

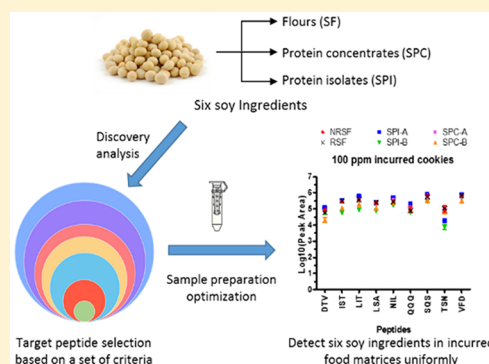
Detection of Six Commercially Processed Soy Ingredients in an Incurred Food Matrix Using Parallel Reaction Monitoring

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S Supporting Information

ABSTRACT: Soybeans are one of the major allergenic foods in many countries. Soybeans are commonly processed into different types of soy ingredients to achieve the desired properties. The processing, however, may affect the protein profiles and protein structure, thus affecting the detection of soy proteins. Mass spectrometry (MS) is a potential alternative to the traditional immunoassays for the detection of soy-derived ingredients in foods. This study aims to develop a liquid chromatography–tandem MS method that uniformly detects different types of soy-derived ingredients. Target peptides applicable to the detection of six commercial soy ingredients were identified based on the results of MS label-free quantification and a set of selection criteria. The results indicated that soy ingredient processing can result in different protein profiles. A total of six soy ingredients were then individually incurred into cookie matrices at different levels. Sample preparation methods were optimized, and a distinct improvement in peptide performance was observed after optimization. Cookies and dough incurred with different soy ingredients at 100 ppm total soy protein showed a similar level of peptide recovery (90% mean signal relative to unroasted soy flour), demonstrating the ability of the MS method to detect processed soy ingredients in a uniform manner.

KEYWORDS: soybean, food allergen, parallel reaction monitoring



INTRODUCTION

Soybean (*Glycine max*), a species of legume from the Fabaceae family, is one of the most important sources of nutrition around the world. However, soybean is also a regulated allergenic food in the United States, European Union, and many other countries.^{1,2} Food allergy is an increasing concern in many countries around the world, affecting an estimated 4–8% of children and 3–4% of the overall population.³ Approximately 0.4% of children are allergic to soy, although about 50% of them outgrow their soy allergy by the age of 7.^{4–6} Because there is no effective treatment for soy allergy, or any other food allergy, complete avoidance of the offending food is the only option for allergic individuals.

Currently, enzyme-linked immunosorbent assay (ELISA) is the major method for detection and quantification of soy proteins in foods. Commercial soy ELISA kits are able to reach very low limits of quantitation (1–5 ppm soy protein). However, a major disadvantage of ELISA is the reliance on the integrity of immunorecognition between antibodies and antigens, which could be affected by treatments that are commonly used in food processing, such as high-pressure, thermal, and proteolytic processing. Moreover, soy ingredient manufacturers often use different combinations of heat treatment, acid precipitation, alkaline precipitation, enzymatic hydrolysis, and acid hydrolysis to achieve desirable flavors and

functional properties in soy ingredient processing.^{7,8} These processing methods, however, may cause changes in the protein profiles and protein structures. The wide variety of soy ingredients used in food products could be particularly challenging for ELISA detection largely because only limited types of ingredients were used to generate antibodies for ELISA. Therefore, to protect soy-allergic consumers, it is crucial to have an accurate detection and quantification method capable of detecting different types of processed soy ingredients. In recent years, mass spectrometry (MS) has been considered a promising food allergen detection and quantification method.^{9–12} One of the major advantages of MS over ELISA is that it relies on the detection of peptides instead of epitopes, thus eliminating the reliance on epitope integrities. Moreover, MS allows the use of relatively harsh extraction conditions, which are not widely adopted in ELISA methods due to the potential damage to immunorecognition.^{13,14} MS, therefore, can provide opportunities for detection of soy proteins derived from different processed soy ingredients.

Among the MS methods applied to food allergen detection, selected reaction monitoring (SRM) has been used in many allergen detection and quantification studies.^{12,15–17} In 2012,

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Peterson et al. proposed a novel targeted method called parallel reaction monitoring (PRM).¹⁸ Compared with SRM, for which only one transition is monitored at a time, in PRM, all fragment ions of a given precursor ion are scanned in parallel in a high-resolution mass analyzer.¹⁹ One of the biggest advantages of developing a PRM assay is that there is no need for selecting transitions ahead of time, thus reducing the time and effort necessary for method development. PRM has been applied to relative quantification of protein abundance and validation of post-translational modification in multiple studies.^{20–22}

The first objective of this research was to select peptide targets from soy that would be representative of a wide range of soy-derived ingredients. The second objective was to develop a qualitative method for the detection of soy-derived ingredients in an incurred cookie matrix using PRM.

■ EXPERIMENTAL SECTION

Reagents and Materials

Ammonium bicarbonate (AMBC), Tris(hydroxymethyl) aminomethane (Tris), iodoacetamide (IAA), and polyvinylpyrrolidone (PVPP) were purchased from Sigma-Aldrich. Sequencing grade modified trypsin was purchased from Promega. Acetonitrile (ultrahigh-performance liquid chromatography–mass spectrometry, UHPLC–MS, grade) and formic acid were from Fisher Scientific. Pierce 660 nm protein assay reagent, trifluoroacetic acid (TFA), dithiothreitol (DTT), and C18 spin columns were purchased from Thermo Fisher Scientific. Urea was purchased from Bio-Rad. Molecular-weight cutoff (MWCO) filter devices (10 kDa and 3 kDa) were purchased from Millipore.

A total of six commercially processed soy ingredients were obtained from Archer Daniels Midland Company, including nonroasted and roasted soy flours (NRSF and RSF), two protein isolates (SPI-A and B), and two protein concentrates (SPC-A and B) (see detailed product information in Table S1). The total protein content of each soy ingredient was determined by the LECO Dumas method. The bleached wheat flour was obtained from General Mills Operations. Other ingredients for the cookie matrix were purchased from a local grocery store.

Soy Protein Extraction Evaluation and SDS-PAGE Analysis

Various extraction buffers were used to compare extraction efficiency. Nonroasted soy flour (0.07 g) was extracted at a 1:20 sample-to-buffer ratio (w/v) using different extraction buffers (see complete information in Table 1). The samples were placed in a 60 °C shaking water bath for 30 min, followed by centrifugation at 13000g for 10 min. Soluble protein concentration was determined using the Pierce 660 nm protein assay. Sample extracts were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition using Tris-glycine gels.

