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Application News

Simultaneous Analysis of Food Allergens, Including Nuts and Fruits, Using a Triple Quadrupole Mass Spectrometer

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User Benefits

- Simultaneous detection of allergens from seven specified ingredients and ten ingredients equivalent to the specified ones is possible, and calibration curves can be prepared from 1 ppm.
- The LC-MS/MS method enables detection of all target ingredients with a single sample preparation.

■ Introduction

Food allergies are caused by excessive immune responses to specific food proteins (allergens) and can occasionally result in severe symptoms. To prevent health risks associated with food allergies, food labeling is strictly regulated in many countries. In Japan, based on the severity and frequency of past health incidents, labeling is mandatory for seven specified allergens and recommended for twenty allergens equivalent to the specified ones in prepackaged processed foods.

In recent years, LC-MS/MS has attracted increasing attention for allergen analysis due to its various advantages. This application news presents an example of the simultaneous analysis of seven specified food allergens and ten allergens equivalent to specified ingredients in processed foods using LC-MS/MS. Specific peptides and corresponding MRM transitions for four types of nuts and four types of fruits were newly selected and developed using Skyline software. In addition, examples of calibration curve preparation using extraction reagents and standard materials optimized for food allergen analysis are presented.

■ Various Analytical Methods for Allergens

The quantity and type of allergens responsible for allergic symptoms vary among individuals; therefore, it is essential to accurately determine the presence or absence of allergenic ingredients in food on an ingredient-by-ingredient basis. In Japan, the enzyme-linked immunosorbent assay (ELISA) has been designated as a quantitative testing method, whereas the polymerase chain reaction (PCR) and Western blotting have been designated as qualitative testing methods. The characteristics of each analytical method are summarized in Table 1.

Table 1 Analytical methods for food allergens

Method	Quantitative	Specific	Simultaneous analysis
ELISA	Possible	Medium	Not possible
PCR	Not possible	High	Not possible
Western blot	Not possible	High	Not possible
LC-MS/MS	Possible	High	Possible

ELISA enables quantitative determination of allergens; however, it carries a risk of false-positive results due to cross-reactivity with homologous proteins. When it is difficult to distinguish true positives from false positives based on ELISA results and manufacturing records, PCR or Western blotting is employed as a complementary technique. Moreover, ELISA cannot analyze multiple ingredients simultaneously, requiring a dedicated kit for each ingredient along with individual sample preparation and measurement procedures.

PCR detects target DNA sequences specific to each ingredient through amplification reactions. This provides highly specific discrimination even among closely related species, such as wheat and barley. In contrast, PCR is not suitable for distinguishing foods that share identical genetic sequences, such as eggs and chicken meat. In such cases, Western blotting is used instead, targeting proteins that are present in the same species but not designated as allergens. Because Western blotting detects proteins via antigen–antibody reactions following electrophoresis, it allows evaluation of molecular weight information in addition to protein detection. Although both PCR and Western blotting exhibit high specificity, quantitative determination of allergenic proteins remains difficult using these methods.

LC-MS/MS, which has recently attracted increasing attention, involves extracting allergenic proteins from food, enzymatically digesting them into peptide fragments, and subsequently analyzing these peptides. The amino acid sequences of the detected peptides enable identification of the corresponding allergens. This approach allows simultaneous analysis of multiple ingredients and provides high specificity because it targets amino acid sequences. Quantification has also become feasible through the use of allergen protein standards. Nevertheless, challenges remain, including the difficulty of selecting appropriate target peptides and MRM transitions to ensure accurate analysis, as well as the lack of an established protein extraction method optimized for LC-MS/MS. This application news introduces sample preparation procedures and analytical methods developed to address these challenges.

■ Standard Materials for Allergen Analysis

Food allergen labeling is determined based on the total protein concentration derived from the raw ingredients contained in the food. Therefore, the reference standard materials for quantification are calibrated according to the total protein concentration of each ingredient. In this application, food-derived allergen extracts (SAIKA Technological Institute Foundation) were used as standard materials for food allergen analysis. These materials contain total proteins and are applicable to LC-MS/MS analysis. Standard powders were prepared with reference to the labeling method specified by the Consumer Affairs Agency. Protein extraction was performed using a food allergen extraction reagent (SAIKA Technological Institute Foundation), which is suitable for LC-MS/MS analysis, and the protein concentrations were then determined after extraction (Fig. 1).

