin digestion.

RESULTS AND DISCUSSION

Detection of Ara h 1, Ara h 2, and Ara h 3. All peanut extracts were digested with trypsin, and the resulting peptides were separated by capillary LC, after which the eluting peptides were measured by nano-ESI Q-TOF operated in the “MS survey scan” mode to characterize the peptide pattern and to detect the presence of specific peptides derived from peanut allergens. The chromatographic part is required to resolve the peptides prior to electrospray ionization and MS detection because of the high amount of peptides present in the samples.

The MS/MS spectra that were obtained were subjected to a search against databases to identify peptides by means of their fragment masses. Masses were calculated to four decimals for all fragments. In addition to this, we utilized a *de novo* sequencing module that calculates an amino acid sequence from MS/MS data when no match is retrieved from the database search. This module traces the sequence that best fits the MS/MS data and assigns a probability score to the peptides (“peptide score”).

Sequence Analysis. The detection of the peanut allergens Ara h 1, Ara h 2, and Ara h 3 by means of the employed MS-based proteomic approach relies on the detection of their peptides. To assess the coverage that theoretically can be achieved, peptide sequence alignment and peptide cutter tools from SwissProt/TrEMBL were used to determine the predicted trypsin cleavage sites.

For Ara h 1 it has been reported that the N-terminal sequence of the mature protein starts with RS(H)PPGERTRG, which implies that the first 72–84 amino acids are cleaved off (length depending on isoform) (19, 36). **Figure 1** shows the two known sequences of Ara h 1 (entry numbers P43238 and P43237) with the N-terminal peptide that is cleaved-off to form the mature protein in italics. The length of the predicted trypsin cleavage products varied from 1 to 35 amino acids. Peptides containing 1–5 amino acids are likely too short to be identified as Ara h 1 specific cleavage products, whereas peptides consisting of more than 15 amino acids are most likely too long to be efficiently detected by the Q-TOF mass analyzer due to poor separation on the RP column. Thus, the trypsin cleavage products consisting of 6–15 amino acids that can potentially be detected cover 293 and 265 amino acid residues, for P43238 (542 amino acids) and P43237 (536 amino acids), respectively.

The N-terminal sequence of the mature Ara h 2 starts with RQQWELQGDRRC (37). In **Figure 2** the signal peptide that is cleaved-off to form the mature protein is indicated in italics. The two known sequences of Ara h 2 were investigated (entry numbers Q941R0 and Q8GV20), and their predicted trypsin cleavage sites are shown. The Ara h 2 trypsin cleavage products consisting of 6–15 amino acids that can potentially be detected cover 84 and 95 amino acid residues for the two isoforms Q941R0 (135 amino acids) and Q8GV20 (151 amino acids), respectively.

Ara h 3 contains an acid and a basic subunit and undergoes extensive proteolytic processing (15). The N terminus RQQPEEN corresponds to the acidic subunit, and the N terminus GIEETIC corresponds to the basic subunit (15, 23). Two known sequences of Ara h 3 were aligned (entry numbers O82580 and Q8LKN1), and their predicted trypsin cleavage sites are shown in **Figure 3**. The Ara h 3 trypsin cleavage products consisting of 6–15 amino acids that can potentially be detected cover 208 and 171

amino acid residues for O82580 (507 amino acids) and Q8LKN1 (515 amino acids), respectively.

Peptide Identification and Sequence Coverage. The analysis of peanut samples was aimed at generating a large number of allergen-derived peptide sequences. The experimentally detected peptides allow a determination of the protein coverage (percent) when MS data is compared with the theoretical assessment described above.

A list of Ara h 1 specific peptides was compiled after data collection, data treatment, and peptide identification. **Figure 1** shows the position of the identified peptides within the two amino acid sequences of Ara h 1. Respectively, 20 and 23 peptides consisting of 6–15 amino acids were identified for both Ara h 1 isoforms, P43238 and P43237 (**Table 1**). The protein coverage was determined by taking the predicted trypsin cleavage products into account, and the peptides that were identified experimentally. This led to coverages of 50 and 64% of the two sequences P43238 and P43237, respectively. Although the majority of peptides are predicted trypsin cleavage products, a number of peptides that were detected do not seem to correlate with trypsin cleavage at both termini (**Figure 1**).

Only a few peptides corresponding to the allergen Ara h 2 were identified in peanut extracts (**Table 2** and **Figure 2**). This might be explained by the fact that Ara h 2 acts as a trypsin inhibitor, the activity of which was found to increase upon roasting (38). **Table 2** lists the five Ara h 2 derived peptides with lengths between 6 and 15 amino acids that were identified (**Table 2**). The protein coverage was determined as 40% of the sequence Q8GV20 (**Table 2**).

Table 3 shows the experimentally detected Ara h 3 derived peptides and **Figure 3** their location within two Ara h 3 amino acid sequences (lacking the signal sequence that is cleaved off from the mature protein). Ara h 3 is known to be cleaved into an acidic and basic subunit, and the N terminus of the basic subunit was defined as GIEETIC... (15). Despite this, we detected next to the peptide GIEETICTASAK also the peptide NGIEETICTASAK (amino acid residues 322–334 and 323–334 of O82580), indicating that some Ara h 3 that is not cleaved at the acidic–basic subunit boundary is present in the peanut extracts. In addition to pure tryptic peptides, a few partial peptides were found that might be generated by aspecific cleavage at either the N-terminal or C-terminal side of the protein chain. Among the peptides found experimentally, tryptic peptides cover 61 and 60 amino acid residues for the acidic and basic subunits of Ara h 3, respectively. The experimental sequence coverage of O82580 that was calculated by taking the predicted trypsin cleavage products into account was 54% and is quite well symmetrically divided over the acidic (129 amino acids, 47% coverage) and basic subunits (135 amino acids, 44% coverage).

Recently, Piersma et al. have described the sequence analysis and peptide identification of Ara h 3 purified from raw peanuts (15). In their study four different amino acid sequences from the NCBI protein database were considered for sequence analysis. MS analysis was utilized to identify peptides and to determine the sequence coverage. The coverage of Ara h 3 (O82580) was determined to be 54% as in our work, but was differently divided over the acidic (35% coverage) and basic subunits (135 amino acids, 89% coverage). The fact that Ara h 3 is not cleaved at the acidic–basic subunit boundary was not found by Piersma et al., who used MS detection after SDS-PAGE (15).

The differences between the theoretically identified tryptic peptides that could potentially be detected on one side and the

