Vietnam Journal of Food Control

A Scientific Journal of National Institute for Food Control

Journal homepage: https://vjfc.nifc.gov.vn

Research Article

Simultaneous analysis of certain tree-nut allergens in cookies by LC-MS/MS

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(Received: 26 Jul 2024; Revised: 26 Aug 2024; Accepted: 26 Aug 2024)

Abstract

In this study, four tree-nut allergens were identified using liquid chromatography-mass spectrometry (LC-MS/MS) with a reverse-phase XSelect HSS C18 SB column (100 mm x 2.1 mm, 3.5 µm) and corresponding guard column. The gradient solvent program employed 0.1% formic acid in water and 0.1% formic acid in acetonitrile, with a flow rate of 0.5 mL/min, a positive ion electrospray ionization (ESI+) source, and a multiple reaction monitoring (MRM) mode. The method was validated following the guidelines of AOAC International. Results showed that the method had good specificity, with calibration curves constructed in the concentration ranges of 30.5 - 1220 µg/g for almonds, 102 - 4080 µg/g for cashews, 128 - 5120 µg/g for hazelnuts, and 122 - 4880 µg/g for pistachios. Limits of detection were 9 µg/g for almonds, 30.6 µg/g for cashews, 38.4 µg/g for hazelnuts, and 36.6 µg/g for pistachios, while limits of quantification were 30.5 µg/g for almonds, 102 µg/g for cashews, 128 µg/g for hazelnuts, and 122 µg/g for pistachios. The repeatability and accuracy of the method met AOAC requirements. The method was used to analyze the content of four groups of nut allergens (almond, cashew, hazelnut, and pistachio) in ten cookie samples from the Hanoi area. Results indicated that three samples contained undeclared allergens on the food labels.

Keywords: *tree-nut allergen, LC-MS/MS, cookies.*

1. INTRODUCTION

Symptoms of food allergies (allergic reactions) can include rash; red or flushed skin; tingling or itching in the mouth; swelling of the face, tongue, lips, throat or vocal cords; vomiting and/or diarrhea; abdominal cramps; coughing or wheezing; dizziness and/or

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Doi: https://doi.org/10.47866/2615-9252/vjfc.4419

lightheadedness; difficulty breathing and even loss of consciousness [1]. While most food allergy symptoms are mild and limited to the skin or gastrointestinal discomfort, some can progress to a severe, life-threatening reaction known as anaphylaxis (characterized by airway constriction in the lungs, throat, and laryngeal swelling leading to suffocation, severe hypotension, and shock) [1]. Long-term food allergies can impact nutritional quality and growth in children [2].

According to the FDA, nine major food allergen groups account for 90% of food allergies are milk, eggs, fish, crustacean shellfish, tree- nuts, peanuts, soybeans, wheat, and sesame seeds [3]. These allergens are commonly found in daily diets, with an increasing variety of processed products derived from them. Additionally, these allergens are prone to cross-contamination during production, and even small amounts can trigger allergic reactions. Children are particularly vulnerable and more likely to experience severe reactions, necessitating the identification of allergens in food.

The FDA has strict regulations for labeling food products that contain potential allergens [3]. In Vietnam, there is Decree No. 43/2017/ND-CP on goods labeling and the Vietnamese Standard TCVN 7087:2013 (CODEX STAN 1-1985, amended in 2010) on labeling pre-packaged food products.

Currently, common methods for analyzing allergens in food include ELISA and PCR. However, these traditional methods have some limitations. ELISA has the disadvantage of being able to cause false positives because of the similarity of some related proteins to activate the binding site of the used antibodies. PCR method can detect the DNA of allergenic tree–nut but PCR is an indirect indicator because processed food has little or no DNA but still have large amount of allergenic protein. Mass spectrometry (MS) overcomes the drawbacks of both methods and is proving to be an effective approach for analyzing food allergens.