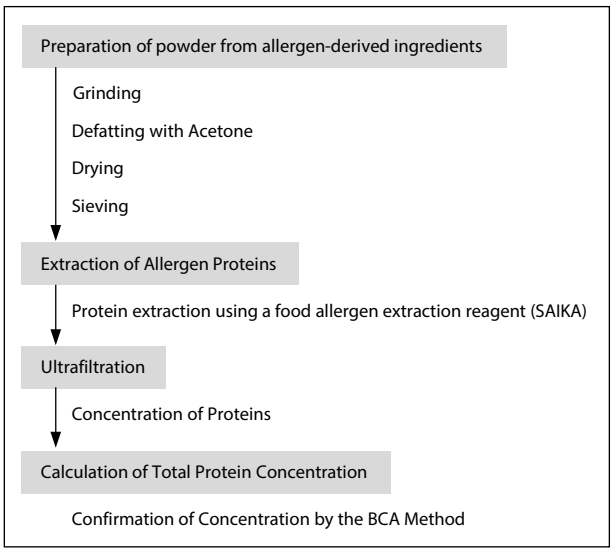

Fig. 1 Example of the preparation procedure for standard materials used in food allergen analysis

■ Development of MRM Transitions

Although several studies have reported the analysis of eight specified allergens using LC-MS/MS, this application describes the development of new analytical methods for walnut, which was newly added to the list of specified ingredients in Japan, as well as for three nuts and five fruits categorized as non-mandatory food allergens (allergens recommended for labeling under Japanese regulation).

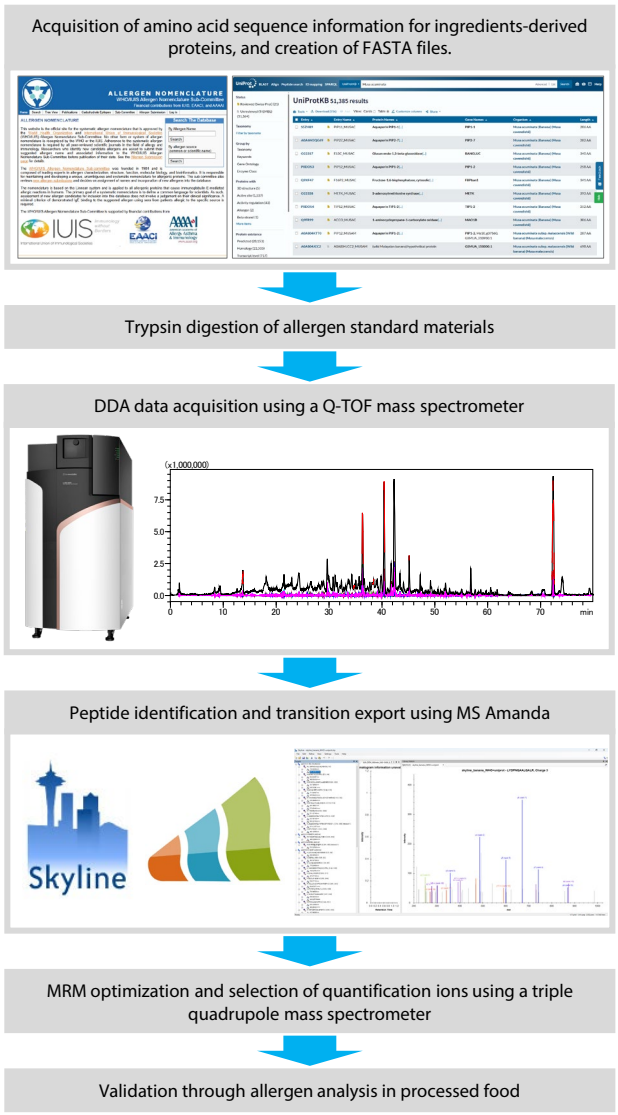

Fig. 2 Method for creating MRM transitions for allergen analysis using Skyline

Food-derived allergen extracts from walnut, almond, cashew nut, macadamia nut, banana, kiwifruit, orange, peach, and apple were used as standard materials. Each standard was digested into peptide fragments using S-Trap (ProtiFi) and trypsin. For MS/MS analysis used in qualitative evaluation, a system consisting of an ultrafast liquid chromatograph Nexera™ X3 UHPLC coupled with a quadrupole time-of-flight mass spectrometer LCMS-9030 was employed, and data were acquired in DDA mode. Data analysis was performed using Skyline, with MS Amanda applied as the peptide identification algorithm for high-accuracy matching. Amino acid sequence information for the proteins derived from each ingredient was obtained from the Allergen Nomenclature Database (www.allergen.org) and UniProt. Candidate MRM transitions obtained were optimized using a triple quadrupole mass spectrometer, and transitions were selected based on sensitivity, specificity, and ionization stability. The workflow is shown in Fig. 2, and the identified peptides and their corresponding source proteins are summarized in Table 2.

