Analysis of Allergen Proteins in Food Sample using LC-MS/MS

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1. Introduction

Food allergies are caused by excessive immune responses to specific proteins, known as allergens, present in food, and in some cases, these reactions can lead to severe symptoms. The types and amounts of allergens that trigger allergic reactions vary among individuals. Therefore, it is essential to accurately determine the presence or absence of allergens derived from specific food ingredients.

To prevent health hazards caused by food allergies, food labeling is strictly regulated in many countries. In Japan, based on the frequency and severity of past health incidents, labeling is mandatory for eight specific allergens and recommended for an additional twenty allergens considered equivalent in risk. For processed and packaged foods, the official analytical methods include ELISA for quantitative testing, and PCR or Western blotting for qualitative testing.

Food allergen labeling is determined by the total protein concentration derived from the specified allergenic ingredients. Accordingly, reference standards used for quantification are calibrated based on the total protein content of each allergen. ELISA enables quantification, but due to the high structural similarity of proteins from closely related species, cross-reactivity may occur, potentially resulting in false positives. In cases where ELISA results are inconclusive and not supported by manufacturing records, PCR or Western blotting is employed. PCR amplifies allergen-specific DNA sequences, allowing for differentiation even among closely related species such as wheat and barley. However, in cases where the DNA is identical—such as between eggs and chicken—PCR cannot distinguish the source, and Western blotting is used instead. Western blotting detects specific proteins based on antigen-antibody interactions following electrophoresis, providing molecular weight information as well.

In recent years, LC-MS/MS has gained attention as a promising method for allergen detection. In this approach, proteins are extracted from food samples, digested enzymatically into peptides, and analyzed by LC-MS/MS. Allergen identification and quantification are performed based on the amino acid sequences of the detected peptides. This method allows for simultaneous analysis of multiple ingredients and offers high specificity by targeting peptide sequences. However, challenges remain, such as the lack of standardized protocols for protein extraction from food matrices and the difficulty of selecting optimal target peptides and MRM transitions, limiting its widespread adoption.

In this study, we developed new MRM transitions for five types of fruits and four types of nuts using allergen standards. We also constructed calibration curves using extraction reagents optimized for food allergen analysis, and we present these findings as examples of LC-MS/MS-based allergen quantification.

2. Methods

2-1. MRM Method Development

Allergen standards (Saika Technological Institute Foundation, preparation method shown in figure 1) were digested into peptide fragments using S-Trap and trypsin. MS/MS analysis of peptides was performed using the LCMS-9030, a Q-TOF mass spectrometer coupled with the Nexera™ X3 UHPLC (Shimadzu Corporation). Skyline software was used for analysis, employing the MS Amanda algorithm for high-accuracy identification. FASTA files were generated from the Allergen Nomenclature database (www.allergen.org) and UniProt (reviewed). MRM optimization was conducted on the detected transitions to create a method for candidate peptide analysis.

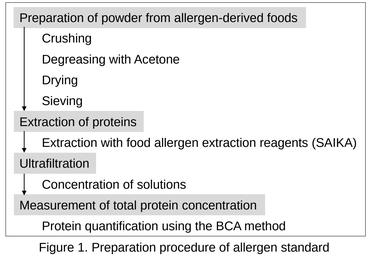

Figure 1. Preparation procedure of allergen standard

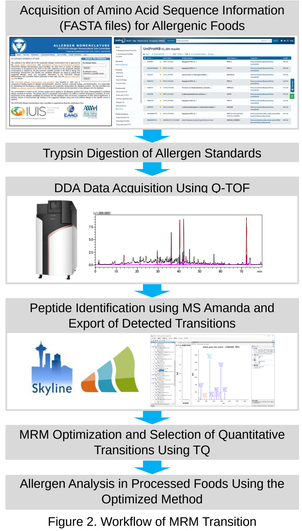

Figure 2. Workflow of MRM Transition Optimization using Skyline

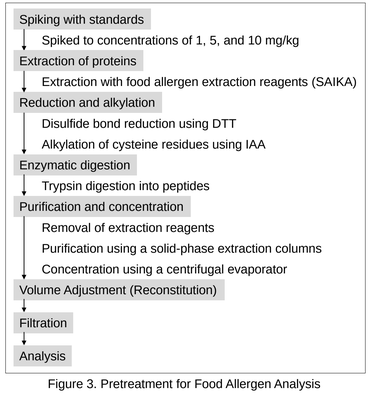

Figure 3. Pretreatment for Food Allergen Analysis

Figure 4. Nexera X3 and LCMS-8060RX

2-2. Investigation of Allergen Quantification in Food

Allergen-free processed food (chicken rice) was spiked with allergen standards at concentrations of 1, 5, and 10 mg/kg and pretreated using the method shown in figure 3. The allergen standards spiked were a total of 16 items, comprising the 8 mandatory labeling allergens in Japan: milk, egg, wheat, buckwheat, crustacean, soybean, walnut, and peanut; as well as 3 types of nuts: almond, cashew nut, and macadamia nut; and 5 types of fruits: banana, kiwifruit, orange, peach, and apple. Extraction was performed using an allergen extraction reagent (Saika Technological Institute Foundation). Analysis was performed using a triple quadrupole mass spectrometer LCMS-8060RX (Shimadzu Corporation) coupled with the Nexera X3 UHPLC (figure 4). The analytical conditions are shown in Table 1.

2-3. Specificity Assessment

High food-specific selectivity is crucial for allergen analysis. Therefore, samples simulating processed foods containing each food protein at a concentration of 500 mg/kg were prepared and analyzed using the same method as in the quantification study.

