



Comprehensive quantification of sesame allergens in processed food using liquid chromatography-tandem mass spectrometry

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ABSTRACT

Sesame (*Sesamum indicum*) is a well-recognized food allergen, and sesame-related allergies have been increasingly reported worldwide. Patients with sesame allergy are specifically allergic to different sesame proteins (Ses i 1–7). Because of the increasing burden associated with allergies to sesame, there is an urgent need to develop sensitive and quantitative analytical methods capable of detecting sesame allergen contaminants. Targeted proteomic approach employing liquid chromatography/tandem mass spectrometry (LC-MS/MS) has emerged as a promising technique that offers increased specificity and reproducibility compared to antibody and DNA-based technologies. In this study, we developed a qualitative and absolute quantification method based on the LC-MS/MS-multiple reaction monitoring (MRM) system utilizing stable isotope-labeled internal standard (SIIS) peptides. We optimized extraction and trypsin digestion conditions and selected signature and absolute quantification (AQUA) peptides for seven sesame allergen proteins respectively. This quantitative method produced a linear relationship ($R^2 > 0.99$) in a wide concentration range (0.4–2000 fmol/μL), and the overall coefficient of variation was < 5% in multiple tests. Limits of detection (LOD) and Limits of quantification (LOQ) for the seven sesame allergens AQUA peptides were determined to range from 0.1 to 140.0 fmol/μL and 0.4–400 fmol/μL respectively. The results showed that the accuracy of 20, 50, 100 ppm incurred protein Ses i 2 were 90%, 92%, 94% in non-processed material and 92%, 95%, 102% in processed material respectively. Overall, this method allowed for rapid and simultaneous absolute quantification of seven sesame allergens. The newly developed LC-MS/MS assay will likely increase the safety of sesame processed foods and add diagnostic value to the sesame allergy testing.

1. Introduction

Sesame (*Sesamum indicum*) is an emerging and well-recognized food allergen and reports of sesame allergy are increasing worldwide. The prevalence of sesame allergy varies from 0.1% to 0.9% based on studies utilizing self-reporting, skin prick test or oral food challenge (Dalal et al., 2002; Derby, Gowland, & Hourihane, 2005; Fox, Logan, Du Toit,

& Foong, 2013; Osborne et al., 2011; Sicherer, Munoz-Furlong, Godbold, & Sampson, 2010; Soller et al., 2015; Zuidmeer et al., 2008) with sesame allergy cases being reported in different countries across the world, including Italy, France, England, Denmark, Sweden, Switzerland and Japan (Dalal et al., 2002; Derby et al., 2005; Fox et al., 2013; Sicherer et al., 2010; Soller et al., 2015). Specifically, in Israel, sesame was reported as one of the most common food allergens and a

Abbreviations: LC-MS/MS, liquid chromatography/tandem mass spectrometry; MRM, multiple reaction monitoring; CV, coefficient of variation; RT, retention time; CE, collision energy; quality control, QC; LOD, limit of detection; LOQ, limit of quantification; SIIS, stable isotope-labeled internal standard; PTMs, post-translational modifications; CAM, carbamidomethylated cysteine; DP, deconvolution potential; AQUA, absolute quantification

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major driver of anaphylaxis (Dalal et al., 2002).

Sesame allergy triggers immediate hypersensitivity mediated via immunoglobulin E antibody, which begins early in life (usually before the age of 2) and persists in 80% of patients (Cohen, Goldberg, Levy, Leshno, & Katz, 2007; Dalal et al., 2002). For individuals with sesame allergy, complete elimination of sesame from the diet is virtually impossible, since an increasing number of bakery items and other oriental food products frequently incorporate sesame as one of the ingredients (Neomi Wolff et al., 2004). Sesame oil can also present a hazard to individuals allergic to sesame seeds, as it is often not very refined and may contain sesame protein residues (Alonzi, Campi, Gaeta, Pineda, & Romano, 2011). Moreover, many foods can come in contact with sesame through cross-contamination and use of shared processing equipment and facilities that routinely process sesame (Koppelman et al., 2015). Given these considerations, many countries or regions (UNION, 2011) have introduced mandatory legislations that require appropriate labeling of foods that contain sesame.

Several allergenic storage proteins have been isolated from sesame and have been partially characterized to date (Leduc et al., 2006; Pastorello, V, Farioli, 2001; N. Wolff et al., 2003). Of these, 7 Ses i proteins, including 2S albumin, 7S globulin, oleosin and 11S globulin, are accepted by the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies (IUIS) (<http://www.allergen.org/index.php>). Multiple reports suggest that Ses i 1 and Ses i 2 (from the 2S albumin family) and Ses i 3, Ses i 6 and Ses i 7 (from the cupin family) are predominantly responsible for serious hypersensitive reactions to sesame (Kirsten Beyer, Bardina, Grishina, & Sampson, 2002; K. Beyer, Grishina, Bardina, & Sampson, 2007; Leduc et al., 2006; Pastorello et al., 2001; N. Wolff et al., 2003). Allergy to sesame can be elicited in patients by very small amounts of sesame (as low as 1 mg) regardless of the kind of processing procedure used (Dano et al., 2015). Additionally, population threshold research indicates that approximately 5 mg of sesame seed protein can trigger an allergic reaction in 10% of the susceptible population (Dano et al., 2015; Gangur et al., 2005).

Over the years, many analytical methods utilizing immunoassays (Husain, Bretbacher, Nemes, & Cichna-Markl, 2010; Redl, Husain, Bretbacher, Nemes, & Cichna-Markl, 2010) and polymerase chain reaction (PCR) (López-Calleja et al., 2017; Pafundo, Gulli, & Marmioli, 2010; Schöringhumer et al., 2009; Waiblinger, Boernsen, Näumann, & Koepfel, 2014) have been specifically developed to detect sesame residue. However, antibody-based immunoassays, like enzyme-linked immunosorbent assay (ELISA), may produce false positive or false negative results due to cross-reaction with similar proteins or because of structural modification caused by food processing or sample preparation (Iqbal et al., 2016; Khuda et al., 2012; Monaci, Brohee, Tregoeat, & van Hengel, 2011). Complex components of the food matrix like polyphenols or tannins can interact with antibodies and proteins. Despite DNA sequences being more stable under heat compared to protein, DNA is not tissue-specific and DNA-based detection methods cannot directly inform on the actual allergenic exposure (Lee & Kim, 2010).

Targeted proteomic approaches based on liquid chromatography coupled to mass spectrometry (LC-MS) have been introduced as more effective determination methods compared to ELISA and PCR-based assays (Ahsan, Rao, Gruppuso, Ramratnam, & Salomon, 2016; De Angelis, Pilolli, & Monaci, 2017; Heick, Fischer, & Pöpping, 2011; Koeberl, Clarke, & Lopata, 2014). Multiple reaction monitoring (MRM) MS can realize provide accurate quantification of multiple target proteins in complex samples with high sensitivity and high-throughput capabilities (Boja & Rodriguez, 2012; Hoffmann, Münch, Schwägele, Neusüß, & Jira, 2017). The precise quantitative determination of target allergen proteins in complex matrices is the greatest advantage of MRM (Heick et al., 2011; Ji et al., 2017). Furthermore, absolute quantification (AQUA) of each individual allergen protein is the ultimate goal for food allergen detection. To achieve protein AQUA, stable isotope-labeled internal standard (SIIS) peptides are spiked into the

samples and the determination of a signature peptide as a stoichiometric surrogate for the target allergen is obtained. Analytical standards of the signature peptides and SIIS are used to develop a quantitative approach based on LC-MS/MS and MRM transitions.

However, recovery and sensitivity of the method can be impaired by the type of food processing affecting protein stability and extractability (Pilolli, De Angelis, & Monaci, 2018). Incurred samples permits simulation of the effects of food processing on target analyte structure which can affect analyte extractability and detectability (Matarozzi & Careri, 2019). This step is strongly recommended in order to assess the marker specificity, excluding the presence of interfering peaks from the matrix, as well as discover potential susceptibility of the peptides to food processing effects, both features that cannot be foreseen *in silico* (Monaci, De Angelis, Montemurro, & Pilolli, 2018). So in the method validation, we added incurred samples with a known amount of allergen to calculate the accuracy.

The potential application of LC-MS/MS-MRM method in food allergen detection has been demonstrated (Koeberl et al., 2014), but an AQUA approach which targets several sesame allergens in a single chromatographic run has never been reported. The objective of this work was to identify biomarkers of exposure and characterize a quantitative approach for sesame seed allergens, particularly in the matrix with other food allergens that would possibly show interference in other assays.

2. Materials and methods

2.1. Materials

Acetonitrile (ACN), formic acid (FA) and water (MS grade) were purchased from Fluka (Sigma, St. Louis, MO, USA) and trypsin protease (MS Grade) was from Pierce (ThermoFisher Scientific, Waltham, MA, USA). Urea, thiourea, sodium dodecyl sulfate (SDS), iodoacetamide (IAA), dithiothreitol (DTT), ammonium bicarbonate (ABC), tris(hydroxymethyl) aminomethane (TRIS), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and hydrochloric acid were from Sigma-Aldrich (St. Louis, MO, USA). Hexane (analytical grade, Fisher Scientific UK Ltd), 0.22 µm polyethersulfone (PES) membrane (Merck Millipore, Burlington, MA, USA) and Amicon® Ultra-0.5 mL 5 K centrifugal filter device (Merck Millipore, Germany) were also used. All foods required for method development and analysis were purchased from the local market (Beijing, China). Peptide standards were ordered from Guoping Pharmaceutical Co., Ltd (Hefei, China), which provides custom peptide synthesis services.

