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Development of a simple and reliable LC-MS/MS method to simultaneously detect walnut and almond as specified in food allergen labelling regulations in processed foods

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ABSTRACT

We developed a simple and reliable analytical method using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) to simultaneously detect walnut and almond as specified in regulations for food allergen labelling in processed foods. Five specific target peptides derived from walnut 2S albumin and 7S globulin and three target peptides from almond 11S globulin were selected by analysing several varieties of walnut and almond, eight kinds of other nuts, and ten kinds of major allergen ingredients or cereals. The limit of detection for the walnut 2S albumin peptide GEEMEEMVQSAR (*m/z* 698.3 [precursor] > 316.1 [product]) was 0.22 ± 0.02 μg/g, and that for almond 11S globulin peptide GNLDFVQPPR (*m/z* 571.8 [precursor] > 369.2 [product]) was 0.08 ± 0.02 μg/g when extracted walnut and almond protein were spiked into butter cookie chocolate ice cream. These peptides had good linearity ($R^2 > 0.999$) for each calibration curve with a range of 0.1–50 μg/mL protein concentration in the sample solutions, and sufficient recovery rates (90.4–101.5%) from the spiked samples. The developed analytical approach is applicable to a wide variety of processed foods for food allergen labelling.

1. Introduction

Food allergies are immunological reactions caused by ingesting foods containing allergens that cause symptoms, and even a very small amount (a few mg) can cause serious health problems such as anaphylactic shock. In recent years, the number of patients with food allergies has been increasing worldwide (Warren et al., 2020) and has become an international issue (Ogura et al., 2019). Therefore, in 1999, the Codex Alimentarius Commission established labelling guidelines for raw materials that cause food allergies. The guidelines state that currently eight raw materials are to be labelled: gluten-containing grains, shellfish, eggs, fish, peanuts and soybeans, milk, nuts, and sulphites with a concentration of 10 mg/kg or more (Codex Alimentarius Commission, 2018). And recently, Codex Committee on Food Labelling (CCFL46) has suggested that sesame should be added in place of soybeans as raw materials to be labelled (Codex Alimentarius Commission, 2021). It is important to provide raw material information on packaging so that consumers can make the right choices when buying food to avoid unexpected food allergies. Since nuts including walnuts and almonds often cause highly severe reactions in those with allergies, the labelling is mandatory in the United States, Canada, the European Union, Australia, New Zealand, and Hong Kong. (FDA, 2004; Government of Hong Kong, 2017; Popping and Diaz-Amigo, 2018; Government of Canada, 2020; FSANZ, 2022). In Japan, some labelling of allergen-inducing raw materials has been mandatory or recommended since 2002, and currently seven items, eggs, milk, wheat, buckwheat, peanuts, shrimp, and crab, are required (Government of Japan Consumer Affairs Agency, 2015). In recent years, since the number of cases of allergies caused by nuts, especially walnuts, has increased rapidly, the mandatory labelling of walnuts is being considered in Japan. Testing methods are important for

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Table 1
LC-MS/MS parameters used to detect walnut and almond peptides.

Nut	Target protein	Amino acid sequences of the target peptide	Precursor ion (*m/z*)	Charge state	Product ion (*m/z*)	Fragment type and number	Retention time (min)	Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)
Walnut	2S Albumin (Jug r1)	QQQQQGLR	493.3	+2	345.2[b]	y3	6.5	67	27	15
					473.3	y4				
					601.3	y5				
		DLPNEC$_{[CAM]}$[a]GISSQR	688.3	+2	477.2[b]	y4	8.4	81	34	15
					647.3	y6				
					807.4	y7				
		GEEMEEMVQSAR	698.3	+2	316.1[b]	b3	9.0	82	34	15
					461.2	y4				
					820.4	y7				
	7S Globulin (Jug r2)	GQEQTLC$_{[CAM]}$[a]R	496.2	+2	186.1[b]	b2	7.3	67	27	15
					549.3	y4				
					806.4	y6				
		ATLTLVSQETR	609.8	+2	620.3	y5	9.3	76	31	15
					719.4[b]	y6				
		QGQGQR	337.2	+2	175.1[b]	y1	8.3	56	21	15
					488.3	y4				
		HESEEGEVK	522.2	+2	246.2	y2	7.6	69	28	15
					690.3[b]	y6				
Almond	11S Globulin (Pru du 6)	GNLDFVQPPR	571.8	+2	175.1	y1	9.6	73	29	15
					369.2[b]	y3				
					596.4	y5				
		YNRQETIALSSSQQR	594.3	+3	605.3	y5	8.2	74	30	15
					692.3[b]	y6				
		QETIALSSSQQR	674.3	+2	605.3	y5	8.3	80	33	15
					692.3[b]	y6				

[a] Cysteine residue methyl-carbamated by iodoacetamide.
[b] Quantifier ion (no mark is qualifier ion).

confirming the validity of allergen labelling in foods, and enzyme-linked immunosorbent assay (ELISA), lateral flow immunoassay (immunochromatography), polymerase chain reaction (PCR), and western blotting are generally used for allergen analysis in foods (Köppel et al., 1998; Saito et al., 2019; Miyazaki et al., 2019; Ross et al., 2019; Pilolli et al., 2020).

According to the official method of food allergen analysis in Japan, after conducting a screening test by ELISA, if the protein of the target raw material is detected in an amount of 10 mg/kg or more, PCR (for wheat, buckwheat, peanuts, shrimp, and crab) or western blotting (for milk and egg) should be performed (Akiyama and Adachi, 2021).

