JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Global Proteomic Screening of Protein Allergens and Advanced Glycation Endproducts in Thermally Processed Peanuts

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Supporting Information

ABSTRACT: Peanuts (*Arachis hypogaea*) are the cause of one of the most prevalent food allergies worldwide. Thermal processing (e.g., roasting) of peanuts and peanut-containing foods results in complex chemical reactions that alter structural conformations of peanut proteins, preventing accurate detection of allergens by most immunochemical and targeted screening methodologies. To improve food allergen detection and support more accurate food labeling, traditional methods for peanut protein extraction were modified to include protein denaturants and solubilization agents. Qualitative characterization by SDS-PAGE and Western blot analyses of raw and variably roasted peanut extracts confirmed improvements in total protein recovery and provided evidence for the incorporation of Ara h 1, Ara h 3, and, to a lesser extent, Ara h 2 into high molecular weight protein complexes upon roasting. Relative quantification of allergens in peanut lysates was accomplished by label-free spectral feature (MS1) LC-MS/MS methodologies, by which peanut allergen peptides exhibiting a differential MS response in raw versus roasted peanuts were considered to be candidate targets of thermal modification. Identification of lysine-modified Maillard advanced glycation endproducts (AGE) by LC-MS/MS confirmed the formation of (carboxymethyl)lysine (CML), (carboxyethyl)lysine (CEL), and pyrraline (Pyr) protein modifications on Ara h 1 and Ara h 3 tryptic peptides in roasted peanut varieties. These results suggest that complex processed food matrices require initial analysis by an untargeted LC-MS/MS approach to determine optimum analytes for subsequent targeted allergen analyses.

KEYWORDS: *peanut allergens, roasted peanuts, Ara h, Maillard reaction, AGE, mass spectrometry*

INTRODUCTION

Peanuts are the cause of one of the most prevalent food allergies worldwide. Epidemiological studies estimate an increase in reported cases of peanut sensitization over the past decade, affecting approximately 1.1% of the general population in the United States.[1] Due to the prevalence of peanut and peanut products in a wide variety of foods, complete avoidance is the only reliable mode of treatment for allergic consumers. To address the growing public health concern for food allergy, clinicians, manufacturers, and regulatory agencies are faced with the challenge of developing reliable methods for the accurate detection of food allergens in a variety of processed food samples.

Traditionally, enzyme-linked immunosorbent assay (ELISA) is the gold standard for food allergen detection, offering a simple experimental design with suitable sensitivity for protein allergens in different sample matrices. Methods for immunochemical detection of peanut allergens have been studied extensively and developed to yield commercial products, including the BioKits Peanut Assay Kit (Tepnel), Veratox for Peanut Allergen (Neogen Corp.), and Ridascreen Fast Peanut (R-Biopharm AG).[2] Although accepted as standard methods for peanut allergen measurement, the design of immunochemical-based methods varies with manufacturer. Antibodies can be raised to individual peanut allergens, such as Ara h 1 in the Tepnel BioKits, or to total peanut protein, as found in both the R-Biopharm AG and Neogen Veratox kits.[3,4] Furthermore, reference standards are typically represented by the raw, unprocessed form of the allergen. Whereas ELISA methods have been shown to be appropriate for the detection of low levels of protein allergens in complex food matrices, discrepancies in quantitative results can arise due to limitations in protein extraction, lack of standard reference materials, variations in batch and cultivar sampling, and epitope modifications due to food processing.[5,6]

Food processing is considered to be any physical, chemical, or mechanical manipulation that a food undergoes from the harvest of raw materials until the time the final product reaches the consumer. Processing examples range from food production to any additional steps used to prolong storage, eliminate microbial contamination, or enhance flavors, colors, and textures. For many food allergens, including peanuts, there are no general rules regarding the consequences of food processing, particularly thermal processing, on protein allergenicity.[7,8] Thermal processing has been shown to alter (increase or decrease) the allergenicity of a protein depending on the structure and chemical properties of the allergen, the mode of thermal processing (wet versus dry), and processing conditions including temperature, duration, and pH.[9] Additionally, interactions with other food matrix constituents may affect the overall allergenicity of the food.[10] Uncertainties in the

Special Issue: Advances in Food Allergen Detection

Received: August 14, 2012
Revised: October 1, 2012
Accepted: October 5, 2012
Published: October 5, 2012

effects of processing on the biophysical and immunological properties of a protein pose a significant challenge in the development of reliable extraction and detection methods, thereby requiring rigorous investigations of protein modifications and interactions within processed food matrices.

For most proteins, thermal heating ruptures the delicate balance of intramolecular forces and induces a drastic reorganization at all levels of protein structure.[11] Hydrophobic groups, originally oriented inward, become solvent exposed during thermal processing, making proteins vulnerable to interactions with other mixture constituents in an aqueous environment. Loss of protein tertiary and secondary structure follows with increasing temperatures, causing proteins to adopt a configuration that approaches a fully unfolded random coil conformation.[12] Additional increases in temperature and heating duration result in the formation of insoluble protein aggregates, oligomers, and reduced protein solubility. As demonstrated during in vitro investigations of raw and roasted peanut extracts, thermal heating yielded changes in protein structure and stability of the common peanut allergens Ara h 1 and Ara h 2.[13] Whereas Ara h 1 was characterized by protein higher order covalent complexes in the formation of protein oligomers,[13] both Ara h 1 and Ara h 2 displayed an increase in protection from protease digestion and denaturation.[14,15] Understanding thermally induced conformational changes in peanut protein allergens not only helps to explain altered patient reactivity but also can be utilized in the development of improved detection methodologies.

In correlation with structural changes in protein conformation at higher temperatures is the formation of irreversible protein alteration by chemical modifications. The most extensively studied food protein modification process is the Maillard reaction, by which proteins are modified in the presence of reducing sugars and heat.[7,8] The Maillard reaction is most commonly known to improve the color and flavoring properties of food at temperatures at or above 100 °C. In this reaction, side-chain amino groups of proteins are modified by nonenzymatic condensation with reducing sugars, ketones, or aldehydes to form glycosylamines.[16] Subsequent processing of reaction intermediates results in the formation of covalent protein cross-links or advanced glycation endproduct (AGE) protein modifications.[17] Despite the multitude of AGEs that can form during thermal processing, only a small number have been identified unequivocally and quantified in foods.[18] Well-characterized AGEs in food include lysine derivatives (carboxymethyl)lysine (CML), (carboxyethyl)lysine (CEL), and pyrraline (Pyr). Information about arginine derivatization and cross-linked AGEs in foods, on the other hand, is limited.