2.2. Sample preparation

Raw sesame (*Sesamum indicum*) samples from 13 different non-genetically modified commercial cultivars are listed as: ZZ13, ZZ20, ZZ34, ZFZ1, WB78, PJ08, KS1, KS2, US1, US2, CD1, CD2, GE1, as shown in Table 1. Sesame varieties were selected to ensure representation of the germplasm currently grown by Chinese and international farmers. Additionally, 15 other species (almonds, soybeans, pistachios, apricot seeds, macadamias, cashews, sunflower seeds, pine nuts, walnuts, pecan, oats, corn, wheat, rice and peanuts) were purchased from the local market (Beijing, China).

All sesame samples were ground using a TissueLyser (Qiagen, Hilden, Germany) and stainless steel beads (5 mm; Qiagen) under liquid nitrogen to obtain a fine homogeneous powder. Next, the resulting powder was defatted in hexane at a ratio of 1:5 (w/v) under constant stirring for 1 h at 4 °C. Following removal of the hexane phase by extraction filtration, the partially defatted powder was allowed to dry in a chemical hood to remove all traces of the residual solvent. The defatting process was repeated three times for each sesame powder. The defatted powders were then ground again for 30 s in a tissue grinder and stored at −80 °C until further use.

Table 1
Sesame varieties information.

Sample code	Varieties	Origin	Color
ZZ13	Zhongzhi 13	China	white
ZZ20	Zhongzhi 20	China	white
ZZ34	Zhongzhi 34	China	white
ZFZ1	Zhongfengzhi 1	China	white
WB78	WB78	China	white
PJ08	PJ08	China	white
KS1	Suwon 117	Korea	white
KS2	Daeduk	Korea	black
US1	u.C.R/82NO205shat	USA	white
US2	u.C.R/82NOINS	USA	white
CD1	White youma	China	white
CD2	Black youma	China	black
GE1	ALNATURA	Germany	white

2.3. Extraction and digestion of trypsin

Proteins were extracted from a total of 1 g of crude powder in a volume of 10 mL of extraction buffer (buffer A: 7 M urea, 2 M thiourea; buffer B: 7 M urea, 2 M thiourea, 4% CHAPS; buffer C: 50 mM NH_4HCO_3 , pH 8.0; buffer D: phenol extraction ammonium acetate precipitation method) by ultrasonication bath for 30 min at 25 °C in a 50 mL plastic tube. To avoid carbamidomethylation and crystallization with urea, the samples were centrifuged at $12,000 \times g$ for 20 min at 25 °C. The supernatants were collected, filtered through a 0.22 μm PES filter and stored at -80°C . Protein was then quantified using the Bradford assay (Kruger, 2002) with bovine serum albumin as a standard. The filter-aided sample preparation assay was used for protein digestion. A 30 μL aliquot of the protein extract ($< 200 \mu\text{g}$) was loaded on a 5 kDa filter (Merck Millipore, Germany). After addition of 120 μL of a solution containing 10 mM DTT (8 M urea, and 0.1 M Tris-HCl, pH 8.5), the samples were incubated at 37 °C for 1 h to reduce proteins. Subsequently, alkylation was performed by adding 120 μL of 50 mM IAA (8 M urea, 0.1 M Tris-HCl, pH 8.5) and samples were incubated for 15 min at 25 °C in the dark. All the sample solution on the filter was spun at $14,000 \times g$ at room temperature for 15 min. Next, the samples were washed with 100 μL of buffer (8 M urea, 0.1 M Tris-HCl, pH 8.0) before equilibration with three washes (100 μL of 50 mM ABC) and subsequent centrifugation under the same conditions. After three times of washing step, there were no solution on the ultrafiltration device, only denatured protein left on it. 50 μL of 50 mM ABC was added on the ultrafiltration device with denatured protein, and vortexed the solution for 1 min. Afterwards, protein digestion was performed by adding 4 μL of 1 $\mu\text{g}/\mu\text{L}$ trypsin (in 50 mM acetic acid) to each sample (in 50 μL of 50 mM ABC) at a 1:50 mass ratio and incubation at 37 °C under slow shaking. For digestion time optimization, sample digestion was quenched at various times (0.5, 2, 4, 6, 8, 10, 12, 14, and 16 h) after incubation with trypsin at 37 °C for the sesame allergen proteins.

For absolute quantification, 50 μL of SIIS peptides (at 80 fmol/ μL) was spiked into protein samples (50 μL) prior to trypsin digestion. Elution of both native and isotope peptides was done with two washes of 100 μL of 25 mM ABC solution. The collected peptide samples were dried in a concentrator machine (Eppendorf, Hamburg, Germany) and redissolved in the buffer (0.1% FA and 2% ACN in water) to a volume of 100 μL , then spun down (1200 rpm, 25 °C, 15 min) to remove particulates and transferred to vials for injection into the LC.

2.4. LC-MS/MS optimization and protein identification using database search

The extracts were separated by Eksigent NanoLC-Ultra 2D plus interfaced with NanoFlex system (Eksigent Technologies, Dublin, CA, USA). The samples were loaded with Nano-RPLC buffer at 2 $\mu\text{L}/\text{min}$ for 10 min. Separation of peptides was performed with an analytical

column (75 $\mu\text{m} \times 15 \text{ cm}$, C18, 3 μm 120 Å, ChromXP; Eksigent Technologies) at a flow rate of 300 nL/min. The column was maintained at 40 °C. Mobile phase A consisted of 2% ACN-98% water with 0.1% v/v FA and mobile phase B consisted of 98% ACN-2% water with 0.1% v/v FA. The elution program was applied as follows: 0.0 min = 5% B; 0.1 min = 9% B; 30 min = 25% B; 40 min = 35% B; 45 min = 80% B; 50 min = 80% B; 51 min = 5% B; 60 min = 5% B. Data acquisition was performed with a Triple TOF 5600 System (AB SCIEX, Foster City, CA, USA) fitted with a Nanospray III source (AB SCIEX) and a pulled quartz tip as the emitter (New Objective Inc, Woburn, MA, USA). Data were acquired under the condition of positive ion detection mode, ion spray voltage of 2.5 kV, nebulizer gas at 6 PSI, curtain gas at 30 PSI, and an interface heater temperature of 150 °C. For information dependent acquisition (IDA), survey scans were acquired in 100 ms and as many as 30 production scans were collected once they exceeded a threshold of 120 counts per second (cps) with a 2 + to 5 + charge state. The mass range was set at 100–1500 m/z. Dynamic exclusion time was set to 20 s. The total cycle time was fixed to 2.0 s. Rolling collision energy (CE) setting was applied to all precursor ions for collision-induced dissociation (CID).

We used ProteinPilot Software v.5.0 (AB SCIEX) to process the data using the following settings: searching as “thorough”; sample type: identification; digestion: trypsin; search effort: rapid ID; ID focus: biological modifications; fixed modification: carbamidomethyl (C); cyst alkylation: IAA; variable modifications: methionine (M) oxidation; peptide tolerance: $\pm 0.05 \text{ Da}$; MS/MS fragments tolerance: 0.03 Da; maximum missed cleavage: 2. The databank was imported from the UniProt Knowledgebase (UniprotKB) (<http://www.uniprot.org>) and searched against the sesame database alone (*Sesamum indicum*). The false discovery rate (FDR) was calculated using the ProteinPilot integrated tools. A total of seven sesame allergen proteins were identified with the ProteinPilot unused score greater than 1.3 (with protein confidence threshold greater than 95). We calculated peptides properties using ProtParam (a web-based tool for calculating the physical and chemical parameters of a protein or peptide, <http://web.expasy.org/protparam/>).

2.5. Selection of biomarker peptides

For the selection of signature peptides as sesame allergens markers, sequence specificity was verified by Basic Local Alignment Search Tool (BLAST) search algorithm (blastp: protein-protein BLAST; database: UniprotKB; scoring parameters: matrix: percent accepted mutation (PAM) 30), to ensure they are unique but specific to the target protein. A Q-value of 0.01 (1% FDR) was set as the cutoff for peptide signal analysis. Criteria for the biomarker peptides required peptides to be between 10 and 20 amino acids long, m/z < 1250, without missed cleavage sites and variable post-translational modification (PTM) sites in the amino acid sequence. Among the selected signature peptides, the most responsive MRM transitions were chosen for each quantitative peptide. For the selection of signature peptides which are stable under roasting or baking conditions, raw sesame seeds were dry roasted at 120 °C or 180 °C for 5, 10, or 20 min. In addition, unheated sesame samples were also used. After the samples cooled down, they were processed as described in Methods section 2.2.

