

FOOD COMPOSITION AND ADDITIVES

Development of a Method for Determination of Buckwheat Allergens Using Liquid Chromatography with Tandem Mass Spectrometry

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An analysis technique using LC with tandem MS (MS/MS) has been developed for the determination of buckwheat proteins, including major allergens. A protein solution extracted from buckwheat was reduced, alkylated, and digested by trypsin. Peptide spectra were obtained using full-scan LC-MS/MS analysis, and peptide sequences were determined through a protein search. Nine peptides of the 13S globulin seed storage protein and one peptide of a 16 kDa allergen were selected as the marker peptides, and multiple reaction monitoring conditions were optimized. Using the conditions, different kinds of buckwheat noodles, powders, and other food ingredients were analyzed. As a result, buckwheat samples present all the fragment peaks, whereas other foods, including *Sesamum indicum*, wheat, and soybeans, are not detected at all. These findings indicate that LC-MS/MS analysis may be applied to the detection of buckwheat food allergens.

Buckwheat (*Fagopyrum esculentum*) is widely consumed in Asia, especially in Japan, Korea, and China. Recently, its consumption in North America and Europe has increased remarkably. Buckwheat is rich in flavonoids, fiber, vitamins, and antioxidants, and is consequently becoming popular as a health food. However, buckwheat is known as a highly allergic food that can cause symptoms typical of type I allergy (1). It has been reported that buckwheat can cause urticaria, vomiting, and anaphylactic shock in the same way as soybean and peanut allergies (2). Buckwheat allergy often develops at around 7 years of age and is almost always a lifelong condition. Thus, it is important to prevent allergic reactions to buckwheat by accidental ingestion. In Japan, buckwheat has been designated as a specific raw material, the presence of which—like egg, milk, crustaceans, and wheat—must be clearly indicated on food product packaging.

The ELISA technique is the official method for the qualification and quantitation of buckwheat allergens in Japan (3, 4). Although the ELISA can be used to detect the allergenic proteins of buckwheat with high sensitivity, it also detects unregulated species. Furthermore, sample processing can disrupt the conformation of allergenic proteins (5, 6), which may affect their binding to antibodies (7) and, thus, assay results. When a sample is judged positive (over 10 µg buckwheat protein/g food

weight) by ELISA, the PCR technique must be used to confirm the result. PCR allows only the indirect determination of the presence of an allergenic food commodity by a DNA marker. However, LC-MS/MS combined with protease digestion is a method that characterizes and identifies the allergen itself, and it is expected to be an effective method for the analysis of food allergens (8–10). In this study, we developed a highly sensitive analytical method for the detection of buckwheat allergens using LC-MS/MS techniques.

Materials and Methods

Materials

Buckwheat samples were purchased from local grocery stores. The buckwheat dry noodle 1 sample contained buckwheat (made in Nagano prefecture, Japan), wheat, and NaCl. The buckwheat dry noodle 2 sample contained buckwheat (made in China) and NaCl. Buckwheat powder 1 (made in Nagano prefecture, Japan) and buckwheat powder 2 (made in China) contained only buckwheat. Boiled noodle 1 contained buckwheat (made in North America) and alcohol. Boiled noodle 2 contained buckwheat (made in China) and wheat. The *Sesamum indicum*, wheat, and soybean samples were pure powders. Porcine pancreatic trypsin (mass analysis grade), trifluoroacetic acid (TFA), formic acid, urea, ammonium bicarbonate, dithiothreitol (DTT), and iodoacetamide were purchased from Sigma-Aldrich, Japan (Tokyo, Japan). Acetonitrile and methanol were purchased from Kanto Chemical (Tokyo, Japan), and Strata X 33 µm polymeric reverse-phase SPE cartridges (200 mg) were purchased from Phenomenex (Torrance, CA). The synthetic peptide (>99%, VQVVGDEGR) was purchased from Eurofins Genomics, Inc. (Tokyo, Japan). All other reagents were purchased from Nacalai Tesque (Kyoto, Japan) or Wako Chemical (Wako, Japan). Unless otherwise specified, all reagents used were of analytical grade. Deionized water was used throughout the study.

Sample Extraction and Trypsin Digestion

A 1.0 g portion of buckwheat or buckwheat processed food was homogenized in 25 mL Tris-HCl (50 mmol/L, pH 10.2) containing 2 M urea by agitation for 60 min using a roller mixer. The resulting extracts were centrifuged for 5 min (2280 × g at 15°C). Aliquots of the supernatants (6 mL) were transferred to 15 mL polypropylene centrifuge tubes. To reduce protein disulfide bonds, 200 µL DTT (1 M) was added to a 6 mL portion of the supernatant, which was then heated for 1 h at 37°C. The sample was cooled to room temperature, and 2 mL 0.5 M iodoacetamide was added for the

reductive alkylation of cysteine. The reaction was then protected from light for 30 min at room temperature. Trypsin solution was added to this solution to give a final trypsin concentration 600 nM. The proteins were then digested overnight at 37°C. The resulting solution was centrifuged at 10°C and $2280 \times g$ for 5 min, and then filtered through a 0.45 μm PTFE filter.

