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Article

Assessment of Peanut Allergen Cross-Contact Risk from Reused Roasting Oil Using a Targeted Proteomics Method

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ABSTRACT: Allergen cross-contact risk can arise when the oil used to roast peanuts is reused. Heat-induced protein denaturation reduces the accuracy of immunochemical methods; therefore, the objective of this study was to develop a targeted proteomics method to measure peanut transfer to roasting oil. Marker peptides for quantification were identified from blanched peanut butter, and method-determined protein levels of peanut butter-spiked oil after 10 min heating at 190 °C ranged between 77% and 94% recovery. Whole peanuts were then batch-roasted in oil, and peanut protein levels were measured up to 405 μg of peanut protein/mL after 10 batches. Filtration trials of the batch-roasted oil revealed that metal sieves with a pore size of ≤25 μm and filter papers with a pore size of ≤35 μm reduced peanut protein concentrations below reporting limits. These results highlight the need for accurate quantitative methods when assessing the peanut allergen risk in thermally processed foods and determining the efficacy of preventive controls.

KEYWORDS: *food allergy, food safety, cross-contact, filtration, mass spectrometry, quantification, detection methods*

INTRODUCTION

Peanut allergy is one of the most common food allergies in Western nations, affecting on average 1 in 50 people.[1−3] From the 1990s to the 2000s, the self-reported prevalence rates of peanut allergy have increased from <1% to about 3% in children and adolescents[4−6] and have continued to remain high despite the adoption of preventative measures such as early introduction of peanut products to the diet.[7,8] Unlike milk or egg allergies, most children (70−80%) will not resolve their peanut allergy as they age[9,10] and so must remain on an avoidance diet throughout their lives. This poses a burden both on the consumer with a peanut allergy and on food manufacturers, who must implement preventative controls during food storage, production, and handling to prevent or minimize the risk of peanut cross-contact.

One common preparation method for peanuts is oil-roasting, wherein batches of whole peanuts are immersed in hot oil in a batch frier or conveyed on tracks through the oil in a continuous fryer. While peanut-allergic populations know to avoid oil-roasted peanut products, the reuse of the roasting oil to roast or fry other foods, such as tree nuts, seeds, or other snack foods, can pose a cross-contact risk as particulates or fragments from the whole peanuts are released into the oil during roasting and may transfer to nonallergenic foods. Indeed, there have been reports of allergic reactions arising from the consumption of foods fried in oil previously used to fry allergenic foods.[11−13] Preventative controls to minimize cross-contact risk can include passive and active filtration of the oil to remove particulates before reuse. However, to ascertain the success of these measures, accurate quantitative methods are needed to measure allergenic food protein concentrations in oil.

Most commonly, allergen detection in foods is carried out through enzyme-linked immunosorbent assays (ELISA),[14] wherein antibodies bind to food proteins and, through an enzymatic reaction, catalyze the generation of a colorimetric signal where the relative absorbance of light corresponds to concentration. Manufacturers produce a suite of kits designed to detect specific food allergens such as peanuts or specific allergenic proteins such as gluten, casein, or β-lactoglobulin. While these kits are user-friendly and economical, comparing results of different kits can be challenging depending on the units that are reported, e.g., whole peanut vs peanut protein.[15] Furthermore, any physicochemical changes to the target food allergen or allergenic protein, such as those that occur during high-heat frying and oil-roasting, may alter the epitopes available for antibody binding and affect quantification.[16] Measuring peanut concentrations in oil used for oil-roasting is particularly difficult since the analyte proteins exist in varying degrees of thermal denaturation as successive batches are introduced into the fryer. Critically, even though thermally denatured peanut proteins are less detectable by ELISA, they can still pose a health risk to peanut-allergic consumers.[17,18]

An alternative method that may be able to overcome the issues impacting the quantification of peanut proteins in oil is targeted proteomics. Unlike immunoassays, which are most accurate when the analyte protein is intact and in the same conformation against which the antibodies were raised,

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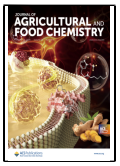

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targeted proteomics quantification is less reliant on protein conformation since it uses proteotypic marker peptides released during enzymatic digestion. These peptides are analyzed with a mass spectrometer, and their signal intensities are compared to a calibration curve, often matrix-matched to the same material and sample preparation conditions. Thus, even if the physical conformation of the allergenic protein is changed as a result of cooking, it may still be extracted and digested into detectable peptides. Targeted proteomics has been used to quantify peanut protein in foods, including cookies, muffins, and ice cream,[19−21] but as oil is a distinct matrix and the processing conditions used to produce these foods are dissimilar, different marker peptides may be required for quantitative accuracy. A targeted proteomics method was recently developed for cashew protein in roasting oil,[22] proving the feasibility of extracting proteins and developing quantitative methods for food allergens in oil.

The main aim of this study was to develop a targeted proteomic method to quantify peanut proteins in the oil used to roast whole peanuts. A matrix-matched calibration curve utilizing unheated cooking oil spiked with peanut butter prepared from blanched, unroasted peanuts (BPB) was created for the method, and its accuracy was tested across different peanut concentrations, heating durations, and standardized peanut sources. The method was then applied to quantifying peanut protein in oil used to batch-roast whole peanuts, and the results were compared with results obtained with peanut-specific ELISA kits and a total protein assay. The developed method was used to evaluate the efficacy of filtration and settling for removing peanut proteins from oil.

MATERIALS AND METHODS

Materials

Dithiothreitol (DTT), iodoacetamide (IAA), and glacial acetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Urea, ammonium bicarbonate (ABC), LC-MS grade acetone, hexane, and acetonitrile (ACN), and trifluoroacetic acid and formic acid (FA) were obtained from Thermo Fisher Scientific (Waltham, MA). Tris-HCl and Trypsin/Lys-C were obtained from Promega (Madison, WI). Vegetable (soybean) cooking oil was produced by Wesson (Memphis, TN).

Standardized Peanut Sources

BPB was made in-house from blanched, unroasted Virginia peanuts (nuts.com, Cranford, NJ) using a WB02 nut butter mill (WEnutbutter, Groningen, Netherlands), aliquoted into plastic bags, and frozen at −20 °C prior to use. The protein content of BPB was determined to be 24.6% by weight using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Additional peanut sources used were National Institute of Standards & Technology peanut butter (NIST PB) (Standard Reference Material 2387; Gaithersburg, MD) containing 22.2% protein by weight and light-roasted 12%-fat peanut flour (LRPF) from Golden Peanut (Alpharetta, GA) containing 50% protein by weight per manufacturer's specifications.

Preparation of Unheated and Heated Peanut-Spiked Oil Samples

Analytical results from all samples are expressed as μg of peanut protein per mL of oil rather than μg of peanut butter or peanut flour per mL of oil. The amount of BPB, NIST PB, and LRPF needed to achieve target peanut protein concentrations in oil were determined by dividing the target peanut protein concentration by the percent protein of the peanut source.