2.6. MRM optimization and analysis

For the MRM analyses, data acquisition was performed with a QTRAP 5500 LC-MS/MS system (AB SCIEX) fitted with a Turbo V electrospray ionization (ESI) source run in positive mode. The LC method for the tryptic digests separation was modified with an Agilent 1260 Infinity HPLC on a Waters XBridge peptide BEH C18, 3.5 μm , 300 Å (150 $\times$ 4.6 mm) column at a flow rate of 400 $\mu\text{L}/\text{min}$. The column was maintained at 40 °C. Mobile phase A consisted of 2% ACN-98% water with 0.1% v/v FA and mobile phase B consisted of 98%

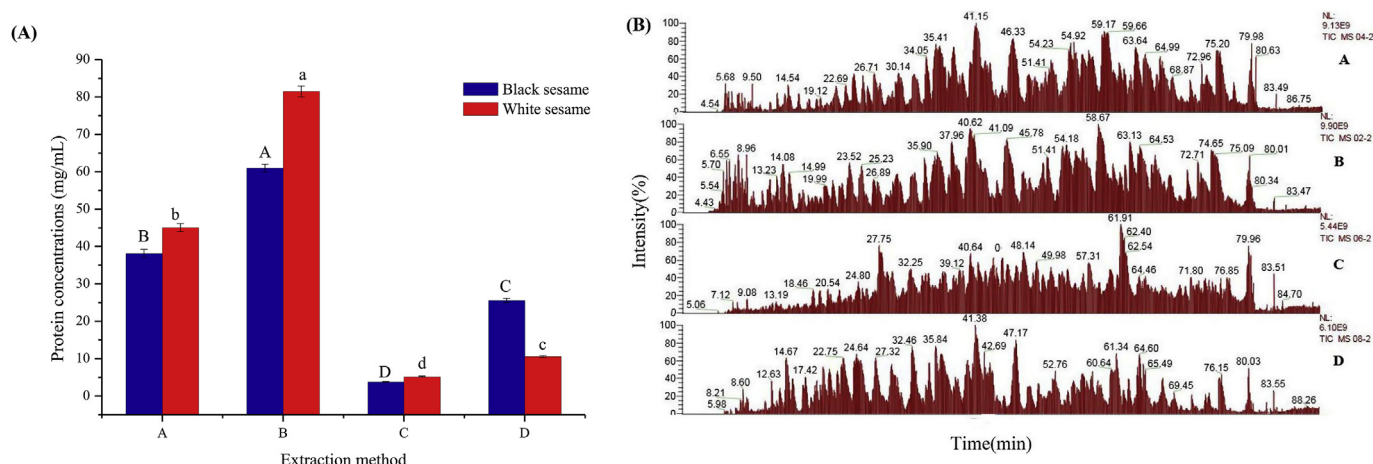


Fig. 1. The effect of four extraction methods on the sesame protein content. (A) and total sesame protein trypsin digestion peptides (B). Extraction method A: 7 M urea, 2 M thiourea; extraction method B: 7 M urea, 2 M thiourea, 4% CHAPS; extraction method C: 50 mM NH_4HCO_3 , pH 8.0; extraction method D: phenol extraction ammonium acetate precipitation. Data points represent the protein concentrations (mg/mL). Data and error bars display mean and standard deviation for three transitions per peptide obtained using duplicate experiments.

ACN-2% water with 0.1% v/v FA. The elution program was applied as follows: 0.1 min = 2% B; 0.5 min = 8% B; 25 min = 22% B; 31 min = 35% B; 33 min = 80% B; 36 min = 80% B; 36.5 min = 2% B; 39.9 min = 2% B.

The MS settings were as follows: ion spray voltage at 5500 V; source temperature at 600 °C; curtain gas at 20 psi; gas 1 at 60 psi; gas 2 at 90 psi; collision gas set to medium. The MRM transition list for signature and SIIS peptides was created using Skyline software (v. 2.5, <https://skyline.ms>). A total of six products (y or b) ions with the most intense signals were chosen as the candidate MRM transitions for each selected peptide. The CEs were optimized for each transition using the predicted CE together with CE-2, CE-4, CE-6, CE + 2, CE + 4, CE + 6. To monitor additional MRM transitions, scheduled MRM was performed with a 120 s MRM detection window at retention times (RT) and 2 s target scan time for the identified peptides, which was the ideal way to maximize multiplexing. Following optimization of the cycle time, scheduled MRM periods enabled collection of sufficient data points (≥ 10) for chromatographic peaks with a mean base width of 30 s. After optimization of all parameters, the three most intense transitions with a significant correlation ($r^2 > 0.9$) in MS response selected from a complex of calibration standards were chosen for quantification of each peptide.

2.7. Preparation of calibration standards and method validation

Standard signature peptides and stable isotope-labeled [^{13}C , ^{15}N] peptides (purity > 99%) were custom synthesized by ChinaPeptides Co., Ltd (Hefei, China) to estimate the level of sesame allergens.

Calibration sample was prepared at a range of concentrations (2000.0, 1600.0, 1200.0, 800.0, 400.0, 200.0, 160.0, 120.0, 80.0, 40.0, 20.0, 16.0, 12.0, 8.0, 4.0, 2.0, 1.6, 1.2, 0.8, and 0.4 fmol/ μL). The SIIS peptide standards were spiked into each calibration sample at 50.0 fmol/ μL assuring equal volumes of the calibration solution. Peak area was integrated, and manually inspected and adjusted as needed. The peak ratios from three transitions were summed to yield the peptide ratios. Ratios of the representative peptides from sesame allergens were plotted against the digestion time to determine the optimal digestion conditions.

Calibration curves were constructed by linear regression analysis of the peak area ratios plotted against the absolute concentrations of the calibration sample to the SIIS peptide. All experiments were performed in duplicate with three replicate injections to assess error and reproducibility. Data are represented as the mean $\pm$ standard deviation (SD). Limit of detection (LOD) and limit of quantification (LOQ) were

assessed using the signal-to-noise ratio value of 3 and 10, respectively. The analytical validation study evaluated assay accuracy, precision (intra- and inter-day repeatability), linearity, and recovery. The experiments were repeated six times one day, a total of 30 times on different days. Different levels of quality control (QC) samples (20, 200, and 2000 fmol/ μL) were used to calculate the precision of the method.

Incurred materials for the quantitative analysis. Here we chose cookies as processed food model containing sesame. Here we chose the purified Ses i 2 (purity > 95%, obtained by alcohol precipitation, shown in Fig. S5) as allergenic protein used for incurred materials preparation. The mixture of wheat flour, sugar, butter and Ses i 2 in solution were mixed in a kneading bowl were homogenized as the non-processed food matrix. Then non-processed food matrix molded into cookies and baked for 15 min at 180 °C, making as the processed food matrix. Both incurred matrixes were fortified with 100 ppm Ses i 2. Blank matrixes (0 ppm) of both processed and non-processed food matrix not fortified with Ses i 2 were also prepared. After baking and homogenization into a fine powder, the 100 ppm incurred matrixes were serially diluted (based on weight) using appropriate blank matrixes to prepare 20 and 50 ppm incurred samples. Then all the mixture was pre-dried over-night at 40 °C in drying oven. All the materials were ground into fine powders in liquid nitrogen with a tissue grinder. Protein extraction procedure followed the same steps as described in Methods section 2.3.

2.8. Sesame allergens quantification in food products

Food products, including hotpot dipping sauce, spicy chili sauce, cookie, cake, candy, were purchased from local market (Beijing, China). All of them (Supplemental Table S1), were ground into fine powders in liquid nitrogen with a tissue grinder. Protein extraction procedure followed the same steps as described in Methods section 2.3. These samples were analyzed using LC-MS/MS-MRM approach.

3. Results and discussion

3.1. Optimization of extraction and trypsin digestion

We used a total of four extraction methods (A, B, C and D) to compare the total yield of the soluble protein from sesame. The highest protein concentration (82.94 mg/mL) was obtained using method B, where 7 M urea, 2 M thiourea and 4% CHAPS were used as an extraction buffer (Fig. 1(A)). CHAPS, a zwitterionic surfactant, is typically used as a non-denaturing detergent in the process of protein

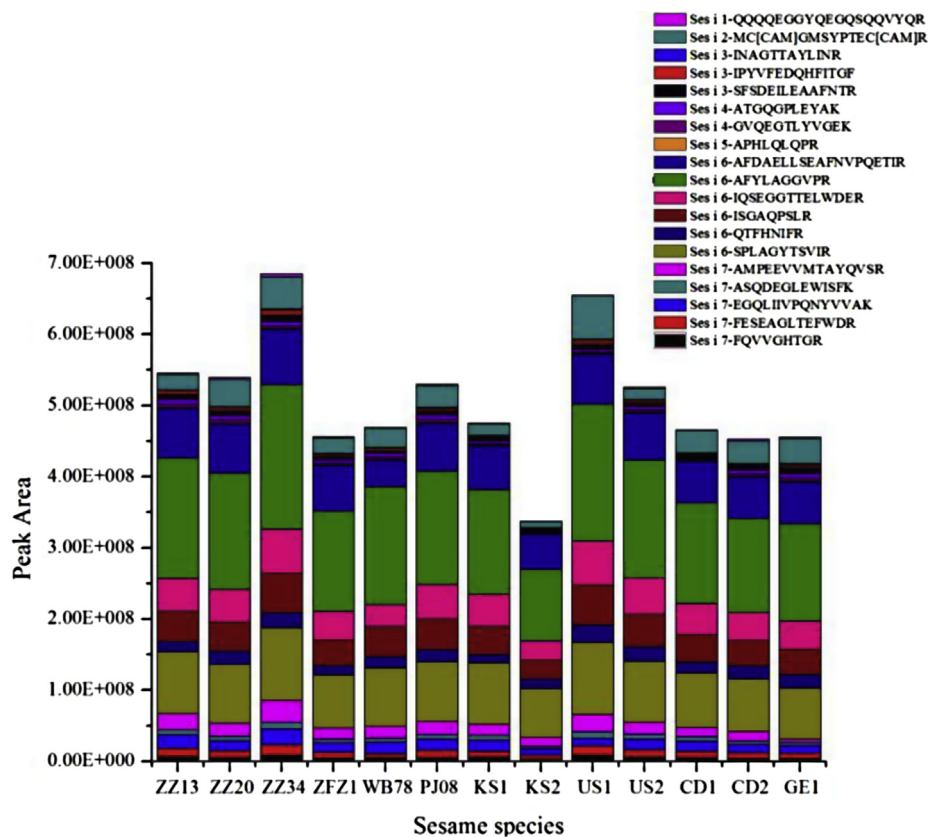


Fig. 2. The mass spectrometry results of signature peptides in different sesame varieties.