Sample Purification

Strata X SPE cartridges (Phenomenex) were conditioned with 6 mL acetonitrile containing 0.1% formic acid, followed by 6 mL water containing 0.5% TFA. The supernatant from the centrifuged sample was loaded onto the SPE cartridge and washed with 0.5% TFA in water. Peptides were eluted with acetonitrile and evaporated to a dry state using a centrifugal evaporator. Next, a 300 μL aliquot of a 5% acetonitrile solution was added to reconstitute the sample residue. The samples were mixed using a vortex mixer for 30 s and sonicated for 30 min to complete the dissolution of the materials. The contents were then transferred to a 1.5 mL tube and centrifuged for 5 min at $20\,000 \times g$. The supernatant was then removed and transferred to a polypropylene HPLC vial for LC-MS/MS analysis.

HPLC and Mass Analysis

Analysis of the digested peptides was performed using a triple quadrupole and linear ion trap MS equipped with a turbo ion spray source (4000 QTRAP; AB SCIEX, Framingham, MA) coupled to an LC system (1200 Series; Agilent Technologies, Santa Clara, CA). The sample was injected onto an L-column2 ODS (2.1×150 mm, 3 μm ; Chemicals Evaluation and Research Institute, Japan) and analyzed in positive ion mode. The mobile phase was water–0.1% formic acid (A) and LC-MS grade acetonitrile–0.1% formic acid (B). The separation run started at 2% B for 2.5 min, followed by a gradient to 40% B over 14.5 min, and then a gradient to 98% B over 18 min. This isocratic run at 98% B was continued for 3 min. Lastly, the column was equilibrated at 2% B for 10 min. The injection volume, flow rate, and column temperature were 20 μL , 0.2 mL, and 30°C, respectively. A source temperature of 500°C, an ion spray voltage of 5.5 kV, and a curtain gas flow of 25 psig were used for the MS experiments. MS/MS was used to monitor nine marker peptides for buckwheat proteins. This was done in the multiple reaction monitoring (MRM) mode, in which the transition from the divalent protonated peptides of interest (the precursor ions) to one of their fragments was monitored. The digested buckwheat standard was analyzed in full survey scan mode, which presents MRM transition data. Information-dependent acquisition was used. Full-scan spectra were recorded from m/z 400 to 1200. The detection criteria included a signal intensity greater than 300 000 counts and a charge state of +2 or +3. Product ion spectra were recorded from m/z 100 to 1700. The International Protein Index databases were used to identify the digested peptides on the basis of MRM transitions. One missed cleavage was allowed. The MS/MS tolerances were set to 0.2 Da.

Results and Discussion

First, peptides from the buckwheat powder that were stably produced by trypsin were screened and submitted to alignment search tool analysis (ProteinPilot Software v4.0; AB SCIEX)

to ensure specificity. The criteria applied for marker peptides were the absence of cysteine and methionine, a peptide size between 5 and 25 amino acids, and no modified amino acids. Figure 1 shows examples of typical product ion spectra. These were identified as the peptides AIPVEVLANSYDISTK and GQILVVPQGFAVVLK from 13S globulin seed storage proteins 1, 2, and 3; and GFIVQARDLK from 13S globulin seed storage protein 2. The 13S globulin seed storage proteins are major allergens in *F. esculentum* (11, 12; Figure 2). The UniProt databases were used (13), and VPEEYEEELQR, VQVVGDEGR, and NDDNAITSPIAGK of 13S globulin seed storage proteins 1 and 3; GQILVVPQGFAVVLK and AIPVEVLANSYDISTK of 13S globulin seed storage proteins 1, 2, and 3; and GFIVQARDLK and NSGANITSPIGGR of 13S globulin seed storage protein 2 were selected as specific peptides. Theoretical digest data and MRMs, which were estimated using Skyline software v1.4.0.4421, were used to detect NAILGPR of 13S globulin seed storage proteins 1 and 3, NAIIGPR of 13S globulin seed storage protein 2, and YAEALSR of the 16 kDa allergen contained in buckwheat, in addition to the peptides mentioned above. None of the peptides contain modified amino acids. Three or four intense fragments of each peptide were chosen as product ions for the MRM transitions. The selected fragments for each peptide are listed in Table 1. The parent ions are all $[M + 2H]^{2+}$ ions, and the fragment ions are all $[M + H]^+$. Because the MWs of leucine and isoleucine are the same, the peak of m/z 370.7 is thought to be NAILGPR or NAIIGPR. Of the selected marker peptides, VQVVGDEGR is thought to be the most sensitive and stable, and, therefore, a candidate for the quantitative (target) peptide. The LOD for VQVVGDEGR was investigated using a commercial synthesized peptide. Six VQVVGDEGR standards with concentrations ranging from 1.5 to 3000 ng/mL were analyzed by this method (Figure 3). VQVVGDEGR shows a linear relationship between the peak area and concentration. The coefficients of determination (R^2) are >0.99 , and the LOD is approximately 1 ng/mL.