Unheated spiked oil samples were prepared by mixing the peanut source (BPB, NIST PB, or LRPF) in oil to target peanut protein concentrations. Heated spiked oil samples were prepared using a countertop deep fryer (Waring, Stamford, CT) filled with 3.79 L of vegetable oil heated to 190 °C. Beakers containing 180 mL of fresh oil were placed in the deep fryer and allowed to come to temperature. Peanut spikes were prepared at a 10× concentration by vortexing the peanut source in 20 mL of oil. The spikes were added to the oil, stirred, and then sampled at 1, 5, and 10 min with stirring immediately prior to sampling.

Batch-roasted peanuts were prepared in oil samples using the procedures used to prepare heated spiked oil samples to understand the levels of peanut protein that transfer to the oil during roasting. A model experiment was first performed to mimic the transfer of peanut protein in oil from whole peanuts using batches of BPB spiked into the oil at known concentrations. For the model experiment, beakers containing 180 mL of fresh oil were allowed to come to a temperature of 190 °C in a countertop deep fryer. A master mix of BPB-spiked oil was prepared at 750 μg peanut protein/mL, and 20 mL was added to the beakers of hot oil to prepare oil with a starting peanut protein concentration of 75 μg/mL. At 5 min intervals, a 20 mL sample was removed from the beaker after stirring, and then another 20 mL spike was introduced.

For experiments aimed at understanding peanut protein transfer to oil during roasting, 400 mL of oil was heated to 190 °C in beakers in the deep fryer, and 20 g batches of blanched unroasted peanuts (ratio of 1:20 w/v peanuts/oil) were roasted in the beakers in 5 min intervals. After each interval, the oil was stirred, a 20 mL oil sample was collected and replaced with 20 mL of fresh oil, and then the roasted peanuts were removed, and a fresh batch of peanuts was added.

Regeneration Treatments for Peanut Roasting Oil

To assess the effect of various regeneration treatments on removing peanut particulates from oil, the beakers from the whole peanut trials were removed from the fryer after all batches were roasted and allowed to cool to room temperature (RT). The oil in the beakers was stirred to disperse the peanut particulates, and 25 mL samples were gravity filtered using metal sieves (Gilson, Middleton, WI) of pore sizes 150 μm, 75 μm, and 25 μm and paper filters of pore sizes 35 μm (Ahlstrom, Helsinki, Finland), 20 μm (Whatman #4, Cytiva, Marlborough, MA), and 11 μm (Whatman #1). Another 25 mL sample was collected and did not receive filtering treatment. The beakers were then allowed to sit undisturbed at RT to allow for settling of the peanut particulates. After 10 min and 1 h, a 25 mL sample was collected via serological pipet at a depth of 1 in from the surface.

Sample Preparation for Proteomics

Multiple methods of extracting peanut protein from oil prior to trypsinization were performed and compared. The main method used to prepare all nontargeted and targeted proteomics samples is described below. Additional methods to prepare BPB, NIST PB, and LRPF samples for targeted proteomics to compare recovery are described in the Supporting Information.

An organic precipitation method, used to remove lipids and precipitate proteins, was accomplished by mixing 200 μL of peanut-spiked oil samples with 800 μL of an ice-cold mixture of 50:50 acetone/hexane. Samples were vortexed briefly and then incubated at −20 °C for 1 h. Samples were then centrifuged at 14,000*g*, RT for 10 min (all centrifugation steps were performed at 14,000*g*, RT unless otherwise noted), and the supernatants were discarded. The pellets were vortexed with 400 μL of the ice-cold acetone/hexane mixture and centrifuged for 10 min; the supernatants were discarded, and this process was repeated once more. The pellets, which contained peanut protein, were frozen at −20 °C until needed.

Protein pellets were resuspended in 400 μL of 2 M urea, 50 mM Tris-HCl, sonicated for 15 min, vortexed for 30 s, and sonicated again for 5 min to ensure homogeneous dispersal of the protein pellet. DTT was added to obtain a final concentration of 10 mM, and then the samples were incubated at 55 °C for 50 min. IAA was then added to a final concentration of 25 mM, and samples were incubated in the dark at RT for 1 h. The samples were centrifuged for 10 min, and the

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supernatants were added to Microcon 10 kDa molecular weight cutoff filters (Millipore, Burlington, MA) and centrifuged in 20 min increments until roughly 20 μL remained on the filters. The filters were placed in fresh microcentrifuge tubes. Trypsin solution, prepared in 50 mM glacial acetic acid at a concentration of 40 ng/mL, was added to each sample at a ratio of 1:100 enzyme-to-protein, and then 50 mM ABC was added to reach a total volume of 80 μL on the filter. The tubes were wrapped in parafilm to prevent evaporative losses and incubated at 36 °C for 16 h. Following digestion, samples were centrifuged for 40 min; then 50 μL of 50 mM ABC was added to the filters, and they were centrifuged again for 40 min. Trypsin activity was terminated by adding trifluoroacetic acid and ACN to concentrations of 0.5 and 5%, respectively, and incubating at 36 °C for 1 h. Samples were centrifuged once more for 10 min, and the supernatant was stored at −20 °C until ready for analysis.

For targeted proteomics, samples for eight-point calibration curves of BPB in unheated oil ranging from 5−1000 μg peanut protein/mL were prepared by diluting two BPB-spiked oil master mixes prepared at 1000 μg/mL and 50 μg/mL. The 1000 μg/mL master mix was used for calibration levels between 1000 and 100 μg/mL, and the 50 μg/mL master mix was used for calibration levels between 50 and 1 μg/mL. Two master mixes were used to reduce compounding errors from the serial dilutions. Blanks were prepared using oil with no BPB. Calibration curves and blanks were prepared simultaneously with each batch of samples using the exact extraction conditions that the samples were prepared with.

Proteomics Analysis

Methods used for nontargeted proteomics analysis of BPB-spiked oil samples to identify candidate marker peptides are described in the Supporting Information. Candidate marker peptides for targeted proteomics analysis were selected from the nontargeted data and are listed in Table 1. C-terminal $^{13}C^{15}N$ Arg/Lys stable isotope-labeled peptides were synthesized by Biosynth (Staad, Switzerland) at >98% purity and resuspended in 5% ACN, 0.1% FA. For all targeted proteomics samples, an equivalent volume of stable isotope-labeled peptides was mixed with the samples to a final heavy peptide concentration of 100 fmol/μL.

Table 1. List of Marker Peptides Used for Targeted Proteomics and Their Parent Peanut Allergen Groups[a]

parent allergen	sequence	retention time (min)	*m/z*	*Z*
Ara h 7	CCNELNR	4	483.2	2
Ara h 7	CCNELN**R**	4	488.2	2
Ara h 7	WDPDR	6.7	344.66	2
Ara h 7	WDPD**R**	6.7	349.66	2
Ara h 2	NLPQQCGLR	10.7	543.28	2
Ara h 2	NLPQQCGL**R**	10.7	548.28	2
Ara h 7	NLPQNCGFR	12.1	553.26	2
Ara h 7	NLPQNCGF**R**	12.1	558.27	2
Ara h 3	QILQNLR	15.1	442.77	2
Ara h 3	QILQNL**R**	15.1	447.77	2
Ara h 2	CCNELNEFENNQR	15.7	576.24	3
Ara h 2	CCNELNEFENNQ**R**	15.7	579.57	3
Ara h 3	SSNPDIYNPQAGSLR	16.3	809.89	2
Ara h 3	SSNPDIYNPQAGSL**R**	16.3	814.89	2

[a]Bolded residues indicate heavy-isotope labeling, and italicized residues indicate carbamidomethylation.