purification, and it had a significant solubilization effect in our experiment. We found that CHAPS was particularly useful in purification of membrane proteins, like sesame allergens Ses i 4 and Ses i 5, which are largely insoluble in aqueous solutions due to their native hydrophobicity (Cladera, Ricaud, Verde, & DuNach, 1997). Based on the total ion chromatogram of digested peptides for the extracted protein profiles (200 µg total protein), the 7 M urea + 2 M thiourea + 4% CHAPS extraction solvent (method B) resulted in the best yield of digested peptides from sesame proteins, as shown in Fig. 1(B). Furthermore, we also optimized the extraction time and found that extraction by ultrasonication bath for 30 min (as shown in Supplemental Fig. S1) was sufficient to extract protein from the buffer. Based on these results, all subsequent extractions were performed using extraction method B. In addition, based on the MRM method (Results section 3.3) the tryptic digest time was evaluated at various times (0.5, 1, 2, 4, 6, 8, 10, 12, 14, and 16 h) in order to determine optimal formation of the target peptides and achieve the best sensitivity (Supplemental Fig. S2). Specifically, we chose the 4 h digestion time as it resulted in a signal intensity that was similar to the 8 and 12 h digestion time, and was significantly better than the 1 h digestion.

3.2. Selection of signature peptides and AQUA peptides

For the signature allergen peptides and AQUA allergen peptides determination, we used proteins that fit specific criteria, including being unique for the selected allergen, between 10 and 20 amino acids in length, no missed cleavage sites after digestion (Koeberl et al., 2014; Rauh, 2012; Zhang et al., 2012), without amino acids affected by PTMs, and no consecutive arginine (R) or lysine (K) residues at the digestive site to allow for complete trypsin digestion (Kamiie et al., 2008). Moreover, the selected peptides had to be thermally stable, follow reproducible digestion, be easily extracted, detectable by MS systems, and should be found at abundant levels in the food matrix.

Based on the identification results of tryptic peptides performed with ProteinPilot software, a list of peptides was generated and all identified peptides were very abundant, were between 10 and 20 amino acids in length, and represented sesame allergens. It is important to stress that sesame can be mixed with other grains and nuts to create a variety of food products frequently found in stores or restaurants. Allergens from these foods share relevant sequence which may produce the same amino acid sequences of tryptic peptides. Therefore, the next criterion in marker peptide selection was to exclude peptides present in other seeds, plants and foods that were subjected to trypsin digestion. The additional level of selection of the signature peptide candidates resulted in high specificity which was verified by using BLAST algorithm in the UniProtKB/Swiss-Prot database. Of note, BLAST requires comprehensive and well-annotated sequence databases which, in a current state, may not contain all the protein information for some foods like gluten (Bromilow et al., 2017). Therefore, the specificity assessment of these peptides was analyzed using a batch of extracts from other common food allergens (almonds, soybeans, pistachios, apricot seeds, macadamias, cashews, sunflower seeds, pine nuts, walnuts, pecan, oats, corn, wheat, rice, peanuts). All of these peptides were analyzed using MRM-based LC-MS/MS multi-method. The extracts from pecan gave a positive response with the same soluble protein extracts when peptide SPLAGYTSVIR (amino acid numbers 410–420, sp|Q9XHP0|11S2_SESIN, Ses i 6) was used as an allergen marker candidate. However, additional studies are needed to confirm this observation. Trypsin digested peptides from pecan were analyzed by TripleTOF® 5600 + LC/MS/MS. Next, the LC-MS/MS data were searched to identify peptide sequences using algorithms in the UniProt database. The results indicated that trypsin digested peptide SPLAGYTSVIR found in pecan and sesame had a score of 18, with the scores of 15 and above representing correct fragmentation and valid interpretation. Typically, a score greater than 10 is widely accepted as the successful protein identification by MS/MS (Sealey-Voyksner,

Zweigenbaum, & Voyksner, 2016). For additional confirmation, comparison of the MS/MS spectra of the peptide from pecan and sesame yielded the same results, suggesting that SPLAGYTSVIR peptide exists in both pecan and sesame proteins indeed. However, according to the current version of the UniProt database (Available online: <http://www.uniprot.org/>, accessed on 10 May 2018.), this peptide sequence exists only in sesame protein Ses i 6, and is not found in pecan proteins. These findings likely suggest that the protein database for pecan is far from being comprehensive. Our results also indicate that SPLAGYTSVIR peptide is not specific enough to be a signature sesame peptide. Based on these observations, we concluded that database blast and negative sample verification were both required for the assessment of peptide specificity. On the other hand, signature peptide should exist in all sesame cultivars. Allergic protein-encoding genes in different cultivars may be conserved, but are likely differentially expressed, with peach lipid transfer proteins being a major example (Ma et al., 2014; Yang et al., 2011). Therefore, we investigated the selected signature peptides in 13 positive samples using the peak area from the MRM assays. Results indicated that all the peptides exist in different sesame species (Fig. 2), although the composition of each peptide/protein varied between different cultivars.

Additionally, selected peptides were also screened for stability of PTMs, including glycosylation under basic conditions. Firstly, the sequences of seven sesame allergen proteins were further analyzed to predict protein glycosylation sites (Blom, Sicheritz-Ponten, Gupta, Gammeltoft, & Brunak, 2004; Franc et al., 2013), so as to eliminate the potential impact of glycosylation on tryptic digestion. The results indicated that only Ses i 3 (Q9AUD0) had two predicted sites of N-linked glycosylation (Fig. 3). And these two sites are out of the selected signature peptides, suggesting that the amino acid 'N' in the selected peptide is not glycosylated. Without reactants, there will be no Maillard reactions (glycation of amino acids). Following digestion of the allergen Ses i 2, there were 148 amino acids, with only five peptides that could be analyzed by the MS system. Three of them were excluded due to short sequences (less than six amino acids) which could result in the lack of specificity. Additionally, one of the sequences had a low response and long sequence (24 amino acids), but it was not used owing to the high cost of synthesis and spread of the ion current over higher number of multiply charged ions (Sealey-Voyksner et al., 2016). Overall, these four peptides were not suitable as signature peptides for

Ses i 2. Instead, MC [CAM]GMSYPTEC [CAM]R derived from MCGM-SYPTECR (amino acid numbers 132–142, Supplemental Fig. S3) was chosen as the target signature peptide for Ses i 2 from all the peptides produced by trypsin digests. In this peptide, the free cysteine thiol groups are carbamidomethylated (CAM, a fixed modification to cysteine) by IAA, which is a very powerful modifying compound that produces very specific and complete reactions with cysteine under appropriate conditions (Franc et al., 2013). Given these considerations, the MC [CAM]GMSYPTEC [CAM]R peptide was deemed suitable as a signature peptide for Ses i 2. No other variable modifications were detected for other selected peptides.

Thermal processing of foods most typically results in glycosylation of R and L residues or denaturation of proteins, leading to a mass shift for tryptic fragment peptides (Poulsen et al., 2013). Our experiments require that the signature peptides representing each specific sesame allergen need to be present in raw and thermally processed forms of sesame. Peptides influenced by temperature-induced or Maillard reactions (glycation of amino acids) that lead to formation of aggregates are not considered (Cucu, De Meulenaer, Bridts, Devreese, & Ebo, 2012). Next, we performed additional selection of signature peptides by comparing the intensity of selected peptides from the raw and thermally processed forms of sesame. Here, the process included a series of roasting times (5, 10, or 20 min) under different temperature conditions (120 °C and 180 °C). As shown by the heat map (Fig. 4), peptides like Ses i 1-QQQQEGGYQEGSQSQVYQR displayed little variation were chosen as signature peptide candidate, while other peptides like Ses i 4-ATGQGPLEYAK, Ses i 5-APHLQLQPR, Ses i 7-AMPEEVMTAYQVSR showed reduction after thermal processing were eliminated.

The selection of target signature peptides was performed according to the response intensity, specificity, variable modification, and temperature stability. Data presented in Table 2 summarize the final peptides discovered for each sesame allergen based on the discussed criteria with the three most intensive transitions per peptide included. Where it was possible, we selected one or two peptides from different allergenic proteins instead of multiple peptides from one protein, to include better protein coverage. However, for Ses i 1, 2, and 5 only one peptide was selected because of their short sequences. In Table 2, two peptides (ISGAQPSLR, AFYLAGGVPR) were identified by other researchers as significant peptide markers (Huschek, Bönick, Löwenstein, Sievers, & Rawel, 2016). While the peptide IQSEGGTTELWDER was

Output for 'tr_Q9AUD0_Q9AUD0_SESIN'

