

Development of a liquid chromatography-tandem mass spectrometry method for simultaneous quantification of hen's egg white allergens Gal d 1–4 in fresh and processed eggs

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ABSTRACT

Hen's egg white allergens, namely Gal d 1–4, cause food allergies worldwide and their intake must be strictly controlled by allergic individuals. However, an efficient method for quantifying these allergens is currently unavailable. We aimed to develop an LC-MS/MS method for simultaneous Gal d 1–4 quantification. Purified Gal d 1–4 proteins were trypsin-digested and the resulting peptides used in LC-MS/MS analysis. The limits of quantification were 9.77–39.1 ng/mL. The Gal d 1–4 recovery in fresh and processed eggs was 68.3–121.3%, and intra- and interassay coefficients of variation were 1.5–15.7% and 2.4–38.1%, respectively, indicating high sensitivity, accuracy, and reproducibility. In addition, the high specificity of this method was confirmed by testing 27 other foods. This newly developed method could provide reliable information to the industrial food and clinical fields, facilitating improved quality of life for individuals with egg allergies.

1. Introduction

Hen's eggs (*Gallus gallus domesticus* eggs, hereinafter simply called eggs) are widely consumed in the human daily diet and, while lacking dietary fiber and vitamin C (U.S. Department of Agriculture [USDA], 2020), contain rich and well-balanced nutrients, including proteins, fats, vitamins, minerals, and bioactive compounds such as carotenoids (Zhu, Vanga, Wang, & Raghavan, 2018). The contents and compositions of the nutrients depend on the strain, diet, age, and environmental breeding conditions of the hens (Kuang, Yang, Zhang, Wang, & Chen, 2018). In addition to being an essential food for a healthy diet, eggs are used for pharmaceutical and cosmetic materials (Lesnierowski & Stangierski, 2018), which play important roles in our lives.

Unfortunately, eggs are a well-known causative food of allergies worldwide (Mine & Yang, 2008). The prevalence of egg allergies tends

to vary by region. In Oceania and Asia, egg allergies are more common than milk allergies, while the opposite is true in the Americas and Middle East, and in Europe, both egg and cow's milk allergies are the most common (Prescott et al., 2013; Samady, Warren, Wang, Das, & Gupta, 2020). The onset of an egg allergy may occur at any age, from infants to adults. Egg has become a common allergy in infants and preschool children, although the allergy often fades by school age (Dhanapala, De Silva, Doran, & Suphioglu, 2015). Individuals with an egg allergy must be cautious with their diet, as accidental ingestion of even small amounts of causative foods may elicit immediate allergic reactions such as itching, vomiting, urticaria, and even severe anaphylaxis through IgE-dependent mechanisms (Eigenmann, 2000; Faeste, Lovberg, Lindvik, & Egaas, 2007). In terms of the number of food allergy cases in Japan, accidental ingestion and initial-onset food allergies accounted for 41.9% and 58.1% of the cases, respectively,

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whereby medical consultation was required. In the former cases, the most causative food among those under 19 years of age was egg (Ebisawa, Ito, & Fujisawa, 2017). Therefore, as mandated by the Japanese government since 2002, the presence of eggs in food products must be declared on the label as is done in other countries, similarly to other causative foods such as milk, wheat, shrimp/prawn, crab, peanut, and buckwheat (Ebisawa et al., 2017).

Egg is comprised of 9.5% (w/v) egg shell and shell membrane, 27.5% egg yolk, and 63.0% egg white (Mine & Yang, 2008). Within the egg yolk and white, six allergens, Gal d 1–6, have been identified according to the official allergen database of the Allergen Nomenclature Subcommittee of the World Health Organization and International Union of Immunological Societies (WHO/IUIS) (WHO/IUIS Allergen Nomenclature Sub-Committee, 2020). Furthermore, egg white comprises 88.5% water, 10.5% protein, and 0.5% carbohydrate (Zhu et al., 2018), and contains potentially higher allergenic components than the egg yolk, namely Gal d 1–4. Gal d 1 (ovomucoid, approximate molecular weight (Mw): 28,000, comprised of 186 amino acids, 9.5–11% in egg white), Gal d 2 (ovalbumin, Mw: 45,000, comprised of 385 amino acids, 54–66%), Gal d 3 (ovotransferrin, Mw: 77,700, comprised of 686 amino acids, 12–13%), and Gal d 4 (lysozyme, Mw: 14,300, comprised of 129 amino acids, 2.3–4.5%) (Faeste et al., 2007; Zhu et al., 2018). Gal d 1 is the most allergenic of the four because of its heat-, acid-, and enzymatic hydrolysis stabilities. Gal d 2–4 are heat-labile, and so cooking the eggs lowers the risk of developing allergic symptoms for individuals who are allergic to Gal d 2–4, although not Gal d 1 (Lin, Wu, Huang, Cheng, & Yeh, 2016). While Gal d 4 is considered less allergenic than Gal d 1–3, it is also an important target in egg allergy research because it is widely used as a food additive and a pharmaceutical component due to its antimicrobial activities (Dhanapala et al., 2015). Thus, identifying the specific egg allergen components that affect allergic individuals may improve the quality of life for such individuals by expanding the array of foods available to them. Since individuals suffering from egg allergies exhibit various patterns of reaction to the allergens (Hoffman, 1983; Lin et al., 2016), in Japan, component-resolved diagnosis, including measurement of the Gal d 1-specific IgE antibody, is conducted to achieve a higher diagnostic accuracy (Ebisawa et al., 2017).

Eggs are a popular food ingredient, thus their complete elimination from the diet is difficult to accomplish. In order to avoid an allergic reaction, eliminating each causative allergen is principally important for allergic individuals (Ebisawa et al., 2017). Therefore, to help individuals be knowledgeable about causative allergens in foods and thereby control accidental ingestion, several quantitative methods for egg allergens have been developed. These methods employ mostly antibody-based techniques, such as a sandwich enzyme linked immunosorbent assay (ELISA) and lateral flow devices, which are available as commercial kits (Ito et al., 2016). These assays have many advantages such as ease of use, rapid results, and high sensitivity. However, the techniques are a single-target assay, mostly targeting Gal d 2 in the case of egg allergies, and have some limitations due to the antibody properties, such as the possibility of matrix effects from the sample, the inability to recognize modified epitopes in food processing, and cross-reactivity (Koeberl, Clarke, & Lopata, 2014).