Peanuts, characterized by high fat and protein contents, are especially prone to AGE formation during cooking.[19] Different types of thermal processing (blanching, boiling, frying, or roasting) will alter the protein structure in a variety of ways, potentially affecting immunochemical response and allergenicity. Whereas dry heat roasting conditions (150−170 °C) have been shown to promote AGE formation by 10−100-fold relative to the uncooked state, thermal treatment at lower temperatures such as boiling (100 °C) and frying (120 °C) impart only minimal Maillard reaction modifications.[19] In dry-roasted peanuts, thermally induced modifications and structural rearrangements of peanut allergens were found to increase IgE binding by 90% as compared to raw peanuts.[20−23] Whereas a complete explanation for the increase in IgE binding and allergenicity of roasted peanut products is not yet known, current hypotheses predict protein structural reorganization that results in an increased availability (or exposure) of epitopes, the formation of new IgE recognition sites as a result of covalent modification during roasting (neo-epitopes), and/or sensitization due to resistance to digestion.[15,23−25]

Understanding the effects of processing on allergen proteins is necessary for the development of reliable methods for allergen detection and quantification. Effective food allergen analysis must take into account processing-induced changes in protein structure, solubility, and thus extraction efficiency. In this work, we investigated protein solubility from raw and thermally processed peanut samples using various buffer extraction conditions followed by complementary protein analysis methods of SDS-PAGE and LC-MS/MS. Qualitative profiling of protein extracts confirmed enhanced recovery of peanut allergens and solubilization of higher molecular weight complexes upon incorporation of protein denaturants into the extraction protocol. Using a comparative global proteomics approach, differentially abundant peptides in raw versus roasted peanuts were identified using MS1 (peptide abundance) methodologies and surveyed for thermal processing reaction products. Identification of CML, CEL, and Pyr allergen protein modifications confirmed the formation of Maillard AGEs in thermally processed peanuts. Depending on conditions and reactants during thermal processing, additional covalent chemistries including lipoxidation, glycosylation, disulfide interactions, and protein−protein complexes may also be considered as potential reaction products. To ensure effective food allergen protein analysis, we propose utilizing a global comparative proteomics approach for profiling protein allergens in raw and variably roasted peanut samples to identify peptide markers of thermal processing. Compiled data from global proteomics analyses of a wide variety of food samples will be used toward building an allergen information library by which we can begin to understand fundamental changes in protein chemistry induced by food processing of peanuts, thereby improving the performance of quantitative methods for peanut allergen detection in complex food systems.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. The Golden Peanut Co. (Alpharetta, GA, USA) provided raw Runner peanuts and partially defatted (12%) roasted peanut flour (light, medium, and dark varieties). Protein precipitation was facilitated using a Bio-Rad ReadyPrep cleanup kit (Hercules, CA, USA). NuPAGE gels, gel reagents, and iBlot Western blotting supplies were purchased from Invitrogen Life Technologies (Grand Island, NY, USA). Polyclonal chicken anti-Ara h 1, chicken anti-Ara h 2, and chicken anti-Ara h 3 antibodies (IgY, custom synthesized by Sigma Immunosys, The Woodlands, TX, USA)[13,26] in addition to purified Ara h 1 and Ara h 2 protein standards isolated from raw peanut[27,28] were graciously provided by Dr. Soheila Maleki (U.S. Department of Agriculture). SuperSignal West Pico enhanced chemiluminescent (ECL) substrate (Pierce, Thermo Scientific, Rockford, IL, USA) was used for the detection of proteins in Western blot applications. Estimated protein concentrations were determined using a Qubit Fluorometer (Invitrogen Life Technologies). Waters RapiGest SF acid labile surfactant (Milford, MA, USA) supplemented porcine trypsin (Promega, Madison, WI, USA) in whole peanut lysate digestions to enhance peptide recovery. Pierce (Thermo Scientific,) C18 spin columns were utilized for sample cleanup and concentration of protein digests. Rabbit phosphorylase B (Waters MassPrep Digestion Standard) was added to each sample digestion as an injection quality

control. Optima grade solvents for liquid chromatographic analysis were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Protein Isolation from Raw Peanut and Roasted Peanut Flour. Raw peanut kernels were frozen with liquid nitrogen, skinned, and ground to coarse flour in a stainless steel blender. To obtain a fine powder, the peanut flour was frozen with liquid nitrogen and milled using an electronic mortar and pestle. Raw peanut and roasted peanut flour varieties were defatted using a 1:10 (w/v) flour:hexane mixture. The hexane layer was decanted, and the defatted peanut flour was vacuum dried.

Defatted peanut flour (50 mg) was extracted with 1000 μL of extraction buffer. Extraction buffers investigated were (1) 10 mM phosphate-buffered saline (PBS), pH 7.4, and 25 mM iodoacetamide and (2) 7 M urea, 1% amidosulfobetaine-14 (ASB-14), 50 mM Tris-buffered saline (TBS), pH 8.0, and 25 mM iodoacetamide. Both extraction buffers included a plant protease inhibitor cocktail, which consisted of 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), bestatin, pepstatin A, E-64, leupeptin, and 1,10-phenanthroline in dimethyl sulfoxide (DMSO). The solubilized protein mixture was vortexed for 15 min, sonicated in a water bath for 15 min at 4 °C, and allowed to rotate end-over-end for 3 h at room temperature. Centrifugation at 12000*g* pelleted the undissolved peanut debris. The protein content of the extraction solution was measured using a Qubit fluorometer.

SDS-PAGE and Western Blot Analysis. Peanut flour lysates were analyzed by discontinuous SDS-PAGE on NuPAGE Novex 4−12% gradient Bis-Tris gels with NuPAGE MES SDS running buffer. Peanut extracts were reduced (50 mM dithiothreitol, 10 min at 70 °C) prior to gel analysis. To compare extraction efficiencies, equal concentrations of raw peanut extracts and corresponding equal volumes of roasted peanut extracts were loaded on SDS-PAGE gels. PAGE gels were stained using SimplyBlue SafeStain and imaged on a Kodak Image Station 2000R. For protein molecular weight referencing, a SeeBlue Plus2 prestained protein ladder was loaded on each gel.

The iBlot Western blotting device was utilized to transfer proteins from the SDS-PAGE gel to a PVDF membrane (0.2 μm). After transfer, membranes were blocked with a 5% nonfat dry milk solution in 5 mM Tris-buffered saline with 0.05% Tween 20 (TBST) followed by primary antibody incubation for 1 h at room temperature. Chicken anti-Ara h 1 (1:5000), chicken anti-Ara h 2 (1:8000), and chicken anti-Ara h 3 (1:5000) antibodies were individually diluted (v/v) in 5% nonfat dry milk in TBST. The membrane was washed (3 × 5 min) with TBST, blotted for 1 h at room temperature with the secondary rabbit anti-chicken/turkey HRP antibody (1:3000), and incubated with an enhanced chemiluminescence (ECL) substrate according to the manufacturer's instructions. Chemiluminescence was measured using a Kodak Image Station 2000R.

Sample Cleanup and Digestion: Whole Peanut Lysate. Equal concentrations (30 μg) of raw peanut protein extracts (PBS or urea) and corresponding equal volumes of roasted peanut protein extracts were isolated by total protein precipitation (Bio-Rad ReadyPrep Cleanup Kit) in the presence of 10 pmol of yeast alcohol dehydrogenase (precipitation internal standard). The protein pellet was reconstituted in RapiGest SF acid labile surfactant (ALS) at a final concentration of 0.1% in 50 mM ammonium bicarbonate and heated at 95 °C for 5 min. A digestion internal standard, yeast enolase (2 pmol), was added to each biological replicate, and the samples were reduced (60 °C for 30 min) and alkylated (room temperature in the dark for 30 min) with dithiothreitol and iodoacetamide at 10 and 25 mM final concentrations, respectively. Samples were digested at a 1:25 trypsin to total protein concentration ratio overnight at 37 °C. The digestions were quenched with the addition of 0.5% TFA and incubated for 1 h at 60 °C to degrade the ALS surfactant. Samples were centrifuged and passed through a Pierce C18 spin column for cleanup. Prior to LC-MS/MS analysis a 12.5 fmol/μL (25 fmol on column) solution of a rabbit phosphorylase B digest standard was spiked into each sample as an injection quality control.