Formulation of Allergen-Incurred Cookies

The formulation of the model sugar cookies was based on the AACC International method no. 10-50.05. The method was modified to include double the mixing time to ensure the even distribution of soy ingredients. All of the ingredients used in the formulation (wheat flour, butter, sugar, salt, baking soda, and dextrose) were tested using the Neogen Veratox soy allergen ELISA kit to ensure the absence of soy. None of these ingredients were found to have soy proteins above the limit of

Table 1. Comparison of the Protein Extraction Efficiency from Unroasted Soy Flour

extraction solution	average soluble protein concentration (mg/mL) ^a
A. 50 mM AMBC, 2 M Gu-HCl, 20 mM DTT, 1% PVPP	12.07 ± 2.36
B. 50 mM AMBC, 2 M Gu-HCl, 1% PVPP	13.30 ± 1.81
C. 50 mM AMBC, 2 M Gu-HCl, 20 mM DTT	12.39 ± 1.20
D. 50 mM AMBC, 2 M Gu-HCl	14.21 ± 2.00
E. 50 mM AMBC, 6 M urea, 20 mM DTT, 1% PVPP	16.24 ± 4.00
F. 50 mM AMBC, 6 M urea, 1% PVPP	16.77 ± 2.84
G. 50 mM AMBC, 6 M urea, 20 mM DTT	14.72 ± 1.22
H. 50 mM AMBC, 6 M urea	17.14 ± 0.81
I. 50 mM Tris-HCl, 2 M Gu-HCl, 20 mM DTT, 1% PVPP	12.66 ± 4.40
J. 50 mM Tris-HCl, 2 M Gu-HCl, 1% PVPP	12.27 ± 2.02
K. 50 mM Tris-HCl, 2 M Gu-HCl, 20 mM DTT	13.05 ± 1.75
L. 50 mM Tris-HCl, 2 M Gu-HCl	13.56 ± 1.91
M. 50 mM Tris-HCl, 6 M urea, 20 mM DTT, 1% PVPP	21.32 ± 2.24
N. 50 mM Tris-HCl, 6 M urea, 1% PVPP	17.87 ± 2.05
O. 50 mM Tris-HCl, 6 M urea, 20 mM DTT	17.32 ± 1.36
P. 50 mM Tris-HCl, 6 M urea	16.11 ± 2.55
Q. PBS, pH 7.4	11.48 ± 1.35

^aMean plus or minus the standard deviation ($n = 6$; 3 extracts were analyzed in duplicate). ^bProtein concentration determined using the Pierce 660 nm protein assay.

quantification of the ELISA (LOQ: 2.5 ppm soy flour). To ensure the even distribution of the soy ingredients, well-mixed 2500 ppm soy protein in flour and 1000 ppm soy protein in flour (micrograms of soy protein per gram of wheat flour) pre-spikes were prepared beforehand for soy flour and other soy ingredients, respectively. The pre-spikes were prepared by adding appropriate amounts of soy ingredients to wheat flour followed by a 25 min thorough mixing in a KitchenAid stand mixer. The pre-spikes were laid out on a flat sheet, and five samples (one from each of the four corners and one from the center) were taken to verify the homogeneity of the pre-spikes. The homogeneity of the pre-spikes was verified using the Veratox soy ELISA kit.

The formulation of cookies was as follows: 47.6% wheat flour, 27.5% sugar, 13.5% unsalted butter, 7% dextrose solution (6%, w/v), 3.4% water, 0.5% baking soda, and 0.4% salt. To produce the desired concentration of soy protein in the model food, the pre-spikes replaced a certain amount of the wheat flour in the formulation. Butter, salt, sugar, and baking soda were mixed in a KitchenAid stand mixer for 3 min, dextrose solution and distilled water were added and mixed for an additional 2 min, and individual pre-spikes and wheat flour were added and mixed for 4 min. The incurred dough samples were prepared at 100 micrograms of soy protein per gram of dough level for all soy ingredients. Following dough production, half of the dough was kept aside for analysis of the raw dough, while the remaining dough was divided into small portions with similar weight (approximately 12 g) and baked on a tray in a conventional oven (205 °C) for 10 min. Moisture loss of the baked cookies was determined by

measuring the weight difference of cookie dough before and after baking (11.5% weight loss on average). Subsequently, all cookies and dough samples were ground using an Osterizer blender (model no. 6640, Sunbeam Products, Inc.) and stored at -20°C until further use.

Sample Preparation for Discovery Proteomics Analysis

Sample extracts were diluted to less than $1\text{ }\mu\text{g}/\mu\text{L}$ prior to reduction (determined by Pierce 660 nm protein assay). Sample extracts were reduced with 5 mM DTT (final concentration) at 95°C for 5 min, followed by alkylation with 10 mM iodoacetamide (final concentration) in the dark for 20 min at room temperature. The alkylated proteins were digested with trypsin at a 1:100 (w/w) trypsin to protein ratio at 37°C for 3 h followed by overnight digestion (at least 12 h) at a 1:50 (w/w) trypsin to protein ratio at the same temperature. Equal amounts of digested and undigested proteins were compared side by side using SDS-PAGE to confirm complete digestion. The samples were cleaned-up with Pierce C18 spin columns following the manufacturer's instructions. Samples were then dried in a vacuum evaporator and reconstituted in 0.1% (v/v) formic acid prior to discovery analysis by LC-high-resolution, accurate-mass (LC-HRAM) MS analysis (described below).

Optimization of Sample Preparation for Targeted MS Detection in Incurred Food Matrices

In the optimized extraction method, 40 min shaking on a rotator at the speed of 200 rpm was added to the original extraction protocol. For the optimization of digestion method, the effect of the filter-aided sample preparation (FASP)²³ method was compared to the original preparation method. Briefly, 105 μL of the sample extracts (approximately 1 microgram of protein per microliter determined by Pierce 660 nm protein assay) were reduced with 5 mM DTT (final concentration) at 95°C for 5 min and alkylated with 10 mM IAA (final concentration) in the dark for 20 min. Samples were then loaded onto 10 kDa MWCO filter devices and centrifuged at 14000g for 15 min, and the flow-through was discarded. Proteins retained on the filter were resuspended in 160 μL of 50 mM AMBC with or without 1 M urea. Samples were then digested by trypsin at a 1:100 (w/w) trypsin to protein ratio at 37°C for 3 h followed by overnight digestion (at least 12 h) at a 1:50 (w/w) trypsin to protein ratio at the same temperature. Following the overnight digestion, the filter devices were centrifuged at 14000g for 15 min to elute digested peptides. The digested samples (100 μL) were cleaned using C18 spin columns following the manufacturer's instructions. Samples were then dried in a vacuum evaporator and reconstituted in 20 μL of 0.1% (v/v) formic acid prior to liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.

LC–HRAM MS Settings: Discovery Analysis

Peptide separation was conducted on a Thermo Fisher Dionex UltiMate 3000 RS ultrahigh performance liquid chromatography (UHPLC) system, in which samples were loaded onto a Hypersil GOLD C18 Selectivity LC Columns (100 mm $\times$ 1 mm i.d.) with 1.9 μm silica particles (175 Å). The column flow rate was maintained at 0.06 mL/min. Peptides were eluted with a linear gradient of 2–40% solvent B over 68 min, where mobile phase A consisted of 0.1% (v/v) formic acid in water and mobile phase B consisted of 0.1% (v/v) formic acid in acetonitrile. The injection volume was 5 μL . The mass

spectrometric analysis was accomplished using a Q Exactive Plus Orbitrap mass spectrometer operated in positive ionization mode. Source parameters were as follows: positive ion spray voltage of 3700 V, heated electrospray ionization (HESI) probe heater temperature of 150°C , sheath gas flow of 20 arbitrary unit (au) and auxiliary gas of 5 au, and S-lens RF of 60 au, and normalized collision energy of 27. Automatic gain control (AGC) target for MS1 was 1×10^6 , and the AGC for MS2 was 1×10^5 . Maximum injection time (IT) for MS1 was 100 ms, and the maximum IT for MS2 was 60 ms. The top 10 abundant peptides were selected for fragmentation with 3 s dynamic exclusion window. Intensity threshold was set at 2.5×10^4 . Mass isolation window for precursor selection was 2.0 m/z with a $-0.4\text{ }m/z$ offset. The resolution for both MS1 and MS2 was 70 000. Charge states that were unassigned and higher than or equal to 6 were excluded.