When MRM transition candidates are generated solely using Skyline, it is known that several transitions that can actually be ionized are not included among the proposed candidates. By exploring transitions from MS/MS analysis data as performed in this method, such omissions can be effectively reduced.

Table 2 Ingredients and peptides with newly developed MRM transitions

Food	Protein name	Peptide sequence
Walnut	Jug r 1	DLPNECGISSQR
	Jug r 2, Jug n 2	ATLTLVSQETR
		SPDQSYLR
	Jug n 4	LVALEPSNR
Almond	Pru du 6-1	TEENAFINTLAGR
	Pru du 6-2	ADFYNPQGGR
		ALPDEVLQNAFR
	Pru du 10	VTGINALR
Cashew nut	Ana o 1	IDYPPLEK
	Ana o 2	ADIYTPEVGR
		GQVQVVDNFGNR
		WLQLSVEK
	Ana o 3	ELYETASELPR
Macadamia nut	Mac i 1-1	QSDNPYYFDER
	Mac i 1-1, Mac i 1-2	ESYNLECGDVIR
		FLQTISTPGQYK
		GPYNLFNK
	Mac i 1-2	EGVIIR
		EILEAALNTQTER
Banana	Mus a 4	ATFEIVNR
		CSYTVWAAAVPGGGR
		TGCSFDGSGR
	Mus a 5	NSNIQVLLDVPR
Kiwifruit	Act d 1	SAGAVVDIK
	Act d 5, Act c 5	IVALSTGWYNGGSR
		VVDECDSR
Orange	Cit s 7	DECPCYR
	Photosystem II protein	AATEAIK
	Pectinesterase 1	NVVDGSTTFK
Peach	Pru p 1	AFVLDADNLVPK
		IAPQAIK
		NVNNLAR
Apple	Mal d 1	AFVLDADNLIPK
		IAPQAVK
		LIESYLK
		LVASGSGSIIK
	Mal d 2	VCPAPLQVK
	Mal d 3	TINGLAR

■ Sample Preparation for Allergen Analysis

The sample preparation procedure for allergen analysis in processed foods is shown in Fig. 3. Standard allergen materials were spiked into food samples, and extraction was performed using a food allergen extraction reagent. After reduction and alkylation, enzymatic digestion was carried out using trypsin. Following removal of the extraction reagent and cleanup using a solid-phase extraction column, the samples were dried to remove the solvent and concentrated. The residues were dissolved in 0.1% formic acid aqueous solution, filtered, and then analyzed with LC-MS/MS.

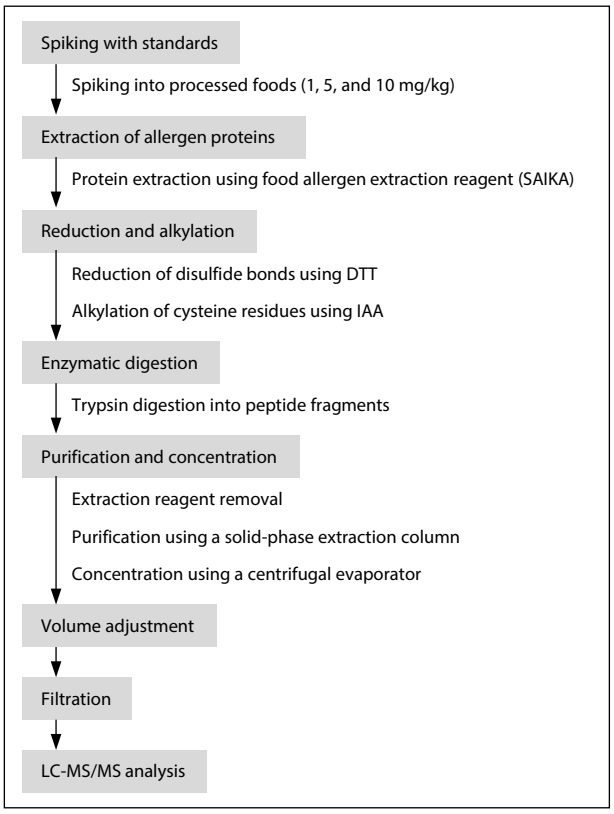

Fig. 3 Sample preparation for food allergen analysis in processed foods

■ Verification of Specificity

In food allergen analysis, it is essential that the analytical method used exhibits high specificity for each target ingredient to avoid false-positive detections. Therefore, the specificity of MRM transitions was verified by examining whether false-positive peaks were detected when analyzing non-target ingredients using the transitions developed for the target allergens.

