arastirma.tarimorman.gov.tr/bursagida
ISSN 1303-3107
Gıda ve Yem Bilimi - Teknolojisi Dergisi / Journal of Food and Feed Science - Technology 28:74-85 (2022/2) e-ISSN 2822-2873

Original Article/Özgün Araştırma

Determination of allergens in several food matrix with proteomics approach and investigation of heat stability of allergen proteins

Bazı gıdalarda alerjenlerin proteomiks tekniği kullanılarak tespiti ve ısıl işlem sonrası alerjenlerin stabilitesinin araştırılması

Nurcan Ayşar Güzelsoy[1*], Filiz Çavuş[1], Yasemin Şahan[2]

[1]*Central Research Institute of Food and Feed Control, BURSA, TÜRKİYE*
[2]*Department of Food Engineering, Faculty of Agriculture Bursa Uludağ University, BURSA, TÜRKİYE*
(Yazar sıralamasına göre)
***ORCID ID:** 0000-0002-6843-6076, Ph.D.*
***ORCID ID:** 0000-0002-8742-1857, Chemical Eng., M.Sc.*
***ORCID ID:** 0000-0003-3457-251X, Prof.*
**Corresponding author/Sorumlu yazar: nurcan.aysarguzelsoy@tarimorman.gov.tr*

Geliş Tarihi : 10.02.2022
Kabul Tarihi : 12.04.2022

Abstract

Objective: Allergic reactions to food are among the major food safety concerns in especially industrialized countries. The primary treatment for food allergy is avoidance of the allergen food. Investigation of allergens in food products is important in terms of ensuring food safety and protecting consumer health. In this study, it was aimed to determine the allergenic proteins in several food matrices using the proteomics technique and to investigate their stability after heat treatment.

Materials and methods: Hazelnut, almond, walnut, pistachio and sesame were used as material. After protein extraction and enzymatic digestion, unroasted samples were analyzed by liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF/MS). Firstly, specific allergen peptides were determined in unroasted samples with the help of MS/MS data and databases. After roasting of samples at 130°C, 150°C and 170°C up to 30 minutes, stability of allergen peptides was investigated.

Discussion and conclusion: After roasting at different time intervals and temperatures, the mass spectrum of the samples were examined in the database and it was observed that some peptide sequences lost their stability, while others continued to exist. Two peptide sequences were determined as markers for hazelnut, almond, walnut, pistachio and sesame, which maintain their stability after roasting.

Keywords: food allergy; allergens; LC-Q-TOF/MS; proteomics

Öz

Amaç: Gıdaya karşı gelişen alerjik reaksiyonlar özellikle gelişmiş ülkelerde gıda güvenliği açısından önemli bir sorun teşkil etmektedir. Günümüzde alerjinin önlenmesinin tek yolu kişilerin alerjen içeren gıdaları tüketmekten kaçınmasıdır. Gıdaların alerjen maddeler yönünden incelenmesi ürün güvenilirliğinin sağlanması ve tüketici sağlığının korunması açısından önemlidir. Bu çalışmada bazı gıdalardaki alerjen proteinlere ait peptit dizilimlerinin proteomiks tekniği kullanılarak tanımlanması ve ısıl işlem sonrası stabilitelerinin araştırılması amaçlanmıştır.

Materyal ve yöntem: Materyal olarak badem, fındık, ceviz, Antep fıstığı ve susam kullanılmıştır. Kavrulmamış örnekler, protein ekstraksiyonu ve enzimatik parçalanma işlemleri sonrası sıvı kromatografisi kuadropol uçuş zamanlı kütle spektrometresi (LC-Q-TOF/MS) ile analiz edilmiştir. İlk olarak kavrulmamış örneklere ait kütle spektrum (MS/MS) verileri, veri tabanlarında incelenerek her örnek için spesifik olan alerjen peptit dizilimleri belirlenmiştir. Daha sonra örneklere 130°C, 150°C ve 170°C'de 30 dakikaya kadar kavurma işlemi uygulanarak peptit dizilimlerinin kararlılığı incelenmiştir.

Tartışma ve sonuç: Farklı süre ve sıcaklıklarda kavurma işlemi sonrası örneklere ait kütle spektrumları veri tabanında incelenmiş ve bazı peptit dizilimlerinin varlığını devam ettirirken bazılarının ise stabilitelerini kaybettiği gözlenmiştir. Fındık, badem, ceviz, Antep fıstığı ve susam için kavurma işlemi sonrası stabilitesini devam ettiren ikişer adet peptit dizilimi marker olarak belirlenmiştir.