However, in ELISA, immunochromatography, and western blotting using an antibody, cross-reactivity may occur with a component other than the target raw material, and a false positive result may be obtained (Saito et al., 2019; Akiyama and Adachi, 2021), and in PCR, an adequate amount of DNA required for testing may not be extracted from some processed foods, and the sensitivity may be insufficient (Linacero et al., 2020).

In addition, these test methods are basically individual analytical methods for each target allergen, and it is difficult to simultaneously analyse multiple allergen proteins. These analytical methods also require skill to obtain reproducible results.

To solve these problems, a method for analysing food allergens using high-performance liquid chromatograph-tandem quadrupole mass spectrometry (LC-MS/MS) has been reported in recent years (Croote et al., 2019; Fallahbaghery et al., 2017; Henrottin et al., 2019, 2023; Huang et al., 2020; Lexhaller et al., 2019; Li et al., 2022; Ma et al., 2020; Neils et al., 2022; Ramachandran et al., 2020; Schalk et al., 2018a,b; Schalk et al., 2018a, 2018; Wang et al., 2021; Xiong et al., 2021). In this method, protein contained in foods is digested into peptides by a protease such as trypsin, and peptides having sequences specific to target raw materials are detected by the mass spectrometer.

It easy to construct an analytical method by obtaining amino acid sequences of the target proteins from public proteome databases, and by using the multiple reaction monitoring (MRM) mode of the mass spectrometer, it is possible to simultaneously detect peptides derived from multiple allergen proteins with high specificity. In previous studies, Voyksner et al. (2016) reported a method for simultaneously detecting peanuts and 11 species of tree nuts using LC-Q-TOF/MS. In addition, Korte and Brockmeyer (2016) reported a method for highly sensitive detection of peanuts and five kinds of tree nuts using the MRM mode of the Q-TRAP system (linear ion trap type LC-MS/MS). Downs et al. (2016) detected roasted walnuts using an Orbitrap-type LC-MS and reported the effect of the heating process on enzyme digestibility. Planque et al. (2017) reported a method using UHPLC-MS/MS to detect 10 allergen proteins, including walnuts and almonds, in incurred food products. Pilolli et al. (2020) reviewed proteotypic peptide marker tracing for six allergenic ingredients (containing almond) in incurred foods by mass spectrometry. And also Pilolli et al. (2021) reported high resolution MS/MS analysis for selection of allergen (containing almond) markers in chocolate and broth powder matrices.

Some of these previous studies partially confirmed specificity and verified quantitativity, however, they have not adequately confirmed cross-reactivity between diverse raw materials, and the differences between multiple walnut and almond varieties have not reported previously. Therefore, there is a need to develop and validate a simple, highly specific, and simultaneously detectable analytical method for walnuts and almonds contained in various processed foods to quantify allergen proteins and obtain further reliable results.

In this study, we developed a reliable and highly sensitive analytical method to simultaneously detect walnuts and almonds contained in processed foods using LC-MS/MS by applying the simultaneous analytical method for wheat and buckwheat that we previously reported (Seki et al., 2021). We confirmed the specificity using raw materials containing major allergens, including nuts, and verified the differences between multiple walnut and almond varieties. In addition, proteins with known concentrations extracted from walnuts and almonds were spiked into processed foods, and laboratory validation was performed to verify the quantitation. Then the applicability of the developed method was confirmed using commercially available processed foods.

2. Materials and methods

2.1. Samples

Five cultivars of *Juglans regia* L. (Chandler [USA origin], Howard [USA origin], Tulare [USA origin], Shinano-Gurumi [Japan origin], Teuchi-Gurumi [Japan origin]), two native species of *Juglans mandshurica* (Oni-Gurumi [Japan origin] and Hime-Gurumi [Japan origin]), four cultivars of *Prunus dulcis* (Nonpareil [USA origin], Butte [USA origin], Marcona [Spain origin], native species [Kyrgyz origin]), and eight other kinds of tree nut (pistachio [*Pistacia vera*], macadamia [*Macadamia integrifolia*], hazelnut [*Corylus avellana*], cashew [*Anacardium occidentale*], pecan [*Carya illinoinensis*], Brazil nut [*Bertholletia excelsa*], pine nut [*Pinus koraiensis*], and coconut [Cocos nucifera]) were obtained from wholesale companies in Japan. Seven kinds of raw ingredients containing major food allergens (wheat flour, bovine milk, whole chicken egg, buck wheat, peanut, shrimp, crab), three kinds of major cereals (rice, soybean, corn), and thirteen kinds of processed foods were purchased at local stores in Japan. The processed foods were divided into four tiers according to their ingredient lists: tier 1, both walnut and almond (two products); tier 2, walnut (three products); tier 3, almond (two products); tier 4, no walnut or almond (eight products).

2.2. Chemicals and reagents

Trypsin from porcine pancreas (cat. no T4799, having activity 1,000–2,000 units/mg dry solid), iodoacetamide (purity ≥99%), and dithiothreitol (purity ≥99%) were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (for Biochemistry), urea (for Biochemistry), sodium hydrogen carbonate (for Molecular Biology), formic acid (LC-MS grade), and acetonitrile (LC-MS grade) were purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). Trifluoroacetic acid (HPLC grade) and methanol (LC-MS grade) were purchased from JUNSEI CHEMICAL CO., LTD. (Tokyo, Japan). Synthetic peptides (purity ≥95%) were purchased from Greiner Bio-One (Tokyo, Japan). Lateral flow immunoassay kits for walnut and almond (*Allergeneye® Immunochromato*) were purchased from Prima Meat Packers, Ltd. (Tokyo, Japan).