Targeted proteomics was performed on a 1290 Infinity II UHPLC system connected to a 6550 iFunnel QToF mass spectrometer (Agilent, Santa Clara, CA). An injection volume of 12 μL was loaded onto a 2.1 mm × 150 mm, 1.8 μm bead ZORBAX Eclipse Plus C18 column (Agilent) and separated using mobile phases consisting of 0.1% FA in water (solvent A) and 0.1% FA in ACN (solvent B). The chromatography gradient held at 5% solvent B for 1 min, increased to 28.2% solvent B by minute 35, increased to 90% solvent B by minute 36, decreased to 5% solvent B by minute 39, and then held at 5% solvent B until minute 40. A 22 min wash was performed after each sample with the following gradient: increase from 5 to 90% solvent B by minute 15, hold at 90% solvent B until minute 19, decrease to 5% solvent B by minute 20, and then hold at 5% solvent B until minute 22.

The first three min of the gradient were directed to waste, after which samples were analyzed in positive ion mode and ionized with a Dual AJS electrospray ionization source. Source parameters consisted of 3500 V capillary voltage, 1000 V nozzle voltage, 290 °C gas temperature, 12 L/min gas flow, 35 psi nebulizer pressure, 350 °C sheath gas temperature, and 11 L/min sheath gas flow. Spectra were acquired using Agilent MassHunter Acquisition version B.09.00 in Auto MS/MS mode. MS spectra were collected at a rate of 8 spectra/s, and MS/MS spectra were collected at a rate of 4 spectra/s with a Narrow isolation width. Precursor ions were fragmented using a variable collision energy (Table S1). A targeted peptide list was created using the *m/z*, retention time, and charge state of the candidate marker peptides selected from the nontargeted data of BPB samples. Reference mass correction was enabled using *m/z* 121 and *m/z* 922 ions from the Agilent API-TOF Reference Mass Solution Kit.

ELISA and BCA Analysis of Oil Used for Batch-Roasting

Peanut protein concentrations of oil samples from batch-roasting whole peanuts were measured using Veratox for Peanut (Neogen, Lansing, MI), Ridascreen Peanut (R-Biopharm AG, Darmstadt, Germany), and BCA kits. Peanut protein extracts from oil were prepared by mixing 1 mL of oil with 4 mL of ice-cold acetone. The samples were vortexed and stored for 6 h at −20 °C and then refrigerated for 18 h at 4 °C. Samples were centrifuged at 4000*g* for 1 h at 4 °C, and the oil layer was discarded, leaving a protein pellet. The protein pellet was resuspended in 1 mL of 10 mM phosphate-buffered saline, and the remaining oil was removed by adding 1 mL of hexane. The tubes were vortexed briefly and then centrifuged at 4000*g* for 5 min. The hexane layer was discarded, and this process was repeated once more. The samples were frozen at −20 °C. The extracts were prepared for ELISA following the kit instructions. Dilution of the extracts was necessary to bring expected protein concentrations within the working ranges of the kits and was performed using deionized water. Absorbance readings were recorded with an ELx808 spectrophotometer (BioTek, Charlotte, VT). To compare results between methods, the reported units from the Veratox and Ridascreen kits of μg whole peanut were converted to μg peanut protein by multiplying by 0.258, which is the average percent of protein in peanut.[23]

Data Analysis

Peanut-spiked oil samples for nontargeted and targeted proteomics were prepared in duplicate and analyzed in duplicate ($n = 4$). Batch-roasting experiments were performed in triplicate and analyzed in duplicate by LC-MS/MS, ELISA, and BCA ($n = 6$). Regeneration treatment experiments were conducted on each batch-roasting trial and analyzed in duplicate ($n = 6$). Data are presented as mean values throughout.

For targeted proteomics, Table 2 shows the selected transitions that were summed to determine the peak areas of the light and heavy peptides. Peak areas were calculated with Skyline version 24.1.0.414 (Seattle, WA). Modifications allowed were carbamidomethylation and C-terminal $^{13}C^{15}N$ Arg and Lys. Data was exported to Microsoft Excel for quantification based on the peak area ratio of the light to heavy peptide and for the construction of figures. Calibration curve quality was assessed for linearity with $R^2 > 0.99$ using a $1/x$ weighting. The minimum reporting limit for each marker peptide was set at the lowest calibration level with a signal-to-noise ratio (S/N) ≥ 10, where the noise was defined as the peak area of the blank (oil with no spiked peanut). Additional peptide quality criteria for samples and calibrants were a retention time window of ±0.25 min, heavy peptide peak area within ±20% of the mean for each sample set, and visual inspection of the relative ion ratios for conformity.

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Table 2. Selected Transitions for Each Peptide Used to Calculate Peak Area during Targeted Proteomics

	most abundant transition		second most		third most		fourth most	
	ion	m/z	ion	m/z	ion	m/z	ion	m/z
CCNELNR	y5	645.33	y3	403.25	y6	805.36	y4	531.29
WDPDR	y3	387.19	y4	502.23	y2	290.15	b2	302.11
NLPQQCGLR	y7	858.43	y5	633.31	y6	761.37	b2	228.13
NLPQNCGFR	y7	878.39	y5	653.28	y6	781.34	b2	228.13
QILQNLR	y5	643.39	y4	530.3	y3	402.25	y6	756.47
CCNELNEFENNQR	y5	660.31	y4	531.26	y6	807.37	y3	417.22
SSNPDIYNPQAGSLR	y7	728.41	y9	1005.51	y8	842.45	y12	1330.68

RESULTS AND DISCUSSION

Identifying Tryptic Marker Peptides from Nontargeted Data

The primary goal of this study was to develop a targeted proteomics method that could accurately quantify peanut protein transferred to oil during oil-roasting of whole peanuts. Marker peptides for targeted proteomics were identified from BPB spiked into oil. A main challenge with establishing accurate analytical methods for food allergen quantification is matching the calibration curves to the samples to be analyzed. BPB was chosen for the calibration curve as it is a homogeneous mixture that most closely represents the particulates transferred to oil during the roasting process from whole peanuts, which are typically blanched in hot water or air to remove the skins before roasting.

NIST PB and LRPF were also considered for calibrants as they are similarly homogeneous, easy to disperse in oil, and have known protein concentrations. However, NIST PB is prepared from roasted peanuts, and LRPF is manufactured from roasted, partially defatted peanuts, whereas BPB is prepared from unroasted peanuts, which were briefly blanched to remove peanut skins. Because quantification relies on peptide signal intensities, any processing-induced changes that affect protein extraction or digestion can compromise the measurement accuracy. Cooking treatments such as roasting are known to induce chemical and structural modifications in food proteins,[24−26] which may reduce digestion efficiency or alter peptide recovery. As both NIST PB and LRPF are roasted, there was greater potential for processing-induced changes to impact the extraction and digestion of their proteins compared to BPB and whole peanuts, thus decreasing their quantitative accuracy.