Second, using LC-MS/MS, different kinds of buckwheat noodles, powders, and other food ingredients were analyzed. The six buckwheat samples present all the fragment peaks, whereas other foods, including *Sesamum indicum*, wheat, and soybeans, are not detected at all (Figure 4). Therefore, it is suggested that monitoring these peptides is suitable for detecting buckwheat proteins. The 24 kDa allergen, which is a fragment of the 13S globulin seed storage protein, is assumed to be a major allergen of buckwheat based on the results of immunoglobulin E binding assays using patient sera (11). It has been recently suggested that the 24 kDa allergen is vulnerable to pepsin digestion and may not be a major allergen in buckwheat proteins (12). The 16 kDa allergen, which is resistant to pepsin, has been reported to be responsible for immediate hypersensitivity reactions to buckwheat (14). Although the trypsin-digestion-based method can detect nine peptides from 13S globulin seed storage proteins, only one peptide, YAEALSR, was detected from the 16 kDa protein. The sensitivity of LC-MS/MS peptide analysis depends on the amount of proteins present and digestion efficiency. Therefore, the 16 kDa allergen might be present in only small amounts or resistant to trypsin, as well as to pepsin. In Japan, the regulation of allergens is based on the total amount of protein (10 $\mu\text{g/g}$) in food, because foods that cause allergic reactions usually contain plural allergens. Commercial ELISA kits for buckwheat target