Each sample was analyzed with duplicate injections in LC–HRAM MS in a randomized order with one blank placed between every injection to avoid chromatographic carry-over. Protein and peptide identification was performed on Proteome Discoverer (version 2.1.1.21), allowing a 1% false discovery rate (FDR) at both the protein and the peptide levels. Precursor mass tolerance was 10 ppm. Fragment mass tolerance was 0.6 Da. The identification is based on a customized protein database, which is the combination of all *Glycine max* protein sequences and common lab contaminant protein sequences (all protein sequences of *G. max* from UniProt (accessed December 8, 2015) and the Global Proteome Machine contaminants database, for a total of 100 342 sequences). The search parameters allowed two missed tryptic cleavages, static carbamidomethyl (C) and variable oxidation (M) modifications. Sequest HT was used as the search algorithm. Label-free quantification was conducted based on MS1 peak area information acquired from Skyline²⁴ (version 3.5.0.9319) using the same parameter settings. The data analysis and presentation were performed using GraphPad Prism (version 7.03). The discovery analysis data have been deposited to the ProteomeXchange Consortium via the PRIDE²⁵ partner repository with the data set identifier PXD008699.

LC–HRAM MS Settings: Targeted Analysis

HPLC and MS instrument parameters were optimized during the course of method development to improve performance in increasingly complex matrices. HPLC conditions were initially maintained as described above. Preliminary targeted MS analyses of soy-derived ingredients were run with an inclusion list consisting of the selected precursors in scheduled 5 min retention time windows. The scan events consisted of MS1 full scan at a resolution of 17 500 from 400 to 2000 m/z followed by PRM scans of the precursors in the inclusion list at the resolution of 17 500. AGC for MS2 was set at 2×10^4 . Fragmentation was performed with the normalized collision energy of 27. Isolation window for MS2 was set to be 1.6 m/z without offset. Maximum IT for MS1 was set at 50 ms and, for MS2, was set automatically.

For the analysis of food matrix samples, similar settings were used with some modifications made to optimize performance. The injection volume was 2 μL . The scan events consist of MS1 full scan at a resolution of 17 500 from 400 to 2000 m/z followed by PRM scans of the precursors in the inclusion list at the resolution of 140 000. AGC for MS2 was set as 2×10^5 . The maximum IT for MS2 was automatically set. Isolation

window for MS2 was set to be 1.6 m/z . After sample preparation optimization (described above), the chromatography gradient was shortened to improve method efficiency, and peptides were eluted with a linear gradient of 20–40% solvent B over 26.5 min (the settings are related to the experiment denoted as “post-optimization evaluation”). The AGC for MS2 was changed to 3×10^6 , and the maximum IT was set automatically. Label-free quantification was conducted using Skyline²⁴ (version 3.5.0.9319) based on the sum of product ion peak areas. All data have been uploaded to PanoramaWeb²⁶ (<https://panoramaweb.org/farrp-soy.url>). The mass spectrometry proteomics data have been deposited to the PRIDE Archive (<http://www.ebi.ac.uk/pride/archive/>) via the PRIDE partner repository with the data set identifier PXD008699 and 10.6019/PXD008699. Targeted proteomics data is available at PanoramaWeb: <https://panoramaweb.org/farrp-soy.url> as well as ProteomeXchange with the ID PXD010843

RESULTS AND DISCUSSION

Selection of Extraction Buffers

The extraction efficiency of proteins is essential for any protein detection and quantification method. In the current study, three buffer systems with different combinations of denaturants (guanidine-HCl or urea) and reducing agent (DTT) were compared. The nonroasted soy flour was used to compare the extraction efficiency of the different buffers as it is the least processed among the six ingredients. The protein yields of the different extraction solutions were largely similar, with some increased protein solubility observed with buffers that included urea (Table 1). Among the 17 different extraction solutions, the highest protein yield was obtained using buffer M: 50 mM Tris-HCl pH 8.6 with 6 M urea, 1% PVPP, and 20 mM DTT. A smaller set of extraction buffers (buffers M, N, and P) were used to compare extraction efficiency in all six soy ingredients (Table S2). Soluble protein contents of the six ingredients extracted using the three buffers were compared, and no distinguished differences were found.

To investigate the protein profiles of the six soy ingredients, SDS-PAGE analysis of the six soy ingredients extracted with buffer M was carried out under reducing conditions (Figure 1). Of the six ingredients, four ingredients showed a similar resolution of the protein bands as well as the number of bands: SPC-B had a slightly lower intensity, and SPI-B consisted mainly of low-molecular-weight proteins and peptides. The latter observation is in agreement with the product information, which indicates that this product has been partially hydrolyzed (Table S-1).

Investigation of the Protein Profiles of Six Commercial Soy Ingredients

The objective of discovery proteomics analysis was to investigate the protein and peptide profiles of different soy ingredients and use the results to inform selection of tryptic peptide markers for future targeted analysis. Although there has been some similar research performed by other authors, most of these studies were conducted on the whole soybean or on limited types of soy ingredients,^{10,17,27,28} which does not necessarily represent the different types of commercially available processed soy ingredients. Therefore, a discovery analysis was performed on six different soy ingredients to investigate the protein profiles and select potential peptide markers representative to all six ingredients.

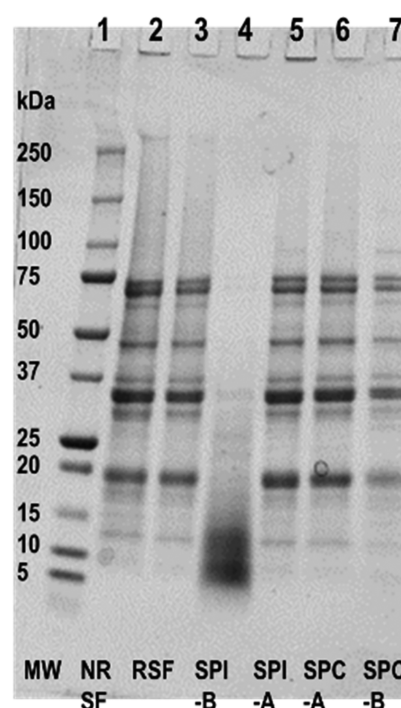


Figure 1. SDS-PAGE of 6 commercial soy ingredients extracted using extraction buffer M (50 mM Tris-HCl at pH 8.6, containing 6 M urea, 1% PVPP, and 20 mM DTT). Samples were loaded based on equal extract volume.

The soy ingredients were extracted using buffer M, and in-solution tryptic digestion and C18 cleanup were conducted as described previously. A discovery analysis of the six soy ingredients was performed, and each ingredient had two extraction replicates and two injection replicates. Overall, a total of 15 332 peptides with different levels of confidence were identified, of which 530 high-confidence (FDR < 1%) peptides were identified in the soy proteome. Seed storage proteins, including those in soybeans, have long been known to be represented by a diverse range of isoforms and proteoforms, and a substantial number of highly identical soy protein sequences are currently in databases such as UniProt. Assigning peptide identifications to individual protein sequence accessions is, therefore, a substantial challenge, and in some cases, it is impossible due to the lack of unique peptide identifications. Because the objective of this work is to identify target peptides representative of the presence of soy proteins in general, rather than an individual soy protein isoform, the discussion of soy proteins will focus on classes of seed proteins, rather than individual sequences.