Anahtar kelimeler: gıda alerjisi; alerjenler; LC-Q-TOF/MS; proteomiks

1. Introduction

Food allergy is an immunological reaction developed against a protein or a different component in some foods and poses a global risk to public health (Costa et al., 2016). Symptoms of an allergic reaction may involve the skin, the gastrointestinal tract, the cardiovascular system, and the respiratory tract (Johansson et al., 2001; Köksel et al., 2011). 5% of children and 3-4% of adults in Western countries are estimated to be affected by food allergy (Meyer et al., 2019; Sicherer and Sampson, 2010). Although it is known that about 200 foods cause allergic reactions; milk, eggs, peanuts, nuts, shellfish, fish, wheat, and soy account for 90% of allergic reactions (Liew et al., 2009). Most of the allergens found in these foods are water-soluble glycoproteins in sizes of 10 to 70 kDa and have stability against heat, acid, and proteases (Jiménez-Saiz et al., 2015). Plant food allergens can be classified based on their structural or functional properties. The most common plant proteins that cause allergic reactions are prolamins, globulins and protein groups involved in the plant defense mechanism (Breiteneder et al., 2004).

Currently there are no proactive treatments available for food allergy beyond the careful avoidance of allergenic foods, and even the consumption of very small amounts of these allergenic foods can be life-threatening for some people. The best way to manage to prevent an allergic reaction is to avoid consuming the food that cause allergy. Therefore, issuing legal regulations regarding the declaration of allergens in foods is important. EC/ 1169/2011 directive of the European Union involves provisions regarding the declaration of allergens on food labels (Anonymous, 2017). In Türkiye, on the other hand, the regulations for the declaration of allergens are included in the Turkish Food Codex Regulation on Labelling and Food Information to Consumers (Anonymous, 2017). Allergens that are mandatory to be declared on the labels of foods are gathered under 14 groups and allergen components or allergen processing aids are required to be specified in the ingredients list by using the allergen substance or product names clearly. Although there are legal regulations, undeclared allergens may also be detected in some food products as a result of inadequate equipment sanitation, cross-contamination or mislabeling (Parker et al., 2015). It is important to identify the allergens present in foods in order to secure the health of individuals with food allergies and to ensure that safe foods are delivered to consumers.

Enzyme-linked immunosorbent assay (ELISA) tests and polymerase chain reaction (PCR) analyzes are among the most preferred methods for the detection of allergens in foods, but these methods have some constraints as well (Prado et al., 2016). Complexity of the food matrix, partial degradation of proteins by microorganisms during fermentation, alterations in the structure of proteins caused by reactions occurring during food processing, such as Maillard reactions, difficulties in isolating DNA from some foods can lead to obtaining false positive or false negative results in allergen analysis by ELISA and PCR (Jayasena et al., 2015; Sancho and Mills, 2010).

In recent years, as an alternative to those techniques, analysis methods based on mass spectrometry have been used extensively in the identification, characterization and quantitation of food allergens. Offering high sensitivity and accuracy, the capability to perform analyzes without being affected by many limitations arising from the structure of proteins, and to perform multiple analyzes of allergens in a single analysis mode are among the reasons for preferring MS technique despite requiring high costs and expertise (Carrera et al., 2018; Dhondalay et al., 2018).

Since MS-based allergen analysis methods rely on tracing selected peptides as markers of allergenic components found in foods, the selection of these markers constitutes one of the most important step in method development and affects the robustness and sensitivity of analytical data (Pilolli et al., 2021). In addition to being specific to the relevant allergen, these marker peptides should be stable during food processing and preferably free from amino acids prone to alterations. However, foods may be exposed to different processes such as heat treatment, fermentation, hydrolysis, ultrasonic applications and irradiation during the production process and some changes may occur in the

allergenicity of foods depending on these applied processing conditions (Onwude et al., 2017; Pushpa et al., 2018; Yao et al., 2015).

Heat treatment applications are widely used in food production and affect allergenicity by causing structural alterations in the protein structure. Although there are some studies on evaluating the stability of allergens after heat treatment for different food matrices; it still constitutes a field of study for researchers, as its effect on allergen proteins will vary depending on the complexity of the food matrix and heat treatment conditions (Chassaigne et al., 2007; Van Boxtel et al., 2008).

In this study, it was aimed to determine the peptide sequences of allergen proteins in hazelnut, almond, walnut, pistachio, and sesame using LC-QTOF/MS and to investigate their stability subsequent to heat treatment.