P43238	1	MRGRVSPLMLLLGILVLASVSATHAKSSPYQKKTENPCAQRCLQSCQQEPDDLKQKACES	60
P43237	1	MRGRVSPLMLLLGILVLASVSATQAKS-PY-RKTENPCAQRCLQSCQQEPDDLKQKACES	60
T T T T TT TT T			
P43238	61	RCTKLEYDPRCVYDPRGHTGTTNQRSPPGERTRGRQPGDYDDDRRQPRREEGGRWGPAGP	120
P43237	59	RCTKLEYDPRCVYD----TGATNQRHPPGERTRGRQPGDYDDDRRQPRREEGGRWGPAEP	114
T T T T T TT T T T			
P43238	121	REREREEDWRQPREDWRRPSHQPRKIRPEGREGEQEWGTPGSHVREETSRRNPFYFPSR	180
P43237	115	REREREEDWRQPREDWRRPSHQPRKIRPEGREGEQEWGTPGSEVREETSRRNPFYFPSR	174
T T T T T T T T			
P43238	181	RFSTRYGNQNGRIRVLQRFQDQSRQFQNLQNHRIVQIEAKPNTLVLPKHADADNILVIQQ	240
P43237	175	RFSTRYGNQNGRIRVLQRFQDQSRQFQNLQNHRIVQIEARPNTLVLPKHADADNILVIQQ	234
TT T T T T			
P43238	241	GQATVTVANGNNRKSFNLDDEGHALRIPSGFISYILNRHDNQNLRVAKISMPVNTPGQFED	300
P43237	235	GQATVTVANGNNRKSFNLDDEGHALRIPSGFISYILNRHDNQNLRVAKISMPVNTPGQFED	294
T T TT T TT T			
P43238	301	FFPASSRDQSSYLQGFSRNTLEAAFNAEFNEIRRVLLEENAGGEQEERGQRRWSTRSSSEN	360
P43237	295	FFPASSRDQSSYLQGFSRNTLEAAFNAEFNEIRRVLLEENAGGEQEERGQRRRSTRSSDN	354
T T T T TT T			
P43238	361	NEGVIVKVSKEHVEELTKHAKSVSKKGSEEEGDITNPINLREGEPLDLSNNFGKLFVEVKPD	420
P43237	355	-EGVIVKVSKEHVQELTKHAKSVSKKGSEEE-DITNPINLRDGEPLDLSNNFGRLFEVKPD	412
TT T T T TT T TT			
P43238	421	KKNPQLQDLDMMLTCVEIKEGALMLPHFSKAMVIVVNVKGTGNLELVAVRKEQQQRGR	480
P43237	413	KKNPQLQDLDMMLTCVEIKEGALMLPHFSKAMVIVVNVKGTGNLELVAVRKEQQQRGR	472
T TT T T			
P43238	481	E---EEEEDEEEEEGSNREVRRTARLKEGDVFIMPAAHPVAINASSELHLLGFGINAEN	537
P43237	473	EQEWEEEEEEEEGSNREVRRTARLKEGDVFIMPAAHPVAINASSELHLLGFGINAEN	532
T T T T			
P43238	538	NHRIFLAGDKDNVIDQIEKQAKDLAFPGSGEQVEKLIKQKESHFVSARPQSQSQSPSSP	597
P43237	533	NHRIFLAGDKDNVIDQIEKQAKDLAFPGSGEQVEKLIKQKESHFVSARPQSQSQSPS----	588
T T T T			
P43238	598	EKESPEKEDQEEENQGGKGPLLILKAFN	626
P43237	589	---SPEKEDQEEENQGGKGPLLILKAFN	614

Figure 1. Sequence of two isoforms of the peanut allergen Ara h 1 and peptides identified by capillary LC nano-ESI Q-TOF analysis of tryptic peptides from peanut extracts (from both raw and roasted peanuts). The peptides are highlighted on a gray background, and the cleaved-off N-terminal peptide is indicated with italics.

experimental data on the other side might be explained in several ways. Predicted trypsin cleavage products that were not found, in either raw or roasted peanut, might be undetectable due to post-translational processing such as protein glycosylation. For example, the Ara h 1 peptide containing the sequence

PQSQSQSPSSPEK (residues 587–599 of P43238) is likely to have undergone proline (P) hydroxylation and O-glycosylation (39), which might explain the fact that this peptide was not detected. In the same manner, N-glycosylation (an N-glycosylation consensus site is at residues 521–523) might explain the



Figure 2. Sequence of two isoforms of the peanut allergen Ara h 2 and peptides identified by capillary LC nano-ESI Q-TOF analysis of tryptic peptides from peanut extracts (from both raw and roasted peanuts). The peptides are highlighted on a gray background, and the cleaved-off N-terminal peptide is indicated with italics.

detection of the peptide SELHLLGFGINA (residues 524–535 of P43238) (**Table 1**), where a longer peptide (residues 506–566) was expected.

Sequence heterogeneities were also observed when experimental data were compared with Ara h 1 and Ara h 3 sequences that are present in the databases. For Ara h 1 variant peptides were identified corresponding to the sequences IVQEAK (residues 214–220 of P43238, substitution V→I), IPSGFISY-ILNR (residues 266–277 of P43238, substitution S→T), and KGTGNLEVAVR (residues 460–471 of P43238, substitution T→S) (**Table 1**). The observed variants are pointing at either genetic variation or the existence of additional Ara h 1 isoforms. In the case of Ara h 3, heterogeneities were found at two sites in the sequence, in the acidic subunit peptide encompassing residues 285–299 and in the basic subunit peptide encompassing residues 436–447 (sequence Q8LKN1) (**Table 3**). For the first peptide a variant peptide was identified corresponding to the sequence GENESDEQGAIVTVR (homologous substitution T→S). For the second peptide two more variants were observed corresponding to the sequence SQSENFYVAFK (E→D and V→I). The E→D substitution corresponds to translated nucleotide sequences of other known Ara h 3/4 isoforms (accession numbers from GenBank AY722686.1, AF125192.2, and AY848698.1), which is not the case for the V→I variant.

Several of the peptides identified in this study are specific for particular isoforms of the peanut allergens. For example, EGEPDLSNNFGK (residues 402–413) and DGEPDLSNNFGR (residues 394–405), which differ for two amino acids, are specific for the two known isoforms of Ara h 1 (respectively, P43238 and P43237). For Ara h 3 the peptide EEEGAIVTVR matches with only one of the two isoforms shown in **Figure 3** (O82580 residues 270–279), whereas GENESDEQGAIVTVR matches with the other isoform (Q8LKN1 residues 285–299).

Another issue of major importance is the question whether the detected peptides are specific for peanut. The local alignment search tool of BLAST-NCBI was used to find regions of local similarity between sequences. A search was performed for all sequence tags found for Ara h 1, Ara h 2, and Ara h 3 (**Tables 1–3**). The program compares protein sequences to sequences present in the databases and calculates the statistical significance of matches. No sequences other than the ones specific for peanut (*A. hypogaea*) had significant similarity to the sequence tags of Ara h 1. Also for Ara h 2, the sequence tags (**Table 2**) were found to be peanut specific. However, in the case of Ara h 3, two sequence tags, QIVQNLR and GIETICT (**Table 3**), were found not to be specific for peanut. Their short lengths, respectively 7 and 8 amino acid residues, might be a limiting

factor for specificity. In addition to this, a significant degree of similarity with other plant species was found for two other Ara h 3 sequence tags, QQPEENACQFQR (83% identity with *Lupinus albus*) and SQSENFYVAFK or SQSENFYVAFK (83% identity with *Glycine max*).

Effect of Processing. In the likely event that processing such as roasting of peanuts is found to affect allergen detection by means of MS analysis, it is of importance to establish a link between the presence of specific peptides and the processing history of peanuts. Potential tracers of processing might be (1) the presence of peptides that are specific for either raw or roasted peanut, (2) the relative signal intensity of selected peptides that can be detected in both raw and roasted peanuts, or (3) a combination of these two. To study the effect of processing it is also essential to establish the minimal amounts of protein required to allow MS detection of the peanut allergens and to determine to what extent processing affects the detection limit of the method.

Presence of Specific Peptides and Effect of Processing. A large number of peptides detected in raw peanuts remain present in the tryptic digest of mild- and strong-roasted peanut extracts and were often assigned similar peptide scores (**Tables 1–3**). For Ara h 1, for example, the sequence tags GTGNLELVAVR (residues 461–471 of P43238), DLAFFPGSGEQVEK (residues 560–572 of P43238), and VLLEENAGGEQEER (residues 335–348 of P43238) remain present as unmodified trypsin cleavage products and have high peptide scores in all three extracts. In the case of Ara h 3, QIVQNLR (residues 258–264 of O82580), TANDLNLILR (residues 355–365 of O82580), and SQSDNFYVAFK (residues 436–447 of O82580) can be highlighted as examples of peptides that can be detected in both raw and processed peanuts in a similar fashion. Such peptides can be selected to determine their relative signal intensity in raw and mild- and strong-roasted peanuts.

Several other peptides were found in the tryptic digest of raw peanut extracts, but their signal intensity was decreased dramatically after roasting or they remained undetected in roasted peanut (**Tables 1–3**). Examples for this are the Ara h 1 derived peptides IVQEAK (residues 214–220 of P43238), NPINLR (residues 396–401 of P43238), and HDNQNLNR (residues 278–284 of P43238), which could only be detected in raw peanut. Other peptides were found to exist as unmodified and modified peptides in parallel, and their modification seems indicative of processing, for example, DQSSYLQGFSSR (residues 308–318 of P43238) with its N-terminally modified peptide that was detected in raw peanuts only.