Among the 530 high-confidence peptides, 445 peptides could be used to infer 20 proteins or protein groups with clear annotation in the soy proteome (with at least two high-confidence peptides per accession, see details in Table S3). The remaining 85 peptides were either ones that failed to pass the protein inference requirement (at least 2 peptides per accession; 15 peptides) or ones that were only identified from uncharacterized proteins (70 peptides from 34 accessions). Among the 445 peptides, 283 soy peptides were identified across all six soy ingredients (identified in at least 2 out of 4 replicates per ingredient). A total of three allergenic protein groups in soybean (exemplified by those sequences listed as allergens by WHO/IUIS), glycinin (Gly m 6), β -conglycinin (Gly m 5), and 2S albumin (Gly m 8) were identified in all of

the ingredients. Other proteins, such as basic 7S globulin, Kunitz trypsin inhibitor, lipoxygenase, lectin, and oleosin were also identified in the soy ingredients.

In addition to evaluating peptides and proteins identified in the six soy ingredients, the abundances of the peptides were also evaluated with label-free MS1-based relative quantification. Figure 2 shows the fold change of protein abundance in

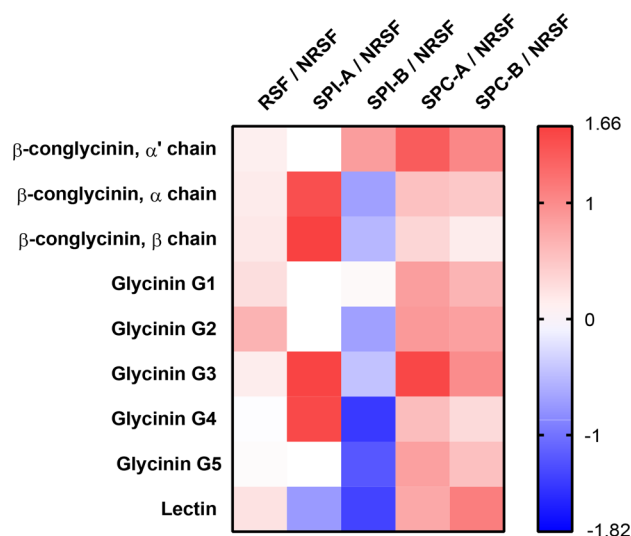


Figure 2. Relative abundance of selected unmodified proteins in soy ingredients. Numbers represent the log 2 value of the fold change in the mean peptide peak area of each soy ingredient compared to NRSF. Protein abundance was calculated from the sum of MS1 peak areas of the top three, unmodified precursors that are unique and isoform-conserved in each protein (some proteins were excluded from the label-free quantification due to the lack of sufficient precursors).

soy ingredients compared to NRSF. Note that the MS1 label-free quantification was performed based on unmodified precursors because it would be very complicated to account for all of the possible modifications that could occur during food processing (Maillard reactions etc.). The sizes of the fold changes on protein relative abundance across different ingredients were mostly within 2-fold. RSF did not show a distinct difference in protein abundance in comparison to NRSF. SPI-A appeared to have a higher amount of proteins but was deficient in lectin, which may be caused by ingredient processing as lectin is a hemagglutinin, which can be easily disrupted by moist heat treatment.⁷ In the food industry, soy

protein hydrolysates are produced by enzymatic hydrolysis processes that involve enzymes from animals, plants, or microorganisms.^{29,30} SPI-B, a partial hydrolysate, appeared to have the lowest protein abundance compared with other ingredients, except for the glycinin G1 and β -conglycinin α' chain, which could be caused by lower degree of hydrolysis on these two proteins.³¹

Selection of Potential Target Peptides

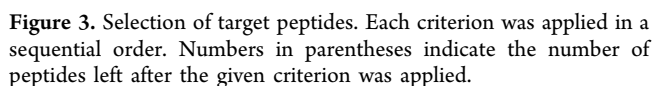
In bottom-up proteomics, peptides serve as surrogates for proteins. Therefore, the robustness and sensitivity of target peptides are critical in MS-based allergen detection methods. A number of researchers have discussed the general recommendations for target peptide selection.^{9,32} In the current study, taking into consideration the characteristics of the samples, the selection of target peptides was made on the basis of the following seven criteria: derivation from major proteins, modifications, length, quality of spectral matches, peptide abundance consistency across soy ingredients, protein polymorphism, and sequence uniqueness. The detailed information for each criterion is described in Table 2.

Based on these criteria, 57 peptides were selected as potential target peptides (Figure 3). The detailed information on the selected marker peptides is listed in Table S-4. The selected peptides were compared with a list of peptides that have been identified in previous studies using data from Allergen Peptide Browser (APB).³⁴ Of the selected peptides, nine peptides have been identified in at least four studies, suggesting a high reproducibility. Because a more-conservative measure was intended to be taken at this stage, peptides with missed cleavage sites were not removed until acquiring more data. Moreover, nine peptides from minor soy seed storage proteins were identified (for example, lectin and protease inhibitors), which have not yet been identified in any of the studies included in the APB.

By comparing the log 2 fold change of peak area [$\log_2(PA_{\text{ingr}}/PA_{\text{NRSF}})$] of the selected peptides across all six soy ingredients (Figure 4), it is evident that based on an equal volume of sample extracts, peptide abundance in SPI-A is higher than the other ingredients, whereas SPI-B showed lower peptide abundances, even though the SPI-B has higher protein solubility according to the manufacturer. The low peptide relative abundance in SPI-B is likely related to the enzymatic hydrolysis that occurred during processing. Enzymes used in the industrial processing of the hydrolyzed protein isolate may have cleavage sites in the middle of these peptides, therefore interrupting the identification of these tryptic peptides.

Table 2. Criteria for Target Peptide Selection

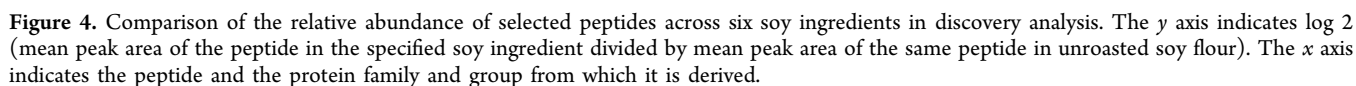
criteria	description
abundance	derived from major soy seed storage proteins (glycinin, β -conglycinin) to make certain that the peptide has sufficient abundance; a few peptides from some less abundant proteins (proteinase inhibitors, 2S albumin, basic 7S globulin, and lectin.) were included for maintaining diverse targets peptide performance evaluation
modification	does not contain methionine (susceptible to oxidation)
length	6–25 amino acids, which is a reasonable length for peptide identification
spectral match	identified as a high-confidence (FDR < 1%) peptide in spectral matches and database searching using Proteome Discoverer
consistent abundance across ingredients	standard deviation of the fold change of peptide peak area of the soy ingredient compared to NRSF ($PA_{\text{ingr}}/PA_{\text{NRSF}}$) across all soy ingredients being less than 1.5
protein polymorphism	Given the polymorphism of seed storage proteins due to post-translational modifications, ³³ peptides conserved across different isoforms were selected; protein isoforms were defined as sharing 90% sequence identity, excluding the short sequence entries (less than 20 aa), as determined by UniRef on UniProt
uniqueness	unique amino acid sequences to avoid false positive results, confirmed with the Basic Local Alignment Search Tool (BLAST) in UniProt



The abundance and reproducibility of the selected peptides in the six soy ingredients were further evaluated using a targeted MS method. Precursors with an isotope dot product (idotp) value lower than 0.7, which indicates an inconsistency between the expected and observed precursor isotope distribution in Skyline,³⁵ were excluded from the inclusion list. For peptides that have both miscleaved and nonmiscleaved versions in the list, those which have lower abundance than their counterparts were also excluded from the list.