2. Material and method

2.1. Material

In the study almond (*Prunus dulcis* L.), hazelnut ((*Corylus avellana* L.), walnut (*Juglans regia* L.), pistachio (*Pistacia vera* L.) and sesame (*Sesamum indicum* L.) were used as materials. Hazelnut samples were procured from Hazelnut Research Institute in Giresun, and almond samples were procured from Pistachio Research Institute in Gaziantep. Walnut, pistachio, and sesame samples were procured from the market. The studies on the detection of allergens in the samples were first performed on unroasted samples. In order to investigate the heat stability of the peptide sequences, the samples were roasted at different temperatures (130ºC, 150ºC, 170ºC) and times (15-30 minutes).

2.2. Chemicals

Acetonitrile (99.9%), methanol (99.9%), ammonium bicarbonate (Bioultra, 99.5%), dibasic sodium phosphate (99%), Tris (hydroxymethyl) aminomethane (TRIS) (99.9%), phosphoric acid (Bioultra, 85%), bovine serum albumin (analytical purity), dithiothreitol (DTT) (98%), iodoacetamide (IAA) (Bioultra), trypsin enzyme (bovine pancreas), and Bradford marker (0.1-1.4 mg/ml protein) were purchased from Sigma Aldrich (St. Louis, USA). hydrochloric acid (HCl; 37%) and formic acid (98-100%) were purchased from Merck (Darmstadt, Germany). Ultra-pure water was obtained using the Milli-Q System (USA).

2.3. Protein extraction

Protein analysis of the samples was performed in accordance with the method of AOAC 990.03 (Anonymous, 2002). In order to determine the ideal extraction solvent for protein extraction in the samples, preliminary experiments were carried out using 200 mM (pH 7.5) TRIS/HCl, 50 mM ammonium carbonate, 50 mM dibasic sodium phosphate and 0.01 M HCl buffers. After homogenization procedure, 3 mL of extraction solution was added to the samples weighing 90 mg and mixed in vortex for 60 min. After centrifugation at 1699 xg for 5 min, the extracts were prepared by filtering through 0.22 µm PVDF (Millipore Millex-HV, Merck). The amount of protein in the extracts was determined according to the Bradford method (Kruger, 2009).

2.4. Optimization of incubation time

In order to obtain peptide from proteins, trypsin enzyme was added to extracts and incubated at 37°C for 2-24 h. The tryptic digests were analyzed by LC-QTOF/MS and the peak areas of peptides at different times were compared to determine the ideal incubation for peptide analysis.

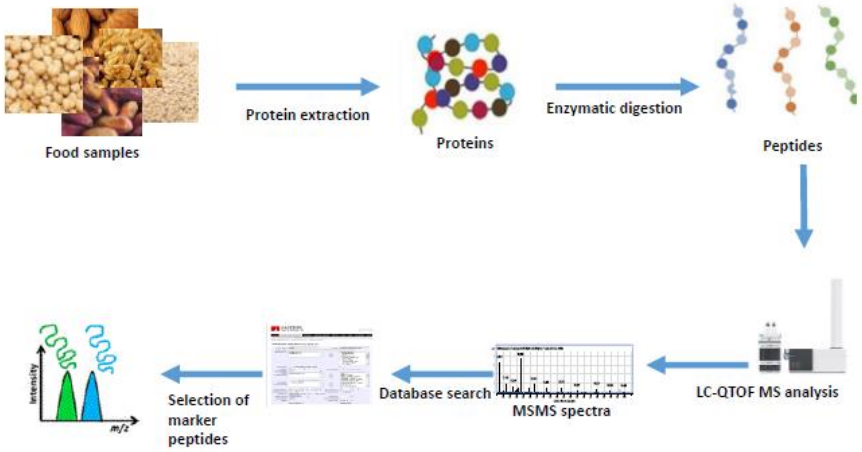

Figure 1. Workflow for selection of marker peptides

2.5. Sample preparation

Sample preparation was carried out using the procedure described by Sealey-Voyksner et al. (2016) with slight modifications. Briefly, an amount of 90 mg of matrix material was extracted with 3 mL extraction buffer (200 mM TRIS/HCl, pH 7.5), mixed in vortex at 20°C for 60 min. Subsequently, DTT solution was added and held at 37ºC for 20 min to facilitate the breaking of disulfide bonds. After the sample cooled down to room temperature, alkylation was carried out with IAA. Then 25µL of a 1 mg/mL trypsin solution prepared in 50 mM sodium phosphate was added for enzymatic digestion and the samples were incubated at 37°C for 18 h. The digested extracts were centrifuged at 1699xg for 5 min and analyzed in the LC-QTOF/MS equipment