Since egg white does not contain DNA, an appropriate method that can directly measure proteins is required to determine the presence of egg white and its corresponding allergens. In the last decade, mass spectrometry (MS) methods have been developed for reliable egg allergen analysis along with immunological techniques to overcome the limitations of antibody-based methods. Previous studies using MS-based techniques measured signature peptides for the qualitative detection of egg allergens (Tolin et al., 2012) or quantitative detection of only one or two egg allergens (mostly Gal d 2 and 4) (Boo, Parker, & Jackson, 2018; Cryar et al., 2013; Kondeková, Maier, Ginterová, Marák, & Ševčík, 2014). However, the amount of information related to multiple egg allergens is important in the context of clinical testing (Meglio

et al., 2013). In addition, Japanese labeling regulations require a numerical criteria for allergens of 10 ppm as raw materials (Ebisawa et al., 2017). Therefore, an efficient, reliable, and quantitative analytical method targeting egg allergen components is needed. Nonetheless, no such efficient multiple allergen analytical method has been reported.

The use of multiple reaction monitoring (MRM) for peptide analysis is a more optimal and universal tool for multiple allergen quantification than antibody-based techniques. Thus, Gal d 1–4 in raw and processed eggs could be more efficiently quantified, with high specificity, sensitivity, certified validation, and simple sample preparation, by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with MRM. In this study, we aimed to develop an LC-MS/MS based method for the simultaneous quantification of the major egg white allergens Gal d 1–4. Using this newly developed method, we assessed Gal d 1–4 levels in raw eggs, soft-boiled eggs, and dried egg white powders.

2. Materials and methods

2.1. Chemicals and reagents

Egg white allergens Gal d 1 (prod. no. T2011, type III-O, free of ovoinhibitor, organic solvent precipitation of proteins, no information on heat treatment), Gal d 2 (prod. no. A2512, Grade VI, $\geq 98\%$ purity by agarose gel electrophoresis, heat-treated during the manufacturing process, no information on heat denaturation), Gal d 3 (prod. no. C0755, $\geq 98\%$ purity by agarose gel electrophoresis, iron-free, heat-treated during the manufacturing process, no information on heat denaturation), and Gal d 4 (prod. no. L4919, $\geq 98\%$ purity by SDS-PAGE, crystallized three times having an enzyme activity, no information on heat treatment) were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris), and sodium bicarbonate were also purchased from Sigma-Aldrich Co. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), dithiothreitol (DTT), 2-iodoacetamide (IAA), acetonitrile, formic acid, acetic acid, ammonium bicarbonate, urea, and cowhide collagen peptide (enzymatic hydrolysate, hereinafter simply called CCP) were purchased from FUJIFILM Wako Pure Chemical Corporation, Ltd. (Osaka, Japan). RapiGest™ SF was purchased from Waters (Milford, MA, USA). Pierce™ Trypsin Protease (MS Grade) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Synthetic peptides (each was $\geq 98\%$ purity as confirmed by HPLC) were purchased from GenScript Corporation (Piscataway, NJ, USA). Ultrapure water was used in all experiments.

2.2. Sample extraction

All food samples were purchased from local stores in Osaka, Japan. The preparation of dried egg white powder is a trade secret, although they are generally heat spray dried. Soft boiled eggs (called *onsen eggs* in Japan) are heated at around 65 °C for 10–20 min (Yamashita, Ishibashi, Hong, & Hirose, 1998). Protein extraction was performed as described previously with some modifications (Yoshimitsu, Kiyota, Kajimura, & Yamano, 2019). Briefly, proteins were extracted from 2.0 g of fresh poultry, livestock, fish, and grain homogenates (hen's eggs, Japanese quail eggs, chicken, pork, beef, cow's milk, yellowfin tuna, salmon, soy sauce pickled salmon roe, soybeans, chickpeas, wheat flour, soft-boiled eggs, and dried egg white powder) using 18 mL of 100 mmol/L Tris buffer (pH 8.0) for 10 min while shaking. Proteins were extracted from 5.0 g of fresh vegetable and fruit homogenates (apple, celery, green kiwifruit, gold kiwifruit, Japanese pear, peach, carrot, tomato, mung bean sprout, watermelon, muskmelon, grape, bell pepper, banana, pineapple, and avocado) using 5 mL of 100 mmol/L Tris buffer (pH 8.0) for 30 min while shaking. The samples were centrifuged at 10–20 °C and more than 10,000 $\times$ g for 5 min. The supernatants were collected and stored as extracts at –20 °C until further use.

2.3. Enzymatic digestion

One hundred microliters of either the extract (diluted at 1:10–1:10,000 with 100 mmol/L pH 8.0 Tris buffer) or Gal d 1–4 mixed standard solution (200 µg/mL each allergen) was mixed in a 1.5 mL Eppendorf Protein LoBind tube with the following components: 5 µL of 1% RapiGest™ SF (acid-labile surfactant), 10 µL of 500 mmol/L DTT, and 10 µL of 1 mol/L Tris buffer (pH 8.0). The mixture was incubated at 95 °C for 15 min. After adding 10 µL of IAA (500 mmol/L) to alkylate the proteins, the mixture was incubated at room temperature (13–20 °C) in the dark for 30 min. For enzymatic digestion, 823 µL of 50 mmol/L ammonium bicarbonate and 2 µL of 1 µg/µL trypsin solution in 50 mmol/L acetic acid were added, and the mixture then incubated at 37 °C for 3 h. To stop trypsin digestion and decompose the RapiGest™ SF, 40 µL of formic acid was added to the mixture. The sample and Gal d 1–4 standard digests were stored at –20 °C. Prior to LC-MS/MS analysis, the sample digests were diluted at 1:1 to contain 2% formic acid and 10% acetonitrile. To generate the calibration curve, the digested Gal d 1–4 standards were diluted 2-fold between 9.77 and 5000 ng/mL, each dilution containing 2% formic acid, 10% acetonitrile, and 50 µg/mL of CCP. The sample and standard digests were transferred to a polypropylene screw neck vial (12 × 32 mm) with a cap and preslit septum (Waters).