In 2011, J. Heck *et al.* developed the first screening method for simultaneous detection of seven allergens by liquid chromatography mass spectrometry to detect milk, egg, soy, hazelnut, peanut, walnut and almond. Protein was extracted from food matrix, followed by enzymatic digestion by trypsin. The detection range is from 10 – 1000 µg/g [4].

In 2018, New LS *et al.* had developed LC–MS/MS method to simultaneously screen the signature tryptic peptides of multiple allergen commodities. The method was capable of detecting egg white, skim milk, peanut, soy, and tree nut (almond, Brazil nut, cashew, hazelnut, pecan, pine nut, pistachio and walnut) at a detection limit of 10 ppm in incurred bread and cookies. The method demonstrated excellent sensitivity with a method quantitative limit of 3 ppm for whole eggs and 10 ppm for the remaining three allergen commodities, as good recovery (60 - 119%) and repeatability (RSDr <20%), with an analytical range of 10 - 1000 ppm for each allergen commodity, and was able to meet the minimum performance requirements of the SMPR 2016.002 [5].

In 2023, Akira Torii *et al.* developed liquid chromatography-tandem mass spectrometry to detect almonds and walnuts as specified in regulations for food labeling in processed food. Akira Torii *et al.* used solid phase extraction with an Oasis HLB column to clean up the sample after tryptic digestion. The limit of detection for the walnut 2S albumin

peptide GEEMEEMVQSAR was 0.22 ± 0.02 µg/g, and that for almond 11S globulin peptide GNLDFVQPPR 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 [6].

In Vietnam, the development of mass spectrometry methods for analyzing food allergens is ongoing. In 2018, Nguyen Thi Ha Binh *et al.* developed a method LC-MS/MS to screen 5 allergens from egg, milk, soybean, walnut, and peanut at concentration ranges from 3 to 20 µg/g [7]. In 2019, Nguyen Thi Minh Hoa *et al.* developed methods to detect casein in milk by HPLC – PDA with LOQ 0.8 g/100g, recovery in the range of 78 – 98%, and RSD in the range of 2.4 – 9.5% and ELISA with LOQ is 3 mg/kg, recovery in range 83 – 109%, RSD is 11% [8].

Therefore, this study was conducted to develop a method for quantifying allergens from tree-nut in cookies using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The results were applied to analyze cookies randomly purchased from the market with/without composition declared on their label.

2. MATERIALS AND METHODS

2.1. Materials and chemicals

Raw almonds, cashews, hazelnuts, and pistachios were purchased from the local supermarket and used as reference materials. The presence of these allergens in food samples is determined through their characteristic proteins. Wheat flour was used as the blank sample.

Acetonitrile (LC–MS grade, Merck, Germany), methanol (LC–MS grade, Merck, Germany), formic acid (LC–MS grade, Merck, Germany), trichloroacetic acid (TCA) (Merck), n-hexane (analytical grade), Tris (hydroxymethyl) aminomethane (TRIS, for Biochemistry, purity ≥ 99%); Dithiothreitol (DTT) (purity ≥ 99%, Merck, Germany), Sodium bicarbonate (Merck, Germany), Hydrochloric acid 37% (Merck, Germany), Urea (for Biochemistry, China), Iodoacetamide (IA) (purity ≥ 99%, Merck, Germany), Trypsin (>250 N.F.U/mg) (Nanjing Duly Biotech, China). Water used in the study was purified from the laboratory's MilliQSP system (Merck, Germany).

2.2. Equipment

The analysis was performed on a liquid chromatography-mass spectrometry system including the Shimadzu HPLC LC20AD XR coupled with the AB Sciex Triple Quad 5500 Mass Spectrometer and a reverse-phase XSelect HSS C18 SB column (100 mm x 2.1 mm, 3.5 µm) column. Other types of equipment used in the experiments included analytical balance (accuracy 0.1 mg) (Mettler Toledo, Switzerland), vortex mixer (IKA, China), Centrifuge Mikro 200R (Hettich, Germany), sample homogenizer (Phillips, Vietnam), incubator (Amerex, United States), 24-position Solid Phase Extraction (SPE) Manifold (24-Port Visiprep™ Vacuum SPE Manifolds, Supelco, United States), Oasis HLB 3cc/60mg SPE Column (Waters, United States), N_2 Evaporation System (Organomation, United States), and other laboratory consumables.