2.6. LC-QTOF/MS analysis

LC-MS analysis of tryptic digests were performed using an Agilent HPLC-1260 (USA) system and an Agilent 6550 high-definition Q-TOF mass spectrometer. Shematic diagram of the workflow was given in Figure 1. Tryptic digests were injected on a Poroshell 120 EC reverse phase C18 analytical column (4.6 × 100 mm, particle size 2.7 µm) (Agilent Technologies, USA). The sample injection volume was 5 µL, at a final flow rate of 0.6 mL/min. Ultra-pure water (0.1% formic acid) and acetonitrile (0.1% formic acid) were used as mobile phase. The applied gradient was as follows: 2 min, 5% B; 2–20 min, 5–60% B; 20–22 min, 60-95% B; 22–23 min, 5% B. The analysis was performed in the positive ionization mode with the mass range setting at m/z 100–2500. The optimized instrument conditions were set as follows; drying gas 200°C at 14 L/min; nebulisation gas 30 psi; sheath gas 350°C at 11 L/min; capillary voltage of 3500 V; nozzle 1000 V; fragmentor 300 V. The reference masses 121.0509 (Purine) and 922.0098 (HP-0921) were used for internal mass calibration. Each sample was analysed in triplicate.

3. Discussion

3.1. Extraction of samples

Extraction solvents including 200 mM TRIS/HCl, 50 mM ammonium carbonate, 50 mM dibasic sodium phosphate and 0.01 M HCl buffer were evaluated for the extraction of proteins in hazelnut, almond, pistachio, sesame and walnut samples. The extraction yield of the different solvent was assessed. Protein recovery was calculated by comparing the experimental protein concentrations (mg/mL) assayed in sample extracts by Bradford assay with total protein content of samples assayed by AOAC 990.03. The extraction yields in percentage obtained for each solvent are given in Table 1. The highest extraction yield in the samples was obtained through 200 mM TRIS/HCl (pH 7.5), while the lowest extraction yield was obtained through 0.01 M HCl buffer.

There are studies conducted on the extraction yield of proteins in foods by applying various extraction solvents at different pH ranges. In a study conducted by Sze-Tao and Sathe (2000), the highest amount of protein in walnut extracts was determined by applying 0.1M NaOH solution, and it was declared that the solubility was minimum when the pH was around 4, on the other hand, the solubility increased when pH < 3 and pH > 6. De Angelis et al. (2018) performed extraction experiments with phosphate buffered saline (PBS), TRIS/HCl, urea-TBS (Tris buffered saline) and ammonium bicarbonate solvents for protein extraction in peanuts and hazelnuts. As a result of their study, the highest extraction yield (53%) in the samples was obtained with TRIS/HCl, while the lowest extraction yield (37%) was obtained by applying phosphate buffered saline. Furthermore, in the studies conducted on the extraction of proteins in nuts by Sealey-Voyksner et al. (2016) and Calinoiu et al. (2013); the highest extraction yield was obtained by applying TRIS/HCl (pH 7.5) solution in parallel with the results of our study.

Table 1. Comparison of extraction yields for samples provided by different buffer compositions

	50 mM Ammonium carbonate	0,01 M HCl (pH 2.5–3)	50 mM Dibasic sodium phosphate (pH 8)	200 mM TRIS/HCl (pH 7.5)
Hazelnut	59.19±1.54 ^{b}	15.68±0.95 ^{d}	49.49±1.57 ^{c}	71.77±1.67 ^{a}
Almond	61.84±1.57 ^{b}	14.78±1.35 ^{d}	47.77±1.39 ^{c}	72.19±0.84 ^{a}
Pistachio	72.32±2.11 ^{b}	13.14±1.55 ^{d}	63.54±1.93 ^{c}	79.28±0.86 ^{a}
Sesame	62.59±1.52 ^{b}	14.57±1.27 ^{d}	55.47±0.76 ^{c}	67.81±1.66 ^{a}
Walnut	53.22±2.71 ^{b}	12.46±1.22 ^{d}	45.53±1.04 ^{c}	62.08±1.52 ^{a}

Values are expressed as the mean ± standard deviation of three replicates. Different letters in the same row indicate a statistically significant difference at p<0.05.

3.2. Optimization of trypsin digestion

Digestion of allergenic proteins to tryptic peptides is required for reproducible LC-MS analysis. In order to determine the ideal incubation time for trypsin digestion, one peptide was selected for hazelnut and almond. Extracts obtained from hazelnut and almond were incubated with trypsin at intervals from 2 to 24 h. Progress of the tryptic digestion was determined via LC-QTOF/MS and peptide peak area was used as a measure of digestion efficiency and compared for different incubation time. Examining the data on the peak areas of the peptides from prunin and Cor a 9 allergens, the optimum time for enzymatic digestion was determined as 18 h to achive the best sensitivity and formation of target peptides (Figure 2). After 18 h, the peak areas of the peptides started to decrease.