A three-phase approach was used to identify candidate peptides. In the first phase, nontargeted proteomics was performed on oil samples spiked with BPB at 1000 μg peanut protein/mL oil that were unheated and heated for 1, 5, and 10 min, as it was critical to find peptides that performed similarly well in both unheated and heated samples. These samples generated 242 unique tryptic peptides, and the list was narrowed down to 28 candidate peptides to proceed into the second phase based on criteria detailed in Figure S1. In the second phase, oil samples spiked at 10, 100, and 1000 μg of peanut protein/mL were analyzed in targeted MS/MS mode using the m/z and retention time values for the candidate peptides generated in phase one. The aim of this phase was to confirm that in the targeted data, when the peptides and their fragment ions were specifically monitored by the mass spectrometer, there was no significant reduction in peak area from the unheated samples to the heated samples across concentrations (Figure S2). The list of candidate peptides was narrowed down to ten based on visual inspection of the data and calculated reductions of <20% in peak area from unheated to 10 min-heated samples. Finally, in phase three, samples of BPB spiked into unheated oil at concentrations ranging from 5−1000 μg peanut protein/mL were prepared to assess the linearity of the peak area (without any internal standard) for each peptide based on $R^2 > 0.95$ (Figure S3). All peptides from phase two had good linearity and so were chosen for synthesis as heavy-labeled internal standards for quantitative analysis. However, the peptides QTVEN, FNLAG, and WLGLS were later excluded after analyzing BPB calibration curves prepared with the heavy-labeled peptides. QTVEN was excluded, as the retention times for the light and heavy peptides were consistently offset by 0.2 min. FNLAG and WLGLS were excluded as they did not produce linear calibration curves, likely due to the heavy peptide internal standard failing to appear in some samples, indicating potential losses from insolubility in the reconstitution buffer or from adhesion to the autosampler vial.

Assessing the Accuracy of the Targeted Proteomics Method

In order for the developed method to be applicable to quantifying peanut protein transferred to oil during roasting, it has to be accurate across several magnitudes of concentration and after extended periods of heating. To assess the accuracy of the method, BPB was dispersed in oil at 9, 90, and 900 μg peanut protein/mL, and the mixtures were heated for 0, 1, 5, and 10 min. A calibration curve ranging from 5−1000 μg peanut protein/mL of BPB in unheated oil was prepared simultaneously, and reporting limits for each peptide were determined by the lowest calibration level with S/N > 10 (20 μg/mL for WDPDR, 5 μg/mL for all others). Subsequently, the proteins were precipitated with organic solvent and extracted with 2 M urea, 50 mM Tris-HCl. The measured peanut protein concentrations based on light/heavy ratio for these oil samples are shown in Figure 1.

In the unheated samples, the method was accurate for all of the marker peptides at the expected concentration of 900 μg/mL, with an average measured concentration of 872 μg/mL (97% recovery). At 90 μg/mL, the average concentration was again accurate at 88 μg/mL (97%). At 9 μg/mL, the WDPDR peptide was below the reporting limit for all replicates, but the remaining peptides measured an average concentration of 9.7 μg/mL (107%). While efforts were taken during the peptide selection phase to ensure the marker peptides performed equally well in the unheated and heated oil samples, there were still significant decreases in concentration after 10 min of heating. At 900 μg/mL, the average concentration across all peptides was 692 μg/mL (77%) after 10 min of heating, with a maximum of 748 μg/mL (83%) by NLPQN. At 90 μg/mL, the

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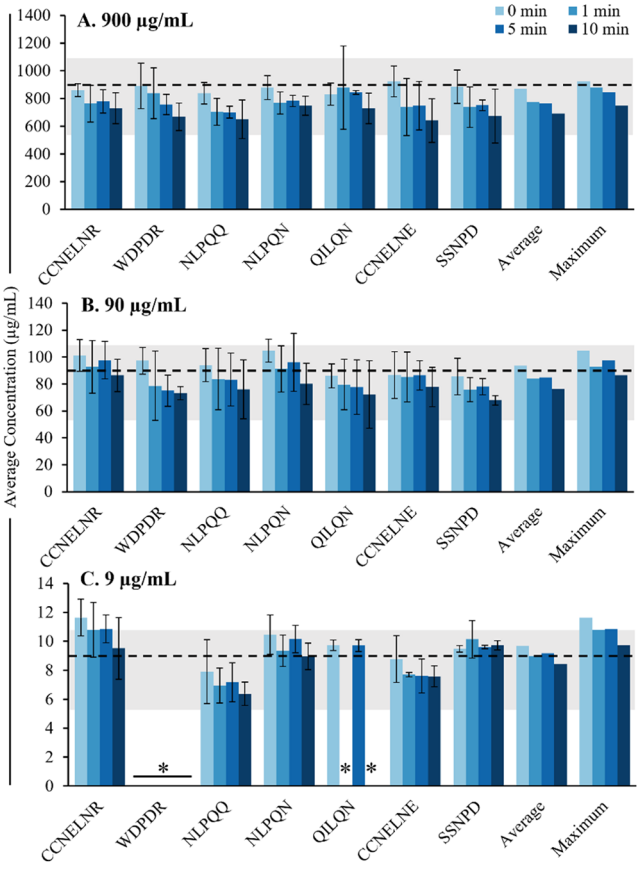

Figure 1. Measured peanut protein concentrations of BPB-spiked oil. To evaluate the accuracy of the developed targeted proteomics method, peanut butter prepared from blanched, unroasted peanuts (BPB) was dispersed in oil at known concentrations and subjected to heating (190 °C) for varying durations (0−10 min), then peanut protein levels were measured. Expected concentrations of peanut protein in oil are (A) 900, (B) 90, and (C) 9 µg peanut protein/mL and are represented by the dotted black line. "Average" represents the average concentration measured across all marker peptides and "Maximum" represents the marker peptide with the highest measured concentration. The gray box represents an acceptable recovery range of 60−120%. Data are shown as the mean ± 95% CI (n = 4). *, The peptide was below the reporting limit (S/N < 10, where the noise is defined as the peak area of the blank oil) in the majority of replicates.

average concentration was 71 µg/mL (79%) with a maximum of 86 µg/mL (96%) by CCNELNR. At 9 µg/mL, most replicates for both WDPDR and QILQN were below their reporting limits. Of the remaining peptides, the average concentration detected was 8.4 µg/mL (94%), and the maximum was 9.7 µg/mL (110%) by SSNPD. The recommended recovery range for mass spectrometry methods for the detection and quantification of peanut allergens is within 60−120%, per AOAC standard method performance requirement (SMPR) 2016.002.[27] Despite heat-induced losses in recovery, all peptides fell within this range across all heating times and concentrations, except for WDPDR and QILQN at 9 µg/mL.