Liquid Chromatography and Tandem Mass Spectrometry. An AB Sciex Eksigent 2D-nanoLC Ultra (Dublin, CA, USA) was operated under 1-D dual-pump trapping mode where samples were loaded using a metered 2500 nL injection onto a Thermo Dionex Acclaim PepMap 100 trapping column (0.5 cm × 300 μm i.d.; 5 μm C18 particles, 100 Å) at 5 μL/min with 97% Optima grade water, 3% acetonitrile, and 0.1% formic acid. Reverse phase separation was completed on a New Objective PicoFrit analytical capillary column (10 cm × 75 μm i.d.) packed with 2.7 μm C18 Halo particles (90 Å) and a 15 μm tip. The column flow rate was maintained at 400 nL/min. Peptides from digested peanut protein lysates were eluted with a step gradient of 3−30% B in 65 min and 30−40% B from 65 to 80 min (100 min total run time). Mobile phase A was prepared with 0.1% (v/v) LC-MS grade formic acid in Optima grade water and mobile phase B with 0.1% (v/v) LC-MS grade formic acid in Optima grade acetonitrile.

Mass spectrometry analysis was accomplished using a Thermo Scientific LTQ-Orbitrap XL instrument (San Jose, CA, USA) with dynamic exclusion data dependent acquisition (DDA) enabled. Each DDA cycle consisted of a high-resolution profile Fourier transform (FT) mass spectrum (scan range m/z 400−2000; resolving power 60000; 1 microscan) that was acquired in parallel with MS/MS centroid spectra for eight of the most abundant precursor ions. Collision-induced dissociation (CID) MS/MS spectra were acquired in the LTQ at one microscan per spectrum with a signal threshold of 1e3 counts and normalized collision energy of 35%. Automatic gain control allowed accumulation of up to 5e5 ions for FTMS scans and 1e4 ions for ion trap MS/MS ($ITMS^n$) scans. The maximum injection time was 100 ms for acquiring FTMS and $ITMS^n$ scans. Dynamic exclusion of previously selected ions was applied for 15 s with a repeat count of 2, a repeat duration of 15 s, and exclusion mass width of 0.6 Da with unassigned and 1+ precursor ion charge states rejected.

All searches of MS/MS spectra were performed using Mascot version 2.3 (Matrix Science, London, UK) search engine against a custom *Arachis hypogaea* database merged with a Swiss-Prot human protein sequence library and common protein contaminants (24,284 sequences). Standard database search parameters were as follows: full trypsin enzyme digestion with up to two missed cleavages, fixed cysteine carbamidomethylation, and variable hydroxyproline and oxidation of methionine. Error-tolerant searches were performed to identify potential AGEs in raw and roasted peanut extracts in which the following Maillard reaction products were added as variable modifications to supplement standard database parameters: CML, CEL, and Pyr. Post-translational modifications were limited to lysine residues. Spectral assignments for all identified AGE peptide modifications were manually validated.

Peptide and protein level parsimony comparisons across multiple experiments were evaluated using MassSieve v. 1.12.[29] For confident identification, assigned peptide sequences had to be represented with a Mascot ion score greater than or equal to the identity score threshold (at $p < 0.05$) and be identified in four of the six replicate injections (triplicate analysis of two biological sample preparations) with a minimum of two peptides identified per protein. Peptides were matched between raw and dark-roast sample conditions on the basis of sequence, modification, charge state, and retention time. Due to extensive sequence homology in peanut proteins, indeterminate identifications, or peptides with MS/MS spectra matched to secondary sequences with an equivalent score, were retained throughout the sample analysis. A detailed parsimony report is presented in Supplemental Table 1 of the Supporting Information. Parsimony type (discrete, differentiable, superset, or equivalent),[29] protein family, number of confidently assigned peptides, and percent coverage are reported for each protein.

Internal standards (precipitation, digestion, and injection) were utilized to normalize peptide abundance. Three tryptic peptides were selected from each internal standard (alcohol dehydrogenase, enolase, and phosphorylase B) and monitored in replicate sample injections. The summed average intensities of the selected peptides were used to calculate a normalization constant to correct for concentration inconsistencies between PBS (correction factor = 1.1) and urea-extracted samples. A comparison of uncorrected summed peptide abundance for each of the top three selected internal standard peptides

in urea-extracted raw and roasted peanut flour is provided in Supplemental Figure 1 of the Supporting Information.

Thermo Proteome Discoverer 1.3 and Thermo SIEVE 2.0.180 were used for differential peptide screening in raw and roasted peanut lysates. Spectra were processed using the same Mascot parameters and confidence thresholds as mentioned above. SIEVE framing conditions were specified according to a user-defined precursor ion mass width, retention time window, and precursor abundance threshold. Differentially abundant peptides were targeted for manual inspection based upon calculated precursor ion abundance ratios between raw and dark roast extracted sample conditions. Peptide ion abundances were considered to be statistically different at a probability threshold of 0.05. Reported precursor ion abundances and reconstructed ion chromatograms were generated from raw data.

RESULTS AND DISCUSSION

Improved Protein Extraction for Thermally Processed Peanuts. *1D SDS-PAGE.* In the development of reliable methods for allergen detection, one of the major limitations is the variable solubility of structurally or chemically modified proteins after thermal processing. For thermally processed foods, ELISA detection of allergens may vary substantially depending on the ability of commercial kits to extract protein from the modified food matrix.[6,30] Several factors may affect the extraction efficiency of protein allergens including protein structure (albumins, globulins, prolamins), hydrophobicity, ionic strength, and pH. As shown in previous evaluations of ELISA, extraction solution compositions with a pH range of 8−11 demonstrated the best protein recovery efficiencies for roasted peanuts.[6,31] Whereas no single extraction condition may be optimally effective for all food allergens,[32] buffer solutions containing 1% sodium dodecyl sulfate (SDS) and 7% 2-mercaptoethanol have been demonstrated as attractive alternatives for protein recovery from processed foods.[30]

In the present study, we evaluated two buffers for the extraction of proteins from raw and roasted peanuts. Figure 1 displays the results of SDS-PAGE separation of proteins from PBS-based (A,i) and urea-based (B,i) extractions of raw (R), light roast (L), medium roast (M), and dark roast (D) peanut varieties. Purified (P) Ara h 1 (~70 kDa) and Ara h 2 (16−18 kDa) protein standards are included for reference. Whereas banding patterns for the PBS- and urea-extracted raw peanut flours are almost identical, roasted peanut extracts reveal a significant change in protein banding profiles by gel electrophoresis between PBS and urea extraction conditions. In both raw and dark roast extracted peanut flour, the total protein extraction yield is approximately 2.5 times greater with urea compared to PBS (data not shown). With increasing roasting time, however, the concentration of solubilized protein steadily decreases such that both the PBS- and urea-extracted dark roast lysates recover approximately half of the total protein reconstituted from the raw flour. In comparing dark roast peanut extracts, PBS is capable of solubilizing mostly low molecular weight protein components, whereas the urea-based protein denaturation provides enhanced recovery of all proteins. Enhanced protein recovery in urea-solubilized dark roast peanut extracts was most notably recognized by gel electrophoresis in the form of thermally induced high molecular weight complexes.