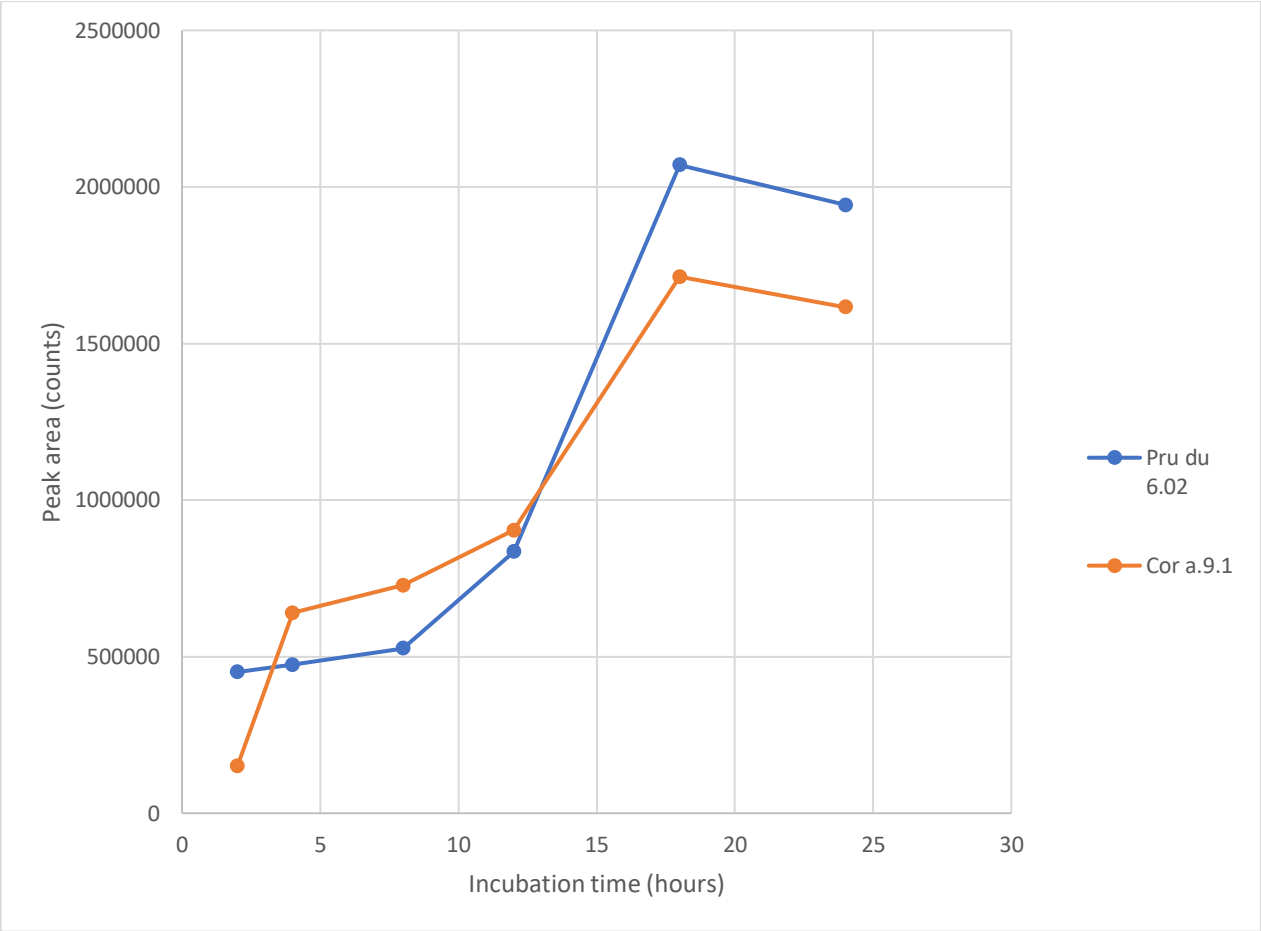

Figure 2. Peak area of peptides belong to almond allergen Pru du 6.02 and hazelnut allergen Cor a 9.1 during incubation.

In a study analyzing egg, milk and peanut allergens in biscuits by LC-MS/MS, protein/trypsin ratio and different incubation times in the range of 2-16 h were investigated. The optimum incubation time for all allergen peptides was determined as 4 h (Boo et al., 2018). Korte and Brockmeyer (2016) identified allergens in different food matrices such as bread and chocolate through the proteomics technique. In order to complete the enzymatic digestion process after protein extraction, they incubated the extracts for 14 h at 37°C, similar to the time applied in our study. In a study conducted by Nitride et al. (2019) investigating the effect of extraction and enzymatic digestion processes on the quantitative determination of egg and milk allergens by mass spectrometry, it was revealed that the peptide density increased after 16 h in the enzymatic digestion of lysozyme and ovalbumin, and after 4 h in the enzymatic digestion of casein.