In the present study, matrix effects of the roasting oil were accounted for by using a matrix-matched calibration curve of BPB in oil without subjecting the mixture to a heating step. The calibration curve was developed as unheated for improved accessibility and ease of preparation. This represents a limitation of the method, as this resulted in an underestimation of the amount of peanut protein in oil samples that were heated, though some marker peptides performed better than others. The reason for these differences is due to the physicochemical effects of heat processing on food proteins. Processing at roasting temperatures and durations will not break the linear sequences of food proteins,[28,29] but it does cause them to lose their secondary and tertiary structures, undergo Maillard reaction, and form less-soluble globular aggregates.[30]

The stability of peanut proteins after heat treatment differs among the allergens, with Ara h 2 best maintaining its structure and solubility,[31−33] thus potentially explaining why the peptides NLPQQ and CCNELNE from Ara h 2 and NLPQN and CCNELNR from Ara h 7 returned better recovery values after heating. On a structural level, Ara h 2 and Ara h 7 are 2S albumin proteins of similar length (~20 kDa) with approximately 50% sequence homology and compact structures,[34] which may confer greater thermal stability compared to the larger, more complex structures of vicilins (e.g., Ara h 1) and legumins (e.g., Ara h 3). Additionally, the CCN peptides contain cysteine residues that participate in a disulfide bond with cysteine in the NLP peptides,[35] which may protect this core region from heat-induced unfolding and aggregation. Similar regional characteristics in other allergens could be exploited to release high-recovery marker peptides that can improve the quantitative accuracy for challenging foods.

Testing the Targeted Method against Other Peanut Protein Sources

During the roasting process of whole peanuts, the heat and agitation of the oil cause particulates to release from the peanut. As finished batches are removed and fresh batches are added, these particulates exist in varying sizes and extents of exposure to the hot oil. Due to the heterogeneous nature of these particulates and difficulties in isolating and characterizing their protein content, it was impossible to directly validate the BPB method for whole peanuts. Instead, the method was applied to NIST PB and LRPF-spiked oil to assess whether the BPB-based calibration curve was applicable to different peanut sources.

NIST PB and LRPF were mixed into oil at 900 µg/mL and heated for 0, 1, 5, and 10 min. The proteins were precipitated with organic solvent and extracted with 2 M urea and 50 mM Tris-HCl. The measured concentrations of peanut protein are shown in Figure 2. As with the BPB, measured concentrations in the unheated NIST PB samples aligned closely with the expected concentration of 900 µg/mL, with an average of 926 µg/mL (103% recovery), although QILQN overestimated the concentration beyond the AOAC upper recommended recovery limit of 120% at 1188 µg/mL (132%). In the LRPF samples, the average measured concentration was lower at 738 µg/mL (82%), and CCNELNE underestimated peanut protein concentration below the AOAC lower recommended recovery limit of 60% at 538 µg/mL (59%).

Despite good agreement in the unheated samples among BPB, NIST PB, and LRPF, the impact of heating on measured concentrations was observed to be more severe with NIST PB and LRPF than with BPB. For NIST PB after 10 min of heating, the average measured concentration was 434 µg/mL (48% recovery) with a maximum of 607 µg/mL (67%) from QILQN, while in LRPF, the average was 418 µg/mL (47%) with a maximum of 542 µg/mL (60%) from CCNELNR. For

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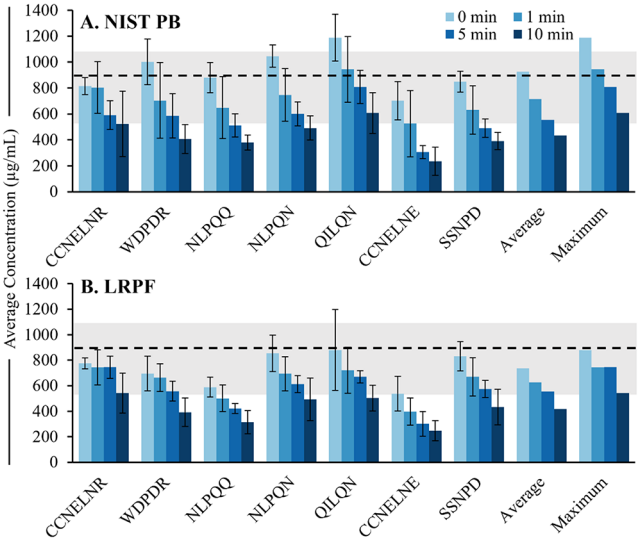

Figure 2. Measured peanut protein concentrations of NIST-PB- and LRPF-spiked oil. To evaluate the applicability of the targeted proteomic method across peanut protein sources, NIST peanut butter (A) and light-roasted peanut flour (B) were spiked into oil at 900 μg peanut protein/mL and concentrations were measured. "Average" represents the average concentration measured across all marker peptides and "Maximum" represents the marker peptide with the highest measured concentration. The dotted black line represents the expected concentration of 900 μg peanut protein/mL. The gray box represents an acceptable recovery range of 60−120%. Data are shown as the mean ± 95% CI (n = 4).

both peanut sources, all peptides except for the maximum recovery peptide were below the lower AOAC-recommended percent recovery after 10 min heating.

Effects of Extraction Conditions on Recovery

Consistently across the three peanut sources, protein recovery decreased as the heating time increased, though some peptides such as CCNELNR and NLPQN were less affected than others. As proteins that are thermally denatured tend to form aggregates and precipitate, different extraction conditions were applied to the BPB, NIST PB, and LRPF samples to determine whether the heated proteins could be more efficiently solubilized, digested, and analyzed. Oil samples were prepared at 900 μg/mL and either not heated or heated for 10 min. Two sets of samples underwent organic precipitation, and then the pellet was resuspended with either a 2 M urea, 50 mM Tris-HCl, 50 mM DTT buffer or an 8 M urea, 50 mM Tris-HCl, 50 mM DTT buffer. Urea is often included in extraction buffers as a chaotropic agent that disrupts protein hydrogen bonds and exposes hydrophilic residues to increase solubility.[36] DTT is also included in extraction buffers as a reducing agent to limit protein oxidation and break disulfide bonds that can complicate peptide identification.[37] A third set of samples was extracted by directly mixing the oil samples with 6 M urea, 50 mM Tris-HCl, 20 mM DTT buffer, as this was identified as the optimal extraction procedure for cashew proteins in roasting oil.[22] Matrix-matched calibration curves were prepared alongside samples by using the same extraction conditions. The measured peanut protein concentrations of these samples are shown in Figure 3.