2.3. Design of targeted peptides for detection

The *Allergome* database (Mari et al., 2009) was used to search for allergenic proteins. Walnut 2S albumin (Jug r1) and 7S globulin (Jug r2) and almond 11S globulin (Pru du 6) were targeted for detection. Targeted peptides derived from trypsin-digested proteins were estimated bioinformatically, and the recommended conditions for LC-MS/MS analysis were obtained through information provided by *Skyline* software tool Ver. 21.2 (MacLean et al., 2010). Peptides targeted for detection were selected from the peaks and intensities observed in the LC-MS/MS chromatogram according to the optimized parameters shown in Table 1.

2.4. Sample preparation for LC-MS/MS analysis

Sample preparation for LC-MS/MS analysis as described in Seki et al. (2021) was used with some modifications. Samples were ground using a food processor, and the ground sample (0.5 g) was solubilized in 5 mL of 100 mM Tris-HCl buffer (pH 8.2) containing 4 M urea and 0.1 M dithiothreitol. To extract proteins, the sample solution was incubated at 37 °C in a water bath (Personal-11SD, TAITEC CORPORATION, Saitama, Japan) for 3 h. After incubation, 1 mL of supernatant was obtained by centrifugation at 1,500×*g* for 5 min. To alkylate the proteins, 100 μL of 4% (w/v) iodoacetamide solution and 4 mL of 50 mM sodium hydrogen carbonate were added to the supernatant and incubated at 37 °C for 1 h. The proteins in the supernatant were digested for 16 h with 0.1 mL of 1% (w/v) trypsin solution at 37 °C. After digestion, 50 μL of TFA was added

Table 2
Varietal differences of targeted peptides between walnut and almond.

Nut	Target protein	Amino acid sequences of the target peptide	Scientific name, Breed name													
			Juglans regia L.	*Juglans regia* L.	*Juglans regia* L.	*Juglans regia* L.	*Juglans regia* L. var. *orientis*	Juglans mandshurica var. sachalinensis	Juglans mandshurica var. cordiformis	Prunus dulcis	Prunus dulcis	Prunus dulcis	Prunus dulcis	Prunus dulcis
			Chandler	Howard	Tulare	Shinano-Gurumi	Teuchi-Gurumi	Oni-Gurumi	Hime-Gurumi	Nonpareil	Butte	Marcona	wildtype	
Walnut	2S Albumin (Jug r1)	QQQQQGLR	+	+	+	+	+	–	–	–	–	–	–	
		DLPNEC[CAM]*GISSQR	+	+	+	+	+	–	–	–	–	–	–	
		GEEMEEMVQSAR	+	+	+	+	+	+	+	–	–	–	–	
	7S Globulin (Jug r2)	GQEQTLC[CAM]R	+	+	+	+	+	–	–	–	–	–	–	
		ATLTLVSQETR	+	+	+	+	+	–	–	–	–	–	–	
		QGQGQR	+	+	+	+	+	+	+	–	–	–	–	
		HESEEGEVK	+	+	+	+	+	–	–	–	–	–	–	
Almond	11S Globulin (Pru du 6)	GNLDFVQPPR	–	–	–	–	–	–	–	+	+	+	+	+
		YNRQETIALSSSQQR	–	–	–	–	–	–	–	+	+	+	+	+
		QETIALSSSQQR	–	–	–	–	–	–	–	+	+	+	+	+

+; peak detected, -; peak not detected.
[a] Cysteine residue methyl-carbamated by iodoacetamide.

Table 3
Specificity of targeted peptides between other nuts, major allergens, and cereals.

Nut	Target protein	Amino acid sequences of the target peptide	Common name, Scientific name							
			Walnut	Almond	Pistachio	Macadamia	Hazelnut	Cashew	Pecan	Brazil nut
			Juglans regia L.	Prunus dulcis	Pistacia vera	Macadamia integrifolia	Corylus avellana	Anacardium occidentale	Carya illinoinensis	Bertholletia excelsa
Walnut	2S Albumin (Jug r1)	QQQQQGLR	+	–	–	–	–	–	–	–
		DLPNEC$_{[CAM]}$[a]GISSQR	+	–	–	–	–	–	–	–
		GEEMEEMVQSAR	+	–	–	–	–	–	–	–
	7S Globulin (Jug r2)	GQEQTLC$_{[CAM]}$[a]R	+	–	–	–	–	–	–	–
		ATLTLVSQETR	+	–	–	–	–	–	–	–
		QGQGQR	+	–	–	–	–	–	–	–
		HESEEGEVK	+	–	–	–	–	–	+	–
Almond	11S Globulin (Pru du 6)	GNLDFVQPPR	–	+	–	–	–	–	–	–
		YNRQETIALSSSQQR	–	+	–	–	–	–	–	–
		QETIALSSSQQR	–	+	–	–	–	–	–	–

+; peak detected, -; peak not detected.
aCysteine residue methyl-carbamated by iodoacetamide.

to terminate the digestion. The resultant mixture was centrifuged at 6, 000×*g* for 5 min. To desalt and purify the sample solution, a solid-phase extraction column (Oasis HLB vac cartridge 150 mg/6 mL, Waters, Milford, MA, USA) was used. The whole amount of the obtained supernatant was loaded onto a column that was pre-conditioned with 5 mL of methanol followed by 5 mL of water. After loading the sample solution onto the column, the column was washed twice with 5 mL of 0.5% (v/v) TFA. The sample was eluted with 5 mL of 70% (v/v) acetonitrile solution twice. The eluate was concentrated using a vacuum evaporator (NVC-2100, TOKYO RIKAKIKAI Co., Ltd., Tokyo, Japan) and then dissolved in 0.5 mL of 5% (v/v) acetonitrile solution (containing 0.1% (v/v) formic acid). The analyte for LC-MS/MS was prepared by filtration using a 0.22 μm PVDF filter cartridge (Millex GV, Merck KGaA, Darmstadt, Germany) and stored in polypropylene vials (Waters, Milford, MA, USA).