3.3. Determination of allergen peptide sequences in samples

Tryptic digests were analysed using LC-QTOF/MS to obtain peak lists from acquired MS/MS data and MASCOT database search tool was used to determine characteristic marker peptides for each sample. Firstly; MS and MS/MS spectra of tryptic digests were obtained. The total ion chromatogram and MS/MS spectra of hazelnut allergen are shown in Figure 3 and Figure 4, respectively.

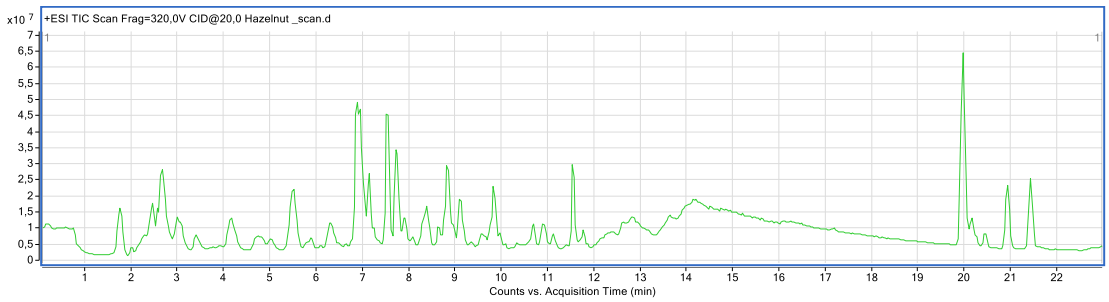

Figure 3. Total ion chromatogram of hazelnut sample

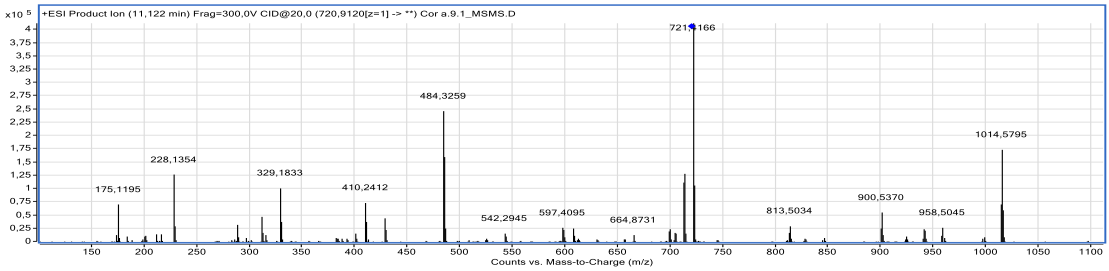

Figure 4. MS/MS spectra of hazelnut allergen Cor a 9

Raw mass spectra were converted to Mascot Generic Format (.mgf) and searched using MASCOT software (Matrix Science, Boston, MA, USA) against the NCBI-Prot/Swiss-Prot database. While making identification in MASCOT software, trypsin enzyme was chosen as the constant modification to ensure consistency among databases and to obtain ease of comparison. In the software, the section of taxonomy was selected as green plants. 1.2 Da peptide mass tolerance, 0.5 Da MS/MS tolerance, carboxymethyl as fixed modification, and 2+, 3+ and 4+ peptide charge states were also selected. For determining the allergen peptide sequences that are markers, particular attention was paid to the selection of peptide sequences that best match the proteins in the SwissProt/NCBIprot libraries. In addition, peptide sequences with less than 6 amino acids were not selected as markers, as their specificity was accepted low. Similarly, peptide sequences with more than 25 amino acids were also eliminated due to their large mass and complications in identifying cleavage ions. The list of potential marker peptides for hazelnut, almond, pistachio, sesame, and walnut allergens is given in Table 2.