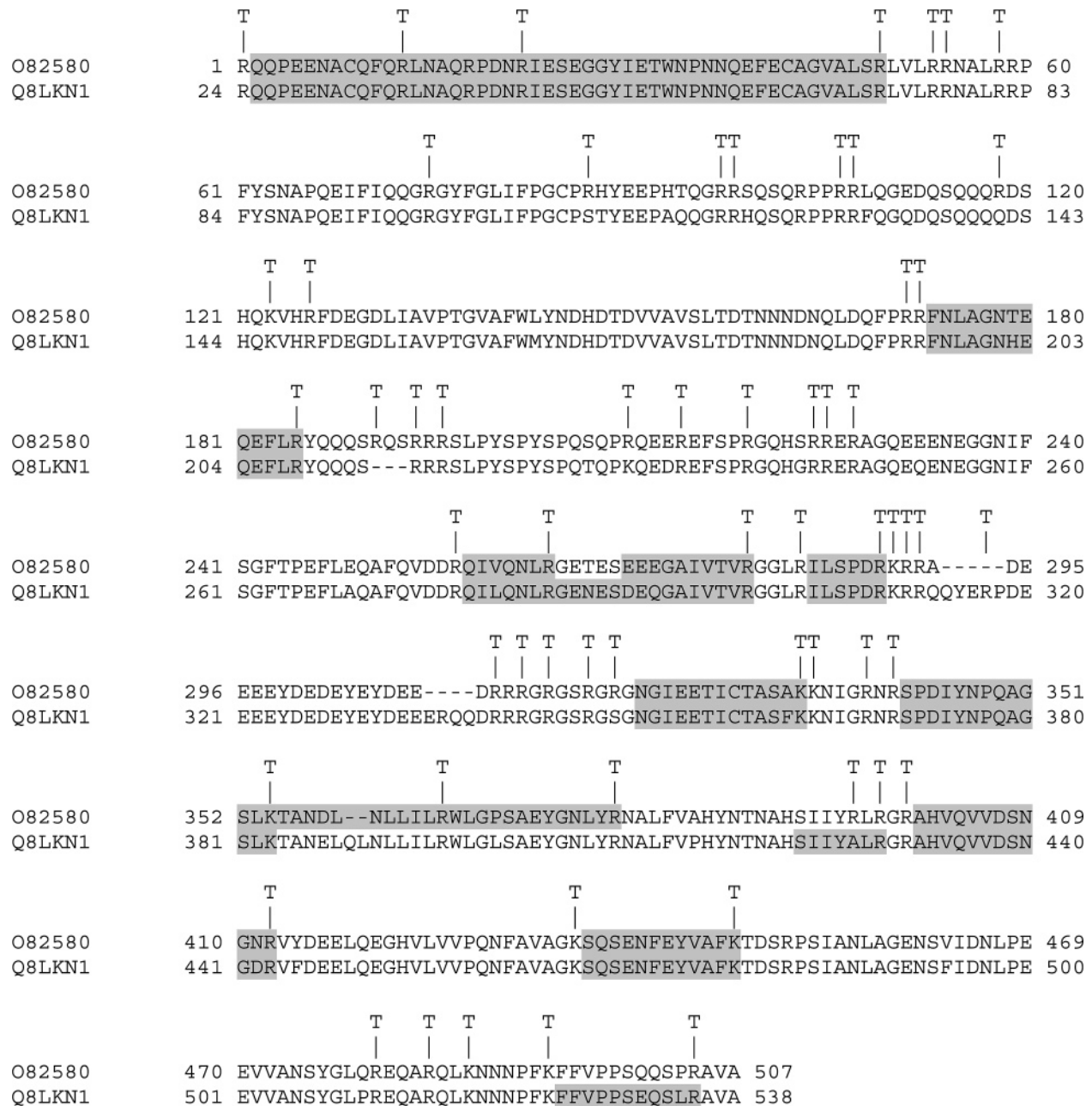


Figure 3. Sequence of two isoforms of the peanut allergen Ara h 3/Ara h 4 and peptides identified by capillary LC nano-ESI Q-TOF analysis of tryptic peptides from peanut extracts (from both raw and roasted peanuts). The peptides are highlighted on a gray background. The cleaved-off N-terminal peptide is not shown.

On the other hand, some peptides were detected only after roasting, or their signal intensity was found to increase with processing (Tables 1–3), such as the Ara h 1 derived peptides QPGDYDDRR (residues 96–105 of P43238) and SFNLDEGHALR (residues 255–265 of P43238), which were detected only in mild- and strong-roasted peanut extracts.

Processing also leads to the detection of peptides that are not known trypsin cleavage products. For example, SELHLLGFGINA (residues 524–535 of P43238), which is not a trypsin cleavage product, was found to be present only in mild-roasted peanut (low peptide score) and not in raw or strong-roasted peanuts.

In the case of Ara h 2, only three peptides could be detected both before and after the roasting process. RQQWELQGDR (residues 22–31 of Q8GV20), QQWELQGDR (residues 23–31 of Q8GV20), and NLPQQCGLR (residues 147–155 of

Q8GV20) all present good stability in terms of peptide scores. On the other hand, some peptides were detected only after roasting, such as CCNELNEFENNQR (residues 103–115 of Q8GV20), which could be detected as a modified peptide only in mild- and strong-roasted peanut extracts.

Similar examples can be given for the Ara h 3 derived peptides with, for instance, VALSRLV (residues 45–51 of O82580), which was found only in raw peanut, and SLPYSPSPSQPR (residues 197–210 of O82580), which was found only in roasted peanut.

A series of peptide sequence tags corresponding to all three allergens was found to be specific for either raw or processed peanut, and their detection might therefore be indicative for peanut processing. On the other hand, several unmodified peptides present a high peptide score independent of their

Table 1. Identified Peptides Derived from the Peanut Allergen Ara h 1 and the Effect of Roasting on Their Detectability^a

Protein Name: **Allergen Ara h 1, clone P41B** [Precursor] Source and accession number: SwissProt/TremBL, P43238
 MW (Da): 71302, pI: 7.018 * as identified by Protein Lynx Global Server v 2.1 **Peptide score: probability based score algorithm, which indicates the reliability of the peptide identification

Peptide sequence	Position in sequence	Modification/ substitution*	Submitted mass	Raw peanut extract	Mild roasted peanut extract	Strong roasted peanut extract
Peptide score ** (scale 0-100)						
QPGDYDDRR	96-105	-	412.87+++		43	43
NNPFYFPSR	172-180	-	571.27	72	74	49
		Hydroxyl DKNP(7), A for S(8)	571.29		67	63
		Carbamyl N-term, Methyl CDEHKNRQ(1)	599.79	81		
QFQNLQNH	205-213	-	395.55+++	77	53	
		Pyrolydine carboxylic acid, N-term (N-term)	584.31		93	
IVQIEAK	214-220	-	429.26	71		
SFNLDEGHALR	255-265	-	420.231		80	55
IPSGFISYLNR	266-277	-	690.41	44	93	89
		Deamidation N(11), Amidation C-Term (C-Term)	690.41	84		69
		Hydroxyl DKNP(11), A for S(7)	690.41		44	
		Carbamyl N-term (N-Term), T for S(3)	718.91	79		
SGFISYLNR	268-277	-	690.41	39	63	39
HDQNLR	278-284	-	455.77	48		
DSQNL	279-284	-	430.23	80		
DQSSYLQGF	308-318	-	644.31	75	69	45
		Carbamyl N-term, Methyl CDEHKNRQ(1)	672.82	82		
NTLEAFAFNEIR	319-333	-	869.96	85	78	68
		Deamidation N(12), K for E(4)	869.96			47
		Deamidation N(12), K for E(10)	869.96			65
		Carbamyl N-term, Methyl CDEHKNRQ(1)	898.45	91		
		Deamidation N(12), Amidation C-term	869.96	59		
		Deamidation N(8), K for E(10)	869.96		65	
		Methyl C-Term (C-Term), V for I(14)	869.96		67	65
		Deamidation N(12), K for E(13)	869.96		85	70
TLAFAFNEIR	320-333	-	898.46	29		
VLEENAGGEQEE	335-348	-	786.88	85	82	58
		Deamidation Q(11), K for E(13)	786.91			63
		Deamidation Q(11), K for E(12)	786.91		62	
		Deamidation Q(11), K for E(10)	786.91		58	
SSENNEGVIVK	357-367	-	588.30	89	53	36
NPINLR	396-401	-	427.26	88		
EGEPDLSNNFGK	402-413	-	653.83	69	70	41
		SMA N-term	653.83			31
		Hydroxyl DKNP(12), A for S(7)	653.83		69	41
		Hydroxyl DKNP(8), A for S(7)	653.83		49	
		Hydroxyl DKNP(9), A for S(7)	653.83		46	
		Deamidation N(9), Amidation C-Term (C-Term)	653.83			
KSGNLELVAVR	460-471	-	621.85	69		
GTGNLELVAVR	461-471	-	564.83	84	76	85
		Carbamyl N-term, Methyl CDEHKNRQ(4)	593.34	69		
SELHLGFGINA	524-535	-	433.58+++		26	
IFLAGDKDNVIDQ	541-553	-	525.63			31
DLAFPGSGEQVEK	560-572	-	688.85	55	57	57
		Methyl C-term (C-Term), D for E(12)	688.85	55	57	57
		Carbamyl N-term, Methyl CDEHKNRQ(1)	717.84	58		