Detection of Soy Proteins in Incurred Food Matrices

Possible causes of the low method performance were investigated by comparing incurred food matrices and spiked



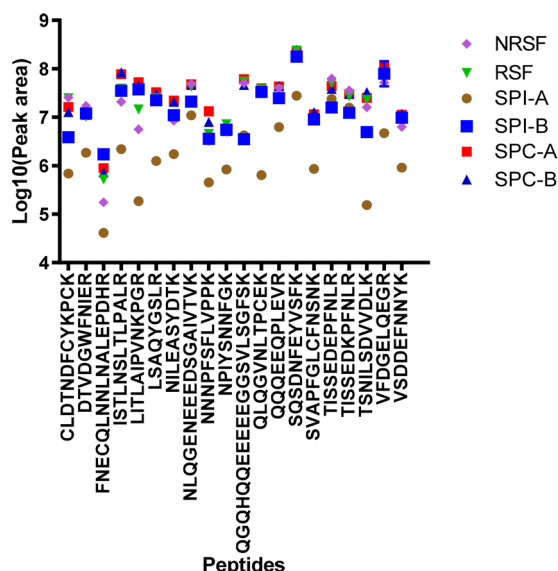


Figure 5. Abundances of selected peptides across 6 soy ingredients using targeted MS. Data reported as mean plus or minus the standard error of the mean ($n = 4$, 2 extractions analyzed in duplicate by MS).

food matrices. Blank matrix samples that were digested and then spiked with an NRSF digest (spiked digest) had higher peptide recoveries compared with blank matrix samples spiked with NRSF before extraction (spiked extract). Samples spiked with NRSF before extraction also showed higher peptide recoveries compared with incurred samples (Figure S2). Moreover, both spiked extract and spiked digest samples showed better peak shape and a higher number of identified fragment ions. Thus, low protein extraction yield and incomplete digestion were identified as the potential causes of the low method performance. Therefore, the optimization of

sample preparation was necessary to improve the method performance.

Optimization of Sample Preparation Techniques

Extraction efficiency is critical to the robustness of any allergen detection method. However, protein cross-linking and changes in protein solubility that occur during the cookie processing pose a challenge to the original extraction method. One of the major challenges of the cookie matrix is that wheat flour exists in bulk in the matrix, accounting for 47% of the total weight of the raw dough. Gluten proteins, the majority of wheat proteins, are largely insoluble in both water and diluted salt solutions. Such characteristics of gluten may have affected the availability of soy proteins for extraction. This could potentially be solved by having longer extraction times and including an additional vigorous shaking extraction to disrupt the structure of the gluten network. Based on this information, an additional 40 min of vigorous extraction at room temperature on a shaker was added to the original extraction protocol.

Protein digestion is another crucial step in sample preparation for MS analysis. Wisniewski et al. proposed a FASP method for proteomic analysis.²³ The application of FASP in allergen detection methods has been reported by Parker et al.¹² This method involves the use of molecular weight cutoff filters, which can help remove impurities, conduct buffer exchange, and concentrate proteins. The workflow of the FASP method is described in the [Experimental section](#). Because most of the soy seed storage proteins are 20 to 30 kDa after reduction, and the digested proteins are less than 10 kDa, 10 kDa MWCO filters were used in this experiment. (The case of the hydrolyzed soy protein isolate will be discussed later.) The performance of the FASP method was compared to the original digestion method with the added vigorous shaking step ([Figure 6](#)). Moreover, the addition of 1 M urea in buffer exchange was also compared with the AMBC

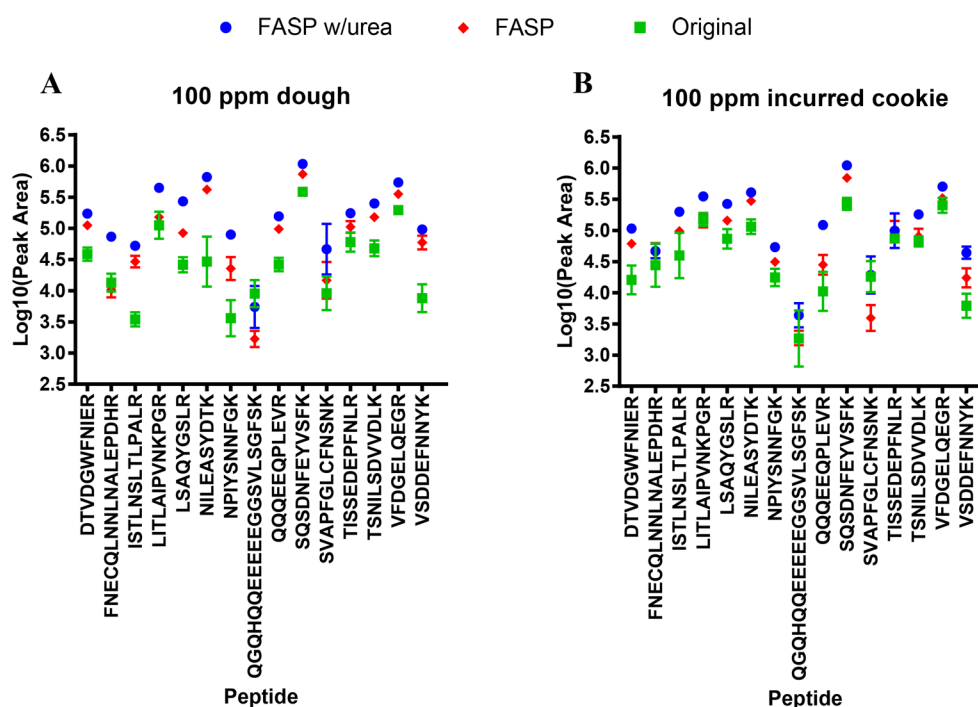


Figure 6. Comparison of peptide abundance in cookies and doughs incurred with NRSF using different digestion methods. Data reported as mean plus or minus the standard error of the mean ($n = 4$, 2 extractions analyzed in duplicate by MS).

only digestion buffer. Figure 6 indicates that the FASP method with urea added in buffer exchange gave better peptide abundance in both 100 ppm cookie and dough samples compared with the previous method and the AMBC-only FASP method, indicating that the addition of urea may have helped with the exposure of trypsin cleavage sites. The increased concentration of proteins and removal of background components were likely to be the reason why such improvement occurred. With the optimized method, soy protein can be detected at the 10 ppm total soy protein incurred level in both cookie and dough samples (Figure S3).