Table 2. List of the peptides discovered in the samples

Samples (Latin name)	Sequences	Accurate mass (m/z)	Charge state	Retention time	Allergen protein name
Hazelnut (*Corylus avellana* L.)	INTVNSNTLPVLR	720.9121	+ 2	11.283	Cor a 9
	ALPDDVLANAFQISR	815.4344	+ 2	9.567	Cor a 9
	VQVLENFTK	539.3021	+ 2	4.213	Cor a 11
	VQVVDDNGNTVFDDELR	967.9582	+ 2	12.127	Cor a 9
	AFSWEVLEAALK	682.7123	+ 2	13.716	Cor a 11
Almond (*Prunus dulcis* L.)	ALPDEVLQNAFR	686.8626	+ 2	13.524	Prunin
	TDENGFTNTLAGR	698.3254	+ 2	9.688	Prunin
	TEENAFINTLAGR	718.3596	+ 2	6.569	Prunin
	VQVVNENGDPILNDEVR	955.4829	+ 2	10.579	Prunin
	QEGGQGQQQFQGEDQLDR	1024.4575	+ 2	8.517	Prunin
Pistachio (*Pistacia vera* L.)	LVLVALADVGNSENQLDQYLR	1165.6205	+ 2	14.107	Pis v 2.0201
	MQIVSENGESVFDEEIR	991.4637	+ 2	11.633	Pis v 2.0201
	IQIVSENGESVFDEEIR	982.4853	+ 2	13.524	Pis v 2.0201
	FVLGGSPQQEIQGSGQSR	937.9731	+ 2	9.215	Pis v 2.0201
Sesame (*Sesamum indicum* L.)	ISTINSQTLPILSQLR	892.5181	+ 2	13.325	Ses i 6
	SPLAGYTSVIR	582.3124	+ 2	9.408	Ses i 6
	IPYVFEDQHFITGFR	623.6494	+ 2	14.512	Ses i 3
	IQSEGGTTELWDER	810.8792	+ 2	13.842	Ses i 6
Walnut (*Juglans regia* L.)	DLPNECGISSQR	659.8064	+ 2	10.395	Jug r 1
	ATLTLVSQETR	609.8381	+ 2	8.842	Jug r 2
	LLGFGINGENNQR	716.3722	+ 2	12.962	Jug r 2
	GEEMEEMVQSAR	698.2989	+ 2	7.867	Jug r 1

In line with our study Ansari et al. (2012), identified marker peptides in hazelnut samples by LC–MS/MS after extraction and enzymatic digestion with trypsin. ALPDDVLANAFQISR and INTVNSNTLPVLR sequences are belong to the Cor a 9 allergen that are found in both studies. In our study, the VQVLENFTK sequence (Cor a 11.0101) from Cor a 11 allergen of hazelnut was determined as marker, and Ansari et al. (2012) identified the peptide sequences of AFSWEVLEAALK and LLSGIENFR of the Cor a 11 allergen as markers. It is believed that the difference in the marker sequences determined in the hazelnut samples may be due to the differences in the cultivars, extraction solvents and equipments used in the studies. Korte et al. (2016) determined marker peptides for almond and hazelnut allergens in chocolate, ice cream and bread matrices using proteomics technique.

In parallel with our results, INTVNSNTLPVLR and VQVVDDNGNTVFDDELR peptide sequences in hazelnut, and ALPDEVLQNAFR and TDENGFTNTLAGR peptide sequences in almonds are found as markers. The peptide IQSEGGTTELWDER was identified also by other researchers as significant peptide markers for sesame (Ma et al., 2000).

3.4. Investigating the heat treatment stability of allergen peptide sequences

Following the determination of the potential marker peptides in the unroasted samples, the heat stability of selected peptides was investigated by applying roasting process at different temperatures and time intervals. Peptide sequences that maintain their stability after roasting process were identified as marker peptide in order to detect allergens in food samples.

The effects of different roasting temperatures on the signals of the peptide sequence of INTVNSNTLPVLR, a hazelnut allergen, and VQVVVNENGDPILNDEVR, an almond allergen, can be seen in Figure 5 and Figure 6, respectively. Analyzing the ion chromatograms, it is seen that although the peak areas decrease with temperature, they still exist at 170°C. It is thought that the decrease in the signals results from decline in the protein content of the extracts and the denaturation of the protein structure after roasting process