Protein Name: **Allergen Ara h 1, clone P17** [Precursor] Source and accession number: SwissProt/TremBL, P43237
 MW (Da): 71302, pI: 7.018 * as identified by Protein Lynx Global Server v 2.1 **Peptide score: probability based score algorithm, which indicates the reliability of the peptide identification

Peptide sequence	Position in sequence	Modification/ substitution*	Submitted mass	Raw peanut extract	Mild roasted peanut extract	Strong roasted peanut extract
Peptide score ** (scale 0-100)						
QPGDYDDRR	90-99	-	412.87+++		43	43
EGEQEWGTPGSEVR	147-160	-	780.88		62	26
		Hydroxyl DKNP (9), A for S(11)	780.88		47	
NNPFYFPSR	166-174	-	571.28	72	74	49
		Hydroxyl DKNP(7), A for S(8)	571.28		67	63
		Carbamyl N-term, Methyl(1)	599.79	81		
QFQNLQNH	199-207	-	395.55+++	77	53	
		Pyrolydine carboxylic acid, N-term (N-term)	584.31		93	
IVQIEAR	208-214	-	443.26	65		
SFNLDEGHALR	249-259	-	420.23+++		80	55
IPSGFISYLNR	260-271	-	690.41	44	93	89
		Hydroxyl DKNP(11), A for S(7)	690.41		33	
		Carbamyl N-term, T for S(3)	781.91	79		
SGFISYLNR	262-271	-	690.41	39	63	39
HDQNLR	272-278	-	455.77	45		
DSQNL	273-278	-	430.23	80		
DQSSYLQGF	302-312	-	644.31	75	69	45
		Deamidation Q(7), Amidation C-Term (C-Term)	644.31			40
		Carbamyl N-term, Methyl CDEHKNRQ (1)	672.82	82		
NTLEAFAFNEIR	313-327	-	869.96	85	78	68
		Deamidation N(12), K for E(10)	869.96	65		65
		Deamidation N(12), K for E(4)	869.96			47
		Methyl C-Term (C-Term), V for I(14)	869.96	67	67	65
		Deamidation N(8), K for E(10)	869.96		65	
		Carbamyl N-term, Methyl(1)	898.45	91		
TLAFAFNEIR	314-327	-	898.46	29		
VLEENAGGEQEE	329-342	-	786.88	85	82	57
		Deamidation Q(11), K for E(13)	786.88			63
		Deamidation Q(11), K for E(12)	786.88		62	
		Deamidation Q(11), K for E(10)	786.88		58	
SSDNEGVIVK	351-360	-	524.28	80		
GSEEDITNPVN	380-391	-	793.92	26	26	
GSEEDITNPINLR	380-393	-	793.90	79	33	
TEEDITNPINLR	381-393	-	793.90	86		
DGEPDLSNNFGK	394-405	-	660.80	62	52	26
		Carbamyl N-term, T for S(1)	660.83	41	41	
		Deamidation N(8), Amidation C-Term (C-Term)	660.83			63
		Hydroxyl DKNP (9), A for S(7)	660.83			25
		Deamidation N(9), Amidation C-Term (C-Term)	660.83			
KGTGNLELVAVR	452-463	-	621.85	69		
GTGNLELVAVR	453-463	-	564.83	84	76	85
		S for T(3)	593.34	69		
		Carbamyl N-term, Methyl CDEHKNRQ (4)	593.34			
SELHLGFGINA	519-530	-	433.58+++		26	
IFLAGDKDNVIDQ	536-548	-	525.63+++			31
DLAFPGSGEQVEK	555-567	-	688.85	55	57	57
		Methyl C-term, D for E(12)	688.85	57	57	57
		Carbamyl N-term, Methyl CDEHKNRQ (1)	717.36	58		

^a Analysis was performed by means of capillary LC nano-ESI Q-TOF analysis of peanut protein extracts. Submitted masses correspond to peptides with charge +2 or +3 (when indicated with +++). Peptides highlighted with a gray background were selected for analysis of their relative intensity. Underlined residues overlap with known IgE epitopes.

Table 2. Identified Peptides Derived from the Peanut Allergen Ara h 2 and the Effect of Roasting on Their Detectability^a

Protein Name: **Allergen Ara h 2.02** Source and accession number: SwissProt/TremBL, Q8GV20
MW (Da): 19755, pI: 5.88 * as identified by Protein Lynx Global Server v 2.1 **Peptide score: probability based score algorithm, which indicates the reliability of the peptide identification

Peptide sequence	Position in sequence	Modification/ substitution*	Submitted mass	Raw peanut extract	Mild roasted peanut extract	Strong roasted peanut extract
Peptide score ** (scale 0-100)						
<u>RQQWELQGDR</u>	22-31	Pirrolidone carboxylic acid N-term (N-term)	439.23+++	82	78	86
<u>QQWELQGDR</u>	23-31		580.284	86	84	88
			571.77	77	58	71
CQSGLER	33-39	Pirrolidone carboxylic acid N-term, Carbamidomethyl C(1)	452.22	61	100	
ANLRPCEQH	40-48	Carboxymethyl C(1), Dehydration ST(3), Palmitoyl CST(6), Gamma-carboxyglutamic acid E(7)	465.59+++		81	84 49
DPYSPSPYDR	82-91	Hydroxyl DKNP (5)	606.78			86
KCCNELNEFENNQR	102-115	Carbamyl N-Term (N-Term), K for R(1)	892.37	81		
CCNELNEFENNQR	103-115	Carbamidomethyl C(1), Carbamidomethyl C(2)	863.88		78	94
CNELNEFENNQR	104-115	Methyl N-Term (N-Term), Farnesyl C(1)	864.37			61
<u>NLPQQCGLR</u>	147-155	Carbamidomethyl C(6)	543.27	84	67	53
RAPQRCDLK	155-163	Amidation C-Term (C-Term), K for E(9)	543.13		33	
CDLVESEGGR	160-169	Carbamyl N-Term (N-term)	554.25		51	
<u>CDLVESEGGR</u>		Carbamidomethyl C(1), D for E(4)	554.26		84	84
		Carbamidomethyl C(1)	561.27	65	78	57
DLEVESEGGR	161-169	SMA N-Term (N-Term), Hydroxyl DKNP(1)	552.76	72	48	44

^a Analysis was performed by means of capillary LC nano-ESI Q-TOF analysis of peanut protein extracts. Submitted masses correspond to peptides with charge +2 or +3 (when indicated with +++). Peptides highlighted with a gray background were selected for analysis of their relative intensity. Underlined residues overlap with known IgE epitopes.