2.3. Experiments

2.3.1. Determination of protein content and preparation of reference samples.

The Kjeldahl method was used to determine the protein content in the nut samples. The tree-nut samples were ground and thoroughly homogenized using a homogenizer. Homogenized tree-nut samples were weighed 0.5 - 1 gram into the Kjeldahl tube, followed by adding 5 grams of K_2SO_4 and 0.5 grams of $CuSO_4.5H_2O$. Then 10 mL concentrated H_2SO_4 was added and heated to 420°C in 70 minutes. After completely decomposing, bring a sample to distillation. Then the sample is titrated by HCl 0.1N to get the result of total nitrogen in the sample. The concentration of protein is calculated by the formula:

$$\%Protein = P\ x\ N$$

P = 6.25 is the conservation factor and N is the concentration of total nitrogen in the sample. The results of protein concentration are shown in Table 1. This study uses each tree-nut with a determined protein concentration as the reference material. The protein extraction solution of each tree-nut is considered as the reference solution for further experiments.

Table 1. *The protein content in each tree-nut allergen*

No.	***Allergen***	***Concentration (%)***
1	Almond	24.27
2	Cashew	20.41
3	Hazel nut	25.65
4	Pistachio	24.42

2.3.2. LC-MS/MS conditions

The specific allergenic proteins of each tree–nut were referenced from the website of WHO/IUIS Allergen Nomenclature Sub-Committee [9]. The protein sequences of allergens in FASTA form were searched from the UniProt protein database and then submitted to the Skyline software to predict the tryptic digested peptide of allergens and the MRM transitions for analysis on LC-MS/MS instrument.

The ESI (+) ionization technique in MRM mode was chosen to identify the parent ions and their fragmentations for each allergen. The ionization source conditions are shown in Table 2.

Table 2. *Ionization source conditions*

Parameters	***Optimized conditions***
Curtain gas (CUR)	35.0 psi
Collision gas (CAD)	8 V
Ionspray Voltage	5500 V
Temperature (TEM)	550°C
GS1 (psi)	60 psi
GS2 (psi)	70 psi

The input parameters and collision energy (CE) are automatically optimized according to the instrument. The optimized results are presented in Table 3.

Table 3. *Optimized mass spectrometry conditions*

Matrix	***Protein***	***Peptide***	***Precursor ion (m/z)***	***Product ion (m/z)***	***CE (eV)***
Almond	Pru du 6	GNLDFVQPPR	571.8	369.2* 596.4	29.0
Cashew	Ana o 2	ADIYTPEVGR	560.8	658.3* 557.3	18.4
Hazel nut	Cor a 9	ADIYTEQVGR	576.2	689.3* 588.3	30.0
Pistachio	Vicilin	VVVLPK	327.7	456.3* 224.2	15.1

(*) Quantitative ion

For each tree–nut allergen, the peptide which was determined to not have signal in the other tree–nut and the highest response are chosen to be the marker for analysis. Based on the optimized results, a quantification ion and a qualification ion are selected, with an IP score of 5 (the IP score is calculated according to the EC 2021/808 standard) [10] required for mass spectrometry analysis (according to AOAC).

The liquid chromatography conditions used are as follows: an XSelect HSS C18 SB column (100 mm × 2.1 mm, 3.5 µm) with a mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile at a flow rate of 0.5 mL/min, injection volume of 20 µL and gradient program in Table 4 (AOAC 2017.17) [11].