To optimize the trypsin treatment time, the samples were subjected to trypsin treatment at 37 °C in series for 0.5, 1, 3, 5, and 22 h, and the peak area values obtained from MRM analysis were compared.

2.4. Selection of peptides for MRM method

For the MRM analyses, Gal d 1–4 was digested with trypsin *in silico* and the MRM transition list of Gal d 1–4 was generated using Skyline software v. 4.1 (<https://skyline.ms/project/home/software/Skyline/begin.view>). For screening of the target peptides, data acquisition was performed with an ExionLC system coupled to an X500R QTOF system and using the SWATH® Acquisition method (AB Sciex, Foster City, CA, USA). Analysis of the data was performed using SCIEX OS software v. 1.3.1. Separation of peptides was performed with a Waters Acquity UPLC HSS T3 column (100 Å, 1.8 µm, 2.0 × 100 mm) at 50 °C and a flow rate of 0.2 mL/min. The mobile phases were water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid. The injection volume was 10 µL. For the positive mode using the TurbolonSpray source, the method parameters were: ion source temperature: 450 °C, ion source gases 1 and 2: 50 psi, curtain gas: 20 psi, CAD gas: 7, ionspray voltage: 5500 V. The scan was performed using the following parameters: for TOF MS, mass range m/z 300–1200 Da with 36 mass windows, accumulation time: 0.1 s, declustering potential: 80 V, declustering potential spread: 0 V, collision energy: 10 V, collision energy spread: 0 V; and for TOF MSMS, mass range: m/z 50–1200 Da, accumulation time: 0.025 s, and charge state: 1. The MRM transition list created by Skyline software for each hen's egg allergen was subjected to the SWATH data using SCIEX OS software for selecting candidate peptides. Some of the candidate peptide ions that were screened based on the peak area value were subjected to the Xevo TQ-S triple quadrupole system. Each target peptide of Gal d 1–4 (Table 1) was selected based on the peak area value of the product ions obtained by LC-MS/MS MRM, the presence of five or more residues for ensuring the specificity, and the avoidance of cysteine residues when possible. In addition, the specificity of each target peptide was confirmed and retrieved from the UniProt database (<https://www.uniprot.org/blast/>).

2.5. LC-MS/MS methods

The LC-MS/MS system, consisting of a Xevo TQ-S triple quadrupole mass spectrometer coupled with an Acquity ultra performance liquid chromatography system (Waters), was used with a Waters Acquity UPLC HSS T3 column (100 Å, 1.8 µm, 2.0 × 100 mm) connected to a

Waters Acquity UPLC HSS T3 VanGuard pre-column (100 Å, 1.8 µm, 2.1 × 5 mm). The injection volume was 2 µL. Peptide separation was performed on the column at 50 °C and a flow rate of 0.4 mL/min with a gradient elution consisting of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The column was initially eluted with a linear gradient from 100% (A) and 0% (B) to 65% (A) and 35% (B) for 10 min, then a linear gradient to 0% (A) and 100% (B) for 1 min, followed by a linear gradient to 100% (A) and 0% (B) for 4 min, and was finally maintained at that gradient for 5 min to avoid any material carry-over to subsequent analyses. The LC-MS/MS parameters were set as cone nitrogen flow: 150 L/h, collision gas flow: 0.15 mL/min, capillary voltage: 1 kV, source temperature: 150 °C, desolvation temperature: 550 °C, and nitrogen flow: 1200 L/h. Acquisition and analysis of the data were performed using MassLynx software v. 4.1 (Waters). MRM detection was performed in positive electrospray ionization mode. The transitions were optimized with infusion using synthetic quantitative peptides including carbamido-methylated VMVLCNR, and are presented in Table 1.

2.6. Method validation

One hundred microliters of the fresh egg extracts (1:20,000 dilution) were spiked with Gal d 1–4 for a final concentration of 625 or 1250 ng/mL, and the egg white powder extracts (1:200,000 dilution) were spiked with Gal d 1–4 for a final concentration of 625, 1250, or 2500 ng/mL. The Gal d 1–4 levels of each spiked extract were measured twice per assay in five independent experiments, which was based on the Japanese official guideline of analytical methods for agricultural chemical residues in foods (Ministry of Health, Labour and Welfare, Japan, 2010). In one laboratory, the average recovery, repeatability, and the intra- and interassay reproducibilities were calculated using one-way analysis of variance (one-way ANOVA). Furthermore, 100 µL of the egg extracts (1:16,000, 1:20,000, and 1:32,000 dilutions) and egg white powder extracts (1:160,000, 1:200,000, and 1:320,000 dilutions) were spiked with Gal d 1–4 to a final concentration of 1250 ng/mL. Multi-dilutional parallelism of the analysis was assessed from the percent of recovery of Gal d 1–4. An eight-point calibration curve was generated in the range having signal-to-noise (S/N) ratios of more than 3 and 10 in the qualitative and quantitative peptide detection, respectively. The percent recovery of Gal d 1–4 was calculated as follows:

$$\text{Gal d 1–4 recovery(\%)} = (C_{SE} - C_0) \times 100 / C_S,$$

where C_{SE} is the concentration of spiked extract; C_0 is the concentration of non-spiked extract; and C_S is the spiked concentration (625, 1250, or 2500 ng/mL). The concentrations obtained from the LC-MS/MS analysis were multiplied by the appropriate dilution factor and converted to units of milligrams per gram of fresh or processed eggs to give the Gal d 1–4 levels.

3. Results and discussion

3.1. Optimization of analytical method and sample preparation

Advanced approaches for food allergen analyses include MS-based systems, such as LC-MS/MS with MRM, LC-qTOF (time-of-flight)/MS, and LC-IT (ion trap)/MS (Koeberl et al., 2014). We used triple quadrupole LC-MS/MS with an MRM system to achieve our aim because it offers precise quantitative performance with a broad dynamic range for complex samples. To the best of our knowledge, this is the first development of an LC-MS/MS method for the simultaneous quantification of Gal d 1–4.