Name: tr_Q9AUD0_Q9AUD0_SESIN Length: 585					
MSOGRLCLVLFALLASAVVASESKDPELKCKKCKCAQQIISKEQKEACIQACKETIRQKHQGEHRCGGDILBEEVW					80
NRKSPILRLRECSRCCEQHQEGREELRQEEYQREKGRDDNPDPKQYQCCRLQCRQEGGGFSREHCERRRE					160
EKYREQQCRGEGRCENYEGREBEQEGCGRIPYVFEDQHFITGFRQHGKRMVLOKFTDRSELLGIDENYRVAILLA					240
EPQFTIYPHWDAESVVFVAKGCTISLVQRDRRESLITKQGDILKINAGTTAYLINDIMMERLVAKLLQPVSTPGEFE					320
LFRGAGGEMPESEFFKSFDEILEAAFNTRRDLQRIFGQRCQVIVKASEEQVRAISRHEBGGIWPFGGSGKTIMIVQQ					400
RPDHSNQGQLHEVDASQYRQLRDLDTVSLANITQANTAPHYNSKATKIALVVDGEGYFEMACPHMSRSGSYQGETR					480
GRPSYQVASESLTRGTVVITPAGHPFVAVASSNQLQVLCFEVIANNEKFLAGRRVIMQLBREAKELAFGMPAREVE					560
EVSRSQEEFFFKGPRQQQGRADA					640
.....					
.....N.....					
.....					
.....					
.....N.....					
.....					
.....					
(Threshold=0.5)					
SeqName	Position	Potential	Jury	N-Glyc	
			agreement	result	
tr_Q9AUD0_Q9AUD0_SESIN	126	NPTD	0.7181	(9/9)	++ WARNING: PRO-X1.
tr_Q9AUD0_Q9AUD0_SESIN	433	NITQ	0.5369	(6/9)	+

Fig. 3. Predicted N-glycosylation sites in sesame allergens (Q9AUD0 protein). The prediction analysis was generated using the NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc/>). The potential score is the averaged output of nine neural networks and the default threshold is 0.5. The jury agreement column indicates the number of networks (out of 9) that support the prediction. Asn-Xaa-Ser/Thr sequons (including Asn-Pro-Ser/Thr) are shown in blue. Asparagines predicted to be N-glycosylated are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

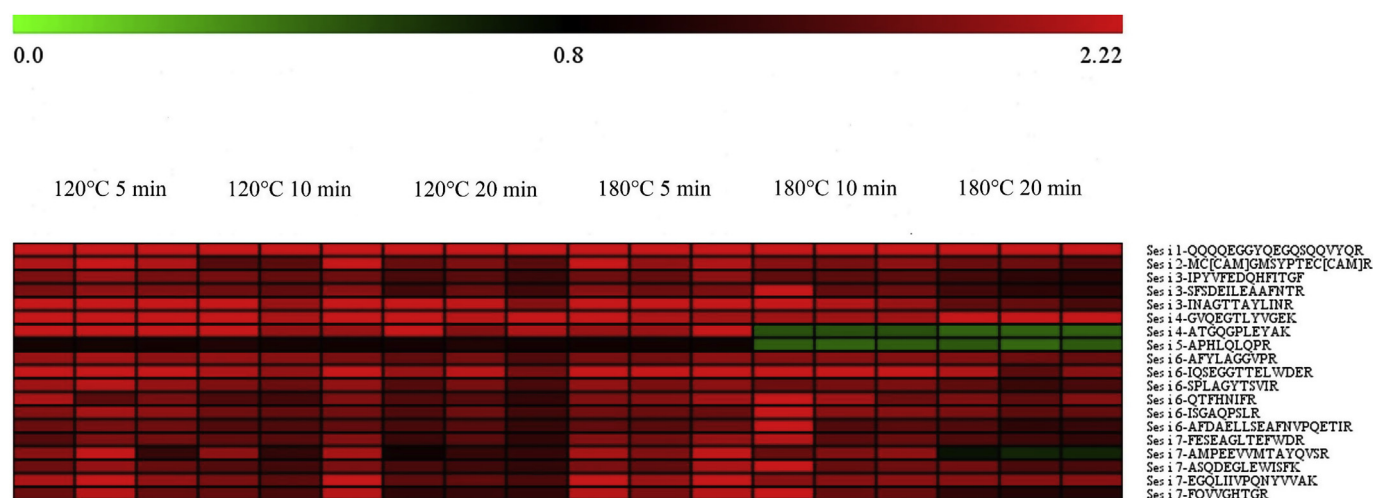


Fig. 4. Heat map representation of peptides obtained from sesame treated by different processing conditions. Data display mean values of peak area ratio of processed to unprocessed samples.

Table 2

Signature peptides and AQUA peptides for sesame allergens obtained using optimized LC and MS/MS parameters.

Allergen	Accession number	Peptide sequence	m/z		CE	RT
			Precursor ion	Product ion		
Ses i 1	2S albumin Q9AUD1	QQQQEGGYQEGSQVYQR	757.012	908.458/821.426/1093.538	35.8/35.8/35.8	7.6
		QQQQEGGYQEGSQV*YQR	759.020	1228.590/1099.55/1042.530	35.8/35.8/35.8	7.6
Ses i 2	2S albumin Q9XHP1	MC[CAM]GMSYPTEC[CAM]R	696.264	662.293/912.388/1100.450	34.0/34.0/34.0	14.0
		MC[CAM]GMSYPTEC[CAM]R*	701.268	672.301/922.396/1110.458	34.0/34.0/34.0	14.0
Ses i 3	7S vicilin-like globulin Q9AUD0	IPYVFEDQHFTGFR	623.649	740.409/593.341/480.257	33.9/33.9/33.9	33.0
		SFSDEILEAAFNTR	800.386	679.352/808.395/921.479	29.5/37.5/41.5	34.2
		SFSDEI*LEAAFNTR	803.890	679.350/808.390/921.480	29.5/37.5/41.5	34.2
Ses i 4	Oleolin Q9FUJ9	GVQEGTLVYGEK	640.330	333.177/866.462/708.393	38.1/30.1/30.1	15.4
		GVQEGTL*YVGEK	643.840	333.180/816.460/715.410	38.1/30.1/30.1	15.4
		ATGQGPLEYAK	567.793	777.414/720.393/962.494	27.6/27.6/23.6	13.9
Ses i 5	Oleolin Q9XHP2	APHLQLQPR	530.307	641.373/754.457/494.788	32.3/32.3/28.3	13.4
		APHLQL*QPR	533.815	648.390/761.47/498.297	32.3/32.3/28.3	13.4
Ses i 6	11S globulin Q9XHP0	AFYLQAGVPR	525.790	556.320/485.283/669.404	28.2/20.2/28.2	25.0
		AFYL*AGGVPR	529.30	556.320/485.28/676.420	28.2/20.2/28.2	25.0
		IQSEGGTTTELWDER	810.879	242.150/605.268/718.352	37.8/37.8/41.8	20.9
		ISGAQPSLR	464.764	728.405/815.437/472.288	22.1/26.1/26.1	9.5
Ses i 7	11S globulin Q9AUD2	FESEAGLTFEWDNR	793.860	853.384/1023.489/623.294	35.3/37.3/37.3	32.5
		EGQLIIVPQNYVVAK	835.977	918.504/1017.573/1243.741	34.7/34.7/38.7	30.9
		EGQL*IVPQNYVVAK	839.490	918.500/1017.570/1243.740	34.7/34.7/38.7	30.9

RT, retention time; CE, collision energy; * denotes stable isotope labeling of amino acid residues; Sequence in bold: the AQUA peptides for each allergen.

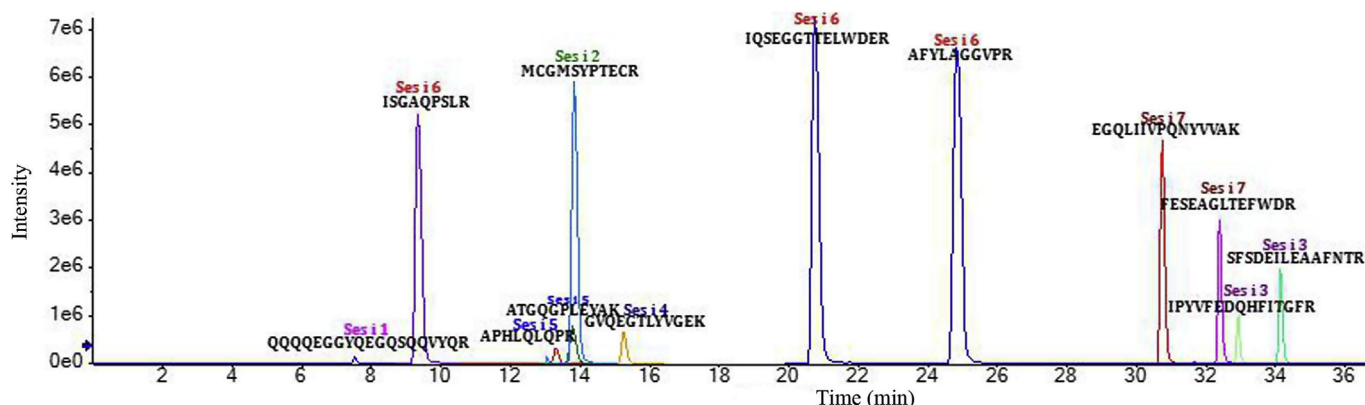


Fig. 5. The extracted ion chromatogram of sesame allergen signature peptides obtained using scheduled LC-MRM analysis.

newly found in our research with good characteristics to be a signature peptide of Ses i 6.