2.5. HPLC and MS/MS conditions

HPLC and MS/MS conditions were described in our previous study (Seki et al., 2021). Liquid chromatography was run with a mobile phase of 0.1% (v/v) formic acid (solution A) and 0.1% (v/v) formic acid/-acetonitrile (solution B) at flow rate of 0.3 mL/min under gradient conditions: 1% (v/v) solution B at 0 min, 1% (v/v) solution B at 4 min, 60% (v/v) solution B at 16.5 min, 95% (v/v) solution B at 20 min, 95% (v/v) solution B at 25 min, 1% (v/v) solution B at 25.1 min, and 1% (v/v) solution B at 35 min. Separation was performed using Nexera X2 (Shimadzu Corporation, Kyoto, Japan) chromatography with a Kinetex C18 column (2.1 × 150 mm, particle size 2.6 μm) (Phenomenex, Torrance, CA, USA). The column temperature was set at 50 °C. The injection volume of each analyte was 2 μL. Mass spectrometry was performed using a QTRAP 5500 spectrometer (SCIEX, Framingham, MA, USA) under the following conditions: scheduled MRM mode, electrospray ionization (+), ion spray at 4.5 kV, turbo gas at 500 °C, curtain gas at 138 kPa, nebulizer gas at 345 kPa, turbo gas at 552 kPa, and nitrogen as the collision gas. To optimize precursor and product ions for each peptide, the 'Compound Optimization' function in *Analyst* software Ver.1.6.2 (SCIEX) was used. Using *MultiQuant* software Ver.3.0.2 (SCIEX), the peaks in the obtained chromatogram were detected, and the signal-to-noise ratios (S/N) were calculated.

2.6. Validation of the method

To evaluate the detection method quantitatively, extracted protein solutions from walnut ('Chandler') and almond ('Nonpareil') were added to butter cookies and chocolate ice cream and analysed in quintuplicate by two people. Walnut and almond were ground by a food processor and defatted by acetone. The total protein concentration in each extracted solution was determined by a Pierce 660 nm Protein Assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and diluted by 100 mM Tris-HCl buffer (pH 8.2) to adjust the walnut and almond protein concentrations in butter cookies and chocolate ice cream to 10 μg/g or 100 μg/g. Finally, the marginal recovery rate, repeatability (RSD_r), and reproducibility (RSD_R) were calculated by the analysis. The instrumental limit of detection (LOD) and limit of quantitation (LOQ) were estimated by S/N of 3 and 10, respectively, according to the following equations after spiking samples at the lowest level validated for each target analyte (Malachová et al., 2014; Ouakhssase et al., 2019). To confirm the applicability of the detection method, fifteen kinds of processed food as mentioned above were analysed by LC-MS/MS and lateral flow immunoassay, and each result was compared with their ingredient lists.

$$LOD = \frac{concentration\ of\ lowest\ spiked\ level}{average\ of\ S/N} \times 3 \quad \text{Eq. (1)}$$

$$LOQ = \frac{concentration\ of\ lowest\ spiked\ level}{average\ of\ S/N} \times 10 \quad \text{Eq. (2)}$$

3. Results and discussion

3.1. Peptides targeted for LC-MS/MS detection

To detect walnut and almond protein, as well as that of their related species in processed foods, 2S albumin (Jug r1) and 7S globulin (Jug r2) for walnut and 11S globulin (Pru du 6) for almond, which are registered as allergens in the *Allergome* database, were targeted for LC-MS/MS detection. Those targeted proteins digested by trypsin were computationally estimated using *Skyline* software. Protein BLAST analysis of the obtained peptide candidates indicated that three peptides from 2S albumin and four peptides from 7S globulin for walnut, and three peptides from 11S globulin for almond are specific to each species (Table 1).

Detection of the synthetic targeted peptides was based on our previously developed LC-MS/MS methods (Seki et al., 2021). All targeted peptides were initially analysed at a very low concentration of 10 ng/mL, and the concentration was increased by 10 times for further analyses. All synthetic walnut and almond peptides were detected at retention times of 6.5–9.5 min and 9.5–10.0 min, respectively. The amounts of sample injected into the MS instrument (Q-Trap 5500) for analysis were increased by using a high injection rate of 20 μL/s for better detectability. Using synthetic targeted peptides, the cone voltage and collision energy for MS were optimized for the analytical conditions. MS parameters such as Declustering Potential (DP), Collision Energy

Common name, Scientific name											
Pine nut	Coconut	Wheat	Buckwheat	Bovine milk	Chicken egg	Peanut	Shrimp	Crab	Rice	Soybean	Corn
Pinus koraiensis	Cocos nucifera	Triticum aestivum	*Fagopyrum esculentum* M.	Bos taurus	Gallus gallus	*Arachis hypogaea* L.	Penaeus monodon	Chionoecetes japonicus	Oryza sativa	Glycine max	Zea mays var. saccharata
–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–
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–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–

(CE), and Collision Cell Exit Potential (CXP) were optimized using *Analyst* software and the sets shown in Table 1. The MS parameters (DP, EP, CE, CXP) of each target peptide were optimized using synthetic peptides.