Performance of the Optimized Sample Preparation

Peptide performance in cookies and dough incurred with different soy ingredients prepared by the optimized sample preparation method were evaluated. The effectiveness of the FASP method was specifically assessed for the hydrolyzed soy protein isolate as it contains mostly low-molecular-weight proteins (as shown in Figure 1), with which a 10 kDa filter may not be compatible. Therefore, peptide abundance in SPI-B prepared using a 10 kDa filter, a 3 kDa filter, and a method using a 3 kDa filter but no filtering in the second centrifugation phase was tested. The results demonstrated that target peptide abundance was similar when using either a 10 kDa filter or a 3 kDa filter (Figure S4). The absence of clear differences among the filter units may be due to the fact that the molecular weight cutoff value claimed by the manufacturer is based on folded proteins, which can go through the filter more easily than unfolded proteins. It has been demonstrated that the 10 kDa filters can in fact retain proteins down to 5 kDa.²³

With the optimized sample preparation method, peptide performance was greatly improved in the FASP with urea method compared to the original method. The performance of each peptide was also evaluated and the peptides that presented robust recovery at 100 ppm incurred food matrices were identified as potential quantitative marker peptides (Table 3). Some other peptides were chosen as qualitative peptides because of their higher variation in peptide abundance across different ingredients, yet they still could be detected at 100 ppm total soy proteins (Figure 7). Further optimization on

the method sensitivity can be focus on selecting product ions that retain the robustness regardless of the food matrix effects.

The final method's performance was also evaluated in cookie and dough samples incurred with six commercial soy ingredients at 100 ppm total soy protein (post-optimization evaluation). The results showed similar peptide detection for several target peptides across all different cookies and dough samples (Figure 7). Such uniformity is difficult to achieve when using antibody-based methods. Several studies have demonstrated that commercial ELISAs produced various responses when it comes to processed foods, especially hydrolyzed proteins.^{27,36–38} The uniformity of peptide detection across different types of processed soy ingredients in incurred food matrices showed the advantages of MS compared with antibody-based methods. The ability of a single method to detect multiple types of soy-derived ingredients will be useful in a number of instances, including when a highly processed soy ingredient is the source of cross-contact and when the type of soy ingredient or processing is unknown. Because these types of processed soy ingredients would still cause allergic reactions in individuals, potential cross-contact must be controlled by food manufacturers. Without adequate detection methods, however, validation of the efficacy of allergen controls is extremely challenging. Broadly applicable methods such as the one developed here will improve the ability of food manufacturers to evaluate controls relevant to a wide range of soy ingredients.

Examination of Peptide Specificity

In the final soy detection method, it was crucial to ensure marker peptides are unambiguously derived from soybean. Although BLAST searches of peptide targets were done in the discovery analysis phase, this approach limits the searching range to those protein sequences that have been reported in public databases. However, the protein sequences of many foods, for example, tree nuts, have not yet been well-established. Therefore, experimental verification of peptide specificity is necessary for an accurate detection method. Six food commodities that have been reported to have cross-reactivity in other ELISA methods were evaluated: walnut, red lentil, peanut, cocoa powder, chickpea, and dry green pea.^{13,39} Each type of food was ground and prepared in the same way as the food matrix samples for MS analysis except for dilution prior to injection to avoid overloading.

Although results indicated that no single food was found to contain all soy marker peptides, three product ions matches from the peptides NILEASYDTK and LITLAIPVKNKGR were found in green pea and chickpea, respectively (Table S5). The extracted ion chromatograms of these two peptides are shown in Figure S5A,B. For the peptide found in green pea (NILEASYDTK), a BLAST search revealed that a similar peptide with one amino acid difference was found in green pea (NILEASYNTK). The substituted asparagine (N) residue in the pea peptide is susceptible to deamidation, resulting in a deamidated pea peptide NILEASYNTK that shares the same m/z as the soy peptide (NILEASYDTK). The precursor and product ion spectra (Figure S5C–E) demonstrated that the peptide identified as the native soy peptide (NILEASYDTK) by Skyline in green pea was in fact the unmodified pea peptide. Specifically, the precursor spectra were consistent with the isotopic distribution of the unmodified pea peptide (576.7984^{2+}), rather than the soy peptide (577.2904^{2+}). In green peas, the product ion spectra generated from the isolated

Table 3. List of Final Target Peptide Selected for the Detection of Soy in a Cookie-Based Food Matrix

peptides	accession no. ^a	protein	m/z	average retention time
LITLAIPVKNKGR	P13916	β -conglycinin α chain	464.63	25.38
NILEASYDTK ^b	P13916	β -conglycinin α chain	577.29	19.22
LSAQYGSRL ^b	P04405	glycinin G2	497.77	15.92
SQSDNFEYVSFK ^b	P04405	glycinin G2	725.83	23.34
VFDGELQEGR ^b	P04776	glycinin G1	575.28	17.01
QQQEEQPLEVR	P11827	β -conglycinin α prime chain	692.34	14.09
ISTLNSLTLPALR	P02858	glycinin G5	699.92	27.84
DTVDGWFNIER	P25273	Kunitz-type trypsin inhibitor KT12	676.32	26.96
TSNILSDVVDLK	P05046	lectin	652.36	28.07

^aRepresentative accession number for the protein group; peptides may also be found in other accessions associated with the protein group. ^bPotential quantitative marker peptides.

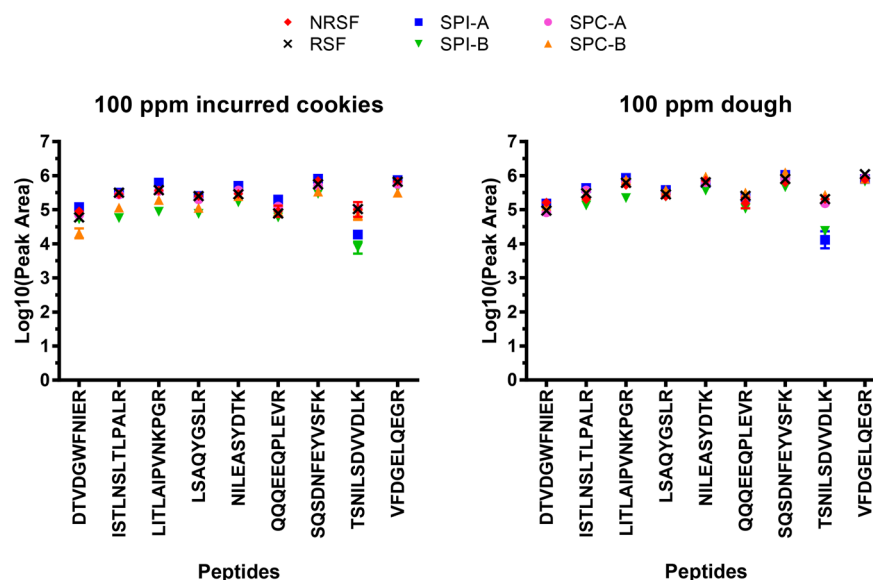


Figure 7. Comparison of peptide detection in cookies and dough incurred with different commercial soy ingredients at 100 ppm total soy protein. Data reported as mean plus or minus the standard error of the mean ($n = 4$, 2 extractions analyzed in duplicate by MS).

precursors included a distinct mass error for the $y8^+$ ion (926.4645 instead of 926.4466), resulting from isolating the $y8$ $[M+1]^+$ ion generated from the unmodified pea peptide (576.7984 $^{2+} \rightarrow$ 925.4606 $^{+}$). The acquisition of full product ion spectra in PRM mode with high-resolution makes the interpretation of these data possible, illustrating an advantage of this type of method for analyzing novel sample matrices. Although the deamidated pea peptide (NILEASYNTK) was not found in these samples, it was found in a subsequent analysis of other pea-derived products, with a relatively low abundance compared to the unmodified version (data not shown). If the deamidated pea peptide were present in a sample undergoing analysis for soy residues, however, it would be impossible to verify whether the signal comes from the deamidated pea peptide or the native soy peptide presence arising from cross-contact. In addition, if method conditions (e.g., mass tolerance, isolation window, transition selection, and RT controls) are not sufficiently stringent or adequately optimized, the native pea peptide may be selected instead of the deamidated pea peptide or native soy peptide.