Several kinds of standards such as purified and recombinant proteins, as well as synthesized peptides, have been used as the source of signature peptides on MS-based systems. Such methods using indicative peptides as an alternative to the target protein require high and stable digestion efficiency to obtain accurate quantitative results, and the

Table 1
MRM parameters of peptides.

Protein (Allergen)	Peptide	m/z		Cone voltage (V)	Collision energy (V)
		Precursor ions (charge state)	Product ions		
Ovomucoid (Gal d 1)	VMVLC* NR	446.23 ⁺⁺	661.3 ⁺ (y5) ^{a)}	35	15
	DVLVC* NK	424.2 ⁺⁺	633.3 ⁺ (y5) ^{b)}	35	15
Ovalbumin (Gal d 2)	HIATNAVLFFGR	449.3 ⁺⁺⁺	639.4 ⁺ (y5) ^{a)}	42	10
	GLWEK	316.7 ⁺⁺	462.2 ⁺ (y3) ^{b)}	35	11
Ovotransferrin (Gal d 3)	YFGYTGALR	524.3 ⁺⁺	737.4 ⁺ (y7) ^{a)}	20	14
	SAGWNIPTLLHR	512.3 ⁺⁺⁺	906.6 ⁺ (y8) ^{b)}	35	17
Lysozyme (Gal d 4)	NTDGSTDYGLQINSR	585.4 ⁺⁺⁺	617.3 ⁺ (y5) ^{a)}	30	16
	GTDVQAWIR	523.3 ⁺⁺	673.4 ⁺ (y5) ^{b)}	35	18

* Carbamidomethylated with iodoacetamide.

a) Quantitative ion.

b) Qualitative ion.

digestion efficiency of the standards and samples should be equivalent. Therefore, we used commercially available highly purified egg proteins as the calibration standards, which were well characterized and useful for confirming reproducibility, increasing the versatility of the method, and comparing the analytical performance with other laboratories.

We started the study using a Gal d 1–4 mixed standard solution (10 µg/mL of each allergen) and optimized the MRM transitions presented in Table 1 for further investigations. Although all the peptides were selected based on the corresponding allergen sequences, immunologically important regions such as immunoglobulin E/T-cell epitopes were not considered, because there were too many for all of them to be covered (Mine & Yang, 2008). Gavage et al. (2019) indicated that incomplete carbamidomethylation on cysteine by IAA was possible, which would be of concern as it would add a bias in the method. Since there were too many cysteines (19) in Gal d 1 to be considered, we selected only the signature cysteine-containing peptides of Gal d 1, VMVLCNR and DVLVCNK. Although there have been reports of Gal d 1 quantification using MS-based methods, Gal d 1, being resistant to urea and a trypsin inhibitor, could nonetheless be measured by enhancing the enzymatic digestion using RapiGest™. Furthermore, the validation was confirmed as described in section 3.2, and thus such carbamidomethylated signature peptides were considered useful, as previously reported (Boo et al., 2018). Prior to MS detection, the HPLC conditions were optimized and each peptide was separated. The approximate retention times (min) of the quantitative and qualitative peptides of Gal d 1–4 were as follows: VMVLCNR, 5.00; DVLVCNK, 4.15; HIATNAVLFFGR, 7.52; GLWEK, 4.93; YFGYTGALR, 6.40; SAGWNIPTLLHR, 8.39; NTDGSTDYGLQINSR, 6.75; and GTDVQAWIR, 6.46 (Fig. S1).

Next, during sample preparation optimization, we compared a Tris-based extraction buffer (pH 8.0) (Qi et al., 2019) with a urea buffer, which is a denaturing agent (Fig. 1). With the exception of Gal d 4, approximately 10 times higher peak area values were obtained for both fresh eggs and dried egg white powder with the Tris-based buffer. Sonication treatment for 15 min at 25 °C did not increase extraction efficiency. Depending on concentration, urea treatment of the dried egg white powder may have resulted in gelation (Totosaus, Montejano, Salazar, & Guerrero, 2002); therefore, the use of a denaturing agent was required for enzymatic treatment of the egg white powder, but was not necessarily required for protein extraction of the egg white powder.

We also optimized the trypsin treatment time (Fig. 2). Peaks were typically reached after 3 h, and then subsequently plateaued or decreased. Therefore, the trypsin treatment was set at 37 °C for 3 h.

Finally, during optimization of the calibration curve, as previously described (Kuppanan, Albers, Schafer, Dielman, & Young, 2011), the reduced peak areas were observed at lower concentrations of the Gal d 1–4 standards due to adsorption of the peptide onto the vial (Fig. S2). This had a negative effect on the linearity of the calibration curve, although the effect was less pronounced at higher concentrations. In

addition, we speculated that the presence of a matrix in the egg samples competitively suppresses adsorption of the target peptide. Thus, to avoid adsorption of the target peptide and maintain the linearity, each dilution of the calibration curve contained 50 µg/mL of CCP as a matrix. CCP was chosen because it is commercially and economically available, and has a simple amino acid sequence, resulting in a simple matrix. CCP would not require the tryptic treatment (namely, no possible interference with tryptic treatment) and would be able to be added to the vial directly.

3.2. Method validation

Although several MRM systems are currently available for the quantification of multiple allergens, they are still relatively new. Therefore, the method design and validation of any MRM system should be carefully verified before use. Using the optimized MRM acquisition parameters, quantitative peptide calibrations curves of trypsin-digested Gal d 1–4 were generated by 2-fold serial dilutions with excellent linearity (r^2 greater than 0.99) and sensitivity, and a wide dynamic range of 39.1–10,000 ng/mL of Gal d 1 and 9.77–10,000 ng/mL of Gal d 2–4. The limits of detection (LOD) and quantification (LOQ) were determined to be the same: 39.1 ng/mL for Gal d 1 and 9.77 ng/mL for Gal d 2–4. The S/N ratio in the qualitative peptide detection was greater than 3. The quantitative peptide at the LOQ had a peak area value of approximately 1000 or greater in order to obtain accurate area integration. This eight-point calibration curve was used for further investigations (Fig. S3). Multi-dilutional parallelism was tested using a series of sample dilutions spiked with 625 or 1250 ng/mL of Gal d 1–4 (Fig. 3). In fresh egg extracts, the recovery of Gal d 1–4 ranged from 50.5% to 118.5% at three different dilutions (1:16,000, 1:20,000, and 1:32,000). In dried egg white powder extracts, the recovery of Gal d 1–4 ranged from 71.5% to 100.7% at three different dilutions (1:160,000, 1:200,000, and 1:320,000). These results indicate a generally good accuracy of Gal d 1–4 detection using this LC-MS/MS method, although relative low accuracies were sometimes observed, especially for Gal d 2 (Fig. 3).