Table 4. *Gradient Program*

Time (min)	***Acid formic 0.1% / H2O (%)***	***Acid formic 0.1% / Acetonitrile (%)***
0	98	2
1.30	98	2
11	60	40
12.5	2	98
14.4	2	98
14.6	98	2
20	98	2

2.4.3. Sample preparation

Based on previous studies [4-8], the proposed sample preparation procedure has three stages:

Stage 1 - Fat removal and protein extraction

Weigh 0.5 g of the sample into a 50 mL centrifuge tube. Vortex for 5 minutes and then shake horizontally for 15 minutes with n-hexane to remove fat. Centrifuge at 1,500 rpm for 5 minutes. Decant the upper layer and evaporate the solvent using a nitrogen evaporator. Add 5 mL of 100 mM Tris-HCl buffer (pH 8.2), 4 M urea, and 0.1 M DTT. Vortex and incubate at 37°C for 3 hours. Centrifuge at 1,500 rpm for 5 minutes. The upper layer at this point is the protein extract.

Stage 2 - Alkylation and hydrolysis

Transfer 1 mL of the protein extract to a 15 mL centrifuge tube. Add 25 µL of iodoacetamide (IA) and 4 mL of 50 mM $NaHCO_3$. Vortex and incubate in the dark at 37°C for 1 hour. Add 250 µL of Trypsin (4 mg/mL). Incubate for 16 hours at 37°C to obtain the hydrolyzed solution.

Stage 3 - Salt removal and clean-up

Add 50 µL of TCA to the hydrolyzed solution. Filter through filter paper and then perform solid-phase extraction. Elute with 95% ACN. Evaporate the solvent to dryness using a nitrogen evaporator. Reconstitute with 1 mL of 5% ACN (0.1% HCOOH). Centrifuge at 13000 rpm for 5 minutes. Filter through a 0.22 µm membrane filter. Transfer the solution to a vial for LC-MS/MS analysis.

2.4.4. Method validation

Perform method validation according to AOAC SMPR 2016.002 guidelines [12]. The parameters validated to evaluate the method include specificity, limit of detection (LOD), limit of quantification (LOQ), linear range and calibration curve, repeatability, and recovery.

The protein extraction of each tree – nuts was collected then diluted by water to the protein concentration equivalent to LOQ, 2LOQ, 4LOQ, 10LOQ and 20LOQ for each tree – nut. 1 mL of each diluted concentration was taken to prepare following the sample preparation protocol.

2.4.5. Data processing methods

Analysis results were calculated and processed using SCIEX Analyst software and Microsoft Excel.

3. RESULTS AND DISCUSSION

3.1. Investigation of sample preparation

Each different optimized conditions were done in triplicate, with each condition. The results of each will be compared based on the peak area or signal intensity of the target analyte.

3.1.1. Protein amount difference between roasted and raw nuts

For each allergen, a roasted process was performed at 180°C for 10 minutes for whole and ground nuts. Afterward, protein extraction from each sample was obtained to compare samples under different processing in Figure 1.

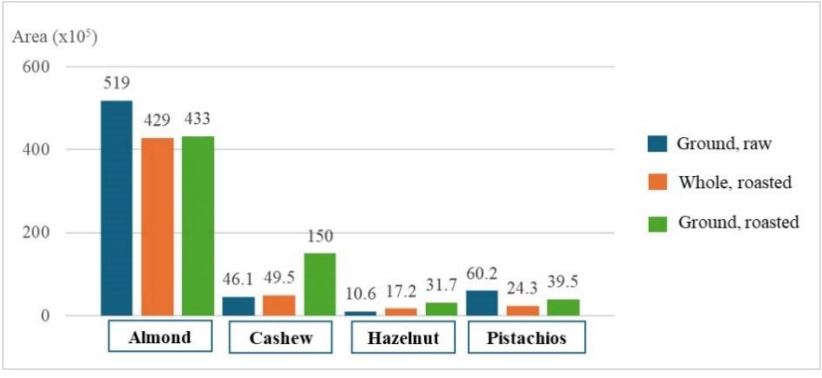

Figure 1. *Difference allergen signal between roasted and raw nuts*

The result revealed that the peak area for almonds, cashews, and hazelnuts was larger when subjected to roasted compared to raw samples, with the exception of smaller for pistachios in Figure 1. This observation can be explained by the fact that roasting may have caused the evaporation of some of the biological liquid/humidity in the samples, leading to an increased protein concentration for almonds, cashews, and hazelnuts. On the other hand, the smaller peak area for pistachios could be due to the thermal degradation of proteins in the nuts.