For the samples extracted with 2 M urea, 50 mM Tris-HCl, and 50 mM DTT (Figure 3A), results from the unheated BPB samples were similar to those obtained with the initial extraction method (Figure 3D). The average concentration across peptides was 933 μg/mL (104% recovery), though CCNELNE overestimated the concentration beyond 120%

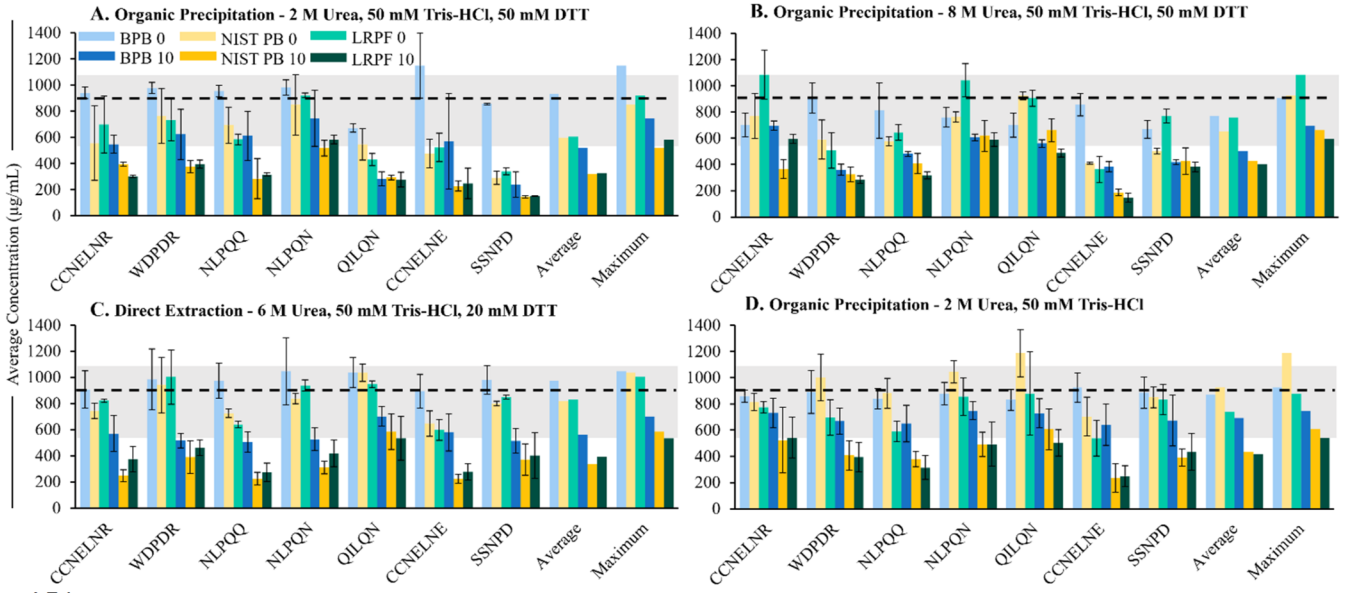

Figure 3. Impact of extraction conditions on peanut protein recovery. Peanut protein concentrations in unheated and 10 min-heated oil spiked with blanched peanut butter (BPB), NIST peanut butter (NIST PB), and light-roasted peanut flour (LRPF) were determined after extraction using (A) organic precipitation with 2 M urea, 50 mM Tris-HCl, 50 mM DTT, (B) organic precipitation with 8 M urea, 50 mM Tris-HCl, 50 mM DTT, and (C) direct extraction with 6 M urea, 50 mM Tris-HCl, 20 mM DTT. (D) Data from the original extraction method is also included to facilitate comparisons. The 0 and 10 following the abbreviations in the figure legend refer to 0 min heating and 10 min heating, respectively. "Average" represents the average concentration measured across all marker peptides and "Maximum" represents the marker peptide with the highest measured concentration. The dotted black line represents the expected concentration of 900 μg peanut protein/mL. The gray box represents an acceptable recovery range of 60−120%. Data are shown as the mean ± 95% CI (n = 4).

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recovery. However, the average recovery for all peanut sources was <60% recovery after 10 min heating. Increasing the amount of urea in the extraction buffer to 8 M (Figure 3B) improved average unheated LRPF recovery to 772 μg/mL (86%), even higher than the recovery obtained with the initial extraction method (Figure 3D), but average unheated BPB recovery was lower than the original, and all peanut sources were below 60% recovery after 10 min heating. Direct extraction by mixing the spiked oil sample into 6 M urea, 50 mM Tris-HCl, 20 mM DTT buffer (Figure 3C) had the best average recovery across all unheated peanut sources, but 10 min-heated BPB was still lower than the initial extraction (Figure 3D) at 559 μg/mL (62% recovery), and heated NIST PB and LRPF recoveries were both below 60%.

None of the different extraction methods had a significant improvement over the original method using 50 mM Tris-HCl and 2 M urea. Direct extraction and/or increasing the concentration of urea and DTT to further unfold proteins did not significantly improve protein recovery in the present study, indicating potential covalently cross-linked thermal aggregates of proteins and Maillard reaction products that contributed to extraction and/or digestion resistance.[38−40] Stronger denaturing reagents such as guanidine hydrochloride or surfactants such as sodium dodecyl sulfate may improve extraction but need additional cleanup as they can interfere with digestion and ionization.[41,42] Deglycosylation by either enzymatic or chemical means may also improve protein solubility by cleaving off covalently linked sugars and freeing the protein backbone from thermal aggregates.[43] Incorporating some of these methods in future extraction procedures may improve the recovery of heated proteins, but will need further testing.

Quantifying the Amount of Peanut Protein in Oil after Batch-Roasting

Oil samples used to batch-roast whole peanuts contain an unknown amount of peanut protein, and no calibrant exists that perfectly represents the particulates released by whole peanuts during batch-roasting. Thus, in order to assess the targeted proteomics method's recovery during batch-roasting, a controlled model experiment was performed to mimic roasting conditions. BPB was spiked into the oil at an initial concentration of 75 μg/mL and heated for 5 min. The oil was sampled, and a fresh spike was introduced and heated, mimicking the roasting of subsequent batches of whole peanuts. The results of the model experiment are shown in Figure 4.

As with the previous results, each peptide had different recoveries once the proteins were heated (Figure 4A). Across all peptides, the average recovery was 119% in the unheated oil sample at B0, with a maximum of 131% by SSNPD. The average recoveries remained >60% until B5, and at B10, the average recovery was determined to be 53%. However, the maximum recovery for each heated batch was within the 60−120% range (93% at B10), and peptides NLPQQ and NLPQN averaged 90% recovery across all of the batches, respectively. CCNELNE also had an average recovery of 96% until B8. These three peptides have been used several times by previous targeted methods as peptide markers for peanut allergen quantification.[21,44−46] The present data indicates that, in heated samples, using the maximum measured concentration from all marker peptides may be the most appropriate when calculating peanut protein concentration in a sample.