The results of a systematic optimization of urea extraction buffer components can be found in Supplemental Figure 2 of the Supporting Information. Compared to the traditional method of protein solubilization (i.e., PBS) that limits protein reconstitution from processed food, a urea-modified extraction

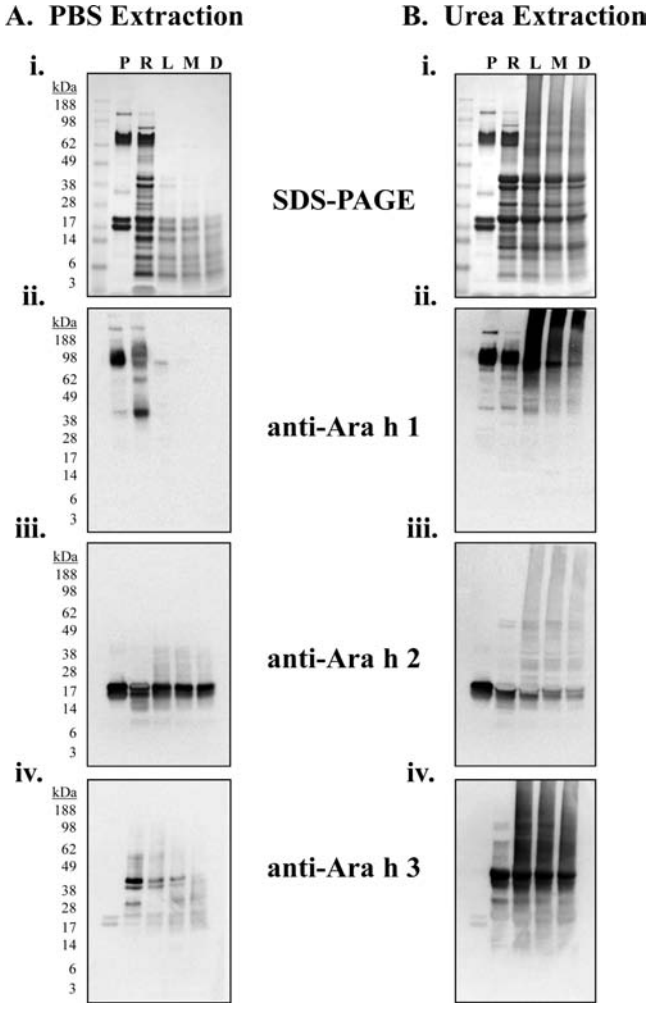

Figure 1. SDS-PAGE and Western blot analyses of reduced raw and variably roasted peanut flours. Sample extractions were completed with (A) PBS or (B) urea (7 M urea, 1% ASB-14, 50 mM TBS, pH 8.0, 25 mM iodoacetamide) extraction solvents. Purified peanut allergen standards (P) are Ara h 1 (~70 kDa) and Ara h 2.01/2.02 (16−18 kDa). Raw (R) and variably roasted (L, light; M, medium; D, dark) peanut sample lanes are labeled accordingly. Stained SDS-PAGE gel images (i) are aligned with corresponding images of Western blots using chicken anti-Ara h 1 (ii), anti-Ara h 2 (iii), and anti-Ara h 3 (iv) antibodies. A SeeBlue Plus2 prestained protein ladder is provided for molecular weight reference.

buffer provides a solution matrix capable of recovering otherwise insoluble protein complexes in thermally processed peanut flour. Utilizing protein fractionation by SDS-PAGE, a rapid, visual, and qualitative assessment of peanut protein extraction efficiencies for PBS- and urea-based buffer systems was obtained. To better characterize changes in protein chemistry generated by thermal processing (i.e., high molecular weight complexes), immunochemical responses for individual peanut allergens were compared by Western blot analyses.

Western Blot Assays. In addition to protein solubilization, changes in protein structure during food processing can alter epitopes and consequently antibody recognition, resulting in detection variability using commercial ELISA kits. Many allergen ELISA methods utilize protein standards and corresponding antibodies for the detection of unprocessed forms of the allergen proteins. Consequently, analytical results obtained with different peanut allergen detection kits and

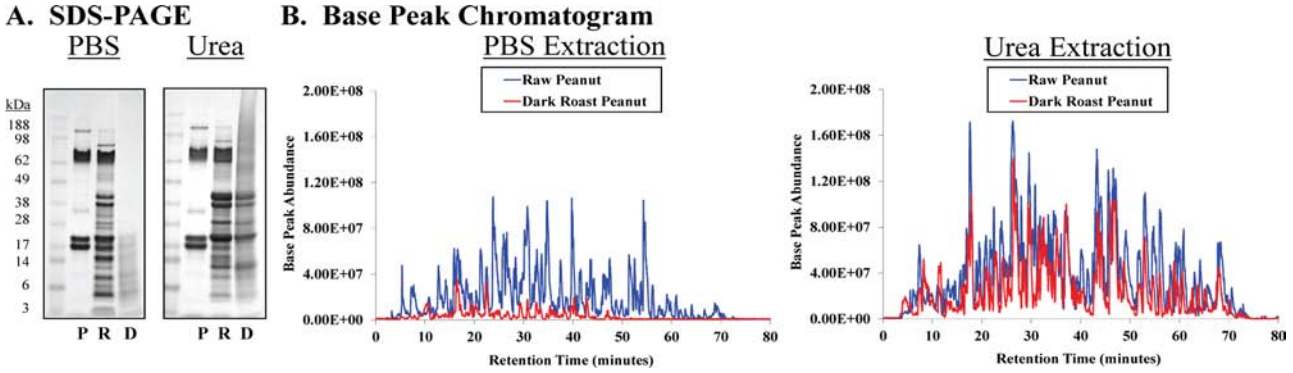

Figure 2. Visual profile of raw and dark roast peanut flour extracts by (A) SDS-PAGE and (B) LC-MS/MS for PBS and urea sample extraction conditions. (A) Purified (P) Ara h 1 (~70 kDa) and Ara h 2.01/2.02 (16−18 kDa) peanut allergen standards are accompanied by raw (R) and dark roast (D) peanut extracts on complementary one-dimensional SDS-PAGE gels. (B) Base peak chromatograms of overlaid raw (blue) and dark roast (red) whole protein tryptic digestions demonstrate enhanced protein recovery with urea-modified extractions. Base peak abundances were normalized with internal standards to correct for discrepancy in total protein column load. As a comparison of extraction efficiencies, equal concentrations of raw peanut extract and corresponding equal volumes of roasted peanut extracts were loaded on a (A) gel or (B) liquid chromatographic column, respectively. A SeeBlue Plus2 prestained protein ladder is provided for molecular weight reference.

assorted methods of food processing can vary substantially.[5] In some cases, highly processed peanuts may not be detected by commercial ELISA kits, whereas the same sample will show high binding capacity by human sera immunoglobulin E (IgE) from peanut allergic individuals.[33]

Western blot images (Figure 1) compare immunochemical responses for the most abundant and potent peanut allergens, Ara h 1 (ii), Ara h 2 (iii), and Ara h 3 (iv), in PBS (A) and urea (B) extractions of raw (R), light roast (L), medium roast (M), and dark roast (D) peanut flour. Whereas protein banding patterns for PBS- and urea-extracted raw peanuts demonstrate almost equivalent antibody responses for Ara h 1, Ara h 2, and Ara h 3, antibody recognition of allergen epitopes in thermally roasted peanut samples yields significant variation between extraction conditions. When extracted with urea, roasted peanut extracts showed evidence of high molecular weight covalent protein complexes for Ara h 1, Ara h 3, and, to a lesser extent, Ara h 2. Interestingly, whereas prominent Ara h 2 antibody binding was not observed for urea-extracted roasted protein aggregates reduced with dithiothreitol (Figure 1B,iii), similar Western assays of the unreduced peanut samples yielded recognition of Ara h 2 epitopes in high-mass entities (Supporting Information, Supplemental Figure 3B,iii).