Table 1. Analytical conditions

Column	: Shim-pack™ GIST-HP C18 (100 mm x 2.1 mm I.D., 3 µm)		
Mobile phase A	: Acetic acid/Water = 2:1000 (v/v)		
Mobile phase B	: Acetic acid/Acetonitrile = 2:1000 (v/v)		
Rinse	: Acetic acid/Acetonitrile/Water = 2:500:500 (v/v/v)		
Flow rate	: 0.3 mL/min (0.5 mL/min only between 18.1-21 min)		
Time program	: B conc. 5% (0-3 min) → 30% (18 min) → 95% (18.1-21 min) → 5% (21.1-25 min) The flow was introduced into the mass spectrometer between 0 to 18 min using a flow switching valve.		
Column temp.	: 50 ºC	Injection vol.	: 5 µL

Table 2. Specificity Assessment of Developed Transitions (* indicates peptides with poor linearity)

Food	Peptide sequence	Rating	Milk	Buckwheat	Wheat	Peanut	Crustaceans	Soybeans	Egg	Walnut	Pecan	Almond	Cashew	Macadamia	Banana	Green Kiwi	Gold Kiwi	Orange	Peach	Apple
Walnut	SPDQSYLR	Good								----										
	DLPNECGISSQR	Unacceptable	++				+			----	+		+				+			
	LVALEPSNR	Unacceptable					++			----										
	ATLTLVSQETR	Acceptable								----								+		
Almond	ADFYNPQGGR	Acceptable	+						+			----					+			
	ALPDEVLQNAFR	Good										----								
	TEENAFINTLAGR	Acceptable										----								+
	VTGINALR	Unacceptable		++	+							----	+	++	+++		++	++	+++	++
Cashew nut	ADIYTPEVGR	Unacceptable	+							+			----	++						
	ELYETASELPR	Good											----							
	GQVQVVDNFGNR	Good											----							
	IDYPPLEK	Acceptable			+	+		+					----					+		
	WLQLSVEK	Acceptable									+		----							
Macadamia nut	EGVIIR	Unacceptable								++++	++++			----						
	EILEAALNTQTER	Good												----						
	ESYNLECGDVIR	Good												----						
	FLQTISTPGQYK	Acceptable									+			----						
	GPYNLFNK	Good												----						
	QSDNPYYFDER	Good												----						
Banana	ATFEIVNR	Good													----					
	NSNIQVLLDVPR	Unacceptable	+				+	+	+					+	----			++		
	CSYTVWAAAVPGGGR	Unacceptable*													----					
	TGCSFDGSGR	Good													----					
Kiwifruit	VVDECDSR	Good														----	----			
	IVALSTGWYNGGSR	Acceptable											+			----	----			
	SAGAVVDIK	Good														----	----			
Orange	DECPCYR	Unacceptable		++			++							++	++			----	++++	++++
	AATEAIK	Unacceptable	+		++	++++	+	+++	+			++	+++	++++			+	----	++	++
	NVVDGSTTFK	Unacceptable			++++		+++		++								++	----		
Peach	NVNNLAR	Good																	----	
	IAPQAIK	Unacceptable			+	+						+							----	++++
	AFVLDADNLVPK	Acceptable										+							----	
Apple	IAPQAVK	Unacceptable																	++	----
	TINGLAR	Good																		----
	LVASGSGSIIK	Good																		----
	VCPAPLQVK	Unacceptable	+		++	+	+	+		+	+		+	+			+		++	----
	ILTDYIK or LIESYLK	Good																		----
	AFVLDADNLIPK	Good																		----

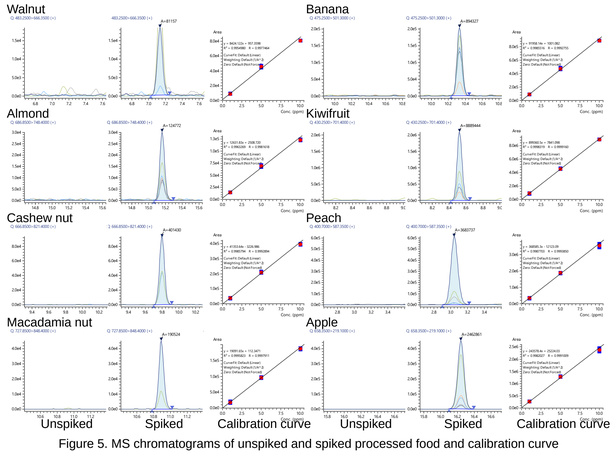

Figure 5. MS chromatograms of unspiked and spiked processed food and calibration curve

3. Results

A total of 16 food allergens were spiked into processed food, and the pretreatment was performed in duplicate. The prepared samples were analyzed to confirm the linearity of the calibration curves and the background. We confirmed that a good calibration curve could be generated from 1 mg/kg for all food allergens. Figure 5 shows the MS chromatograms and calibration curves for the pretreated samples of processed food spiked with nuts and fruits.

For the 16 food allergens, we checked for cross-reactivity of the MRM transitions. Table 2 shows the results for the newly developed transitions for 4 types of nuts and 5 types of fruits. The observed false positive peak intensities were evaluated on a four-tier scale relative to the peak area of the target food: + (0.1–1%), ++ (1–5%), +++ (5–10%), and ++++ (≥10%). Peptides showing no false positive peaks and good linearity of the calibration curve were evaluated as "Good", those with slight false positive peaks as "Acceptable," and those with significant false positive peaks or poor linearity of the calibration curve as "Unacceptable." We successfully developed transitions with good linearity and specificity for all foods except orange. We will continue to work on creating MRM transitions for orange.

4. Conclusions

- Specific MRM transitions were successfully developed for 4 types of nuts and 4 types of fruits, with the exception of orange.
- Further attempts will be made to create alternative transitions for orange.