Digestion conditions significantly influence the concentration of

peptides, highlighting the need to optimize reaction conditions (Mattarozzi et al., 2014). For this reason, we confirmed the completeness and reproduction of the digestion over a range of sesame

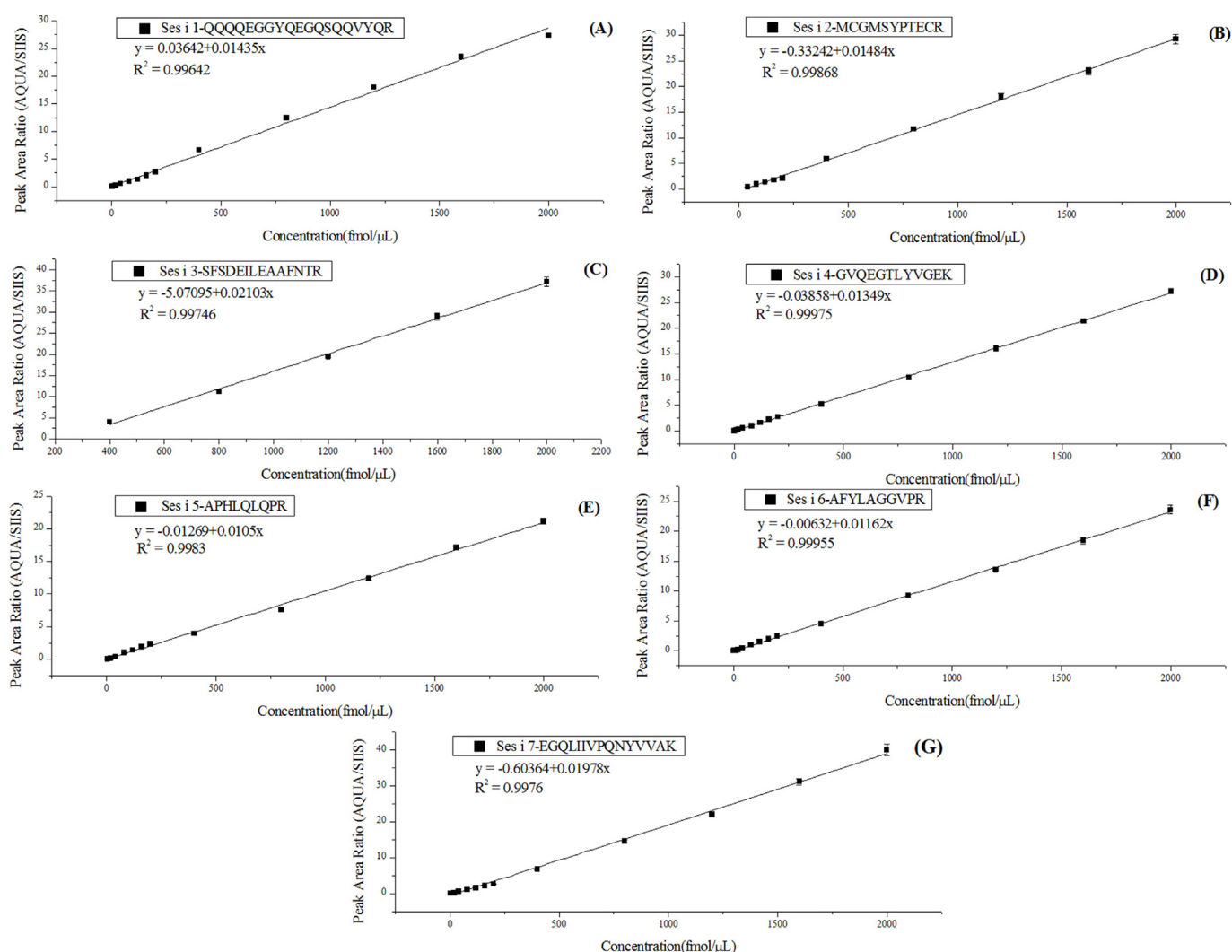


Fig. 6. Linearity of the LC-MS/MS measurement peak area ratios (AQUA/SIIS) assessed with various concentrations of the AQUA peptide.

Table 3

Linearity validation of calibration curves (Fig. 6) and validation for method sensitivity.

AQUA peptide	Linear equations	R^2	LOQ (fmol/μL)	LOD (fmol/μL)
Ses i 1-QQQQEGGYQEGSQVYQR	$y = 0.03642 + 0.01435x$	0.99642	0.4	0.1
Ses i 2-MC(CAM)GMSYPTEC(CAM)R	$y = -0.33242 + 0.01484x$	0.99868	40	15.0
Ses i 3-SFSDEILEAAFNTR	$y = -5.07095 + 0.02103x$	0.99746	400	140.0
Ses i 4-GVQEGTLYVGEK	$y = -0.03858 + 0.01349x$	0.99975	0.4	0.1
Ses i 5-APHLQLQPR	$y = -0.01269 + 0.0105x$	0.99830	4	1.0
Ses i 6-AFYLAGGVPR	$y = -0.00632 + 0.01162x$	0.99955	0.4	0.1
Ses i 7-EGQLIIVPNQYVAK	$y = -0.60364 + 0.01978x$	0.99760	4	1.0

AQUA, absolute quantification; LOQ, limit of quantification; LOD, limit of detection.

concentrations prior to accurate quantification. First, we assessed the linearity of the signature sesame peptides (data was not shown). The AQUA peptide for each allergen based on the best intensity, linearity, thermal stability, reproducibility and sensitivity, is shown in Table 2 in bold.

For the signature and AQUA peptides selection, the Q-TOF offered a significant advantage in the initial database searching due to its mass accuracy and it also provided reliable MS/MS peptide spectrum matches. Compared with *in silico* trypsin digestion by PeptideCutter or Skyline software, the LC-MS/MS analysis combined with ProteinPilot identification can provide a lot of useful information, including the missed cleavage sites and PTMs for each peptide. Some peptides may have partial modifications, which have little influence on the

identification of allergens but have a significant impact on the quantification process (Huschek et al., 2016).

3.3. Selection of MRM transitions

Upon collision induced dissociation, charged peptides fragment into a multitude of small bits, leading to the production of y-ions and b-ions (peptide backbone fragment ions retaining a positive charge at the N- and C-terminal), resulting in multiple peaks for every peptide. In order to obtain highly sensitive measurements, it is important to produce robust MS/MS transitions with high specificity and intensity for each fragment. Additionally, studies suggest that at least three to four transitions should be screened per signature peptide (Rauh, 2012; Tolin

Table 4
Summary of inter-day and intra-day validation for method precision (Variability for the quantitative determination of Ses i proteins in cookie).

Allergens	Day	Concentration (nmol/g matrix weight)	%CV	n
Ses i 1	1	1.6	4.0	6
	2	1.5	4.4	6
	3	1.5	3.7	6
	4	1.6	3.1	6
	5	1.7	3.0	6
	1–5	1.6	3.7	30
Ses i 2	1	188.0	4.6	6
	2	179.0	3.8	6
	3	186.0	4.2	6
	4	198.0	3.1	6
	5	179.0	2.5	6
	1–5	186.0	3.7	30
Ses i 3	1	63.0	3.6	6
	2	60.0	2.8	6
	3	62.0	4.2	6
	4	63.0	4.1	6
	5	62.0	3.5	6
	1–5	62.0	3.6	30
Ses i 4	1	32.0	2.7	6
	2	29.0	4.7	6
	3	31.0	4.5	6
	4	33.0	3.1	6
	5	30.0	2.1	6
	1–5	31.0	3.6	30
Ses i 5	1	7.6	4.6	6
	2	7.5	3.8	6
	3	7.9	4.2	6
	4	8.2	3.1	6
	5	7.9	2.5	6
	1–5	7.8	3.6	30
Ses i 6	1	595.2	3.7	6
	2	581.8	4.9	6
	3	591.0	4.5	6
	4	610.8	3.2	6
	5	597.2	3.1	6
	1–5	595.2	3.9	30
Ses i 7	1	244.6	2.3	6
	2	240.7	2.8	6
	3	242.7	3.6	6
	4	255.0	4.5	6
	5	250.0	4.9	6
	1–5	246.6	3.6	30

Table 5
Average recovery values of sesame AQUA peptides peptides.

Standards	Accuracy (%)		
	QC-low 20 fmol/μL	QC-mid 200 fmol/μL	QC-high 2000 fmol/μL
Ses i 1-QQQQEGGYQEGQSQQVYQR	94.05	95.00	97.90
Ses i 2-MC(CAM)GMSYPTEC(CAM)R	95.45	94.56	93.90
Ses i 3-SFSDILEAFAFNTR	92.50	97.25	99.75
Ses i 4-GVQEGTLYVGEK	94.75	98.00	98.50
Ses i 5-APHLQLQPR	91.00	94.50	96.50
Ses i 6-AFYLAGGVPR	90.00	100.80	102.50
Ses i 7-EGQLIIVPQNYVVAK	93.90	102.30	104.00

et al., 2012). Afterwards, the transitions that provide the best intensity and selectivity should be chosen for the MS/MS quantification to further improve the sensitivity. Reports indicate that the MRM transition for the signature peptides should be designed with the following conditions in mind (Yocum & Chinnaiyan, 2009): doubly charged precursor ion for the first quadrupole (Q1), preference for y-ions instead of b-ions

and larger m/z of product ions compared to the precursor ions. According to the mass fragments generated by the Q-TOF-LC/MS/MS combined with Skyline analysis, we selected six relevant MRM fragments per signature peptide. Following optimization, the final LC-MRM method was capable of monitoring 19 signature peptides (in isotope-labeled and native forms), each with three y-ion fragments, resulting in a total of 57 MRM transitions (Table 2). Isotope-labeled peptides (with incorporated ^{15}N and ^{13}C) likely co-elute together with the native peptides, hence the intensity ratios of the transitions should be the same. Previous studies suggest that at least three transitions of both analyte and the isotope-labeled peptides should be selected for monitoring (Abbatiello, Mani, Keshishian, & Carr, 2010), which can greatly reduce the noise and improve the overall sensitivity of the assay.