Because epitope-binding domains for custom antibodies are not often known, disulfide-dependent variability in Ara h 2 antibody response is interpreted as a conformational change in protein structure resulting in differential recognition of protein linear epitopes or as potential high molecular weight disulfide-linked protein complexes. Although informative in providing allergen-specific detection of mass-based fractionated proteins and protein complexes, gel-based immunochemical measurements are limited in the unambiguous identification of protein targets for antibody recognition and quantification. To obtain a more complete characterization of protein chemistries in thermally processed peanuts, a global proteomics liquid chromatography (LC)−tandem mass spectrometry (MS/MS) approach was undertaken.

LC-MS/MS. Optimal conditions for ELISA extraction buffer composition must balance protein solubilization with immunochemical detection compatibility to deliver reproducible allergen quantification. Extraction conditions using traditional PBS-based methodologies, however, often result in limited protein recovery from processed food and an underestimation of allergen quantity. Working outside the confines of antibody recognition, mass spectrometry enables the use of stronger extraction conditions for protein solubilization when used in conjunction with adequate sample cleanup procedures. As visualized in SDS-PAGE and Western blot images in Figure 1, Tris-buffered saline (pH 8.0) accompanied by urea and ASB-14 yielded enhanced recovery of otherwise insoluble protein components in roasted peanuts compared to a traditional PBS extraction. Focusing the analysis on raw and dark roast peanuts, Figure 2 supplements gel-based methodologies (A) with LC-MS/MS whole lysate chromatographic alignments (B) in the comparison of PBS- and urea-extracted peanut flour. Whereas complementary protein banding profiles and peptide responses were observed in both gel and MS analyses, PBS reconstitution in dark roast samples limited protein recovery to only lower molecular weight proteins (Figure 2A), yielding an overall base peak abundance (Figure 2B) 4 times lower than that of the urea-extracted dark roast peanut digest. Improvements in total protein recovery and solubilization of protein complexes in urea-extracted roasted peanut are qualitatively demonstrated by increased base peak ion signal levels in LC-MS analyses and evidence of higher molecular weight protein entities (discrete bands and smears) in SDS-PAGE

Whole protein lysate analyses by LC-MS/MS can be combined with urea-based extractions to provide a methodology for molecularly specific profiling of peanut protein allergens. To expand on base peak chromatographic comparisons, a detailed report of identified proteins for the raw and dark roast sample lysates is provided in Supplemental Table 1 of the Supporting Information. Comparing the number of peptides identified and percent coverage for each protein family in peanut flour lysates, improvements in protein recovery can largely be attributed to enhanced urea-based solubilization of the 11S seed storage proteins Ara h 3 and to a lesser extent Ara h 1. Whereas urea extractions of Ara h 2 and Ara h 3 protein allergens yielded comparable numbers of peptides identified in raw and roasted peanut varieties, a pronounced decrease in protein coverage is observed for Ara h 1 peptides at longer roasting intervals. This observation suggests roasting-induced reactions of Ara h 1 yield intractable products even under harsh denaturing conditions.

Implementation of a LC-MS/MS platform to assess thermal processing effects on peanut allergens allows individual peptide ion responses to be monitored and screened as reliable analytes for targeted peanut allergen analysis in food. Several groups have developed single[34−37] and multiple allergen[38] MS-based screening methods based on selected reaction monitoring (SRM) for peanut allergen detection and quantification in foods. Selection of peptide analytes is based on criteria such as ionization and fragmentation efficiency, tryptic cleavage reproducibility, and amino acid composition (e.g., known post-translational modifications and hydrophobicity).[39] Although peptide prediction is a rapid method for the selection of tryptic peptides in SRM experiments, a global proteomics analysis is critical for the determination of allergen peptides that are present in, and extractable from, food matrices.

Investigating base peak chromatograms of raw and roasted peanut flour (Figure 2B), ion abundances of individual peanut allergen peptides can be monitored to determine relative differences in quantification. Figure 3 displays reconstructed ion chromatograms (RICs) of three peptides, previously reported in peanut protein SRM analyses,[34,35,37] for peanut allergens Ara h 1 (NNPFYFPSR), Ara h 2 (CCNELNEFENNQR), and Ara h 3 (SQSENFEYVAFK). Spectral feature (MS1) peptide ion abundance ratios (dark roast/raw peanut) were calculated to evaluate extraction efficiencies of PBS- and urea-extracted peanut flour. For raw peanut, peptide abundances were comparable for PBS- and urea-based extractions with the exception of the peanut allergen Ara h 3, which showed a notable increase in recovery from raw peanut upon urea extraction. In contrast to raw peanut, PBS extracts of dark roast peanut samples yielded a considerable reduction in protein recovery, compared with urea, for peanut allergens Ara h 1, Ara h 2, and Ara h 3. Such inconsistencies in protein recovery limit traditional methods (e.g., PBS extractions) for allergen quantification. To combat such constraints, a urea-enhanced extraction of raw and roasted peanut flour provided comparable protein recovery efficiencies, enabling direct comparisons of peptide abundances. These results demonstrate that improved protein extraction allows for a more thorough LC-MS/MS analysis of roasted peanuts, which provides an effective strategy for future experimentation with peanut-containing processed food and the development of targeted methods for allergen quantification.

Proteomics Screening of Peanut Protein Allergens. *Comparative Proteomic Analysis: Raw versus Roasted Peanuts.* With an optimized urea-based protein recovery method, raw and dark roast peanut flours were investigated in greater detail by global proteomics. Figure 4A presents a Venn diagram comparing the number of peptides identified from a LC-MS/MS analysis of raw and dark roast trypsin-digested samples. A total of 655 peptides were reproducibly detected in biological and technical replicates and confidently matched to sequences in our protein database. Of the 376 peptides identified in both the raw and dark roast sample lysates, 211 were identified with raw:dark roast ion abundance ratios in the range from 0.51 to 2.00, defining peptides that are unchanged upon roasting. As a quality control to authenticate peptide response ratios, peptide internal standards were monitored during different stages in sample preparation to account for losses during protein precipitation, digestion, and instrumental changes over time.