3.1.2. Extraction buffer

Prepare the reference sample of each tree-nut for optimizing the protein extraction conditions using different extraction buffers:

Extraction Buffer 1: Extract protein using 100 mM TRIS-HCl pH 8.2.

Extraction Buffer 2: Extract protein using 100 mM TRIS-HCl pH 8.2 with 4 M urea and 0.1 M DTT.

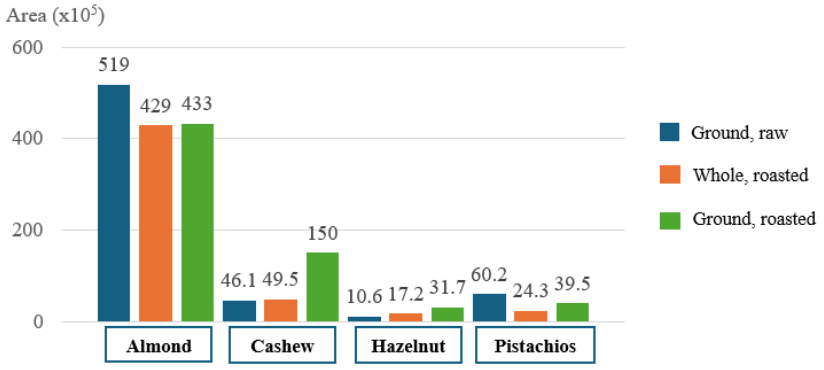

Figure 2. *Effect of protein extraction buffer*

The signal for allergen analysis with extraction buffer 2 is higher than with extraction buffer 1 in Figure 2. Urea can break hydrogen bonds and hydrophobic interactions between amino acids, disrupting the tertiary and quaternary structures of proteins. This facilitates better solubility of proteins in the solution, improving extraction efficiency. Additionally, the presence of DTT, a strong reducing agent, can break disulfide bonds (–S–S–) between cysteine residues in proteins. Disulfide bonds often stabilize tertiary and quaternary protein structures or link proteins together. By reducing these bonds, DTT enhances protein flexibility and solubility. Therefore, TRIS-HCl 100 mM pH 8.2 with 4 M urea and 0.1 mM DTT was selected for the analytical method.

3.1.3. Acetonitrile ratio for solid phase extraction elution

The multi-purpose flour was used as a blank to spike protein extraction solution at a concentration of 100 mg/g protein of each tree nut. At the final stage of the solid-phase extraction process, ACN solutions at concentrations of 90%, 95%, and 100% were used for the elution. Protein needs water to dissolve effectively. A certain ratio of water in the solution helps maintain hydrophilic interactions on the protein's surface and prevents precipitation or denaturation. The optimized results showed that 95% ACN used as the elution solution yielded the highest peak area for the analyte in Figure 3. Thus, 95% ACN was selected for the elution step in the solid-phase extraction process of the method.

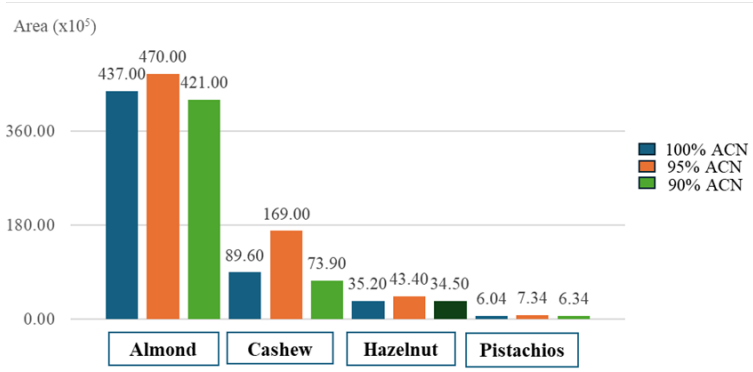

Figure 3. *Results of the ACN concentration survey for elution*

3.2. Method validation

3.2.1. Specificity

The specificity of the method was evaluated based on the following criteria:

IP Score: Each allergen has one parent ion and two product ions, resulting in an IP score of 5, which meets the requirements for mass spectrometry analysis (EC 2021/808) [10].