Table 3. Identified Peptides Derived from the Peanut Allergen Ara h 3 and the Effect of Roasting on Their Detectability^a

Protein Name: **Allergen Ara h 3 Glycinin** [Fragment] Source and accession number: SwissProt/TremBL, O82580
MW (Da): 58314, pI: 5.88 * as identified by Protein Lynx Global Server v 2.1 **Peptide score: probability based score algorithm, which indicates the reliability of the peptide identification

Peptide sequence	Position in sequence	Modification/ substitution*	Submitted mass	Raw peanut extract	Mild roasted peanut extract	Strong roasted peanut extract
Peptide score ** (scale 0-100)						
<u>QQPEENACQFQR</u>	2-13	Carbamidomethyl C(8), Amidation C-Term	767.86	28	41	38
		Pyrrolidone Carboxylic acid N-Term, Carbamidomethyl C(8)	759.36		82	59
LNAQRPDNR	14-22	-	361.86+++	77	60	
IESEGGYIETWNPNN	23-49	Carbamidomethyl C(20)	1024.17+++	36		
QEFECAGVALSR						
PNNQEFCAG	35-44	D8 C(8), Methyl C-Term (C-Term)	786.93			29
VALSRLV	45-51	V for L(6), I for V(7)	379.22	52		
NLAGNTEQELFR	174-185	Myristoyl N-Term, Gamma-Carboxyglutamic acid (7)	549.29+++	56		
SLPSPSPSQSQPR	197-210	Hydroxyl DKNP(6), Hydroxyl DKNP(13)	819.93		51	37
<u>QIVQNLR</u>	258-264		435.76	87	87	87
		Formyl N-term (N-term)	449.75	65		
		Pirrolidone carboxylic acid N-term (N-term)	427.26	74		
		Carbamyl N-Term, Methyl CDEHKNRQ(1)	464.28	74		
ETEESEEGAIIVTK	266-279	SMA N-Term (N-Term), K for R(14)	824.42	68		
EEGAIVTVR	270-279	-	551.79	78		
ILSPDR	284-289	-	350.70	76	76	76
NGIETICTASAK	322-334	Propionamide C(8)	704.35	31		
<u>GIEETICT</u>	323-330	d0 C(7)	654.35	51	38	51
		d0 C(7), Methyl C-Term (C-Term)	661.36	59	65	54
GIEETICTAS	323-332	Farnesyl C(7), Phosphoryl STY(10)	654.35			41
GIEETICTASAK	323-334	Propionamide C(7)	647.32		38	33
		Propionamide C(7), Methyl CDEHKNRQ(12)	654.37			44
		Carbamidomethyl C(7), Methyl CDEHKNRQ(12)	647.32	56		
		Carbamidomethyl C(7), R for K(12)	654.33	70	75	
		S-pyridylethyl C(7), R for K(12)	678.34	43		
<u>SPDIYNPQAGSLK</u>	342-354		695.35	91	87	84
		Hydroxyl DKNP(13), A for S(11)	695.40			51
<u>TANDLNLILR</u>	355-365	Carbamyl N-Term(N-Term), T for S(1)	723.87	88		
			628.38	85	91	96
		Methyl CDEHKNRQ (4)	635.39	96	80	96
		Methyl C-Term (C-Term), V for L(10)	628.39			89
		Methyl C-Term (C-Term), V for I(9)	628.39	58	64	51
		Carbamyl N-Term, Methyl CDEHKNRQ(3)	656.89	76		
		Acetyl N-Term (N-Term), S for A(2)	657.39	35		
		Acetyl N-Term (N-Term), Hydroxyl DKNP (3)	657.39	49		
		AGE-lysine 1 (N-Term)	657.39	35		
DLNLLILRYL	358-367	Myristoyl N-Term (N-Term), Y for W(9)	485.97+++	35		
SAEYGNLYR	370-378	Deamidation N(6), Amidation C-Term	536.80			40
AHVQVDSNGNR	401-412	-	432.57+++		61	61
		Carbamyl N-Term, Methyl CDEHKNRQ(2)	451.56+++	64		
		Hydroxyl DKNP(9), A for S(8)	648.36		31	46
YDEELQEGHVLVVPQ	413-435	Carbamyl N-Term (N-Term)	866.81+++	52		
NFAVAGK						
<u>SOSENFYVAFK</u>	436-447		724.84	95	82	80
		Deamidation N(5), K for E(4)	724.87		80	
		Carbamyl N-Term, Methyl CDEHKNRQ(2)	753.36	85		
		I for V(9)	731.86	69		
		D for E(4)	717.85	64	71	69

^a Analysis was performed by means of capillary LC nano-ESI Q-TOF analysis of peanut protein extracts. Submitted masses correspond to peptides with charge +2 or +3 (when indicated with +++). Peptides highlighted with a gray background were selected for analysis of their relative intensity. Underlined residues overlap with known IgE epitopes.

technological processing history and can be seen as potential markers for the detection of peanut traces.

Relative Signal Intensity of Selected Peptides. Markers for the detection of peanut traces by means of MS analysis can

ideally be detected in raw peanuts as well as in peanuts that have undergone processing steps such as roasting. To investigate whether information on the processing history can be extrapolated from the MS data for a selection of such markers, we

Table 4. Relative MS Signal Intensity for 17 Selected Peptide Ions Measured in Raw and Mild- and Strong-Roasted Peanut Extracts^a

Sequence accession number (SwissProt/TremBL): ¹ P43238; ² P43237; ³ Q8GV20; ⁴ O82580
 * as identified by Protein Lynx Global Server v 2.1

Peptide sequence	Position in sequence	Modification/ substitution*	Raw peanut extract	Mild roasted peanut extract	Strong roasted peanut extract
			Relative intensity with SD (%)		
NNPFYFPSR	172-180 ¹	-	27.4 (12.0)	18.5 (3.0)	12.3 (7.5)
IPSGFISYILNR	266-277 ¹	-	4.4 (1.2)	7.1 (2.8)	7.4 (2.5)
NTLEAAFNAEFNEIR*	319-333 ¹	-	8.6 (4.7)	2.2 (0.5)	10.6 (5.4)
VLEENAGGEQEER*	335-348 ¹	-	13.8 (6.0)	15.2 (5.0)	16.0 (1.7)
SSENNEGVIVK	357-367 ¹	-	4.9 (1.5)	4.4 (0.7)	8.5 (1.8)
EGEPDLSNNFGK*	402-413 ¹	-	3.9 (0.5)	4.3 (1.0)	16.6 (2.0)
GTGNLELVAVR	461-471 ¹	-	24.9 (10.2)	27.3 (6.6)	13.9 (1.9)
DLAFPGSGGEQVEK*	560-572 ¹	-	12.0 (4.9)	21.0 (3.9)	14.6 (2.1)
DGEPDLSNNFGR	394-405 ²	-	5.0 (1.2)	2.4 (0.3)	1.5 (0.3)
RQQWELQGDR*	22-31 ³	-	8.1 (2.8)	19.5 (5.3)	8.5 (2.5)
QQWELQGDR*	23-31 ³	-	10.0 (4.2)	2.6 (1.2)	1.8 (0.6)
QQWELQGDR*	23-31 ³	Pirrolidone carboxylic acid N-term (N-term)	30.9 (5.0)	3.2 (2.8)	2.1 (1.3)
NLPQQCGLR*	147-155 ³	Carbamidomethyl C(6)	45.4 (7.1)	65.2 (7.4)	81.0 (5.1)
CDLDVESGGR*	147-155 ³	Carbamidomethyl C(1)	5.6 (4.1)	9.6 (3.5)	6.6 (1.8)
QQPEENACQFQR	2-13 ⁴	Carbamidomethyl C(8), Amidation C-Term	8.0 (0.8)	5.9 (0.3)	8.6 (0.7)
QIVQNLR	258-264 ⁴	-	15.0 (7.6)	26.8 (23.6)	5.1 (3.3)
GIEETICT	323-330 ⁴	dO C(7)	13.3 (7.3)	13.2 (6.5)	13.8 (5.2)
SPDIYNPQAGSLK	342-354 ⁴	-	11.5 (3.4)	27.5 (12.6)	28.9 (5.5)
TANDLNLILR	355-365 ⁴	-	33.5 (13.3)	10.8 (5.9)	21.9 (10.3)
SQSDNFEYVAFK	436-447 ⁴	-	11.0 (6.7)	7.5 (0.3)	11.5 (3.8)
SQSDNFEYVAFK	436-447 ⁴	D for E(4)	7.7 (1.7)	8.2 (3.0)	10.3 (4.8)