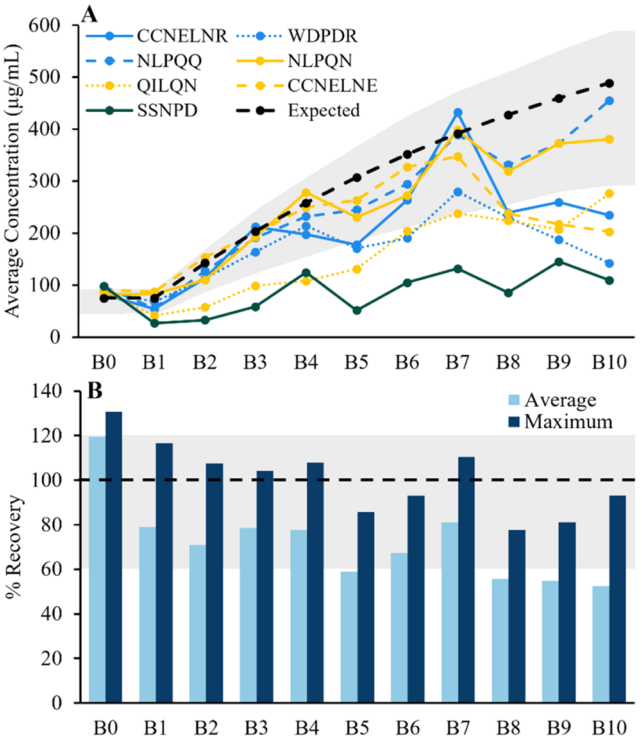

Figure 4. Peanut protein recovery in a model experiment to mimic batch-roasting. Batches of blanched peanut butter were spiked into oil and roasted for 5 min, mimicking conditions used to roast whole peanuts. (A) The average peanut protein concentrations of the oil samples as determined by each marker peptide after each batch. Batches are shown on the *X*-axis, with B0 representing the initial spike into unheated oil, B1 representing the initial spike after 5 min heating at 190 °C, B2 representing the second spike, and so on. The expected concentration of peanut protein is shown by the dotted black line. Data are shown as the mean (n = 6). (B) The % recovery of peanut protein from the oil samples after each batch. "Average" represents the average concentration measured across all marker peptides (n = 7) and "Maximum" represents the marker peptide with the highest measured concentration at each batch. For both figures, the gray region represents an acceptable recovery range of 60−120%.

Next, whole peanuts were batch-roasted in oil, and the protein concentration was measured using ELISA, BCA, and targeted proteomics (Figure 5). Visual inspection of the oil samples clearly indicated the increasing presence of peanut particulates based on the cloudiness of the oil (Figure 5A). Peanut-specific ELISA kits from Neogen and Ridascreen were used to measure peanut protein concentrations, and those results are shown along with the BCA results in Figure 5B. While BCA measures total protein and not peanut protein specifically, the unused oil had no detectable protein in it prior to roasting peanuts; thus, any measured protein had to be from the whole peanuts. BCA results show increasing protein concentration as each batch of peanuts was roasted, matching the visual appearance of the oil samples, which shows an increase in oil turbidity. The maximum measured concentration was 377 μg/mL at B10. The two ELISA kits show increasing concentration until B2, at which point they both level off at around 165 μg/mL for Neogen and 108 μg/mL for Ridascreen kits.

From the targeted proteomics data (Figure 5C), the trend of increasing peanut protein concentration with each batch more closely resembled the visual and BCA results, particularly for

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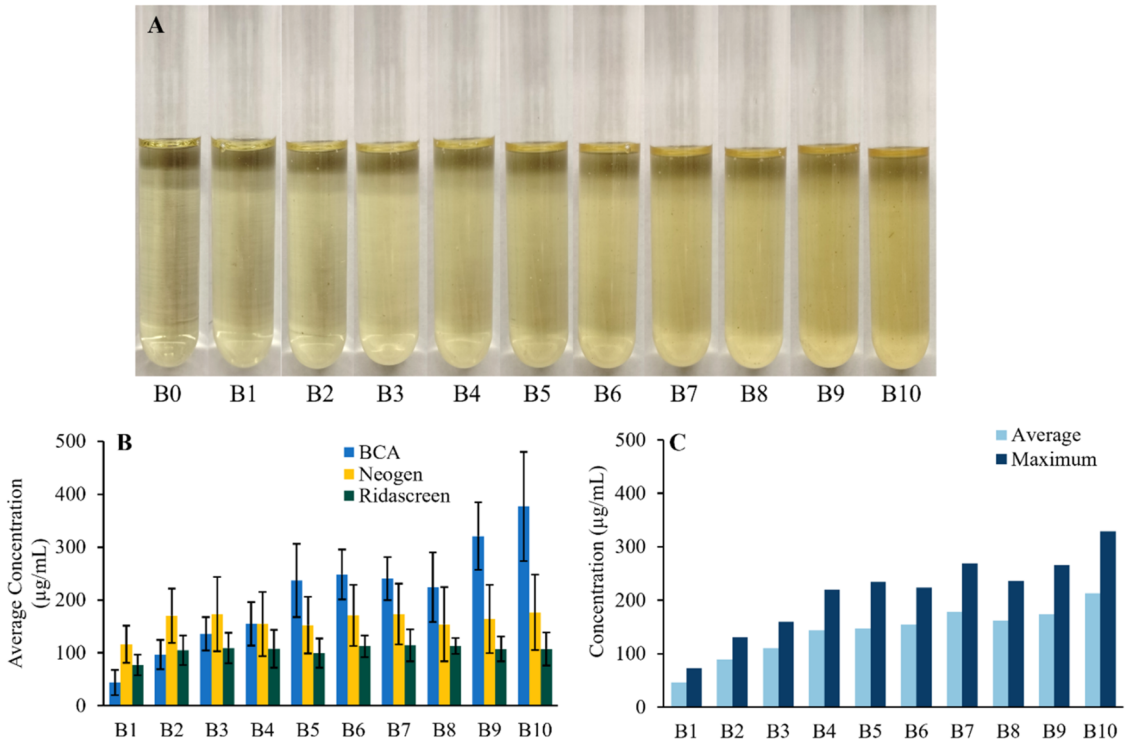

Figure 5. Peanut protein concentrations of oil used to batch-roast whole peanuts. Whole blanched peanuts were batch-roasted in oil, and oil samples were collected after each batch to assess peanut protein transfer to oil. (A) Photographs of the oil samples collected after each batch from one of the trials. B0 is the fresh oil (negative control), B1 represents the oil collected after the first batch of whole peanuts, B2 represents the oil after the second batch, and so on. (B) The measured peanut protein concentrations of the oil after each batch as determined by BCA and peanut-specific ELISA analyses (Neogen and Ridascreen kits). Data are shown as mean ± 95% CI (n = 6). (C) The measured peanut protein concentrations of the oil after each batch as determined by targeted proteomics. "Average" represents the average concentration measured across all marker peptides (n = 7) and "Maximum" represents the marker peptide with the highest measured concentration at each batch.

the maximum concentrations. Each marker peptide again responded differently to heat (Figure S4), with the average across all peptides increasing to 213 μg/mL and the maximum concentration increasing to 329 μg/mL at B10. Based on the above model experiment, which determined the average recovery of 53% and a maximum of 93% after 10 min, we can correct the average measured amount to be estimated at 405 μg/mL, and correcting the maximum in the same way results in 353 μg/mL (Table 3). However, it should be noted that in the BPB model, the dispersed particulates experience the full thermal treatment duration, whereas particulates from the whole peanuts are released gradually throughout roasting. This likely results in differential thermal exposure, aggregation, and precipitation of peanut proteins, and the corrected value of 405 μg/mL represents a conservative, safety-focused estimate.