Prepare spiked sample: blank sample was defatted by n–hexane following by spike amount of protein extraction of each tree–nut.

Analysis of blank sample, reference sample, and spiked sample: The chromatographic results assessing the specificity show that no signal from the analyte was detected in the blank sample, and the spiked blank and reference samples exhibited signals with retention times differing by no more than 2%. Thus, the method meets the specificity validation criteria for analysis (according to AOAC). The chromatograph is shown in Figure 4.

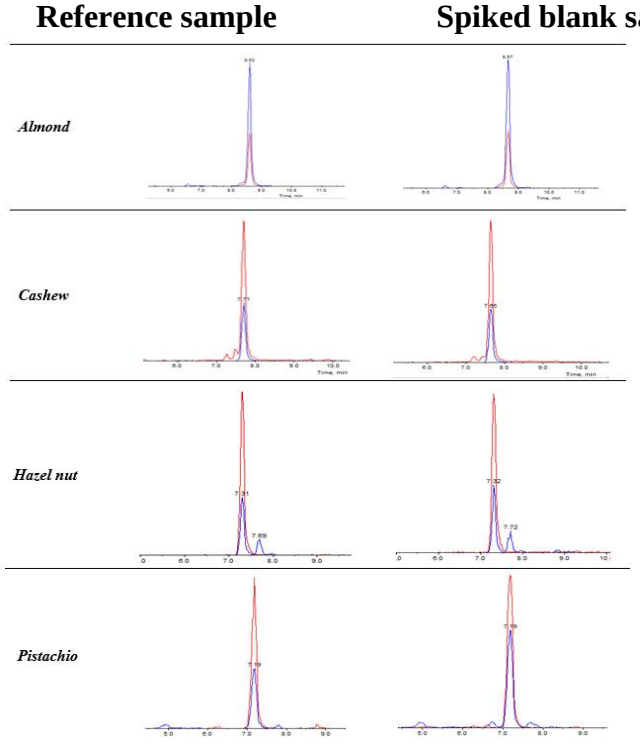

Figure 4*. Chromatograms of each allergen in the reference solution and spiked blank sample*

3.2.2. Limit of detection (LOD) and limit of quantification (LOQ)

Analyzing spiked blank samples for each allergen at the concentration where the signal-to-noise ratio (S/N) is closest to 10 for detecting the analyte was performed, with six replicates for each analysis to ensure the reliability of the LOQ value. The LOD was extrapolated from the LOQ using the formula (LOD = LOQ/3.33). In this study, the LOD was determined as the limit for detecting food allergens (almonds, cashews, hazelnuts, and pistachios) in food samples. The results are summarized in Table 5 and Figure 5.

Table 5. *Limit of detection (LOD) and limit of quantification (LOQ)*

No.	*Allergen*	*LOQ (µg/g)*	*LOD (µg/g)*
1	Almond	30.5	9
2	Cashew	102	30.6
3	Hazel nut	128	38.4
4	Pistachio	122	36.6