^a Nine selected peptides specific to Ara h 1 sequences (P42238 and P42237 sequences) (highlighted in gray in **Table 1**); five selected peptides specific to Ara h 2 sequence Q8GV20 (highlighted in gray in **Table 2**); seven selected peptides specific to Ara h 3 sequence O82580 (highlighted in gray in **Table 3**). Underlined residues overlap with known IgE epitopes.

analyzed the relative intensity of selected markers that can be detected in raw and mild- and strong-roasted peanuts. For this purpose a series of tryptic peptides was chosen. For Ara h 1 nine peptide ions were selected (highlighted in gray in **Table 1**), for Ara h 2 three ions (**Table 2**) and for Ara h 3 seven ions (**Table 3**). The 19 ions were measured in the MS mode (multiple ion monitoring with 19 selected channels), and the MS/MS fragmentation of the ions was performed in parallel to confirm the identity of the measured ions. In three replicate experiments the relative MS signal intensity (normalized to 100% for each of the three samples analyzed, being raw, mild-roasted, and strong-roasted peanuts) was recorded for the peptide ions. Experiment-to-experiment variation was within 15% over the set of experiments.

Table 4 illustrates the changes in signal intensity of the 19 selected peptides (submitted masses highlighted in gray in **Tables 1–3**) as caused by roasting. For some peptides the relative intensities remain quite constant, whereas others show clear alterations in their relative intensities. The data for the Ara h 1 specific peptides shown in **Table 4** indicate that the intensity of the peptide VLEENAGGEQEER remains stable, whereas that of the peptide EGEPDLSNNFGK (specific to P43238) increases with the degree of processing. The inverse was apparent for the peptides DGEPDLSNNFGR (specific to P43237) and NNPFYFPSR, the relative intensities of which drop after roasting. The ratio of such peptides can be indicative for the degree of processing. An example of this is the ratio of the signal intensity of NNPFYFPSR and VLEENAGGEQEER, which has a value of 2.0 for raw peanut, 1.2 after mild roasting, and 0.8 after strong roasting. For Ara h 2, the intensity of the peptide NLPQQCGLR increased, whereas that of QQWELQGDR decreased as a result of processing. Therefore, the ratio of the intensity of the two peptides can also be used as an indication of food processing. For Ara h 3 the relative intensities of the peptide SQSDNFEYVAFK and GIEETICT remain quite stable, but none of the selected peptides shows an increase or a decrease in its relative intensity that is clearly correlated with processing. Notwithstanding this, the relative intensity of the peptide

SPDIYNPQAGSLK increases more than 2-fold in roasted peanuts (mild- or strong-roasted); therefore, the ratio of the intensity of, for example, SPDIYNPQAGSLK and the sum of the intensities of SQSDNFEYVAFK and GIEETICT can be used to differentiate raw from roasted peanuts.

A series of peptide sequence tags corresponding to all three allergens under study can be detected with good signal intensity in raw and processed peanuts. Additionally, the relative intensity or the ratio of the intensities of specific peptides can be indicative of the processing history of this peanut material.

Detection Limits of the Method. Detection limits (LOD) of the MS method were established for the peptide ions measured in the MS mode (multiple ion monitoring of the 19 selected ions in **Figures 1–3**). For raw peanut, the LOD was set at 7 ng of protein used for tryptic digestion and loaded on the capillary LC column, because analysis of this quantity still allows identification of peptide sequence tags corresponding to all three allergens. Detection limits were found to be similar in the case of mild-roasted peanut extract (10 ng of protein), whereas in the case of strong-roasted peanut, the limits of detection were a factor of 4 higher than that of mild-roasted peanut (40 ng of protein).

Among the selected peptides (highlighted in gray in **Tables 1–3**), a differentiation was made between peptides that were identified at the detection limits and the ones that could no longer be detected. Of the nine sequence tags specific to Ara h 1, seven could still be detected. The peptides IPSGFISYILNR and SSENNEGVIVK were totally absent in raw and roasted peanuts. In the case of Ara h 2 only one of the five selected peptides (RQQWELQGDR) was still detected in raw and roasted peanuts. This shows that the possibility of identifying the presence of this allergen is considerably reduced in the diluted samples. In the case of Ara h 3, six ions were measured, two of which (QIVQNLR and QQPEENACQFQR) were found in raw but not in mild- and strong-roasted peanuts. GIEETICT was not measured, either in raw or in roasted peanut. The fact that QIVQNLR and GIEETICT remain undetected is not concerning

because neither is a reliable marker for the presence of peanut allergens as they are not peanut specific.

The sequence coverage was recalculated by taking these new settings into account. The peptides that were found experimentally at the above-mentioned detection limits of the MS method covered 48 and 62% for the two Ara h 1 sequences P43238 and P43237, respectively. For Ara h 2, the sequence coverage was considerably decreased to 28% for the sequence Q8GV20. For Ara h 3, the experimental sequence coverage of O82580 calculated was 51% and was decreased for the acidic subunit (129 amino acids, 38% coverage), whereas it remained unchanged for the basic subunit (135 amino acids, 44% coverage).

Lower detection limits could be reached for raw peanut using the MS-based method; however, this resulted in the measurement of only a few peptides. The lower detection limit was set at 1 ng of protein. Analysis of this quantity allowed the identification of only three peptides, two of which being specific for Ara h 1 (VLEENAGGEQEER and GTGNLELVAVR) and one for Ara h 3 (SPDIYNPQAGSLK) (Tables 1 and 3). For the detection of peanut traces the possibility to measure only a few peptides might be sufficient under the condition that the selected peptides are reliable peanut-specific peptide markers.

Peanut-Specific Peptide Markers. For the future development of a Q-TOF MS-based confirmatory method for the detection of peanut allergens within peanut-containing food products or in nonintentionally contaminated processed food, the choice of good peptide markers is essential.

The candidate peptides should fulfill several requirements such as the abundance of their ions in raw as well as processed peanuts, the relative intensity of their MS signal according to the established LOD, their ability to be fully characterized by MS/MS fragmentation (good peptide score), their specificity to peanut or to a specific allergen, and preferably they should cover known IgE binding epitopes to establish a correlation between allergenic potential and the MS-based confirmatory method.

The potential of employing a combination of liquid chromatography with mass spectrometry (LC-MS/MS) for the detection/confirmation of Ara h 1 in a food matrix has recently been shown by Shefchek and Musser (7, 40). The food matrices investigated in their two studies were ice cream and dark chocolate spiked either with the purified protein Ara h 1 (7) or with pure peanut protein (40). A number of Ara h 1 specific peptides were identified by Shefchek and Musser, who selected four peptides as specific biomarkers for peanut on the basis of their intensity and the fact that they were found to be unique for Ara h 1. Those were NNPFYFPSR, SFNLDEGHALR, NTLEAAFNAEFNEIR, and IFLAGDKDNVIDQIEK, corresponding to isoform P43238 (7). The first three peptides were found in our study, but the latter one was detected only as a partial tryptic cleavage product in our work. In a later work of Shefchek and Musser the doubly charged ions of the tryptic peptides VLEENAGGEQEER and DLAFPGSGEQVEK were preferred (40). The thermostability of the above-mentioned markers has not been reported, and the lack of a guarantee that they can be detected in both raw and highly processed peanuts undermines their suitability as markers for the detection of peanut traces in food products. Our work addresses this issue and allows an evaluation of those markers in terms of processing effects. The two peptides NNPFYFPSR (residues 172–180) and NTLEAAFNAEFNEIR (residues 319–333) may be suitable markers because they were stable in terms of peptide score and they were detected in raw as well as roasted peanuts. On the other hand, both SFNLDEGHALR (residues 255–265) IFLAGDKDNVIDQIEK (residues 541–556) show different

results; the first peptide was found to be present only in roasted peanut, whereas the latter was detected in strong-roasted peanut only. In our work, the intensity of the doubly charged ions of the two last proposed peptide markers (40) VLEENAGGEQEER (residues 335–348) and DLAFPGSGEQVEK (residues 560–572) was shown to remain quite stable as a function of processing (Table 4).