3.2. Specificity of peptide detection for varieties

To test specificity for varieties, seven different varieties of walnut (*J. regia* L. and *J. mandshurica*) and four different varieties of almond *P. dulcis* (almond) as well as other nuts were examined as test samples (Tables 2 and 3). Four peptides selected from walnut 2S albumin (Jug r1) and 7S globulin (Jug r2) were specifically detected for *J. regia* L., but they were not detected in other species (Tables 2 and 3). The presence of walnut was also confirmed by qualitative PCR methods (data not shown). The 2S albumin GEEMEEMVQSAR peptide was detected in all seven different *Juglans* varieties, including Chandler, Howard, Tulare, and Shinano-Gurumi, and in the *Orientis* variety (Teuchi-Gurumi) of *J. regia* L., and it was also detected in *sachalinensis* and *cordiformis* varieties of *J. mandshurica* (Table 2, Fig. 1A). The two varieties of *J. mandshurica* belong to the same species as *J. regia* L., but they are considered to be walnuts native to Japan, suggesting that some proteins may have different amino acid sequences from the western walnuts of *J. regia* L. In addition, the 11S globulin GNLDEVQPPR peptide targeted for almonds in this study was detected within four varieties of *P. dulcis* (Table 2, Fig. 1B). This result suggests that there are no significant differences between varieties in the amino acid sequence of the targeted almond protein (Pru du 6).

3.3. Specificity of peptide detection for walnut and almond

To confirm the specificity of the selected target peptides, we analysed eleven nuts (walnut, almond, pistachio, macadamia, hazelnut, cashew, pecan, peanut, Brazil nut, pine nut, and coconut), seven mandatory allergen labelled raw materials in Japan (wheat, buckwheat, milk, egg, shrimp, crab, and peanut), and five major crops (wheat, buckwheat, rice, soybean, corn) that are often used in foods. In walnuts, the QGQGQR peptide, which has a small precursor ion (m/z) that easily overlaps with interfering peptides in food, and the HESEEGEVK peptide detected in pecans were excluded from the target peptides. Pecans (*Carya illinoiensis*) belong to the same Juglandaceae family as walnuts, and because of their similar protein composition, there are cross-reactivities with walnuts (Weinberger and Sicherer, 2018). HESEEGEVK peptide was detected in both walnuts and pecans, but other marker peptides of walnut protein were not detected in pecans (Table 3). Thus, in this proposed analytical method, the results suggested that when only the HESEEGEVK peptide is detected, the sample contains pecan, and when the other peptides are detected together, the sample contains walnut. Since the other target peptides were only detected in walnuts and almonds, respectively, we confirmed that the target peptides in the proposed analytical method have sufficient specificity for walnuts and almonds.

3.4. Method validation and LOD and LOQ

Labelling of allergens in foods is recommended when the protein in the raw material containing the allergen is contained at a certain amount. For example, the Codex Alimentarius Commission has stated that cereals containing gluten, shellfish, eggs, fish, peanuts, soybeans, milk, tree nuts, and sulphites should be labelled as such (Codex Alimentarius Commission, 2018). In Japan, when seven ingredients, wheat, milk, egg, shrimp, crab, buckwheat, or peanut, are at several μg/g or more (generally 10 μg/g or more) of protein derived from the raw material, labelling is mandatory (Government of Japan Consumer Affairs Agency, 2015). Therefore, an analytical method to confirm the presence of these allergens in foods needs to be quantitative. In this study, protein solutions of known concentrations extracted from walnuts and almonds were spiked into processed foods to determine the trueness (recovery rate), repeatability (RSD_r), reproducibility (RSD_R), detection limit (LOD), and quantification limit (LOQ). Protein concentrations in solutions extracted from defatted, ground walnut (Chandler) and almond (Nonpareil) as described in the Materials and Methods section were measured with a Pierce 660 nm Protein Assay kit and determined to be 21,008 ± 837 μg/mL for walnuts and 17,729 ± 518 μg/mL (mean ± standard deviation of n = 3) for almonds. Trypsin digests of the diluted protein solutions were analysed using LC-MS/MS, and calibration curves were prepared in a range of protein concentrations from 0.1 to 50 μg/mL. The coefficients of determination (R^2) of the calibration curves for both walnuts and almonds were 0.999 or higher, showing sufficient linearity (Supplementary Fig. 1). Walnut- and almond-derived proteins were spiked in butter cookies and chocolate ice cream at levels of 10 μg/g and 100 μg/g (concentrations in the measurement solution were 1 μg/mL and 10 μg/mL, respectively). Table 4 shows the recovery rate (trueness), repeatability (relative standard deviation), and reproducibility of n = 5 × 2 people. Both walnuts and almonds meet the standard criteria of the AOAC and Codex-based chemical analysis method validation guidelines (AOAC Int, 2002; Codex Alimentarius Commission, 2016), confirming that the developed analytical method has sufficient accuracy, repeatability, and reproducibility.