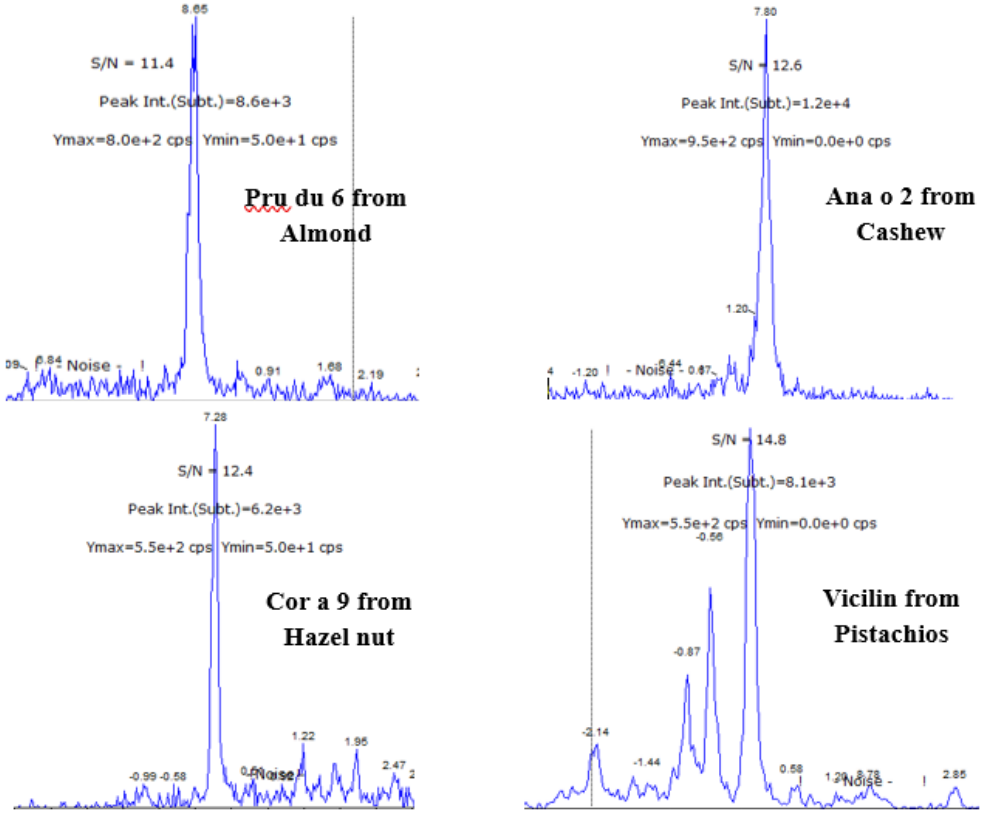

Figure 5. *Chromatograms of allergens at LOD*

3.2.3. Linear range

Based on the optimized conditions, the calibration curve for the four analytes was constructed using LC-MS/MS at six concentration levels (mg/kg): Almond (30.5; 152.5; 305, 610; 1220); Cashew (102; 510; 1020; 2040; 4080); Hazelnut (128; 640; 1280; 2560; 5120); Pistachios (122; 610; 1220; 2440; 4880). The calibration curve was developed by plotting the peak area against the corresponding concentration in Table 6.

Table 6. *Linear Curve and Correlation Coefficient for Analytes in Solvent*

Allergen	***Linear range (ppm)***	***Linear curve***	***Correlation Coefficient (R)***	***Bias (%)***
Almond	30.5 – 1220	y = 7160x + 86500	0.9990	-7.3 – 4.0
Cashew	102 – 4080	y = 341x -1570	0.9997	-1.6 – 5.0
Hazelnut	128 – 5120	y = 309x + 3570	0.9985	-9.1 – 4.0
Pistachio	122 – 4880	y = 593x - 3810	0.9991	-5.1 – 6.0

The results given in Table 6 show that the standard curve equations of the four analytes all have correlation coefficients R > 0.995. Therefore, in the investigated concentration range, there is a linear dependence between peak area and corresponding concentration. The bias at all concentration points does not exceed 15%, and the bias at the standard point with a value equal to LOQ does not exceed 20%. Therefore, the method meets the linearity requirements according to AOAC.

3.2.4. Repeatability and recovery

Perform repeatability analysis 6 times at each concentration level during the analysis. Prepare samples at 4 concentration levels (mg/kg): Almond (30.5; 152.5; 305, 610); Cashew (102; 510; 1020; 2040); Hazelnut (128; 640; 1280; 2560); Pistachios (122; 610; 1220; 2440).