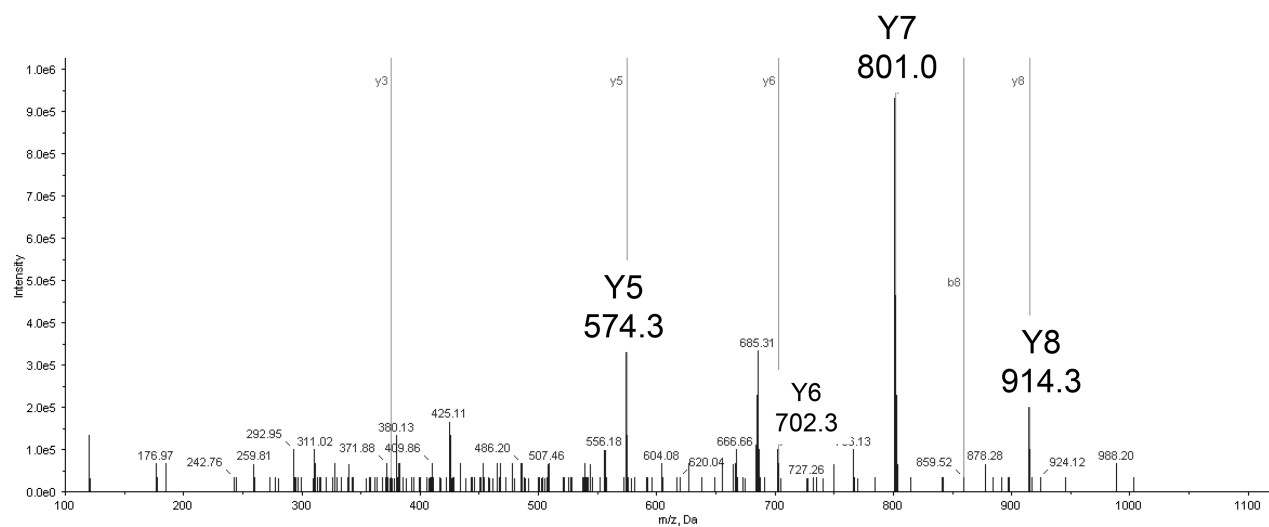
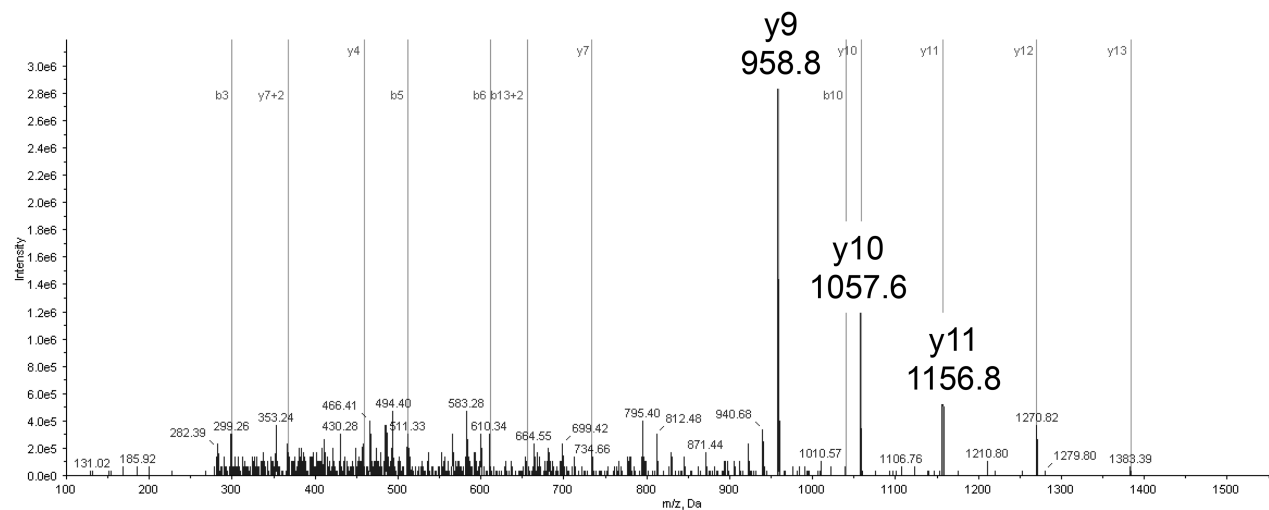
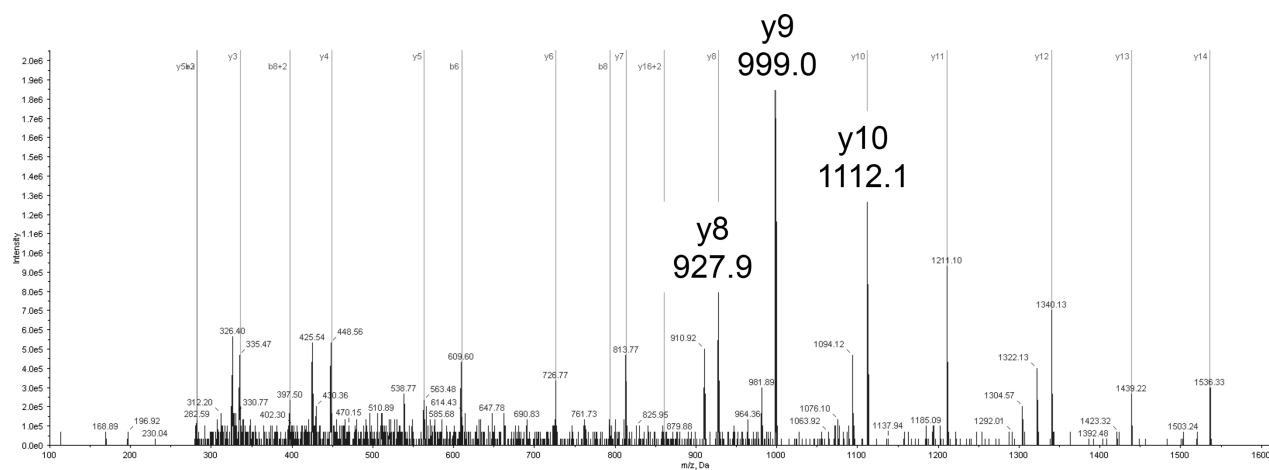


Figure 1. (A) Product spectra of AIPVEVLANSYDISTK, $[M + 2H]^+2$, m/z 860.5, generated from buckwheat; (B) product spectra of GQILVVPQGFAVLK, $[M + 2H]^+2$, m/z 784.5, generated from buckwheat; and (C) product spectra of GFIVQAQDLK, $[M + 2H]^+2$, m/z 559.6, generated from buckwheat.

13S globulin seed storage protein 1 (Accession :O23878)

MSTKLILSFLCLMVLSCSAQLLPWRKGQSRPHRGHQFHHQCDVQRLTASEPSRRVRSEAGVTEIWDNDTPEFRCAGFVAVRVVIQPGGLLLP
 SYSNAPYITFVEQGRGVQGVVPGCPETFQSESEFEYPSQRDQSRQSESEESSRGDQRTQSESEEFSGDQRTQSESEEFSGDQRTQSE
 SEEFSGDQRTQSESEEFSGDQHQKIFRIRDGDVIPSPAGVVQWTHNDGNDLISITLYDANSFQNLQDGNVNRNFFLAGQSKQSRDRRSQR
 QTREEGSDRQSRESDDDEALLEANILTGQFQDEILQEIFRNVDQETISKLRGNDQDQGFIVQARDLKLRLVPEEYEEELQREGRDRKGGSGRSN
 GLEQAFCNLFKQNVNRPVRADVFNPGRAGINTVNSNLPILFIQLSAQHVVLYKNAIIGPRWNLNAHSALYVTRGEGRVQVVGDEGRSVFDD
 NVQRGQILVVPQGFAVVLKAGREGLEWVELKNDNNAITSPIAGKTSVLR AIPVEVLANSYDISTKEAFRLKNGRQEVFVLPFQSRDEKERERF