Each standard material was individually pretreated using the same procedure applied for food allergen analysis. Samples were prepared for each ingredient at final concentrations equivalent to those containing 500 mg/kg of protein derived from the respective raw materials in food, and were analyzed by LC-MS/MS. The peptides examined and the corresponding results for each ingredient are summarized in Tables 3 and 4.

When the peak area of a false-positive signal was 0.1-1% relative to that of the target ingredient, it was indicated as "+"; 1-5% as "++"; 5-10% as "+++"; and greater than 10% as "++++". Peptides showing no apparent false positives and good linearity in the calibration curve were evaluated as "Good"; those with minor false positives as "Acceptable"; and those with pronounced false positives or poor linearity as "Unacceptable".

As shown in Tables 3 and 4, transitions exhibiting good linearity and high specificity were successfully developed for all food ingredients except orange. Because false-positive peaks were observed for all peptides derived from orange, further optimization and development of MRM transitions for this ingredient are considered necessary.

■ Allergen Analysis in Processed Foods

To verify the quantitative capability of the analytical method for food allergen analysis, calibration curves were prepared and evaluated. Allergen-free processed food (chicken rice) was spiked with standard allergen materials at concentrations of 1, 5, and 10 mg/kg. After sample preparation (n = 2), LC-MS/MS analysis was performed, and the background and linearity of the calibration curves were assessed.

As standard materials, protein fractions extracted from 17 food ingredients were used, consisting of eight specified allergens (milk, egg, wheat, buckwheat, shrimp, crab, walnut, and peanut) and nine allergens categorized as equivalent to the specified ingredients under Japanese labeling regulation (soybean, almond, cashew nut, macadamia nut, banana, kiwifruit, orange, peach, and apple).

Quantitative analysis was conducted using a triple quadrupole mass spectrometer LCMS-8060RX coupled with a Nexera X3 UHPLC system (Fig. 4). A Shim-pack™ GIST-HP C18 column was employed, and the analysis was performed under a 25-minute method including column washing and equilibration. The analytical conditions are summarized in Table 5.

In all food samples, good calibration curves were obtained within the range of 1 to 10 mg/kg. Fig. 5 shows the MS chromatograms and calibration curves of nuts and fruits, comparing samples without spike and those spiked at 10 mg/kg.

No peaks derived from raw ingredients were detected in the non-spiked samples, whereas in the spiked samples, not only the quantifier ion but also multiple qualifier ions were successfully detected.

Fig. 4 Nexera™ X3 and LCMS-8060RX

Table 5 LC–MS/MS analytical conditions

[HPLC conditions] Nexera X3	
Column	: Shim-pack GIST-HP C18 (100 mm x 2.1 mm I.D., 3 µm)*1
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)
Gradient 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˚C
Injection volume	: 5 µL
[MS conditions] LCMS-8060RX	
Ionization	: ESI, Positive mode
Interface voltage	: 1 kV
Ion Focus voltage	: 2 kV
Nebulizing gas	: 3 L/min
Heating gas	: 10 L/min
Drying gas	: 10 L/min
DL temp.	: 150˚C
Interface temp.	: 250˚C
Heat block temp.	: 300˚C
Probe position	: +1 mm

*1 P/N: 227-30046-02

Table 3 Verification of specificity of the developed MRM transitions for specified ingredients

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

* Peptides for which no false positives were detected but poor linearity was observed in the calibration curve.

Table 4 Verification of specificity of the developed MRM transitions for nuts and fruits (including ingredients equivalent to the specified ones)

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

* Peptides for which no false positives were detected but poor linearity was observed in the calibration curve.