Fresh egg extracts were spiked with 625 and 1250 ng/mL of Gal d 1–4. Dried egg white powder extracts were spiked with 625, 1250, and 2500 ng/mL of Gal d 1–4. The validation parameters, including recovery and reproducibility, were evaluated in five independent experiments (Table 2). The recovery ranges of Gal d 1–4 were determined to be acceptable and were as follows: Gal d 1, 89.9–121.3%; Gal d 2, 68.3–92.6%; Gal d 3, 78.4–104.2%; Gal d 4, 80.3–98.0%. The repeatability and reproducibility were high, as seen by the intra-assay coefficients of variation (CVs) (Gal d 1, 4.6–15.7%; Gal d 2, 8.2–13.6%; Gal d 3, 3.5–9.8%; and Gal d 4, 1.5–4.6%) and the inter-assay CVs (Gal d 1, 8.0–22.7%; Gal d 2, 9.7–38.1%; Gal d 3, 6.8–18.2%; and Gal d 4, 2.4–9.8%) for fresh egg and dried egg white powder extracts. With some exceptions at low spiking levels, the intra- and interassay CVs

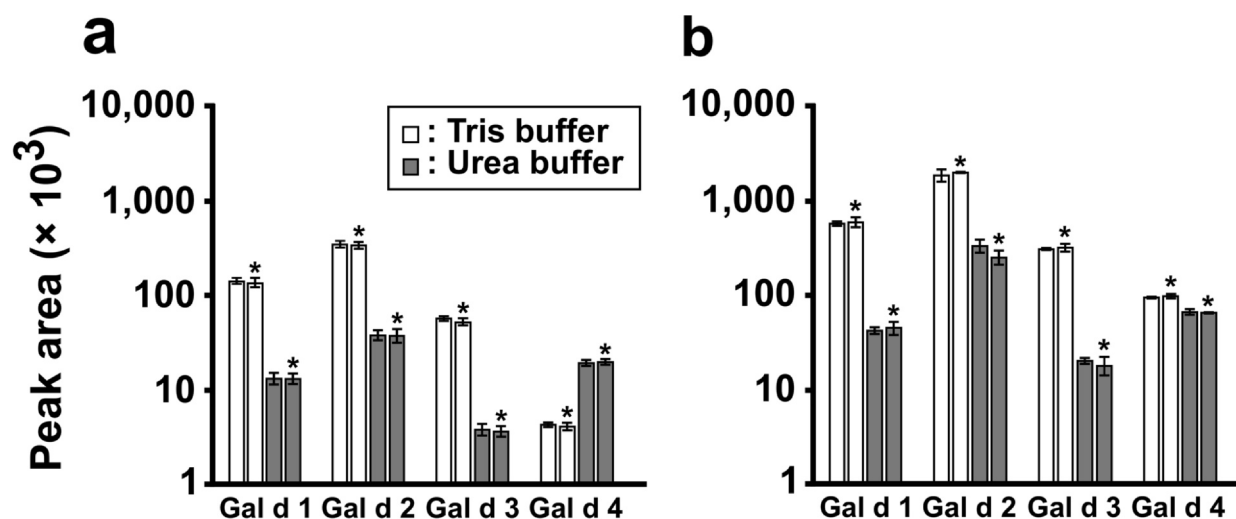


Fig. 1. Comparison of Gal d 1–4 extraction efficiency by quantitative detection of peptides in a) fresh egg extract (1:20,000 dilution) and b) dried egg white powder extract (1:40,000 dilution). The samples were extracted using 0.1 mol/L Tris buffer (pH 8.0) or 8 mol/L urea buffer at a 1:9 sample:buffer (w/v) ratio and shaking for 10 min. To avoid gelation with 8 mol/L urea, dried egg white powder only was extracted at a 1:99 sample:buffer (w/v) ratio, and the resulting peak area value was multiplied by 10. The supernatants were then trypsin-digested as described in the materials and methods section. Values were determined at $n = 3$. The data and error bar represent the mean and standard deviation, respectively. * Sonication treatment for 15 min at 25 °C was performed before the shaking step.

were below 15% and 20%, respectively, which are considered acceptable and are similar to those reported by Sun et al. (2019).

To confirm the specificity of the signature peptides *in silico*, we also tested 8 animal and 19 plant foods for Gal d 1–4, which were compatible with egg for cooking and sweets (Table S1). Gal d 1–4 allergens were not detected in any of the samples with the exception of chicken breast, where a small amount of Gal d 3 (approximate 80 µg/g) was detected. This was due to the fact that chicken bone marrow in the breast contains a serum transferrin that is highly homogeneous to Gal d 3. They share a common gene and amino acid sequence, and differ only in the 473–476 and 618–621 glycosylation sites on the C-terminal lobe (Giansanti, Leboffe, Pitari, Ippoliti, & Antonini, 2012), which did not overlap with the signature peptide sites of our method (YFGYTGLR: 540–548 and SAGWNIPIGTLHR: 141–154). Nevertheless, the developed method could successfully distinguish Gal d 3 derived from eggs and chicken breast by simultaneously monitoring Gal d 1–4, because Gal d 1, 2, and 4 are not inherently present in chicken breast (Dhanapala et al., 2015). In contrast, antibody- and polymerase chain reaction-based methods have difficulty in distinguishing Gal d 3 because of the high possibility of cross-reactivity and because the eggs and bone marrow share the same DNA sequence, respectively. Furthermore, although we detected Gal d 3 and Gal d 4 homologues in Japanese quail eggs using the quantitative peptides, it was possible to distinguish quail