Although the majority of the peptides were detected with near-equivalent raw and dark-roast ion abundances, we found

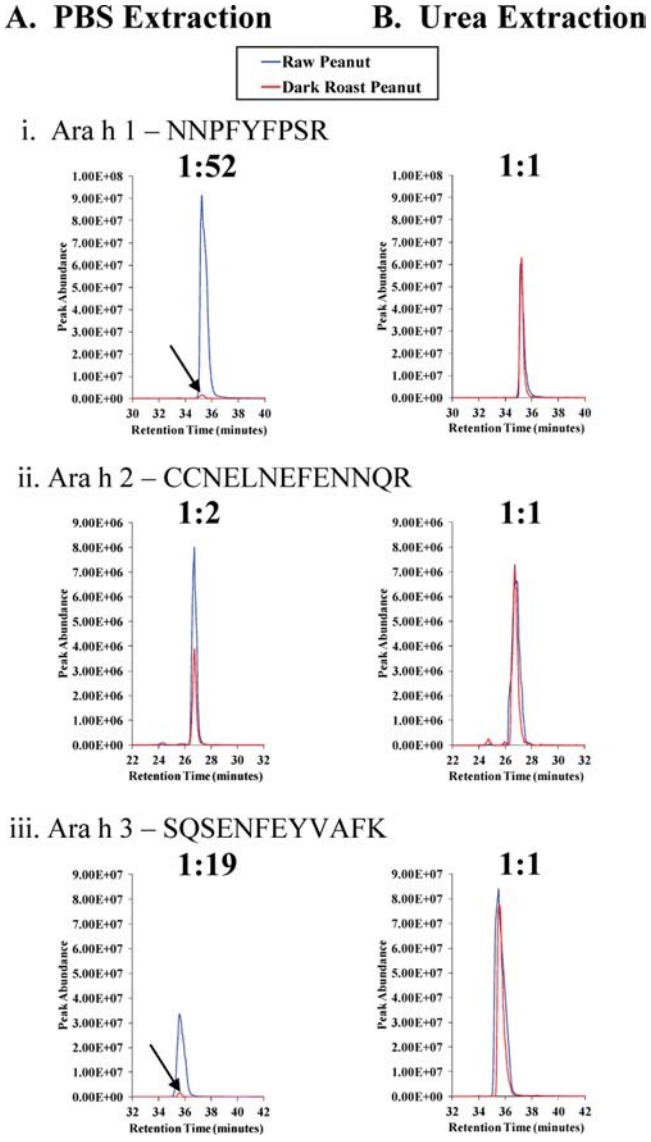

Figure 3. Reconstructed ion chromatograms from LC-MS/MS analyses of raw (blue) and dark roast (red) peanut flour extracts for (A) PBS and (B) urea sample extraction conditions. Ara h 1 (i), Ara h 2 (ii), and Ara h 3 (iii) peptides were selected on the basis of previous literature reports of targeted peanut allergen screening. Peak abundances for selected precursor ion m/z were normalized by internal standards to correct for discrepancies in total protein loaded on column. Ratios of dark roast to raw ion abundances for individual peanut allergen peptide pairs are reported.

many examples of peptides preferentially or uniquely detected in either the raw or dark roast sample conditions. A label-free differential analysis based on peptide ion abundances was used to compare multiple raw and dark roast tryptic lysate data sets. Differentially abundant peptides were selected on the basis of the magnitude of change between raw and dark roast samples and the reproducibility of ion abundance ratios in replicate data sets. Representative reconstructed ion chromatograms are depicted in Figure 4, panels B and C, for peptides demonstrating preferential ion abundances in raw and dark roast samples, respectively

With 140 peptides identified only in the raw peanut lysate, the question arises if the absence of peptide detection in the dark roast sample is due to inefficient protein extraction or

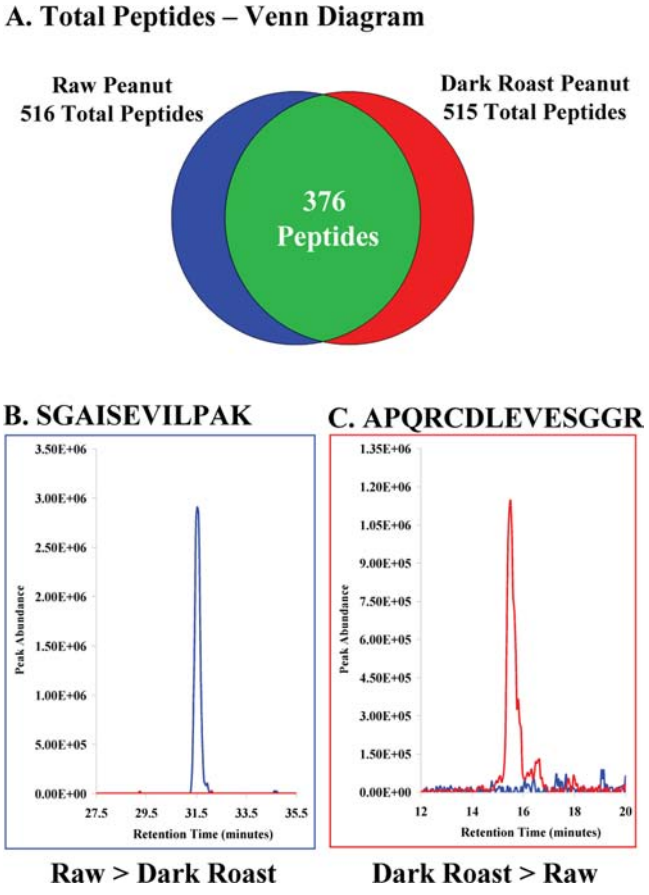

Figure 4. Global proteomics evaluation of urea-extracted raw and dark roast peanut flour. (A) A Venn diagram displays the number of total, common, and unique peptides identified in replicate raw and dark roast whole protein lysates. Reconstructed ion chromatograms are shown for peptides (B) SGAISEVILPAK from peanut lipoxygenase (Q4JME6_ARAHY) and (C) APQRCDLEVESGGR from peanut Ara h 2 in raw (blue) and dark roast (red) tryptic digests. Panels B and C represent differential peptide responses preferential to raw and dark roast lysates, respectively.

peptide modification from thermal processing. Peptide modifications include glycation or lipoxidation post-translational modifications, disulfide-bond rearrangements, structural reorganization resulting in missed-cleavage or semitryptic peptides, and/or covalent protein cross-linking. A representative example of a peptide uniquely identified in the raw peanut flour is shown in Figure 4B. The SGAISEVILPAK peptide belongs to the lipoxygenase (Q4JME6_ARAHY) protein found in peanuts. Although not characterized as an allergenic protein in peanuts, lipoxygenase acts as a catalyst to oxidize polyunsaturated fats in plants. At elevated temperatures, most enzymatic processes become inactive, which might result in protein degradation or the formation of advanced lipoxidation modifications, thereby yielding reduced lipoxygenase peptide abundances in roasted samples.

In contrast, Figure 4C shows the Ara h 2 peanut allergen peptide APQRCDLEVESGGR, which is unique to the dark roast peanut extract. In its native form this C-terminal Ara h 2 peptide is linked via an intraprotein disulfide bond to the tryptic peptide CMCEALQQIMENQSDR.[40] Upon thermal processing, structural changes in protein conformation may occur due to gradual protein unfolding and rearrangement. As a result, such modified proteins may have blocked or eliminated tryptic cleavage sites, which, upon digestion, yield new missed cleavage fragments such as APQRCDLEVESGGR. Consistent with this explanation is the detection of the fully tryptic form of this peptide, CDLEVESGGR, with a greater abundance in the raw lysate (data not shown). Many additional missed cleavage peptides were identified for several peanut proteins in the dark roast lysate. Evaluation of precursor ion abundances at varied roasting time points provides a dynamic profile of changes in protein chemistries during thermal processing. Although interpretation of differential peptide abundances is a nontrivial task, identification of thermal modification products using LC-MS/MS will enable the development of improved allergen detection methods.