In the case of LITLAIPVKNKGR, the reason why this peptide was found in chickpea is less clear, mainly because the BLAST search did not reveal high sequence similarity in chickpea. However, the retention time of the peptide found in chickpea was shorter than the true soy peptide (Figure S5C). In this case, internal heavy peptides would be useful for aligning the retention time with a true soy peptide instead of the peptide of chickpea origin and therefore reduce the possibility of false positive.

These results indicated the necessity of using at least three product ions for each peptide and multiple marker peptides to determine the presence of soy proteins. The selected product ions should also have sufficient length to avoid cross-reactivity. Apart from this, some quantitative indicators can also be used for the accurate identification of marker peptides and soy proteins. For example, product ion ratios (denoted as library dot product, dotp, in Skyline) can be used as an indicator for differentiating false-positive detection. For instance, although all three product ions of peptides NILEASYDTK and LITLAIPVKNKGR were detected in green peas and chickpeas,

respectively; the dotp value for peptide NIL in green peas (0.76) and LIT in chickpeas (0.6) were relatively suboptimal numbers compared with values commonly seen in a true positive sample (dotp of >0.85). Furthermore, the retention time can also be used as a confirmation of peptide identification because the selected quantitative peptides have chromatographic consistency. An internal heavy peptide standard would also play an important role in retention time alignment. The co-elution of all three product ions is also essential because product ions from the same precursor should appear and be analyzed at the same time.

Our finding suggested that peptide specificity examination is necessary when developing criteria to avoid false positives because protein database searches may be insufficient. Coincidences caused by unexpected modifications like deamidation in the pea peptide mentioned above could lead to misinterpretation, and insufficient protein sequence data on foods need to be taken into account when evaluating peptide specificity for food allergen detection methods.

CONCLUSIONS

In the food industry, soy ingredients are often processed in different ways to achieve various functionalities. However, soy ingredients that have gone through different processing have remained major challenges for many antibody-based detection methods. Mass spectrometry provides a promising alternative because it does not rely on the integrity of epitopes for detection. Discovery analyses of six commercial soy ingredients revealed that the content of some minor proteins was reduced by food processing in some ingredients, indicating that the results of antibody-based methods targeting these minor proteins may be biased when testing these ingredients. Sample preparation was shown to have a great influence on protein recovery, with longer extraction time and FASP substantially improving protein recovery and the method sensitivity. The experimental examination of target peptide specificity indicated that caution must be taken in data analysis regarding retention time and the number of peptides and product ions needed to confirm the presence of soy. Such examination is not commonly seen in other MS method development but is

worth being considered as part of standard practice. Ultimately, the target peptide selection strategy and PRM method described in this article successfully selected peptides that showed uniform detection across all selected soy ingredients, including the partially hydrolyzed soy protein isolate, present in an incurred cookie matrix. The PRM-based method that has been developed can be utilized to detect a wider array of soy-derived ingredients than existing methods and will enhance the ability of end users to detect soy in a variety of relevant circumstances.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00689.

Tables showing product information on the soy ingredients used in the study, extractability of a subset of buffers, total number of peptides, potential target peptides selected from discovery analysis, and the total number of sample replicate; figures showing examples of peptide performance, extracted ion chromatograms for peptide VFDGELQEGR detected in dough and cookies, determination of the appropriate MWCO of filter for hydrolyzed soy protein isolate, and a peptide specificity examination (PDF)

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Notes

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■ REFERENCES

- (1) Food and Agricultural Organization. Technical Consultation on Food Allergies; Food and Agricultural Organization: Rome, Italy, 1995.
- (2) European Union European Parliament and Council Directive 2007/68/EC. Amending Directive 2000/12/EC as Regards the Indication of the Ingredients Present in Foodstuffs. *Off. J. Eur. Union* **2007**, No. L310, 11–14.
- (3) Sicherer, S. H.; Sampson, H. A. Food Allergy: Epidemiology, Pathogenesis, Diagnosis, and Treatment. *J. Allergy Clin. Immunol.* **2014**, *133* (2), 291–307 e5.
- (4) Sampson, H. A.; McCaskill, C. C. Food Hypersensitivity and Atopic Dermatitis: Evaluation of 113 Patients. *J. Pediatr.* **1985**, *107* (5), 669–675.
- (5) Sampson, H. A.; Scanlon, S. M. Natural History of Food Hypersensitivity in Children with Atopic Dermatitis. *J. Pediatr.* **1989**, *115* (1), 23–27.
- (6) Savage, J. H.; Kaeding, A. J.; Matsui, E. C.; Wood, R. A. The Natural History of Soy Allergy. *J. Allergy Clin. Immunol.* **2010**, *125* (3), 683–686.
- (7) Liu, K. *Soybeans. Chemistry, Technology and Utilization*; Springer US: Boston, MA, 1997.
- (8) Singh, P.; Kumar, R.; Sabapathy, S. N.; Bawa, A. S. Functional and Edible Uses of Soy Protein Products. *Compr. Rev. Food Sci. Food Saf.* **2008**, *7* (1), 14–28.
- (9) Johnson, P. E.; Baumgartner, S.; Aldick, T.; Bessant, C.; Giosafatto, V.; Heick, J.; Mamone, G.; O'Connor, G.; Poms, R.; Popping, B. Current Perspectives and Recommendations for the Development of Mass Spectrometry Methods for the Determination of Allergens in Foods. *J. AOAC Int.* **2011**, *94* (4), 1026–1033.
- (10) Planque, M.; Arnould, T.; Dieu, M.; Delahaut, P.; Renard, P.; Gillard, N. Advances in Ultra-High Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry for Sensitive Detection of Several Food Allergens in Complex and Processed Foodstuffs. *J. Chromatogr. A* **2016**, *1464*, 115–123.
- (11) Monaci, L.; Visconti, A. Mass Spectrometry-Based Proteomics Methods for Analysis of Food Allergens. *TrAC, Trends Anal. Chem.* **2009**, *28* (5), 581–591.
- (12) Parker, C. H.; Khuda, S. E.; Pereira, M.; Ross, M. M.; Fu, T.-J. J.; Fan, X.; Wu, Y.; Williams, K. M.; DeVries, J.; Pulvermacher, B.; et al. Multi-Allergen Quantitation and the Impact of Thermal Treatment in Industry-Processed Baked Goods by ELISA and Liquid Chromatography-Tandem Mass Spectrometry. *J. Agric. Food Chem.* **2015**, *63* (49), 10669–10680.
- (13) Pedersen, M. H.; Holzhauser, T.; Bisson, C.; Conti, A.; Jensen, L. B.; Skov, P. S.; Bindeslev-Jensen, C.; Brinch, D. S.; Poulsen, L. K. Soybean Allergen Detection Methods—a Comparison Study. *Mol. Nutr. Food Res.* **2008**, *52* (12), 1486–1496.
- (14) Watanabe, Y.; Aburatani, K.; Mizumura, T.; Sakai, M.; Muraoka, S.; Mamegosi, S.; Honjoh, T. Novel ELISA for the Detection of Raw and Processed Egg Using Extraction Buffer Containing a Surfactant and a Reducing Agent. *J. Immunol. Methods* **2005**, *300* (1–2), 115–123.
- (15) Newsome, G. A.; Scholl, P. F. Quantification of Allergenic Bovine Milk As1-Casein in Baked Goods Using an Intact 15N-Labeled Protein Internal Standard. *J. Agric. Food Chem.* **2013**, *61* (24), 5659–5668.
- (16) Lutter, P.; Parisod, V.; Weymuth, H. Development and Validation of a Method for the Quantification of Milk Proteins in Food Products Based on Liquid Chromatography with Mass Spectrometric Detection. *J. AOAC Int.* **2011**, *94* (4), 1–17.
- (17) Houston, N. L.; Lee, D.-G.; Stevenson, S. E.; Ladics, G. S.; Bannon, G. A.; McClain, S.; Privalle, L.; Stagg, N.; Herouet-Guicheney, C.; MacIntosh, S. C.; et al. Quantitation of Soybean Allergens Using Tandem Mass Spectrometry. *J. Proteome Res.* **2011**, *10* (2), 763–773.
- (18) Peterson, A.; Russell, J.; Bailey, D. J.; Westphall, M. S.; Coon, J. J. Parallel Reaction Monitoring for High Resolution and High Mass Accuracy Quantitative, Targeted Proteomics. *Mol. Cell. Proteomics* **2012**, *11* (11), 1475–1488.
- (19) Gallien, S.; Bourmaud, A.; Kim, S. Y.; Domon, B. Technical Considerations for Large-Scale Parallel Reaction Monitoring Analysis. *J. Proteomics* **2014**, *100*, 147.
- (20) Khristenko, N. A.; Larina, I. M.; Domon, B. Longitudinal Urinary Protein Variability in Participants of the Space Flight Simulation Program. *J. Proteome Res.* **2016**, *15* (1), 114–124.
- (21) Li, S.; Nakayama, T.; Akinc, A.; Wu, S.-L.; Karger, B. L. Development of LC-MS Methods for Quantitation of Hepcidin and Demonstration of siRNA-Mediated Hepcidin Suppression in Serum. *J. Pharmacol. Toxicol. Methods* **2015**, *71*, 110–119.
- (22) Rauniyar, N. Parallel Reaction Monitoring: A Targeted Experiment Performed Using High Resolution and High Mass Accuracy Mass Spectrometry. *Int. J. Mol. Sci.* **2015**, *16* (12), 28566–28581.