eggs from hen's eggs where the sequence homologies were high in avian egg white (Giansanti et al., 2012; Thammasirak et al., 2007). The qualitative peptide for Gal d 4 (GTDVQAWIR: 135–143), which had a substitute at Gln139Asn compared with the homologue, was negative in quail eggs (Fig. S4). This identification is difficult to do using antibody-based methods because of cross-reactivity. Although we could not compare the Gal d 3 amino acid sequence with the homologue due to a lack of information in the UniProt database, we speculated that at least one residue in the qualitative peptide of Gal d 3 (SAGWNIPIGTLHR: 141–154) was different from the quail egg homologue. Since Japanese quail eggs are subject to labeling regulations in Japan, it is important to detect and distinguish between different egg types to ensure consistency in labeling, and to determine a causative food in the case of accidental ingestion. The developed method may be useful for screening if it is possible to prepare standards of the corresponding homologue and design the qualitative peptide. In this way, we confirmed the high specificity of this method, indicating it is useful for overcoming the limitations of antibody-based methods.

3.3. Gal d 1–4 concentration measurements

The newly developed method was used to assess Gal d 1–4 levels in fresh and processed eggs under the assumption of quantitative protein

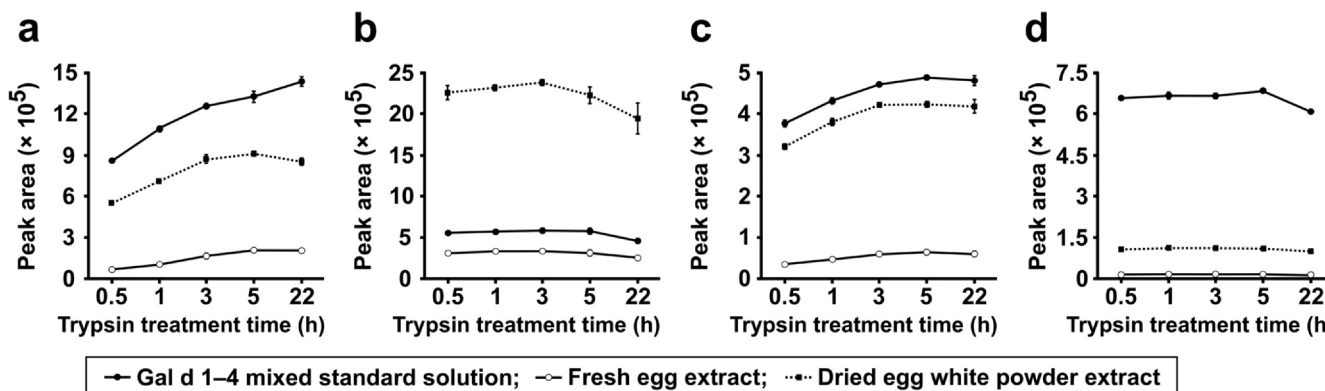


Fig. 2. Optimization of trypsin treatment time for a) Gal d 1, b) Gal d 2, c) Gal d 3, and d) Gal d 4. The peptides were quantitatively measured using 5000 ng/mL Gal d 1–4 mixed standard solution, fresh egg extract (1:20,000 dilution), and dried egg white powder extract (1:40,000 dilution). The extracts were prepared with 0.1 mol/L Tris buffer. Values were determined at $n = 3$. The data and error bar represent the mean and standard deviation, respectively.

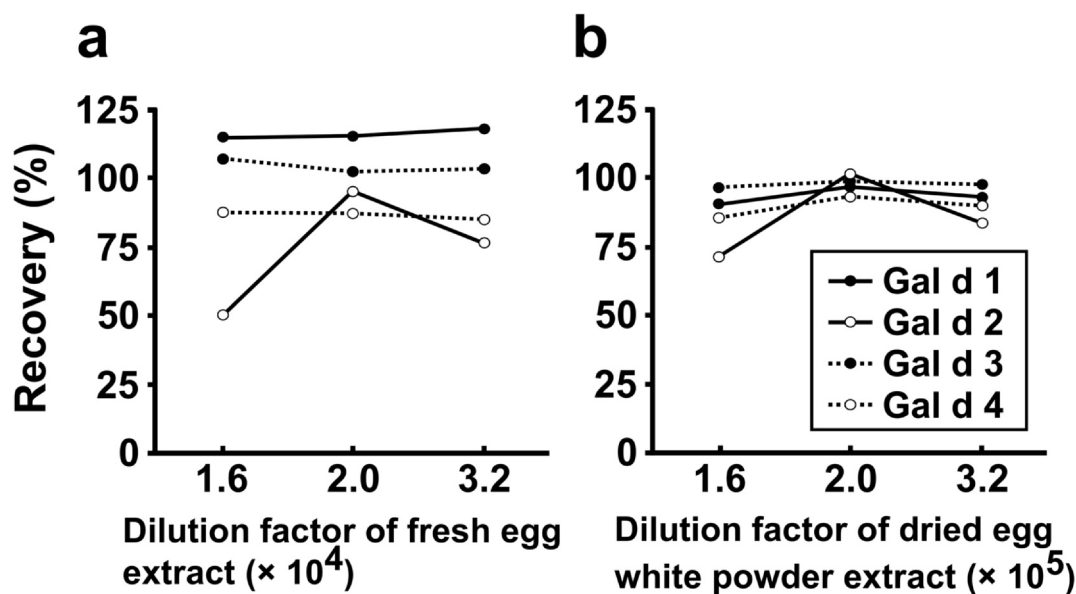


Fig. 3. Multi-dilutional parallelism of the LC-MS/MS method. The percent recovery of Gal d 1–4 using a) fresh eggs and b) dried egg white powder extracts, which were spiked with 625 and 1250 ng/mL, respectively, of Gal d 1–4, are presented.

Table 2
Recovery and coefficients of variation of the LC-MS/MS method.