The target peptides used for quantification were GEEMEEMVQSAR (m/z 698.3 [precursor] > 316.1 [product]), which was also detected in Japanese native varieties for walnuts, and GNLDFVQPPR, which had the best sensitivity and selectivity for almonds (m/z 571.8 [precursor] > 369.2 [product]). In addition, the average LOD and LOQ values of each target peptide calculated in the measurement (n = 10) of spiked samples at protein concentrations of 10 μg/g were LOD 0.22 ± 0.02 μg/g and

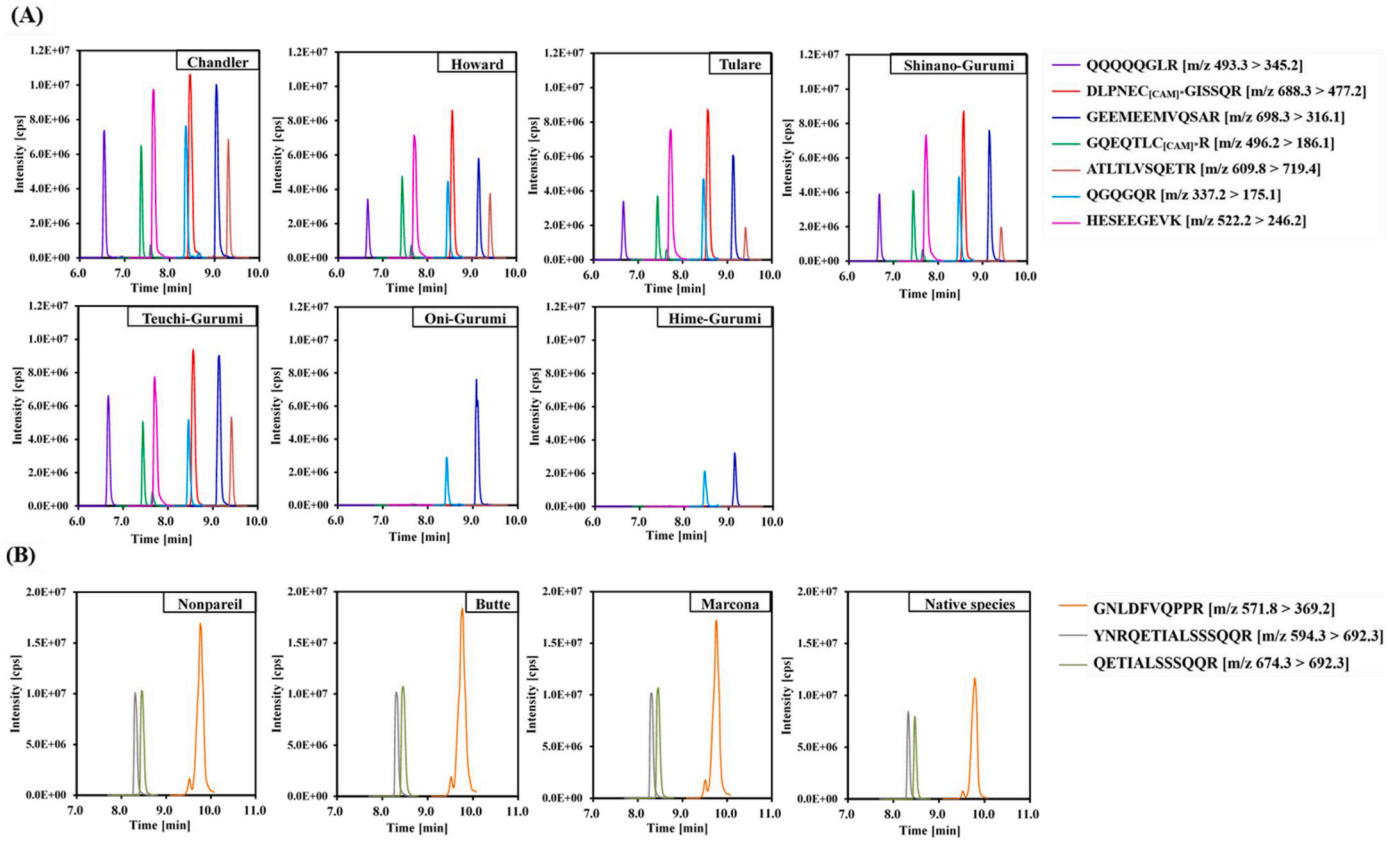

Fig. 1. Representative LC-MS/MS chromatograms (extracted ion chromatograms) obtained from (A) seven varieties of walnut and (B) four varieties of almond.

Table 4
Validation parameters[a] of the LC-MS/MS method as calculated by deduplicated (quintuplicated, two days) analysis of processed food spiked with protein from walnut and almond.

Target nut	Processed food	Concentration of spiked protein (μg/g)	Average recovery (%)	Repeatability (RSD_r, %)	Reproducibility (RSD_R, %)
Walnut	Butter cookie	10	91.1	4.5	4.9
		100	90.4	3.5	3.6
	Chocolate ice cream	10	97.9	2.7	2.9
		100	99.1	1.3	1.4
Almond	Butter cookie	10	101.5	4.6	6.8
		100	96.8	3.2	3.3
	Chocolate ice cream	10	97.7	3.2	3.4
		100	98.8	3.6	4.5

[a] The targeted peak area of walnut was the 2S albumin peptide, GEEMEEMVQSAR (*m/z* 698.3 [precursor] > 316.1 [product]), and that of almond was the 11S globulin peptide, GNLDFVQPPR (*m/z* 571.8 [precursor] > 369.2 [product]).