The identification of IgE binding epitopes of the three peanut allergens Ara h 1, Ara h 2, and Ara h 3 as reported by Shin et al. (41) and Bannon and Ogawa (42) allows an analysis of the peanut allergen derived peptide sequence tags described in this study in terms of their capability to trigger allergic reactions in sensitized individuals. Several of the tryptic cleavage products that were detected overlap with different immunologically relevant epitopes of Ara h 1, Ara h 2, and Ara h 3 (shown as underlined residues in Tables 1–3).

Some of those can serve as good markers and display good thermostability. The proposed Ara h 1 peptide marker VLEENAGGEQEER overlaps with IgE epitope EQEERGQRRW (residues 344–353 of P43238), whereas DLAFPGSGEQVEK has an overlap with IgE epitope KDLAEPGSGE (residues 559–569 of P43238) (42). These two peptides are both specific to the peanut protein Ara h 1 and are shared by the two Ara h 1 isoforms. On the other hand, the doubly charged ions at m/z 653.83 and 660.80 of the two tryptic peptides EGEDPLSNNFGK and DGEPLSNNFGR present a good peptide score, and their relative intensities can serve as indicators of the processing history. Additionally, the peptide EGEDPLSNNFGK overlaps partially with an epitope of Ara h 1 NNFGKLFEVK (residues 409–418 of P43238) (42).

To our knowledge, no data have been published on the selection of peptide markers for the detection of the peanut allergens Ara h 2 and Ara h 3. In the case of Ara h 2, all peptides mentioned in Table 4 overlap with known IgE epitopes. For example, the triply charged ion m/z 439.23 of the unmodified peptide RQQWELQGDR partially overlaps with three different IgE epitopes, HASARQQWEL (residues 18–27 of Q8GV20), QWELQGDRC (residues 24–33 of Q8GV20), and DRRC-QSGLER (residues 30–39 of Q8GV20) (42) (underlined residues in Table 2). In addition to this, this characteristic ion was the only one measured at the LOD of the method.

For Ara h 3 the doubly charged ions at m/z 695.35 and 724.84 corresponding to the peptides SPDIYNPQAGSLK and SQSENFYVAK were found to have a high peptide score and were shown to remain relatively stable as a function of processing. In addition to this those two peptides were measured at the LOD of the MS method. Consequently, they may be good allergen markers and simultaneously provide an indication of the processing state of the peanut (underlined residues in Table 3). The peptide EEGAIVTVR overlaps partially with the epitope VTVRGGLRILSPDRK (residues 276–290 of O82580); this epitope is recognized by the sera of all Ara h 3 allergic patients analyzed in a study by Rabjohn et al. (34). However, our work shows that the doubly charged ion m/z 350.70 corresponding to the tryptic peptide EEGAIVTVR was detected only in raw peanut but not after roasting. Therefore, this peptide is obviously not suitable as a marker for peanut allergen detection, but it can be used as an indicator of the processing of this peanut sample.

On the basis of the above-listed requirements for the selection of peptide ions and the data presented in this study, we propose five (unmodified) peptides that can serve as specific markers for peanut allergen detection. For Ara h 1, two peptide markers are proposed, namely, VLEENAGGEQEER (residues 335–348

of P43238) and DLAFPGSGEQVEK (residues 560–572 of P43238), whereas for Ara h 2 only one peptide, RQQWELQGDR (residues 28–27 of Q8GV20) was found to satisfy the required conditions. Finally, for Ara h 3, the two specific peptides SPDIYNPQAGSLK (residues 342–354 of O82580) and SQSENFYVAFK (residues 436–447 of O82580) are proposed.

Conclusions. Nanoelectrospray Q-TOF MS/MS in conjunction with capillary LC allows the simultaneous detection of a multitude of peptide tags derived from the three major peanut allergens, Ara h 1, Ara h 2, and Ara h 3, in both raw and processed peanuts. This simultaneous detection of multiple peanut-specific allergens increases the confidence in the correct identification of peanut traces in analytical samples. This is further increased by the detection of a large number of peptides per protein. The determination of the protein coverage, in which the predicted trypsin cleavage products has to be taken into account, has shown that clearly >50% of coverage could be achieved for Ara h 1 and Ara h 3. The slightly lower coverage of 40% for Ara h 2 is likely due to its trypsin inhibitory properties. The high sensitivity and mass accuracy of Q-TOF MS/MS also allow for the identification of isoform-specific peptides as well as peptide modifications.

Peanut roasting strongly influences the detectability of a large number of ions derived from Ara h 1, Ara h 2, and Ara h 3. This processing effect was studied in detail, and a list of peptides that remain stable after peanut processing was established. Some peptides were proposed as tracers for the roasting process. Nineteen ions corresponding to sequence tags of Ara h 1, Ara h 2, and Ara h 3 that remain detected after processing were measured in the MS/MS mode. The relative signal intensity of the ions was established and was shown to be indicative for the degree of processing. The ratio of the intensity of the selected peptides is proposed to serve as tracers for food processing. Roasting also affects the LOD of the MS-based method, with roughly a 5-fold increase observed for strong-roasted peanut compared to its raw counterpart.

The results described here allowed for the selection of five peptide tag markers specific for the three major peanut allergens, taking their thermal stability into account. The proper selection of peptide markers is crucial and constitutes the basis for the development of a Q-TOF MS/MS confirmatory method for the detection of food allergens within food matrices. The availability of such confirmatory methods is also required to analyze commercially available food products that might contain peanuts as a nonintentional contamination and will assist in the protection of the health of allergic consumers.