13S globulin seed storage protein 2 (Accession :O23880)

MSTKLILSFLCLMVLSCSAQLWPWQKGGSRPHHGRQHQFHHQCDIQLRTASEPSRRVRSEAGVTEIWDHDTPEFRCTGFVAVRVVIQPG
 GLLPSYSNAPYITFVEQGRGVQGVVPGCPETFQSDSEFEYPSQRGRHSRQSESEESSRGDQHQKIFRIREGDVIPSPAGVVQWTHNDGND
 DLISVTLDDANSYHKQLDENVRFFLAGQSQRETREEGSDRQSRESDDDEALLGANILSGFQDEILHELFRDQVRETISKLRGENDQDGFIVQAQD
 LKLRVPQDFEEYERERGRRRRGQGGSGRSN
 GVEQGFNCNLFRRNFNTPTNTYVFNPRAGRINTVNSNLPILFIQLSAQHVVLYKNAIIGPRWNLNAHSALYVTRGEGRVQVVGDEGKSVFDD
 KVQRGQILVVPQGFAVVLKAGREGLEWVELKNSGNAITSPIGGRTSVLR AIPVEVLANSYDISTKEAYKLKNGRQEVFVRFPFQSRDEKERERFSI
 V

13S globulin seed storage protein 3 (Accession : Q9XFM4)

MSTKLILSFLCLMVLSCSAQLLPWQKGGSRPHHGRQHQFHHQCDIQLRTASEPSRRVRSEAGVTEIWDHDTPEFRCTGFVAVRVVIQPGGLLLP
 SYSNAPYITFVEQGRGVQGVVPGCPETFQSGSEFEYPSQRDQSRQSESEESSRGDQSRQSESEESSRGDQSRQSESEEFSGDQHQKIFRI
 RDGDVIPSPAGVVQWTHNNGDNDLISITLYDANSFQNLQDENVRNFFLAGQSKQSRDRRSQRQRTREEGSDRQSRESQDDEALLEANILSGFED
 EILQEIFRNVDQETISKLRGENDQDQGFIVQARDLKLRLVPEEYEEELQREGRDRKGGSGRSN
 GLEQAFCNLFKQNVNRPVRADVFNPGRAGRINTVNSNLPILFIQLSAQHVVLYKNAIIGPRWNLNAHSALYVTRGEGRVQVVGDEGRSVFDD
 NVQRGQILVVPQGFAVVLKAGREGLEWVELKNDNNAITSPIAGKTSVLR AIPVEVLANSYDISTKEAFRLKNGRQEVFVRFPFQSRDEKERERFSI
 V

16kDa allergen (Accession : Q2PS07)

MKLFIILATATLLIAATQATYPRDEGFDLGETQMSSKCMRQVKMNEPHLKKCNRYIAMDILDDKYAEALSRVEGEGCKSEESCMRGCCVAMKE
 MDDECVCEWMKMMVENQKGRIGERLIKEGVRDLKELPSKCLSELECGSRGNRYFV

Figure 2. Amino acid sequence of buckwheat major proteins.