Identification of Advanced Glycation Endproducts. Whereas some peptides are preferentially identified in the raw or dark roast peanut lysate, other peptides are identified in both sample types and are detected with ion abundances that vary with the duration of roasting. Figure 5A(i) displays reconstructed ion chromatograms for the Ara h 1 peptide IFLAGDKDNVIDQIEK measured in the raw and three variably roasted (light, medium, and dark) samples. Identification of the IFLAGDKDNVIDQIEK peptide is confirmed by interpretation of the MS/MS spectrum shown in Figure 5A(ii), which shows excellent sequence coverage of y- and b-ions. For the processing timeline investigated, the Ara h 1 peptide IFLAGDKDNVIDQIEK was detected with a sequential decrease in ion intensity at longer roasting durations showing an overall 5.7-fold abundance decrease in the dark roast sample relative to that of the raw sample. The decrease in Ara h 1 protein coverage (Supporting Information, Supplemental Table 1) and reduction in ion abundance upon roasting indicate IFLAGDVKDNVIDQIEK as a potential marker of thermally induced Ara h 1 modifications.

A well-studied modification of proteins is glycation. In roasted peanut samples, Maillard reactions are hypothesized as one of the principal means of formation of insoluble protein complexes.[13] At extended roasting durations, covalent high molecular weight aggregates are recognized by human serum IgE from peanut-allergic individuals with enhanced overall peanut protein allergenicity as a result of thermal processing.[13] Although it is unlikely that the actual AGE modification itself is allergenic,[24] protein allergen structural rearrangements and covalent modifications produced during the roasting process likely expose previously unavailable sites and/or result in the formation of new IgE-binding domains (neo-epitopes).[15,23−25]

In a global bottom-up proteomics investigation of raw and roasted peanuts, many MS/MS spectra were not identified due to insufficient fragmentation and restrictive database search parameters. To identify potential AGE-modified peptides, unmatched spectra were searched for variable modifications that correspond to common AGE modification products of CML, CEL, and Pyr. From these results, the IFLAGDKDNVIDQIEK Ara h 1 peptide was identified with a pyrraline modification to the side chain of the lysine that is the missed cleavage residue (Figure 5B(i)). This AGE-modified peptide, shown as IFLAGDK*DNVIDQIEK (where K* is the pyrraline-modified lysine), was absent in the raw lysate and shown to have the strongest signal intensity in the light roast extract, decreasing with increasing duration of thermal processing (roasting). The 24-fold lower abundance of the modified versus unmodified form of this peptide indicates that AGE modifications are only a minor contributing factor to the change in peptide abundance upon processing. Because the

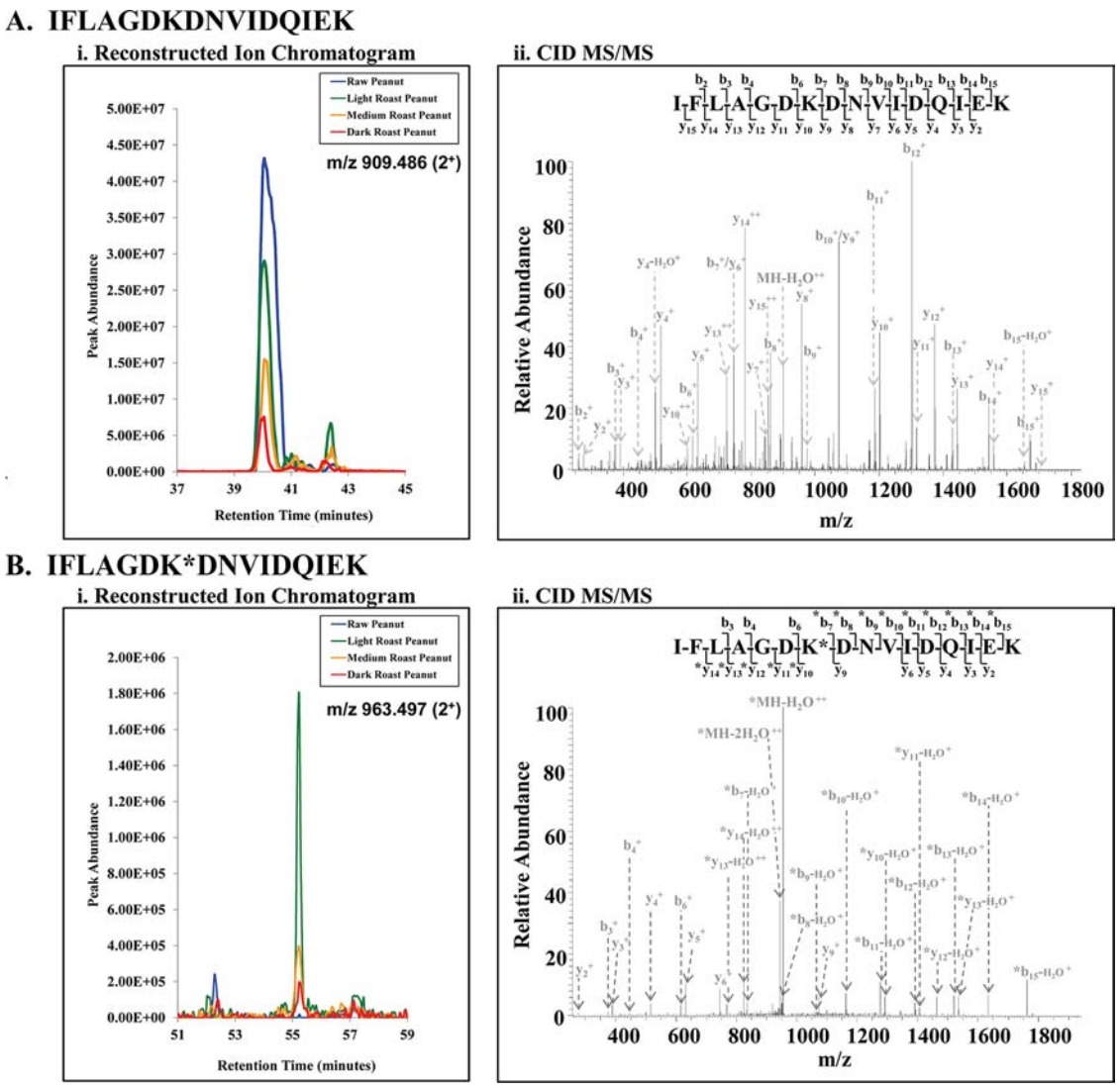

Figure 5. Investigation of Maillard reaction advanced glycation endproducts (AGE) in urea extracts of raw and variably roasted flour. (A) (i) Reconstructed ion chromatogram of the Ara h 1 IFLAGDKDNVIDQIEK peptide in raw, light roast, medium roast, and dark roast sample varieties and (ii) corresponding CID MS/MS fragmentation spectrum. Fragment ion assignments are identified for b- and y-ions. (B) (i) Reconstructed ion chromatogram of the AGE modified IFLAGDK*DNVIDQIEK peptide in roasted peanut sample extracts. K* represents modification of the lysine residue with a pyrraline advanced glycation reaction product. (ii) CID MS/MS fragmentation of the modified Ara h 1 peptide shows H_2O neutral loss as the predominant fragmentation pathway for ions with pyrraline-containing residues (*).

AGE-modified form of the protein decreases with increasing roasting duration, we hypothesize that the Ara h 1 peptide IFLAGDKDNVIDQIEK is modified by additional Maillard-based reactions, which result in new products, interactions, and higher order protein complexes. Located in an IgE binding domain of the protein allergen Ara h 1,[41] AGE modification of IFLADKDNVIDQIEK and other Ara h 1 peptides may further contribute to an increase in patient reactivity upon protein structural reorganization, aggregation, and/or protection from proteolytic digestion.