Food	Allergen	Spiked allergen concentration (ng/mL)	Recovery (%) ^{a)}	Intra-assay CV (%)	Inter-assay CV (%)
Fresh egg extracts (1:20,000 dilution)	Gal d 1	625	121.3	8.1	13.5
		1250	115.6	4.6	19.5
	Gal d 2	625	92.6	13.3	13.3
		1250	82.4	9.7	9.7
	Gal d 3	625	104.2	7.0	12.4
		1250	100.5	3.5	13.2
	Gal d 4	625	98.0	4.6	5.7
		1250	94.2	1.5	2.4
Dried egg white powder extracts (1:200,000 dilution)	Gal d 1	625	89.9	15.7	22.7
		1250	90.7	10.6	14.5
		2500	94.2	8.0	8.0
	Gal d 2	625	68.3	12.6	38.1
		1250	70.4	13.6	16.1
		2500	73.8	8.2	15.0
	Gal d 3	625	78.4	9.8	18.2
		1250	84.7	5.7	14.7
		2500	92.5	6.8	6.8
	Gal d 4	625	80.3	3.1	9.8
		1250	87.7	1.6	7.8
		2500	90.4	4.0	4.0

a) Mean recovery (%) from five independent experiments.

The levels of Gal d 1–4 were quantified using each calibration curve having a range of 39.1–5000 ng/mL.

extraction, with the results summarized in Table 3. The mean Gal d 1–4 concentrations were determined to be as follows: for fresh eggs, Gal d 1, 11.5 mg/g; Gal d 2, 42.4 mg/g; Gal d 3, 12.0 mg/g; and Gal d 4, 2.1 mg/g; for dried egg white powders, Gal d 1, 70.0 mg/g; Gal d 2, 422.0 mg/g; Gal d 3, 99.3 mg/g; and Gal d 4, 14.6 mg/g; and for soft-boiled eggs, Gal d 1, 13.0 mg/g; Gal d 2, 59.1 mg/g; Gal d 3, 2.4 mg/g; and Gal d 4, 0.3 mg/g. For comparison with previously reported values, we converted each value to a percentage of allergen (mg) per milligram of egg white protein. Using a ratio of 63.0:27.5 egg white:yolk (Mine & Yang, 2008) and 109 mg of protein/g of fresh egg white (USDA, 2020), these values were estimated as follows: for fresh eggs, Gal d 1, 15.1%; Gal d 2, 55.9%; Gal d 3, 15.8%; and Gal d 4, 2.8%. For example, if the Gal d 1 concentration of a fresh egg was determined to be 11.46 mg/g, the corresponding value of 15.1% was calculated as follows:

Table 3
Gal d 1–4 levels in fresh and processed eggs.

Food ^{a)}	Manufacturer No. ^{b)}	Concentration (mg/g)			
		Gal d 1	Gal d 2	Gal d 3	Gal d 4
Fresh egg ^{c)} (1:20,000 dilution)	1	13.3	39.2	13.2	3.7 ^{d)}
	1	10.8	41.2	10.8	2.1 ^{d)}
	1	10.2	46.9	12.0	0.6 ^{d)}
	Mean	11.5	42.4	12.0	2.1
Dried egg white powder (1:200,000 dilution)	2	73.8	408.8	104.8	17.6
	3	66.5	404.2	84.6	17.3
	4	69.7	452.9	108.5	8.8
	Mean	70.0	422.0	99.3	14.6
Soft-boiled egg ^{c)} (1:20,000 dilution)	5	16.0	67.0	2.6	0.4 ^{d)}
	5	12.0	58.7	2.3	0.4 ^{d)}
	5	11.1	51.7	2.2	0.2 ^{d)}
	Mean	13.0	59.1	2.4	0.3

a) Values determined at $n = 1$ under the assumption of quantitative protein extraction from the three products.

b) Different numbers indicate a different manufacturer.

c) Whole eggs without the shell and shell membrane.

d) Quantified using a calibration curve having a range of 9.77–1250 ng/mL. Other samples were quantified using a calibration curve having a range of 39.1–5000 ng/mL.

$11.46 \times 90.5/63.0$, multiplied by 100/109. Although individual differences among eggs still need to be interpreted, the Gal d 1–4 concentrations in fresh eggs determined by our method was in approximate agreement with those of previous reports: Gal d 1, 9.5–11% (amount in egg white), Gal d 2, 54–66%, Gal d 3, 12–13%, and Gal d 4, 2.3–4.5% (Zhu et al., 2018). The amount of Gal d 3 in soft-boiled eggs was lower than that in fresh eggs. We speculate that only Gal d 3 coagulated or was otherwise involved in thermal gelation at the heating temperature of around 65 °C used for soft-boiled eggs (Yamashita et al., 1998), resulting in the decrease in extraction efficiency, while the other allergens start to denature above this heating temperature. The protein concentration in dried egg white powder is known to be 6.5 times higher than that in whole fresh eggs (USDA, 2020), which is in approximate agreement with the 6–10 times higher Gal d 1–4 levels of dried white powder determined using this method.

3.4. Applications of the study and future tasks

In this study, we confirmed the analytical performance of a method for quantifying Gal d 1–4 in fresh eggs and dried egg white powders assumed as allergy test materials (Itoh-Nagato et al., 2018). For more reliable diagnoses, therapy, and risk assessment of egg allergy, this method could be used to standardize Gal d 1–4 levels in allergy test materials. Moreover, egg allergen components can be beneficial to our lives. For example, Gal d 2 (ovalbumin) is used as a moisturizer in cosmetics, and Gal d 4 (lysozyme) is used as an antibacterial protein in medicine. In the cosmetic and pharmaceutical industries, where such allergens are used, careful attention must be paid to allergen contamination in the production line. For the cosmetic and pharmaceutical industries, this method may be used to determine egg allergen residues in production lines.

In the absence of available purified or recombinant proteins as egg yolk allergen standards, future studies should be able to quantify egg yolk allergens such as Gal d 5 and Gal d 6 by applying this method, which are also potential problems for individuals suffering from egg allergy (Dhanapala et al., 2015). In addition, expanding the application of this method to allergen contamination inspection in conjunction with current antibody-based methods would be a powerful tool for enhancing safety and reliability for allergic individuals, food industries, and regulatory authorities.