Table 1. Selected peptide markers and MRM transition used in the MS/MS method

Protein	Peptide	Precursor m/z (charge state)	RT, min ^a	Product m/z
13S globulin seed storage protein 1, 3	³⁵³ VPEEYEEELQ ³⁶⁵	710.8 (+2)	14.1	1224.6 (y9) 1095.6 (y8) 966.5 (y7) 674.4 (y5)
	⁵⁷ NAIIGPR ⁶³	370.7 (+2)	13.2	442.3 (y4) 555.4 (y5) 412.3 (b4)
	⁸¹ VQVVGDEGR ⁸⁹	479.8 (+2)	12.3	731.4 (y7) 632.3 (y6) 533.2 (y5)
	¹²⁶ NDNNAITSPIAGK ¹³⁸	658.3 (+2)	14.1	673.4 (y7) 786.5 (y8) 857.5 (y9)
		439.2 (+3)		673.4 (y7) 485.3 (y5) 572.3 (y6)
13S globulin seed storage protein 1, 2, 3	⁹⁹ GQILVVPQGFAVVLK ¹¹³	784.5 (+2)	18.4	958.8 (y9) 1057.6 (y10) 1156.8 (y11)
	¹⁴⁴ AIPVEVLANSYDISTK ¹⁵⁹	860.5 (+2)	17.2	999.0 (y9) 927.9 (y8) 1112.1 (y10)
		574.0 (+3)		726.4 (y6) 609.4 (b6) 813.4 (y7) 927.4 (y8)
	⁵⁷ NAIIGPR ⁶³	370.7 (+2)	13.2	442.3 (y4) 555.4 (y5) 412.3 (b4)
13S globulin seed storage protein 2	²⁷⁵ GFIVQAQDLK ²⁸⁴	559.6 (+2)	15.4	702.3 (y6) 801.0 (y7) 574.3 (y5) 914.3 (y8)
	⁴³⁹ NSGANITSPIGGR ⁴⁵¹	622.3 (+2)	13.8	800.4 (y8) 499.3 (y5) 871.5 (y9)
16 kDa allergen	⁶⁵ YAEALSR ⁷¹	405.2 (+2)	12.4	446.3 (y4) 575.3 (y5) 646.5 (y6)

^a RT = Retention time.

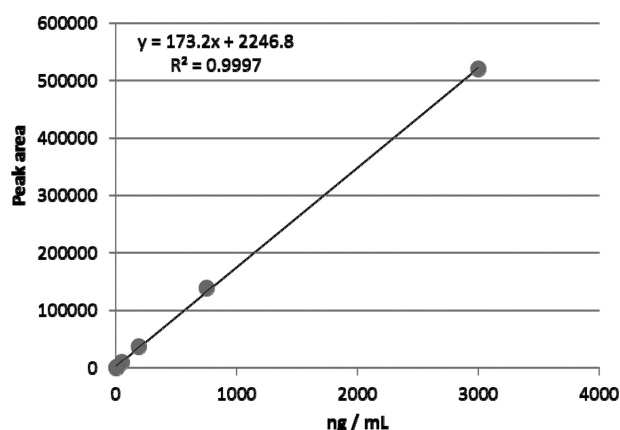


Figure 3. Calibration curve of VQVVGDEGR peptide, and slope, intercept, and R^2 value.