Compared with that of the unmodified peptide, the MS/MS spectrum for IFLAGDK*DNVIDQIEK shows significantly reduced y- and b-ion intensities, with the main cleavage product corresponding to a water loss from the precursor ion. Fragment ions containing the pyrraline modification were further characterized by a water loss, rendering challenges in identification of low abundant AGE-modified peptides. A similar phenomenon was observed for other pyrraline-modified peanut peptides (data not shown) and has been reported in the literature.[42−45] Whereas many MS/MS spectra for AGE-modified peptides may be inefficient for manual confirmation (if targeted at all), the dominant fragment ion corresponding to a neutral molecule loss can be utilized as a marker for screening modified peptides in lysate digestions.

Successful identification of an AGE-modified Ara h 1 peptide confirms the presence of Maillard reactions during thermal processing of roasted peanuts. Other similar modifications were identified for CML-, CEL-, and Pyr-modified allergens in Ara h 1 and Ara h 3 (data not shown). Interestingly, many AGE-modified peptides were detected as more than one glycation product. Figure 6 plots the average precursor ion abundances for the Ara h 3 missed cleavage peptide SPDIYNPQAGSLK-TANDLNLLILR in replicate injections of raw, light roast, medium roast, and dark roast lysate samples. Absent in the raw lysate, the precursor ion abundance for this peptide decreased with longer roasting duration, yielding the lowest signal intensity for the dark-roast urea extract. Although the individual peptide components of SPDIYNPQAGSLKTANDLNLLILR

dx.doi.org/10.1021/jf303554t | J. Agric. Food Chem. 2013, 61, 5638−5648

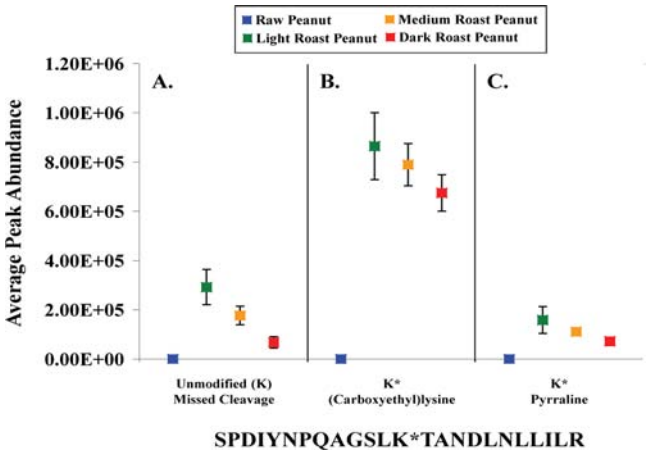

Figure 6. Database aided identification of an allergen peptide target prone to multiple advanced glycation endproduct (AGE) modifications in peanut flour urea extracts. Average peptide abundance values are calculated from biological and technical replicate data sets of each sample condition. Precursor ion abundances corresponding to the unmodified and AGE modified forms of the Ara h 3 tryptic peptide SPDIYNPQAGSLKTANDLNLLILR are reported for raw, light roast, medium roast, and dark roast protein lysates. K* represents modification of the internal lysine residue with an advanced glycation reaction product (carboxyethyl and pyrraline).

(SPDIYNPQAGSLK and TANDLNLLILR) were shown to exhibit only minimal changes in abundance upon thermal processing (raw:dark roast ion abundance ratio 1.00 ± 0.25) (data not shown), the roasting time-dependent decrease in ion abundance suggests that the peptide SPDIYNPQAGSLK-TANDLNLLILR is a marker of thermal processing.

The addition of AGE modifications to our search parameters enabled identification of SPDIYNPQAGSLK*TANDLNLLILR with a CEL and a Pyr AGE modification to the amino side group of the internal lysine residue (K*). In all cases, peptide modifications were detected at low abundance levels, resulting in a small number of acquired MS/MS spectra. Following in accordance with the IFLAGDK*DNVIDQIEK peptide, the missed cleavage modified peptides were most abundant in the light roast lysate with decreasing intensity at longer roasting times and were absent in the raw sample extract.

From this work, it may be concluded that identification of every low-level AGE modification is not practical in individual roasted peanut lysates due to variations in low-level modified products formed from different Maillard reaction pathways. Whereas the link between thermal processing and the formation of Maillard reaction products has been demonstrated in peanuts,[20,21] identification of specific AGE-modified peptides in peanut allergen proteins has not been reported prior to this work. Using a comparative LC-MS/MS proteomics platform, peptide allergen targets prone to AGE modification (i.e., IFLAGDK*DNVIDQIEK and SPDIYNPQAGSLK*-TANDLNLLILR) can be identified. Processing-dependent variability in ion abundance responses, however, suggests that such peptides are not reliable targets for allergen quantification assays.

Global Biomarker Development for Peanut Allergens. To identify peptides that will serve as allergen biomarkers for thermally processed food matrices, more stringent protein extraction protocols must be developed. As demonstrated in Figure 1, incorporation of urea and ASB-14 protein denaturants into the extraction protocol for thermally processed roasted peanut flour enabled solubilization of high molecular weight protein complexes and improvements to total protein recovery as compared to a more traditional PBS extraction. Western blot screening for the presence of Ara h 1, Ara h 2, and Ara h 3 identified a strong association of covalent protein aggregates involving protein allergens Ara h 1 and Ara h 3 with less significant contributions of Ara h 2. Removing the bias of antibody-based Western blot interpretations, MS analysis of whole protein peanut lysates provided molecularly specific protein identifications and the ability to assess differentially abundant peptides ("biomarkers") using MS1-based comparative analysis methodologies. By comparison of ion abundance ratios between raw and roasted peanut flour varieties, peptide targets exhibiting differential ion abundance ratios were determined. Global screening of unidentified precursor ions in raw and roasted peanut flour samples followed by manual MS/MS validation confirmed the identification of CML, CEL, and Pyr AGE modifications to specific Ara h 1 and Ara h 3 peanut allergens. In contrast, a similar differential evaluation of dark roast PBS extracts was not able to identify prominent AGE modifications, likely due to ineffective extraction of modified and/or cross-linked proteins.

In the future we will apply a similar approach to processed food samples incurred with or containing peanut. Together with our knowledge of peptide response in raw and roasted peanut samples, we can begin to build a database enabling peptide profiling in processed foods. Within a whole food system, allergenicity becomes dependent on the contribution of several allergens that may react differently to processing and heat treatments. The conditions (intensity, duration, and form) of heat treatment may also affect differentially the structure of allergenic proteins and interactions with other constituents of the food matrix. Comparing preferentially modified peptides found in roasted peanut flour to peptides identified in other processed food sources may provide insights that will advance the understanding of the complex chemistries involved in the peanut proteome. The information learned throughout these studies can be applied toward the development of robust methods for peanut quantification, as well as aid in the understanding and expansion of methods for other similar plant proteomes.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Table 1 and Figures 1−3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge Bruce Kotz of the Golden Peanut Co. in Alpharetta, GA, for providing the raw and variably roasted Runner peanut reference samples. We express kind regards to Jinxi Li and Kevin Shefcheck for their early work in the peanut allergen project during their tenure in our group at the FDA. We also thank Dr. Soheila Maleki at the U.S.

Department of Agriculture for providing Ara h 1, Ara h 2, and Ara h 3 purified protein reagents and antibodies.

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