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LITERATURE CITED

- (1) Ortolani, C.; Ipsano, M.; Scibilia, J.; Pastorello, E. A. Introducing chemists to food allergy. *Allergy* **2001**, *56*, 5–8.
- (2) Woods, R. K.; Stoney, R. M.; Raven, J.; Walters, E. H.; Abramson, M.; Thien, F. C. Reported adverse food reactions overestimate true food allergy in the community. *Eur. J. Clin. Nutr.* **2002**, *56*, 31–36.
- (3) Mills, E. N. C.; Jenkins, J. A.; Alcocer, M. J. C.; Shewry, P. R. Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. *CRC Rev. Food Sci. Nutr.* **2004**, *44*, 379–407.
- (4) Sampson, H. A.; Mendelson, L.; Rosen, J. P. Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *N. Engl. J. Med.* **1992**, *327*, 380–384.
- (5) Sicherer, S. H.; Muoz-Furlong, A.; Burks, A. W.; Sampson, H. A. Prevalence of peanut and nut allergy in the United States of America determined by a random digit dial telephone survey. *J. Allergy Clin. Immunol.* **1999**, *103*, 559–562.
- (6) Emmett, S. E.; Angus, F. J.; Fry, J. S.; Lee, P. N. Perceived prevalence of peanut allergy in Great Britain and its association with other atopic conditions and with peanut allergy in other household members. *Allergy* **1999**, *54*, 380–385.
- (7) Shefcheck, K.; Musser, S. Confirmation of the allergenic peanut protein, Ara h1, in a model food matrix using liquid chromatography/tandem mass spectrometry (LC-MS/MS). *J. Agric. Food Chem.* **2004**, *52*, 2785–2790.
- (8) European Parliament and Council. Directive 2000/13/EC of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs. *Off. J. Eur. Communities* **2000**, *L109*, 29–42.
- (9) European Parliament and Council. Directive 2003/89/EC of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. *Off. J. Eur. Union* **2003**, *L308*, 15–18.
- (10) van Hengel, A. J.; Anklam, E.; Taylor, S. L.; Hefle, S. L. Analysis of food allergens and practical application. In *Food Toxicant Analysis*; Picó, Y., Ed.; Elsevier: Amsterdam, The Netherlands, 2006; pp 189–229.
- (11) Poms, R. E.; Klein, C. L.; Anklam, E. Methods for allergen analysis in food: a review. *Food Addit. Contam.* **2004**, *21*, 1–31.
- (12) Natale, M.; Bisson, C.; Monti, G.; Peltran, A.; Garoffo, L. P.; Valentini, S.; Fabris, C.; Bertino, E.; Coscia, A.; Conti, A. Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry. *Mol. Nutr. Food Res.* **2004**, *48*, 363–369.
- (13) Maleki, S. J.; Kopper, R. A.; Shin, D. S.; Park, C. W.; Compadre, C. M.; Sampson, H.; Burks, A. W.; Bannon, G. A. Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *J. Immunol.* **2000**, *164*, 5844–5849.
- (14) Dunwell, J. M. Cupins: a new superfamily of functionally-diverse proteins that include germins and plant seed storage proteins. *Biotechnol. Genet. Eng. Rev.* **1998**, *15*, 1–32.
- (15) Piersma, S. R.; Gaspari, M.; Helge, S. L.; Koppelman, S. J. Proteolytic processing of the peanut allergen Ara h 3. *Mol. Nutr. Food Res.* **2005**, *49*, 744–755.
- (16) Burks, A. W.; Williams, L. W.; Helm, R. M.; Caunaughton, C.; Cockrell, G.; O'Brien, T. J. Identification of a major peanut allergen, Ara h 1, in patients with atopic dermatitis and positive peanut challenges. *J. Allergy Clin. Immunol.* **1991**, *88*, 172–179.
- (17) Koppelman, S. J.; Vlooswijk, R. A. A.; Knippels, L. M. J.; Hensing, M.; Knol, E. F.; van Reijssen, F. C.; Bruijnzeel-Koomen, C. A. F. M. 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* **2001**, *56*, 132–137.
- (18) Burks, A. W.; Cockrell, G.; Stanley, J. S.; Helm, R. M.; Bannon, G. A. Recombinant peanut allergen Ara h I expression and IgE binding in patients with peanut hypersensitivity. *J. Clin. Invest.* **1995**, *96*, 1715–1721.
- (19) Wichers, H. J.; De Beijer, T.; Savelkoul, H. F. J.; van Amerongen, A. The major peanut allergen Ara h 1 and its cleaved-off N-terminal peptide; possible implications for peanut allergen detection. *J. Agric. Food Chem.* **2004**, *52*, 4903–4907.
- (20) Burks, A. W.; Sampson, H. A.; Bannon, G. A. Peanut allergens. *Allergy* **1998**, *53*, 725–730.
- (21) Burks, A. W.; Williams, L. W.; Caunaughton, C.; Cockrell, G.; O'Brien, T. J.; Helm, R. M. Identification and characterisation of a second major allergen, Ara h 2, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J. Allergy Clin. Immunol.* **1992**, *90*, 962–969.
- (22) Hales, B. J.; Bosco, A.; Mills, K. L.; Hazell, L. A.; Loh, R.; Holt, P. G.; Thomas, W. R. Isoforms of the major peanut allergen Ara h 2: IgE binding in children with peanut allergy. *Int. Arch. Allergy Appl. Immunol.* **2004**, *135*, 101–107.

- (23) Koppelman, S. J.; Knol, E. F.; Vlooswijk, R. A. A.; Wensing, M.; Knulst, A. C.; Helfe, S. L.; Gruppen, H.; Piersma, S. Peanut allergens Ara h 3: isolation from peanut and biochemical characterisation. *Allergy* **2003**, *58*, 1144–1151.
- (24) Jung, R.; Scott, M. P.; Nam, Y. W.; Beaman, T. W. The role of proteolysis in the processing and assembly of 11s seed globulins. *Plant Cell* **1998**, *10*, 343–357.
- (25) Kleber-Janke, T.; Cramer, R.; Appenzeller, U.; Schlaak, M.; Becker, W. M. Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *Int. Arch. Allergy Immunol.* **1999**, *119*, 265–274.
- (26) Boldt, A.; Fortunato, D.; Conti, A.; Petersen, A. Analysis of the composition of an immunoglobulin E reactive high molecular weight protein complex of peanut extract containing Ara h 1 and Ara h 3/4. *Proteomics* **2005**, *5*, 675–686.
- (27) Mondoulet, L.; Paty, E.; Dumare, M. F. Influence of thermal processing on the allergenicity of peanut proteins. *J. Agric. Food Chem.* **2005**, *53*, 4547–4553.
- (28) Koppelman, S. J.; Bleeker-Marcelis, H.; Duijn, G.; Hessing, M. Detecting peanut allergens. The development of an immunochemical assay for peanut proteins. *World Inged.* **1996**, *12*, 35–38.
- (29) Chassaing, H.; Brohée, M.; Nørgaard, J. V.; van Hengel, A. J. Investigation on sequential extraction of peanut allergens for subsequent analysis by ELISA and 2D gel electrophoresis. *Food Chem.* **2007**, in press.
- (30) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76–85.
- (31) Viquez, O. M.; Konan, K. N.; Dodo, H. W. Structure and organization of the genomic clone of a major peanut allergen gene, Ara h 1. *Mol. Immunol.* **2003**, *40*, 565–571.
- (32) Kolarich, D.; Altmann, F. N-glycan analysis by matrix-assisted laser desorption/ionization mass spectrometry of electrophoretically separated nonmammalian proteins: application to peanut allergen Ara h 1 and olive pollen allergen Ole e1. *Anal. Biochem.* **2000**, *285*, 64–75.
- (33) Viquez, O. M.; Summer, C. G.; Dodo, H. W. Isolation and molecular characterization of the first genomic clone of a major peanut allergen, Ara h 2. *J. Allergy Clin. Immunol.* **2001**, *107*, 713–717.
- (34) Rabjohn, P.; Helm, E. M.; Stanley, J. S.; West, C. M.; Sampson, H. A.; Burks, A. W.; Bannon, G. A. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J. Clin. Invest.* **1999**, *103*, 535–542.
- (35) Viquez, O. M.; Konan, N.; Dodo, H. Genomic characterization of the third major peanut allergen gene Ara h3/4. *Proc. Am. Peanut Res. Educ. Soci. (APRES)* **2002**, *S94*, 19.
- (36) Burks, A. W.; Shin, D.; Cockrell, G.; Stanley, J. S.; Helm, R. M.; Bannon, G. A. Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. *Eur. J. Biochem.* **1997**, *245*, 334–339.
- (37) Gruber, P.; Becker, W. M.; Hofmann, T. Influence of the Maillard reaction on the allergenicity of Ara h 2, a recombinant major allergen from peanut (*Arachis hypogaea*), its major epitopes, and peanut agglutinin. *J. Agric. Food Chem.* **2005**, *53*, 2289–2296.
- (38) Maleki, S. J.; Viquez, O.; Jacks, T.; Dodo, H.; Champagne, E. T.; Chung, S. Y.; Landry, S. J. The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. *J. Allergy Clin. Immunol.* **2003**, *112*, 190–195.
- (39) Tan, L.; Leykam, J. F.; Kieliszewski, M. J. Glycosylation motifs that direct arabinogalactan addition to arabinogalactan-proteins. *Plant Physiol.* **2003**, *132*, 1362–1369.
- (40) Shefcheck, K.; Callahan, J. H.; Musser, S. Confirmation of peanut protein using peptide markers in dark chocolate using liquid chromatography–tandem mass spectrometry (LC-MS/MS). *J. Agric. Food Chem.* **2006**, *54*, 7953–7959.
- (41) Shin, D. S.; Compadre, C. M.; Maleki, S. J.; Kopper, R. A.; Sampson, H.; Huang, S. K.; Burks, A. W.; Bannon, G. A. Biochemical and structural analysis of the IgE binding sites on Ara h 1, an abundant and highly allergenic peanut protein. *J. Biol. Chem.* **1998**, *273*, 13753–13759.
- (42) Bannon, G. A.; Ogawa, T. Evaluation of available IgE-binding epitope data and its utility in bioinformatics. *Mol. Nutr. Food Res.* **2006**, *50*, 638–644.

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