In conclusion, we have developed an LC-MS/MS method for the simultaneous quantification of four major egg white allergens, Gal d 1–4. This method demonstrated excellent sensitivity, dynamic range, accuracy, repeatability, reproducibility, and specificity. Gal d 1–4 levels in fresh and processed eggs were measured and the values of fresh eggs were similar to those of previous reports. Although the application to allergen contamination inspection was left for a future study, this newly developed method can provide reliable information to the cosmetic and pharmaceutical industries and clinical fields, and may facilitate a quality of life improvement for egg allergic individuals and families who share their diet.

CRediT authorship contribution statement

Kyohei Kiyota: Conceptualization, Validation, Investigation, Data curation, Formal analysis, Writing - original draft, Funding acquisition. **Masato Yoshimitsu:** Conceptualization, Methodology, Software, Resources, Writing - review & editing. **Kotaro Uchida:** Writing - review & editing, Visualization. **Keiji Kajimura:** Writing - review & editing, Supervision, Project administration.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2020.128022>.

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Table S1. Specificity of LC-MS/MS method.

Cultivar (<i>n</i> = 1)			Gal d 1–4 homologue concentration (µg/g)			
			Gal d 1	Gal d 2	Gal d 3	Gal d 4
Avian ^{a)} (1:20,000 dilution)	Japanese quail egg	<i>Coturnix japonica</i>	N.D.*	N.D.	N.D.	N.D.
Livestock ^{b)} (1:2000 dilution)	Chicken breast	<i>Gallus gallus domesticus</i>	N.D.†	N.D.	82.2 ^{d)}	N.D.
	Pork	<i>Sus scrofa domesticus</i>	N.D.†	N.D.	N.D.	N.D.
	Beef	<i>Bos taurus domesticus</i>	N.D.†	N.D.	N.D.	N.D.
	Cow milk	<i>Bos taurus domesticus</i>	N.D.†	N.D.	N.D.	N.D.
Fish ^{b)} (1:2000 dilution)	Yellowfin tuna	<i>Thunnus albacares</i>	N.D.†	N.D.	N.D.	N.D.
	Salmon	<i>Oncorhynchus keta</i>	N.D.†	N.D.	N.D.	N.D.
	Soy sauce					
	pickled salmon roe	<i>Oncorhynchus keta</i>	N.D.†	N.D.	N.D.	N.D.
Pulses ^{b)} (1:2000 dilution)	Soybean	<i>Glycine max</i>	N.D.†	N.D.	N.D.	N.D.
	Chickpeas	<i>Cicer arietinum</i>	N.D.†	N.D.	N.D.	N.D.
Cereal ^{b)} (1:2000 dilution)	Wheat flour	<i>Triticum aestivum</i> L.	N.D.†	N.D.	N.D.	N.D.
Vegetables and Fruits ^{c)} (1:40 dilution)	Apple	<i>Malus domestica</i>	N.D.‡	N.D.	N.D.	N.D.
	Celery	<i>Apium graveolens</i> var. <i>dulce</i>	N.D.‡	N.D.	N.D.	N.D.
	Green kiwifruit	<i>Actinidia deliciosa</i>	N.D.‡	N.D.	N.D.	N.D.
	Gold kiwifruit	<i>Actinidia chinensis</i>	N.D.‡	N.D.	N.D.	N.D.
	Japanese pear	<i>Pyrus pyrifolia</i> var. <i>culta</i>	N.D.‡	N.D.	N.D.	N.D.
	Peach	<i>Prunus persica</i> L.	N.D.‡	N.D.	N.D.	N.D.
	Carrot	<i>Daucus carota</i> subsp. <i>sativus</i>	N.D.‡	N.D.	N.D.	N.D.
	Tomato	<i>Solanum lycopersicum</i> L.	N.D.‡	N.D.	N.D.	N.D.
	Mung bean sprout	<i>Vigna radiata</i>	N.D.‡	N.D.	N.D.	N.D.
	Watermelon	<i>Citrullus lanatus</i>	N.D.‡	N.D.	N.D.	N.D.
	Muskmelon	<i>Cucumis melo</i> L.	N.D.‡	N.D.	N.D.	N.D.
	Grape	<i>Vitis labrusca</i> L.	N.D.‡	N.D.	N.D.	N.D.

Bell pepper	<i>Capsicum annuum</i> L.	N.D. [‡]	N.D.	N.D.	N.D.
Banana	<i>Musa sapientum</i>	N.D. [‡]	N.D.	N.D.	N.D.
Pineapple	<i>Ananas comosus</i>	N.D. [‡]	N.D.	N.D.	N.D.
Avocado	<i>Persea americana</i> Mill.	N.D. [‡]	N.D.	N.D.	N.D.

N.D.: * <800 µg/g, † <80 µg/g, ‡ <1.6 µg/g, a) <200 µg/g, b) <20 µg/g, and c) <0.4 µg/g. For example, N.D.* was calculated by 39.1 (ng/mL) x 20,000 x (1 mL/g) = 782,000 ng/g, therefore N.D.* was listed as <800 µg/g.

Neither the quantitative ($S/N > 10$) nor the qualitative ion ($S/N > 3$) was detected.

b, c) Each dilution value was set higher to ensure more specificity.

d) Quantified using the Gal d 3 dose-response curve in the range of 9.77–1250 ng/mL.

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Figure S1.

Extracted ion chromatogram of 39.1 ng/mL standard Gal d 1 signature peptides (1a and 1b) and 9.77 ng/mL standard Gal d 2–4 signature peptides (2a–4b) obtained using LC-MS/MS MRM analysis. The quantitative and qualitative peptides were Gal d 1: 1a and 1b, Gal d 2: 2a and 2b, Gal d 3: 3a and 3b, and Gal d 4: 4a and 4b, respectively.

Figure S2.

Comparison of the quantitative peptides with the linearity of Gal d 1–4 standard curves, each quantitative peptide having a range of 9.77–312.5 ng /mL with CCP (50 µg/mL) and water added.

Figure S3.

Typical eight-point calibration curves of Gal d 1–4 for quantification, each having a range of 39.1–5000 ng/mL.

Figure S4.

Alignment of a) Gal d 4 amino acid sequence (UniProt database, accession number: P00698) with b) the homologue protein from Japanese quail egg (UniProt database, accession number: P00701), highlighting the position of different residues. Single and double underlines with bold show the quantitative and qualitative peptides, respectively, for Gal d 4 detection.