3.4. LC/MS/MS optimization

Signature peptides were synthesized and LC-MS/MS measurement optimization was performed, including chromatographic separation, RT, declustering potential (DP) and CE. For peptide identification, NanoLC approach (2 μL/min) was used to improve the LOD. Some reports suggest that this system is not as robust as normal flow systems which may limit its application in the clinical setting (Rauh, 2012). We used normal flow rates to detect sesame peptides in the LC-MRM assay, given its reliability in detecting target analytes from the tryptic digests (Agger, Marney, & Hoofnagle, 2010). Monitored signature peptides displayed both hydrophilic and hydrophobic properties, as determined by the grand average of hydropathy (GRAVY) values, which were obtained using ProtParam. (a web-based tool for calculating the physical and chemical parameters of a protein or peptide, <http://web.expasy.org/protparam/>) (Kyte & Doolittle, 1982). The LC gradient was specifically designed to minimize peptide co-elution, and obtain MRM acquisition, as described in Method section 2. The CE and DP values varied by ± 10 in 5 steps of 2 relative to the default equations. A single run consisting of a series of MRM transitions at differing CE and DP was created. The same CE and DP values were used for both native and isotope-labeled versions of each signature peptide. We were able to test only a few peptides in a single analysis because of the scan speed, chromatographic peak width, software limitations and other considerations. Importantly, scheduled MRM performed with a detection window can increase the number of peptides in a single analysis. Therefore, scheduled MRM was performed with a detection window set as 120 s at RTs and 2 s target scan time to maximize multiplexing for the identified peptides. Moreover, RTs were incorporated together with the three most intense MRM transitions (retention time variability within $\pm 5\%$). Chromatographic separation of chosen sesame allergens signature peptides obtained using scheduled LC-MRM analysis is shown in Fig. 5. In Table 2, the sesame allergen target peptides are reported together with the MRM fragments and optimized LC-MS/MS parameters. The amino acid sequences listed in Table 2 were for qualitative measurement, the ones in bold are used for absolute quantification of the sesame allergic proteins (Ses i 1–7). The extracted ion chromatogram of sesame allergen SIIS peptides using scheduled LC-MRM analysis were shown in Supplemental Fig. S4.

3.5. Linearity of the LC-MS/MS assay

The linearity of the developed LC-MS/MS assay for the AQUA peptides produced a concentration range of 0.4–2000 fmol/μL which was spiked with a constant amount of SIIS peptides. Linear regression analysis was performed on the LC-MRM analyzed peak area ratios (AQUA/SIIS) versus the absolute concentration for all target peptides (Fig. 6). All peak area values were linear ($R^2 > 0.99$) and the coefficient of variation (CV) values were all under 15%. AQUA/SIIS peak area ratios were calculated to determine the corresponding peptides contents. The contents of targeted sesame allergenic proteins in samples were calculated based on the molar equivalent relationship between the

Table 6
Content of sesame allergens in different sesame products.

Allergen	Content (nmol/g weight)				
	Hotpot dipping sauce	Spicy chili beer sauce	Cookie	Cake	Candy
Ses i 1	28.7 ± 0.9	20.9 ± 0.6	7.8 ± 0.2	5.2 ± 0.2	14.2 ± 0.4
Ses i 2	3284.0 ± 46.0	2644.0 ± 37.0	921.2 ± 12.9	542.8 ± 7.6	1673.2 ± 23.4
Ses i 3	1034.0 ± 22.7	829.0 ± 18.2	308.7 ± 6.8	195.3 ± 4.3	560.7 ± 12.3
Ses i 4	586.0 ± 11.1	402.0 ± 7.6	156.8 ± 3	79.2 ± 1.5	284.8 ± 5.4
Ses i 5	146.8 ± 4.3	97.8 ± 3.8	37.2 ± 1.5	25.6 ± 1.0	67.6 ± 2.6
Ses i 6	9913.6 ± 3271.5	7720.6 ± 2547.8	2916.5 ± 962.4	1800.1 ± 594.0	5280.3 ± 1742.5
Ses i 7	4202.8 ± 96.7	3160.8 ± 72.7	1168.5 ± 26.9	768.3 ± 17.7	2186.9 ± 50.3

The data are shown as average results and the relative standard deviation is less than 5% (n = 6).

AQUA peptides and original proteins.

3.6. Method validation

Validation for method sensitivity were summarized in Table 3. The limit of detection (LOD) was determined as the concentration at a signal: noise (S/N) = 3:1. The limit of quantification (LOQ) was with S/N ratio of 10:1 and as close as possible to lowest point on the calibration curve. LOD and LOQ for the seven sesame allergens AQUA peptides were determined to range from 0.1 to 140.0 fmol/μL and 0.4–400 fmol/μL respectively.

Precision was evaluated by assessing intra- and inter-day repeatability. Intra-day repeatability was assessed by performing LC-MS/MS analysis of sesame allergen peptides six times on the same day. Inter-day variability of our method was evaluated by performing quantitative detection with trypsin treatment and LC-MS/MS measurements on five different days. The reproducibility was high with the intra-day CVs in the 2.5–4.9% range. For the inter-day repeatability, we also achieved very good results with CVs in the 3.6–3.9% range. These data are summarized in Table 4, where the reproducibility of trypsinization and the LC-MS/MS analysis is shown for multiple sesame-containing food products.

Accuracy of LC-MS/MS analysis was evaluated and established over three concentration levels of QC samples. Results (Table 5) showed that the range of accuracy of detection of the 20, 200, and 2000 fmol/μL standards spiked into gluten protein solution (sesame free) were 90–104% (see Table 5).

Although we choose the AQUA peptide for each allergen based on the best intensity, linearity, thermal stability, reproducibility and sensitivity, we still need to permits simulation of the effects of food processing on target analyte (allergen protein) structure affecting analyte extractability and detectability with incurred samples. Finally, we calculated the absolute content of Ses i 2 in non-processed and processed matrixes. According to the recovery, we further analysis the accuracy with incurred samples. The results showed that the accuracy of 20, 50, 100 ppm added purified Ses i 2 were 90%, 92%, 94% in non-processed material and 92%, 95%, 102% in processed material respectively. SIIS peptide standards was added to the samples at early stage of the analytical process and they can thus correct for analytical variabilities due to upstream sample handling (on the condition that they behave similarly to their protein targets during sample processing). The recoveries of incurred materials indicated that sesame allergens in processed food matrix have good extractability and detectability.

3.7. Determination of the sesame allergen content in food items

What we did in this research was to detected each sesame allergen respectively. Each allergen has its own qualitative and quantitative peptides. In addition, no references of calculation factors for sesame allergic are available based on any peptide or allergic protein. In our research, we also found that all the signature peptides exist in different

sesame species (Fig. 2), but the composition of each peptide/protein varied between different cultivars. We express the content of each sesame allergic protein in the form of mol/g. It could be converted as ppm allergenic food with respect to EU legislation (European Parliament, 2011) and international assessment (Taylor et al., 2014) if calculation factors were obtained. One of the advantages of the present method is that it can quantify multiple proteins simultaneously. We can obtain the total sesame allergic proteins using this approach.

The data presented in Table 6 show the contents of sesame allergens in several sesame products (hotpot dipping sauce, spicy chili sauce, cookie, cake, candy) which were purchased from the local market. Although these allergenic proteins may be detected in various kinds of sesame products, baking is the most common processing in sesame products. Our developed method allows for detection of allergic proteins in heat-treated sesame products and the selected signature peptides are thermally stable. The ratios of Ses i 1–7 are different in different products since the sesame varieties were different, as shown in Fig. 2.

4. Conclusions

The trypsin digestion based LC-MS/MS analytical strategies employed in this study allowed for sensitive and specific quantification of seven sesame allergens in a single analysis. Based on our results, we wish to stress two critical outcomes of our study. Firstly, we discovered conserved and thermally stable signature and AQUA peptides. With the discovery of these peptides, we will be able to successfully detect each sesame allergen in a wide variety of food products. Secondly, we were able to perform AQUA for multiple sesame allergens simultaneously. We found that the contents of each sesame allergen varied in different sesame varieties, pinpointing the necessity of AQUA for each allergic protein. Our developed assay demonstrated high reproducibility, sensitivity, and specificity. The highly specific signature peptides we discovered in our study likely minimize the potential for cross-reactivity (present in ELISA assays) and limit the issues associated with indirect protein identification as seen in PCR approaches. For evaluating the levels of the sesame allergens in different varieties and food products, we developed and optimized our approach using SIIS peptides. In summary, our optimized LC-MS/MS approach can likely increase the safety of sesame processed foods and add diagnostic value to the sesame allergy testing.

Conflicts of interest

The authors declare that no competing interests exist.

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Appendix A. Supplementary data

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Supplemental Files

Figure S1. Effect of extraction time on yield of sesame protein.

Figure S2. Time course analysis showing peptide release and stability of the EGQLIIVPQNYVVAK peptide.

Figure S3. (A) Product ion spectrum of the peptide MC[CAM]GMSYPTEC[CAM]R. (B) Amino sequence of sesame allergen Ses i 1.

Figure S4. The extracted ion chromatogram of sesame allergen SIIS peptides using scheduled LC-MRM analysis.

Figure S5. SDS-PAGE analysis of purified sesame allergic proteins. Abbreviations: Marker: Molecular weight standard marker; F1: 20% alcohol precipitation fraction; F2: 30% alcohol precipitation fraction; F3: 40% alcohol precipitation fraction; F4: 50% alcohol precipitation fraction (purified Ses i 2); Sesame: crude protein extracts from sesame; F5: 20% alcohol supernatant fraction; F6: 30% alcohol supernatant fraction; F7: 40% alcohol supernatant fraction; F8: 50% alcohol supernatant fraction.