Table 3. Average and Maximum Concentrations of Whole Peanut Protein in Oil Measured with the Marker Peptides with and without Correction Factors Calculated from the Model Experiment

	measured AVG (μg/mL)	measured MAX (μg/mL)	corrected AVG (μg/mL)	corrected MAX (μg/mL)
B1	46	73	58	63
B2	88	131	125	122
B3	110	160	141	153
B4	144	220	185	205
B5	147	235	249	274
B6	155	224	230	240
B7	179	269	221	244
B8	162	236	291	304
B9	174	266	317	328
B10	213	329	405	353

Understanding the amount of peanut protein in cooking oil after roasting is important for determining the potential transfer of allergenic proteins to other fried foods due to cross-contact. Developing and validating methods for the quantification of peanut in all possible combinations of fried food types and processing conditions is currently impractical. Until direct methods can be developed, the risk can be indirectly estimated from the allergen content of the oil and the amount of oil that the fried food absorbs. As water evaporates during the frying process, oil fills the voids in the food structure, with the amount depending on the fried food, oil type, temperature, duration, and cooling.[47] French fries absorb around 10% by weight of oil,[48,49] thus a 100 g serving of French fries fried in oil used to roast ten batches of peanuts could contain 4.1 mg of peanut protein, based on the present study's results. This value exceeds the reference dose (2 mg) for unintended peanut presence associated with appreciable risk to health in individuals with IgE-mediated peanut allergy.[50]

Quantifying the Amount of Peanut Protein in Oil after Regeneration

Accurate quantification of peanut protein in roasting oil is also necessary to assess the effectiveness of regeneration treatments for removing peanut from the oil. In industrial and food service settings, common regeneration practices for removing food particulates that transfer to oil during frying include settling

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(allowing particulates to drift to the bottom of the container) and passive filtration (utilizing sieves and filters to remove particulates of specific sizes), among others.[51] The majority of quality control measures for oil regeneration have primarily focused on evaluating parameters related to oil quality, such as lipid oxidation, color, and viscosity, rather than allergen content.[52]

After 10 batches of peanuts were roasted, the oil was allowed to cool to RT, and then aliquots of it were prepared following common oil regeneration procedures to determine their efficacy in removing peanut protein (Figure 6). Allowing the

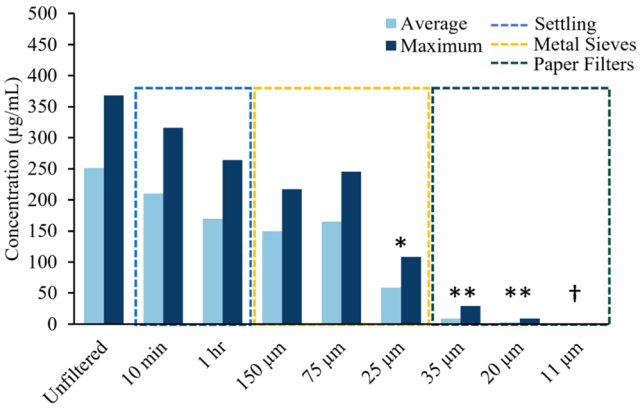

Figure 6. Impact of filtration on reducing peanut protein levels in oil. Oil used to roast 10 batches of whole blanched peanuts was filtered using various regeneration treatments, and the peanut protein concentrations of the treated oil were determined by targeted proteomics. "Average" represents the average concentration measured across all marker peptides (n = 7) and "Maximum" represents the marker peptide with the highest measured concentration at each batch. *, 1 peptide was below the reporting limit in the majority of replicates and was excluded from the average (n = 6); **, 4 peptides were below reporting limits (n = 3); †, all peptides were below reporting limits.

peanut particulates to settle for 10 min reduced the maximum measured peanut concentrations from 368 μg/mL in the unfiltered oil to 316 μg/mL (395 to 340 μg/mL, with the correction factor from the model experiment). Settling for 1 h further reduced the maximum measured protein concentration to 264 μg/mL (284 μg/mL corrected). These results indicate that settling is not an effective means of removing food allergens, as only minor reductions of peanut concentration in the oil were identified.

Filtering the oil through stainless steel sieves with pore sizes of 150 and 75 μm achieved similar protein concentrations as 1 h settling with maximum concentrations of 217 and 246 μg/mL (234 and 264 μg/mL corrected), respectively. Filtering through 25 μm sieves further decreased the concentration to a maximum of 108 μg/mL (116 μg/mL corrected), with most replicates for WDPDR falling below its reporting limit of 20 μg/mL. Finally, filtering the oil through paper filters with pore sizes of 35 and 20 μm reduced protein concentrations to a maximum of 29 and 8.6 μg/mL (32 and 9.3 μg/mL corrected), respectively, with four peptides below the reporting limit for each. Filter papers with a pore size of 11 μm reduced peanut protein levels to below the reporting limits for all of the peptides.

For passive filtration, choosing the correct size filter is important to achieve adequate reduction of protein levels. Although the sieves tested in this study were effective in removing the larger particulates, enough peanut particulates were passed through the 150 and 75 μm sieves that could result in >2 g peanut protein in a 100 g serving of French fries prepared in oil at that peanut protein concentration. The paper filters performed better than the sieves even at larger pore sizes, likely due to some of the finer particulates adsorbing to the paper matrix.[53] While some of the marker peptides were still detectable at 35 and 20 μm, the concentration was low enough that oil reuse would likely pose little risk to a consumer with a peanut allergy.

The targeted proteomics method developed in this work and results from the whole peanut experiment provide crucial data for understanding allergen transfer to cooking oil and improving control measures around cooking oil reuse. Future applications of this method should be used to assess peanut cross-contact risk after active filtration of roasting oil and in industrial-scale fryers, which may alter the levels of peanut protein in oil. Further refinement of the protein extraction protocol may also improve the accuracy and sensitivity of the method, as it is applied to more diverse processing environments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.5c15844.

Materials and Methods, collision energies used for fragmentation during proteomics analysis (Table S1); decision tree flowchart used to narrow down peptides identified during nontargeted analysis of BPB-spiked oil samples (Figure S1); average peak areas of phase one candidate peptides extracted from BPB-spiked oil that was unheated or heated (Figure S2); average peak areas of phase two candidate peptides extracted from BPB-spiked prepared at 5−1000 μg/mL (Figure S3); measured peanut concentrations of oil used to roast whole peanuts by individual marker peptide (Figure S4) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assays; BPB, blanched peanut butter; DTT, dithiothreitol; IAA, iodoacetamide; ABC, ammonium bicarbonate; ACN, acetonitrile; FA, formic acid; BCA, bicinchoninic acid; NIST PB, NIST peanut butter; LRPF, light-roasted peanut flour; RT, room temperature; S/N, signal-to-noise ratio; SMPR, standard method performance requirement

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