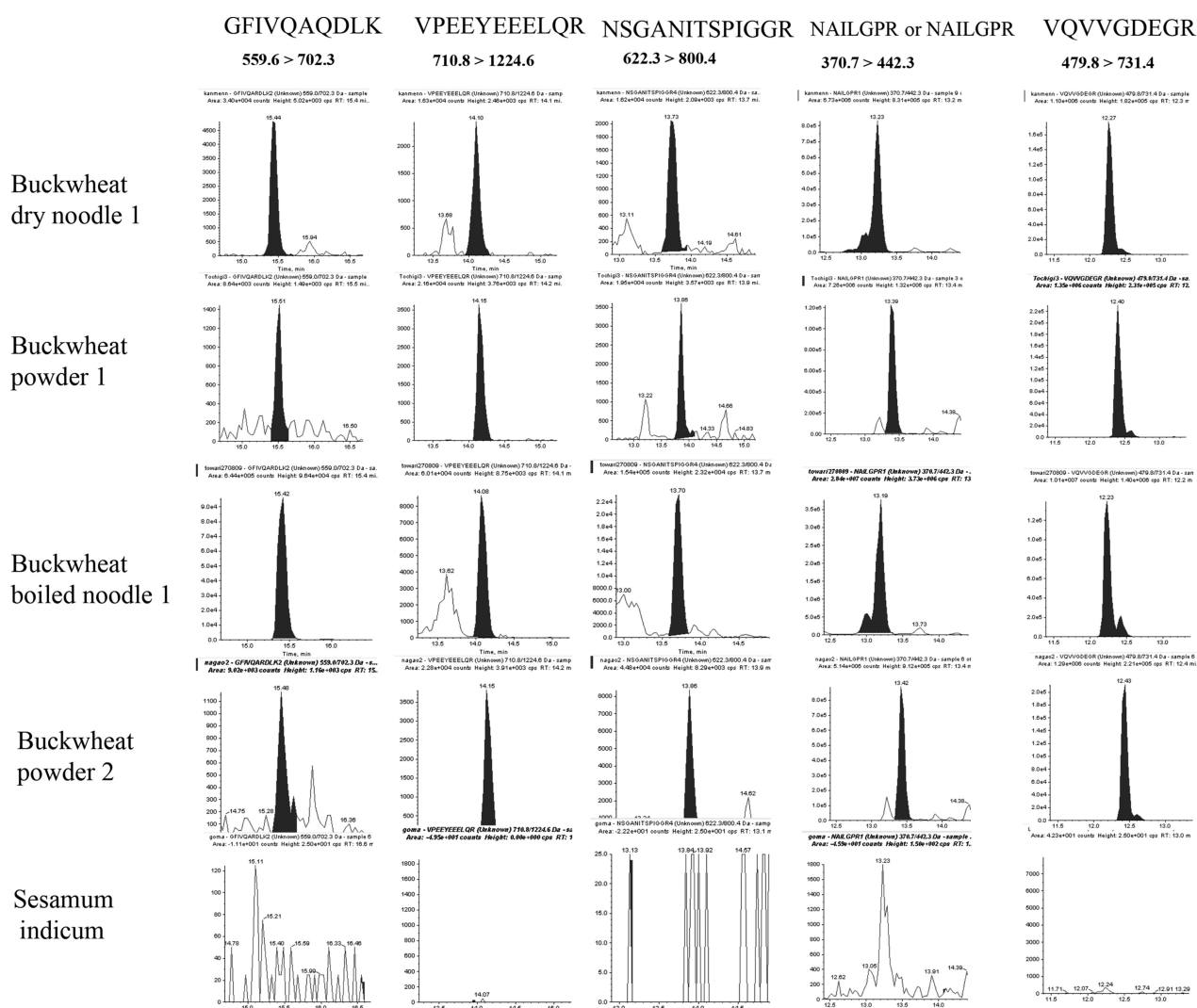


Figure 4. MRM chromatograms of each marker peptide from a digested various buckwheat or other food sample.

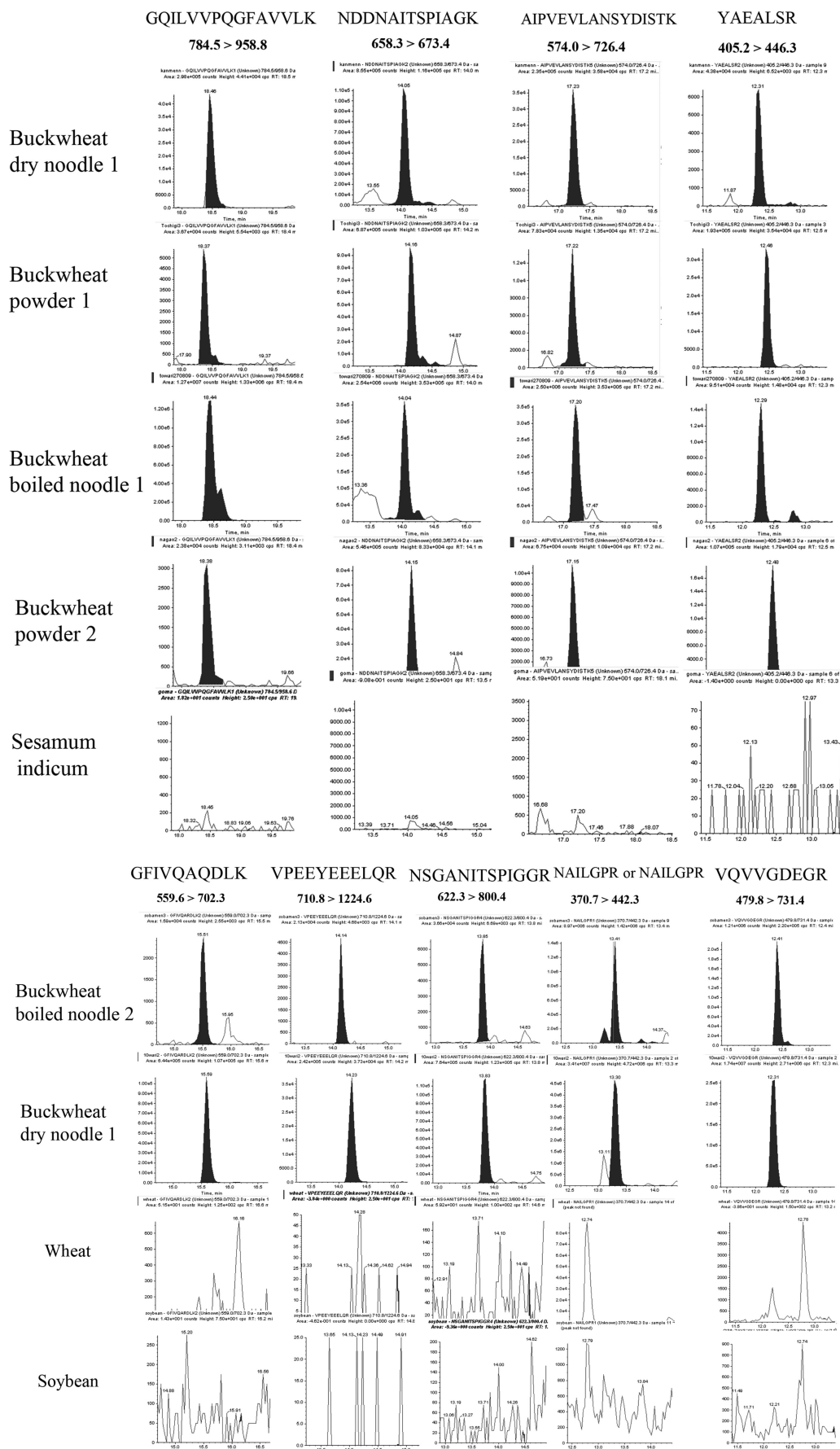


Figure 4. (continued)