- (23) Wisniewski, J. R.; Zougman, A.; Nagaraj, N.; Mann, M. Universal Sample Preparation Method for Proteome Analysis. *Nat. Methods* **2009**, *6* (5), 359–362.
- (24) MacLean, B.; Tomazela, D. M.; Shulman, N.; Chambers, M.; Finney, G. L.; Frewen, B.; Kern, R.; Tabb, D. L.; Liebler, D. C.; MacCoss, M. J. Skyline: An Open Source Document Editor for Creating and Analyzing Targeted Proteomics Experiments. *Bioinformatics* **2010**, *26* (7), 966–968.
- (25) Vizcaino, J. A.; Csordas, A.; del-Toro, N.; Dienes, J. A.; Griss, J.; Lavidas, I.; Mayer, G.; Perez-Riverol, Y.; Reisinger, F.; Ternent, T.; et al. 2016 Update of the PRIDE Database and Its Related Tools. *Nucleic Acids Res.* **2016**, *44* (D1), D447–D456.
- (26) Sharma, V.; Eckels, J.; Taylor, G. K.; Shulman, N. J.; Stergachis, A. B.; Joyner, S. A.; Yan, P.; Whiteaker, J. R.; Halusa, G. N.; Schilling, B.; et al. Panorama: A Targeted Proteomics Knowledge Base. *J. Proteome Res.* **2014**, *13* (9), 4205–4210.
- (27) Heick, J.; Fischer, M.; Kerbach, S.; Tamm, U.; Popping, B. Application of a Liquid Chromatography Tandem Mass Spectrometry Method for the Simultaneous Detection of Seven Allergenic Foods in Flour and Bread and Comparison of the Method with Commercially Available ELISA Test Kits. *J. AOAC Int.* **2011**, *94* (4), 1060–1068.
- (28) Monaci, L.; Pilolli, R.; De Angelis, E.; Carone, R.; Pascale, M. LC-Tandem Mass Spectrometry as a Screening Tool for Multiple Detection of Allergenic Ingredients in Complex Foods. *ACTA IMEKO* **2016**, *5* (1), 5–9.
- (29) Endres, J. G.; Wayne, F. *Soy Protein Products Characteristics, Nutritional Aspects, and Utilization*; The American Oil Chemists' Society: Urbana, IL, 2001; p 53.
- (30) Adler-Nissen, J. *Enzymic Hydrolysis of Food Proteins*; Elsevier Applied Science Publishers: Barking, Essex, 1986.
- (31) Tsumura, K.; Saito, T.; Kugimiya, W.; Inouye, K. Selective Proteolysis of the Glycinin and β -Conglycinin Fractions in a Soy Protein Isolate by Pepsin and Papain with Controlled PH and Temperature. *J. Food Sci.* **2004**, *69* (5), C363–C367.
- (32) Liebler, D. C.; Zimmerman, L. J. Targeted Quantitation of Proteins by Mass Spectrometry. *Biochemistry* **2013**, *52* (22), 3797–3806.
- (33) Shewry, P. R.; Napier, J. A.; Tatham, A. S. Seed Storage Proteins: Structures and Biosynthesis. *Plant Cell* **1995**, *7* (7), 945–956.
- (34) Croote, D.; Quake, S. R. Food Allergen Detection by Mass Spectrometry: The Role of Systems Biology. *Nat. Publ. Gr.* **2016**, *2* (1), 1.
- (35) Schilling, B.; Rardin, M. J.; MacLean, B. X.; Zawadzka, A. M.; Frewen, B. E.; Cusack, M. P.; Sorensen, D. J.; Bereman, M. S.; Jing, E.; Wu, C. C.; et al. Platform-Independent and Label-Free Quantitation of Proteomic Data Using MS1 Extracted Ion Chromatograms in Skyline. *Mol. Cell. Proteomics* **2012**, *11* (5), 202–214.
- (36) Downs, M. L.; Taylor, S. L. Effects of Thermal Processing on the Enzyme-Linked Immunosorbent Assay (ELISA) Detection of Milk Residues in a Model Food Matrix. *J. Agric. Food Chem.* **2010**, *58* (18), 10085–10091.
- (37) Platteau, C.; Cucu, T.; De Meulenaer, B.; Devreese, B.; De Loose, M.; Taverniers, I. Effect of Protein Glycation in the Presence or Absence of Wheat Proteins on Detection of Soybean Proteins by Commercial ELISA. *Food Addit. Contam., Part A* **2011**, *28* (2), 127–135.
- (38) Cao, W.; Watson, D.; Bakke, M.; Panda, R.; Bedford, B.; Kande, P. S.; Jackson, L. S.; Garber, E. A. E. Detection of Gluten during the Fermentation Process To Produce Soy Sauce. *J. Food Prot.* **2017**, *80* (5), 799–808.
- (39) Cucu, T.; Devreese, B.; Kerkaert, B.; Rogge, M.; Vercruysse, L.; De Meulenaer, B. ELISA-Based Detection of Soybean Proteins: A Comparative Study Using Antibodies Against Modified and Native Proteins. *Food Anal. Methods* **2012**, *5* (5), 1121–1130.