LOQ 0.74 ± 0.08 μg/g for walnut, and LOD 0.08 ± 0.02 μg/g and LOQ 0.26 ± 0.02 μg/g for almond. Fig. 2 shows an example of an extracted ion chromatogram when walnut- and almond-derived proteins were spiked into processed foods at concentrations of 10 μg/g and 100 μg/g. It has been reported that the detection limit concentrations of immunochromatography, ELISA, and PCR methods for detecting walnuts and almonds are about 2 μg/g, 1 μg/g, and 10 μg/g, respectively (Niemann et al., 2009; Yano et al., 2007; 3 MTM Company, 2021). The detection limit concentration of the proposed method is about four times as low, indicating that the proposed method is more sensitive than the others.

In practice, it is more preferable to calculate LOD and LOQ using an incurred sample, because thermal denaturation of proteins due to food processing and extraction efficiency of proteins should be taken into consideration. On the other hand, processing effects vary depending on ingredients and/or processes (baking, boiling, deep-frying, freezing etc.), and it's not practical to prepare all incurred samples for each process and ingredient. Therefore, in this study, LOD and LOQ were calculated using spiked samples instead of incurred samples and the applicability of this analytical method was confirmed using a variety of commercial processed foods. Abbot et al. (2010) also reported that spiked samples can be used for method validation of allergen detection instead of incurred samples in unavoidable cases. Testings using incurred samples may further validate our proposed method.

3.5. Applicability to processed foods

We examined the applicability of the developed detection method to different types of processed foods. Fifteen kinds of processed food were selected based on labelling information on the package: tier 1 (nut milk, biscuits) that contain both walnut and almond on the ingredient label of the package, tier 2 (cereals, dressings, and bean curd) that contain only walnut, tier 3 (almond cookie, almond ice cream) that contain only almond, and tier 4 (rice cracker, meat sauce, rice seasoning, curry powder, premixed flour, dried soup, butter cookie, and chocolate ice cream) that contain neither (Supplementary Table 1). The analytes for the developed LC-MS/MS method were prepared following procedures identical to those when raw materials were examined (Tables 2 and 3). Identical samples were also measured by the lateral flow immunoassay,