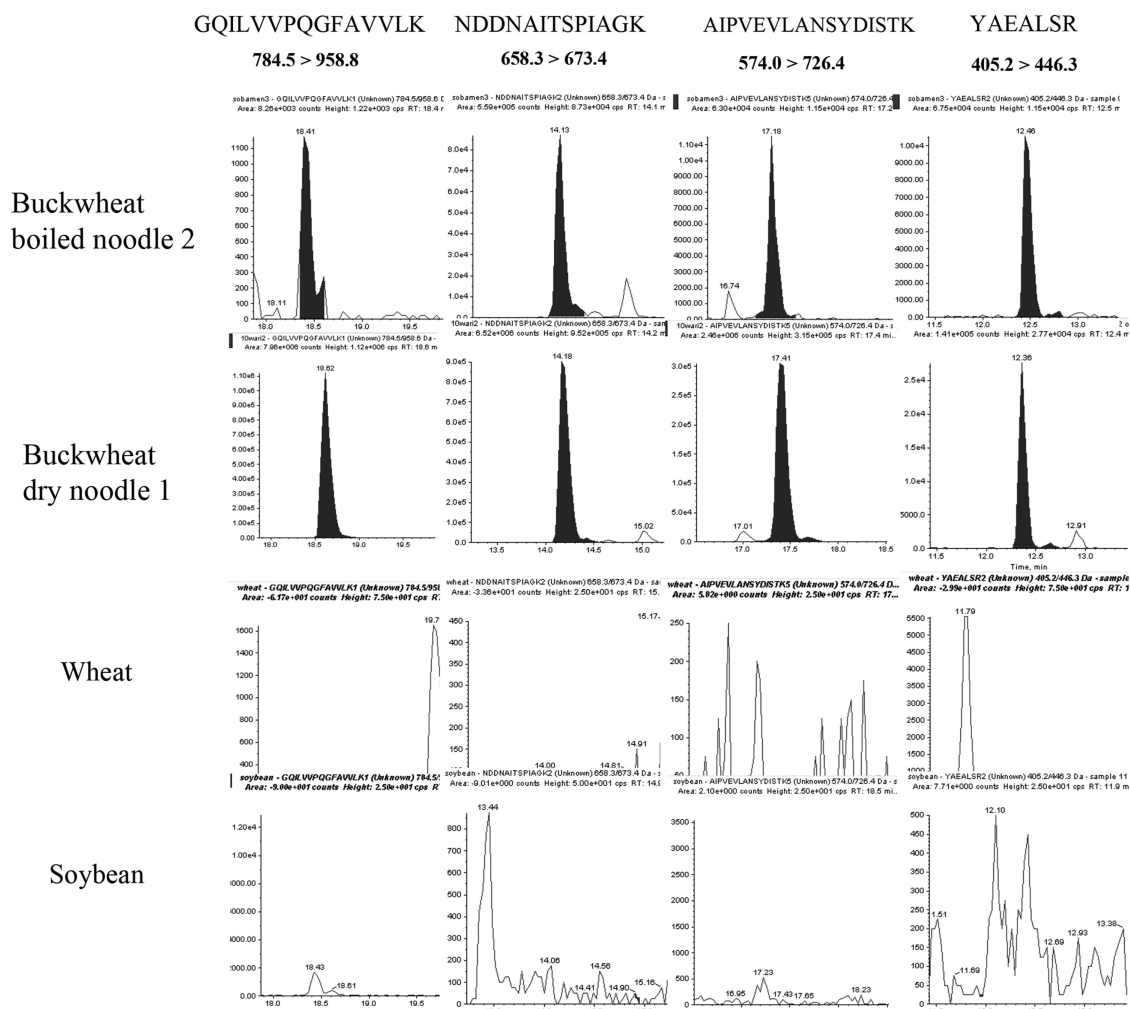


Figure 4. (continued)

different proteins, so the use of two or more kits with different specificities is recommended. However, the LC-MS/MS method can detect plural buckwheat allergens simultaneously and is, therefore, remarkably useful. The LC-MS/MS approach for analyzing allergens may be widely applied in the future.

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