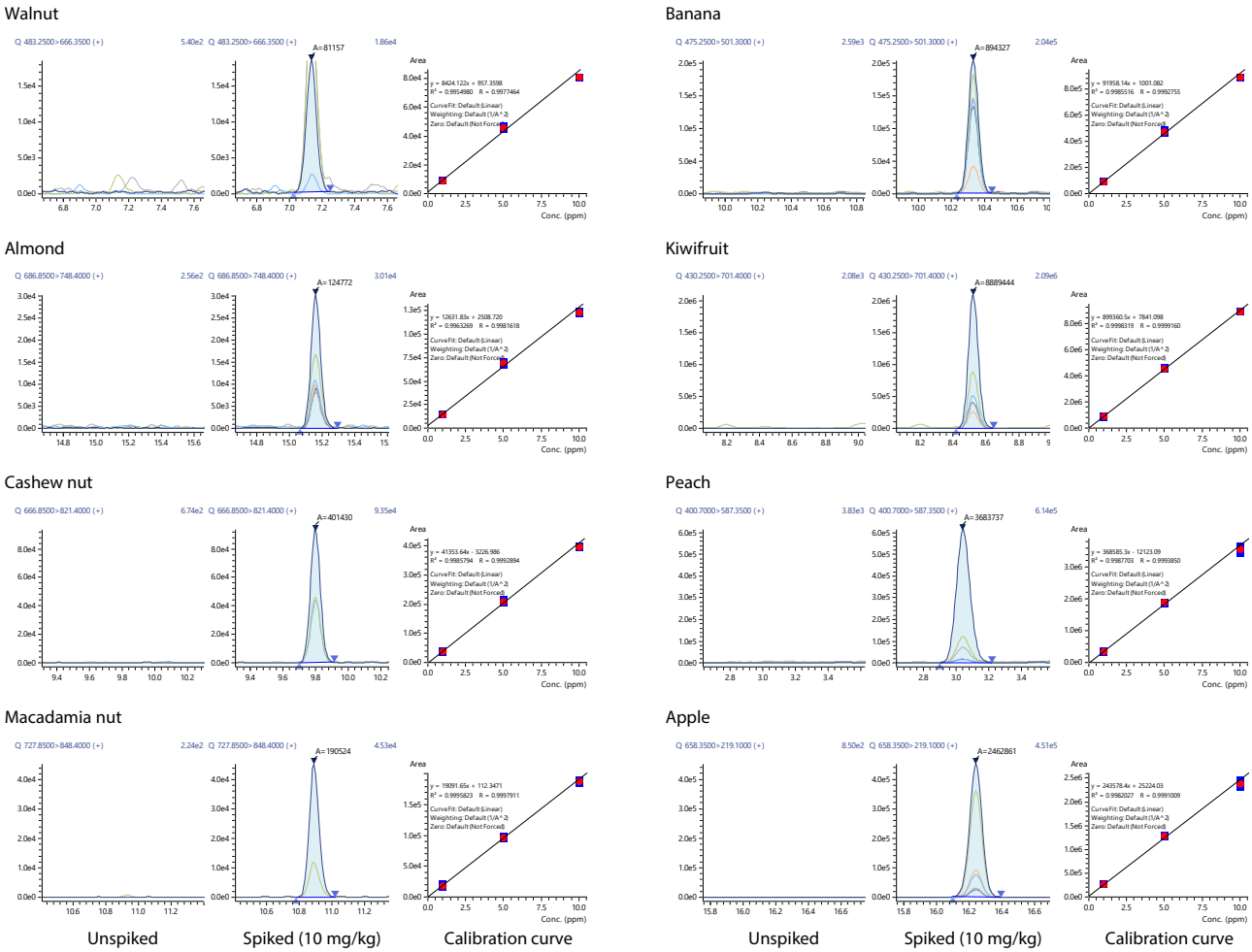

Fig. 5 MS chromatograms of unspiked and spiked (10 mg/kg) processed food and calibration curve

■ Conclusion

New MRM transitions for LC-MS/MS analysis were developed for the specified allergen walnut and eight additional ingredients equivalent to specified ones (almond, cashew nut, macadamia nut, banana, kiwifruit, orange, peach, and apple). Using a triple quadrupole mass spectrometer, spiking experiments were conducted with standard materials for a total of 17 food allergens in processed foods, confirming that good calibration curves could be obtained. The specificity of each peptide was also evaluated, and analyses without false-positive detections were achieved for all ingredients except orange.

These results demonstrate that the developed LC-MS/MS method enables simultaneous analysis of 17 food allergens, including those equivalent to the specified ones, in processed foods.

＜Acknowledgments＞

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＜ Related applications ＞

1. 01-00665-EN Development of a Simultaneous Analysis Method for Allergens in Food Using a Triple Quadrupole Mass Spectrometer

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