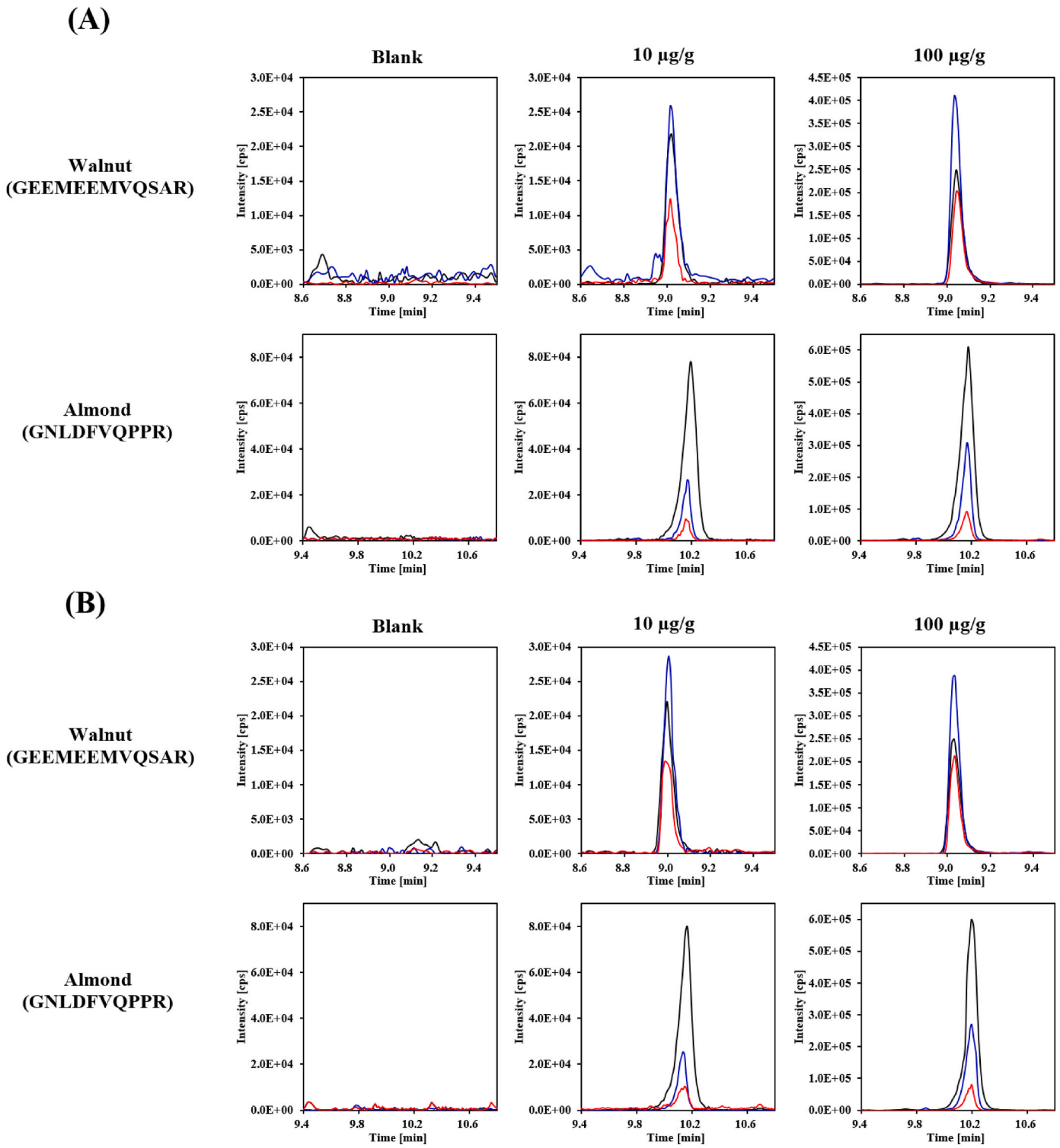

Fig. 2. Representative LC-MS/MS chromatograms (extracted ion chromatograms) obtained from processed foods analysed as spiked test samples. Extracted proteins from walnut ('Chandler') and almond ('Nonpareil') were spiked into two processed foods ((A) butter cookie and (B) chocolate ice cream) at 10 μg/g and 100 μg/g. Blank samples were not added for walnut and almond protein. The analysed target peptides of those samples were GEEMEEMVQSAR (black line; quantifier ion (m/z 698.3 > 316.1), blue line; qualifier ion 1 (m/z 698.3 > 461.2), red line qualifier ion 2 (m/z 698.3 > 820.4)) for walnut, and GNLDFVQPPR (black line; quantifier ion (m/z 571.8 > 369.2), blue line; qualifier ion 1 (m/z 571.8 > 596.4), red line; qualifier ion 2 (m/z 571.8 > 175.1)) for almond. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and the results were compared with this analytical method. Table 5 shows the measurement results by the LC-MS/MS method and the immunochromatography method. In all the processed foods, the detection peaks of walnut and almond target peptides in the proposed method were consistent with the labelling of the raw materials of each food. In addition, the detection of bands in the immunochromatography method for walnut and almond were consistent with the peaks for each target peptide in the LC-MS/MS method. The proposed method accurately

Table 5
Applicability of LC-MS/MS detection method to processed foods.

Processed foods [b]	Allergen labelling on food package		LC-MS/MS method (amino acid sequences of target peptides)									Lateral flow immunoassay (immunochromatography)	
			for walnut					for almond				for walnut	for almond
	Walnut	Almond	QQQQQ-GLR	DLPNEC$_{[CAM]}$[a]-GISSQR	GEEMEEM-VQSAR	GQEQTL-C$_{[CAM]}$[a]R	ATLTLVS-QETR	GNLDFVQ-PPR	YNRQETIA-LSSSQQR	QETIALSSS-QQR			
Nut milk	yes	yes	+	+	+	+	+	+	+	+		+	+
Biscuits	yes	yes	+	+	+	+	+	+	+	+		+	+
Cereal	yes	no	+	+	+	+	+	–	–	–		+	–
Dressing	yes	no	+	+	+	+	+	–	–	–		+	–
Walnut curd	yes	no	+	+	+	+	+	–	–	–		+	–
Almond cookie	no	yes	–	–	–	–	–	+	+	+		–	+
Almond ice cream	no	yes	–	–	–	–	–	+	+	+		–	+
Rice cracker	no	no	–	–	–	–	–	–	–	–		–	–
Meat sauce	no	no	–	–	–	–	–	–	–	–		–	–
Rice seasoning	no	no	–	–	–	–	–	–	–	–		–	–
Curry powder	no	no	–	–	–	–	–	–	–	–		–	–
Premixed flour	no	no	–	–	–	–	–	–	–	–		–	–
Dried soup	no	no	–	–	–	–	–	–	–	–		–	–
Butter cookie	no	no	–	–	–	–	–	–	–	–		–	–
Chocolate ice cream	no	no	–	–	–	–	–	–	–	–		–	–

+; peak or band detected, - ; peak or band not detected.
[a] Cysteine residue methyl-carbamated by iodoacetamide.
[b] Ingredients labelled on the commodity package are listed in Supplementary Table 1.

determined the protein of walnut and almond contained in the processed foods according to their label indications. Therefore, the proposed method has sufficient applicability for various processed foods.

4. Conclusions

We developed a simple and reliable analytical method using LC-MS/MS to simultaneously detect walnut- and almond-derived proteins contained in processed foods. The specificity of the proposed method using raw materials containing other nuts and other major food materials was confirmed, and target peptides digested from walnut- and almond-derived proteins for LC-MS/MS analysis were determined with excellent sensitivity and selectivity. The proteins extracted from walnut and almond were spiked into processed foods for analysis, and we showed that the proposed method has sufficient quantification and high sensitivity compared to the other analytical methods. By applying the proposed analytical method to other raw materials, it would be possible to simultaneously detect more types of allergens. It should be a useful analytical tool for confirming the accuracy of allergen labelling of foods. Further studies are anticipated for the development of an LC-MS/MS method that enables the detection of all potential food allergens simultaneously from a variety of processed foods. However, since foods are manufactured through various processes, measurements using incurred foods containing allergen proteins to investigate the qualitative and quantitative abilities of the proposed method are necessary.

CRediT authorship contribution statement

Akira Torii: Data curation, Formal analysis, Methodology, Validation, writing (original draft), Writing – original draft. **Yusuke Seki:** Data curation, Formal analysis, Methodology, critical review, Writing – review & editing. **Chisato Arimoto:** Validation, critical review, Writing – review & editing. **Naomi Hojo:** Validation, critical review, Writing – review & editing. **Ken Iijima:** Conceptualization, Project administration, critical review, Writing – review & editing. **Kosuke Nakamura:** critical review, Writing – review & editing. **Rie Ito:** critical review, Writing – review & editing. **Hirohito Yamakawa:** Conceptualization, Project administration, critical review, Writing – review & editing. **Hiroshi Akiyama:** Conceptualization, Project administration, writing (corresponding authors), critical review, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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