Figure S6. The extracted ion chromatogram of thermally treated sesame allergen AQUA peptides using scheduled LC-MRM analysis.

Table S1 Food items information.

Food items	Chief Ingredient	Nutritional information (100 g)	Company
Hotpot dipping sauce	Chili, Peppery, Sesame, Soybean, Canola oil, Edible salt, white sugar	Protein 7.3 (g), Fat 10.5 (g), Carbohydrate 5.7 (g), Sodium 5051 (mg)	Meile Food Co., Ltd.
Spicy chili sauce	Soy bean oil, pepper, Peanut, Sunflower seeds, sesame, Edible salt, white sugar, monosodium glutamate, pepper, star anise	Protein 2.6 (g), Fat 60.8 (g), Carbohydrate 28.5 (g), Sodium 1899 (mg)	Chengdu Baibaibei Food Co., Ltd.
Cookie	Wheat flour, Sesame, Soybean, Sugar, Palm oil	Protein 10.0 (g), Fat 23.0 (g), Carbohydrate 63.0 (g), Sodium 143 (mg)	DASHING INDUSTRIAL Co., Ltd.
Cake	Refined wheat flour, White kidney beans, Black sesame, granulated sugar, refined vegetable oil	Protein 7.3 (g), Fat 24.2 (g), Carbohydrate 48.3 (g), Sodium 50 (mg)	Shaanxi Redstar Food Group Co., Ltd.
Candy	Sugar, Cocoa butter substitute, Cocoa powder, Sesame	Protein 8.0 (g), Fat 42.4 (g), Carbohydrate 48.3 (g), Sodium 50 (mg)	Shandong Huifuyuan Food Co., Ltd.

Figure S1.

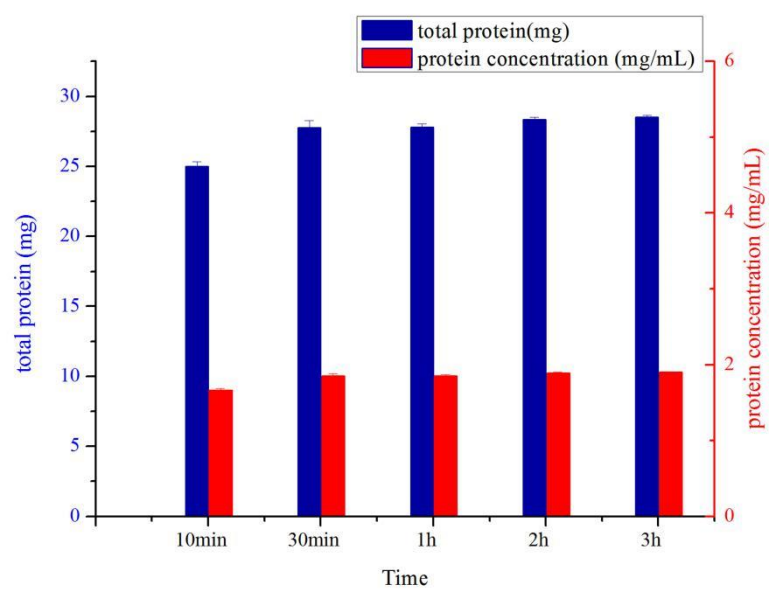


Figure S2.

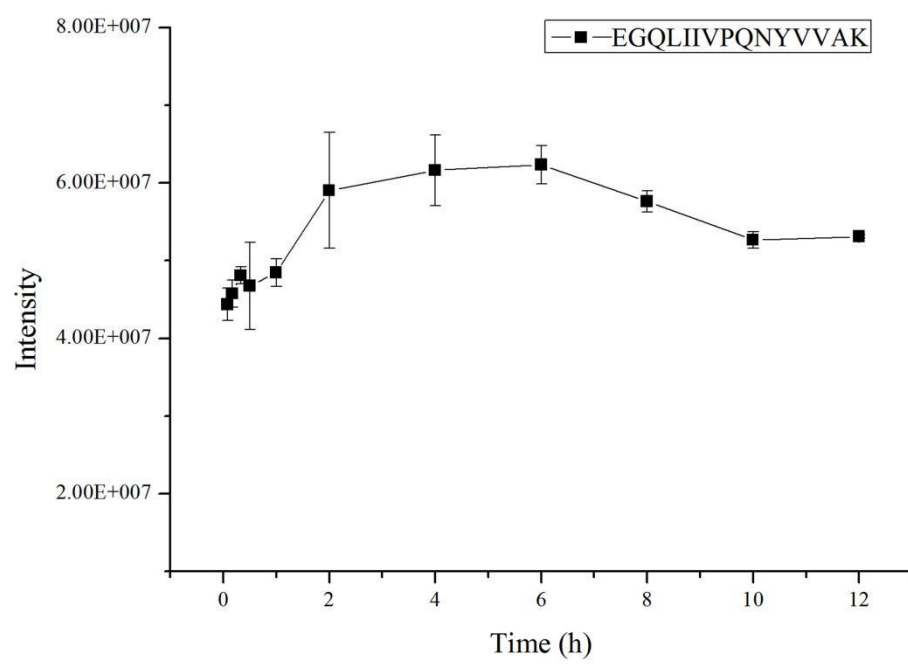


Figure S3.

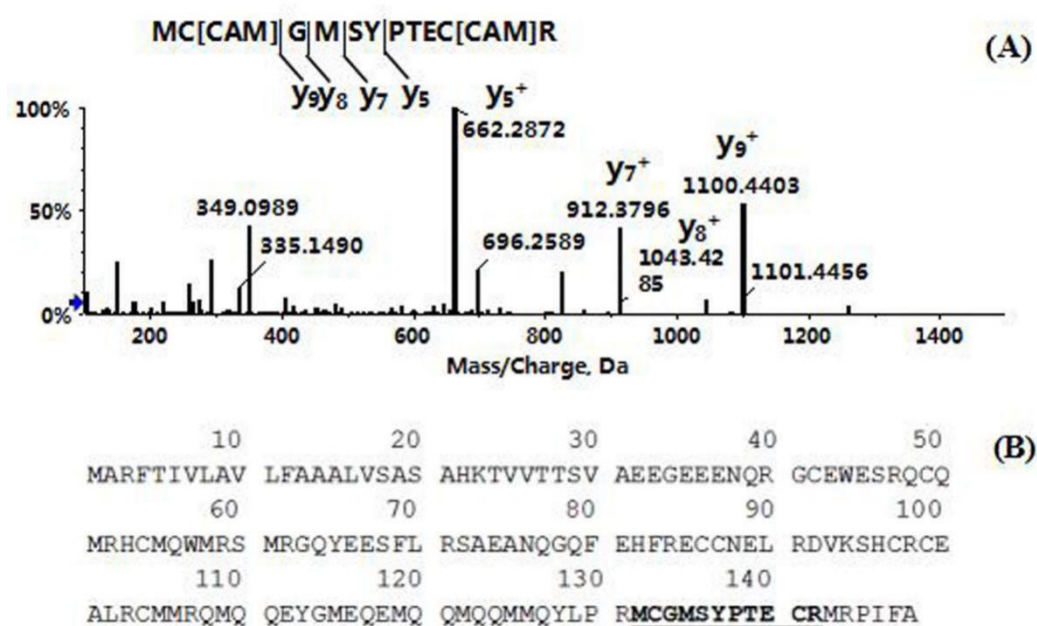


Figure S4.

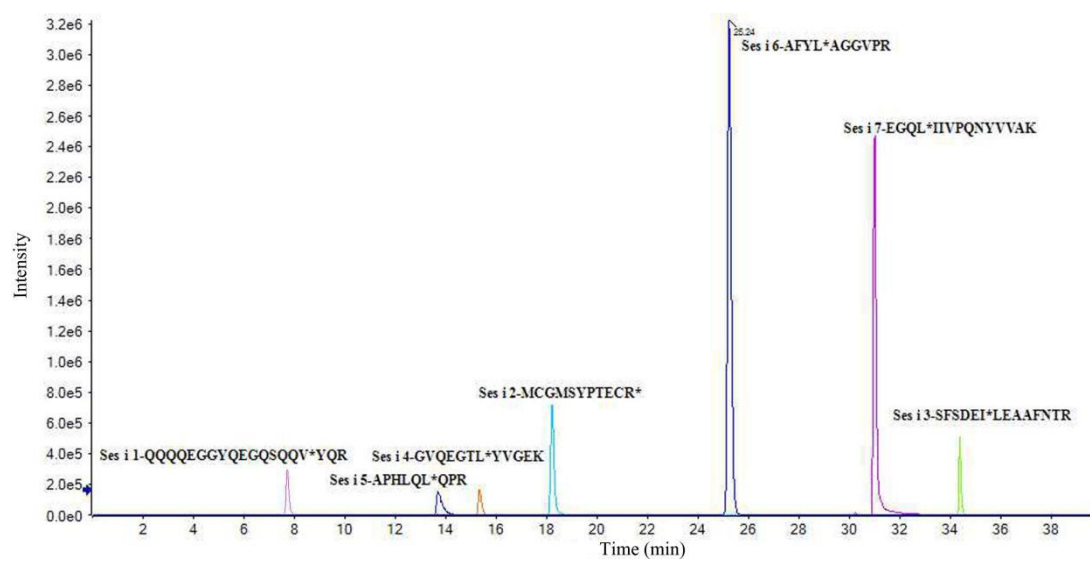


Figure S5.



Figure S6.