Table 7. *Repeatability and Recovery of each allergen on a blank matrix*

Allergen	***Recovery (R) %***	***Repeatability (RSD) %***
Almond	77.3 - 117	10.06 – 14.46
Cashew	81.6 - 118	10.77 – 14.38
Hazelnut	80.1 - 120	7.31 – 13.54
Pistachio	80.1- 117	11.6 – 15.05

According to the Performance Criteria for Food Allergen Detection and Quantification Method Standard (SMPR 2016.002) by AOAC, with concentrations ranging from 10-1000 ppm, recovery values for analysis should be between 60-120% and relative standard deviations should not exceed 20%. The results show that the recovery values for all 4 analytes are within the range of 77.3% – 120%, and the relative standard deviations range from 7.31% – 15.05%, the result showed in Table 7.

These results indicate that the method's accuracy meets the performance criteria set by the AOAC Standard Method Performance Requirements for Food Allergen Detection and Quantification (SMPR 2016.002) [12].

3.3. Sample analysis application

The method has been applied to determine the allergen content of almonds, cashews, hazelnuts, and pistachios in 10 randomly collected cookie samples from the Hanoi market. The results are presented in the Table 8.

From the results obtained in Table 8, among the ten candy samples analyzed, two samples that did not declare their ingredients on the label were found to contain almond allergen (concentration 43.5 µg/g and 34.4 µg/g), and one biscuit sample that did not declare

its ingredients on the label was found to contain hazelnut allergen (concentration below the quantification limit).

Table 8. *Results of Allergen Analysis in Real Samples*

Sample	***Food Matrix***	***Ingredient Declaration***	***Amount (µg/g)***			
			Almond	*Cashew*	*Hazelnut*	*Pistachios*
B1	Cookie	Cashew	ND	1690	ND	ND
B2	Cookie	Pistachio	ND	ND	ND	ND
B3	Cookie	Non-declaration	43.5	ND	ND	ND
B4	Cookie	Almond, Cashew	917	201	ND	ND
B5	Cookie	Cashew	ND	438	<LOQ	ND
B6	Cookie	Non-declaration	34.4	ND	ND	ND
B7	Cookie	Non-declaration	ND	ND	ND	ND
L1	Dry provisions	Cashew	ND	1090	ND	ND
B7	Cookie	Non-declaration	ND	ND	ND	ND
B8	Cookie	Non-declaration	ND	ND	ND	ND
B9	Cookie	Non-declaration	ND	ND	ND	ND

Note: Not detected (ND)

4. CONCLUSION

The study developed and validated a method for simultaneous analysis of four allergens (almond, cashew, hazelnut, pistachio) in food using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method utilized a reverse-phase XSelect HSS C18 SB column (100mm x 2.1 mm, 3.5 µm) and a corresponding guard column, operating with a gradient mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in ACN over 20 minutes at a flow rate of 0.5 mL/min, with an injection volume of 20 µL. MS/MS analysis was conducted on a triple quadrupole instrument, fragmenting proteins with an ESI (+) source and monitoring reactions in MRM mode, selecting 1 parent ion and 2 daughter ions specific to each protein. The method was validated, meeting AOAC requirements (quantification limits ranging from 30.5 to 128 µg/g depending on the allergen; recovery values between 77.3 - 120%, and relative standard deviation from 7.31 – 15.05%). Protein extraction was carried out using a protein extraction buffer. Analysis of ten cookie samples from the market revealed that three samples contained undeclared allergens. However, the quantifying method uses the tree- nuts obtained in local market and considered as the referenced material, this can lead to differences because the protein content in nuts can change based on geography or the time of harvest. Therefore, the certified reference materials for these allergens need to be unified for further research in Vietnam as well as in the world.

ACKNOWLEDGEMENT

The authors are grateful for the support from the National Institute for Food Control in facilities and conditions for the team to conduct this research.

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