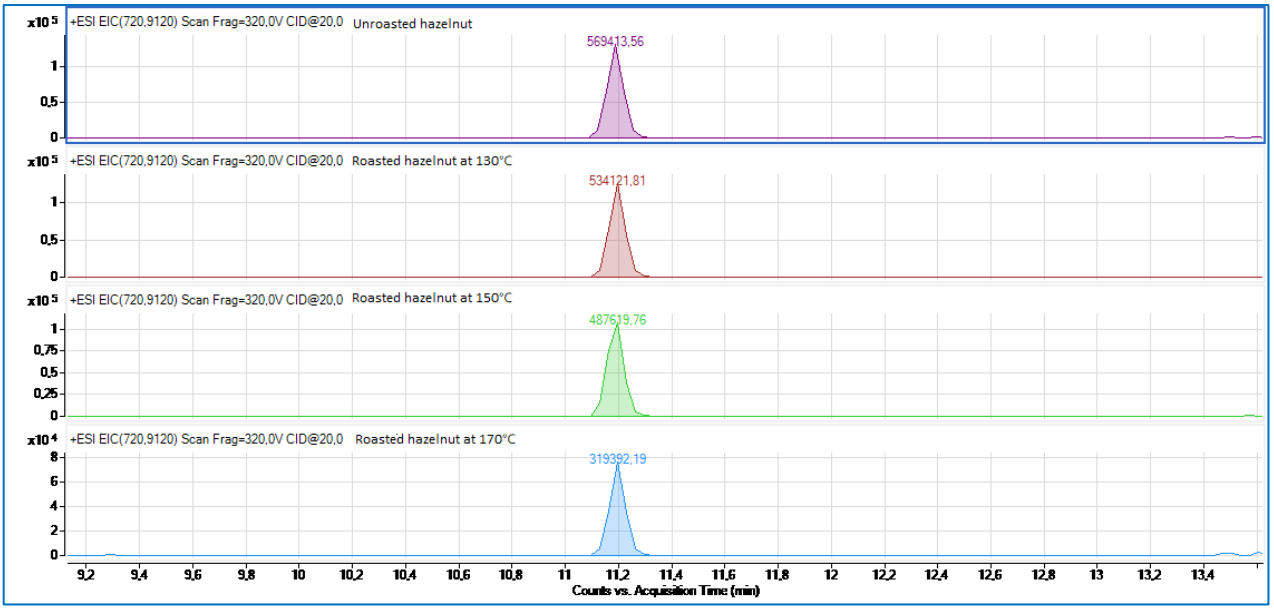

Figure 5. Extracted ion chromatogram of INTVNSNTLPVLR peptide at different roasting temperatures

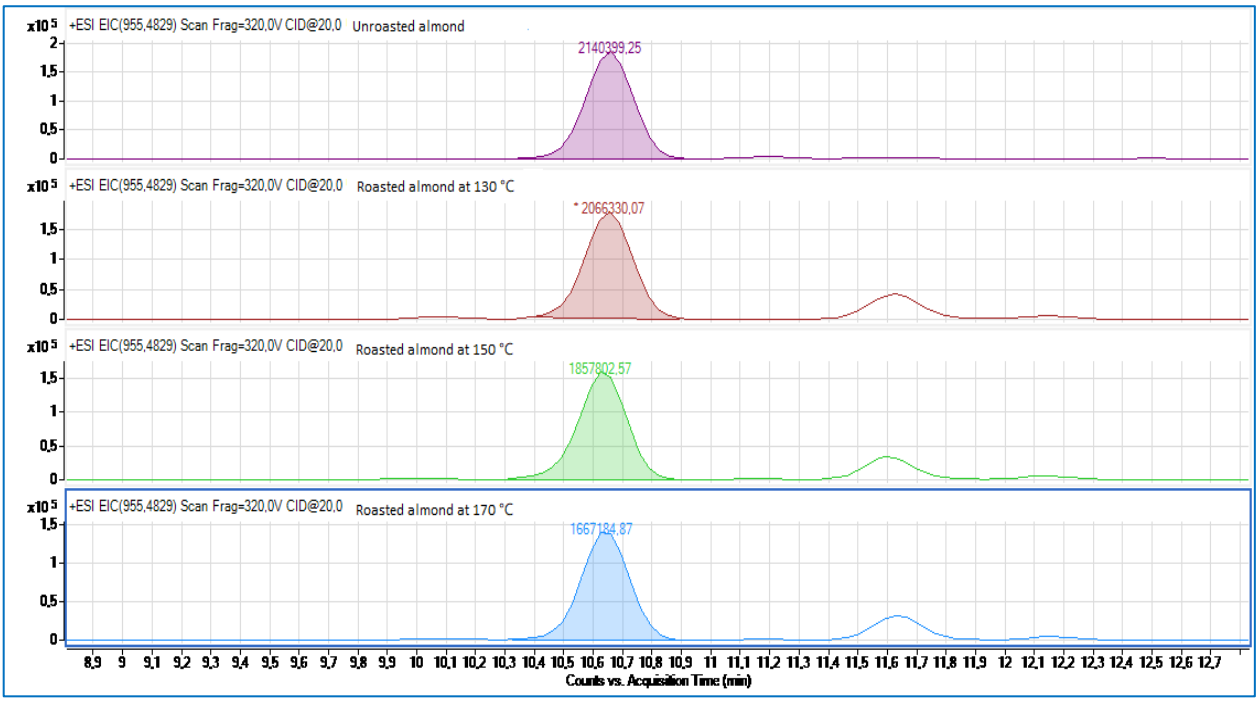

Figure 6. Extracted ion chromatogram of VQVVNENGDPILNDEVR peptide at different roasting temperatures

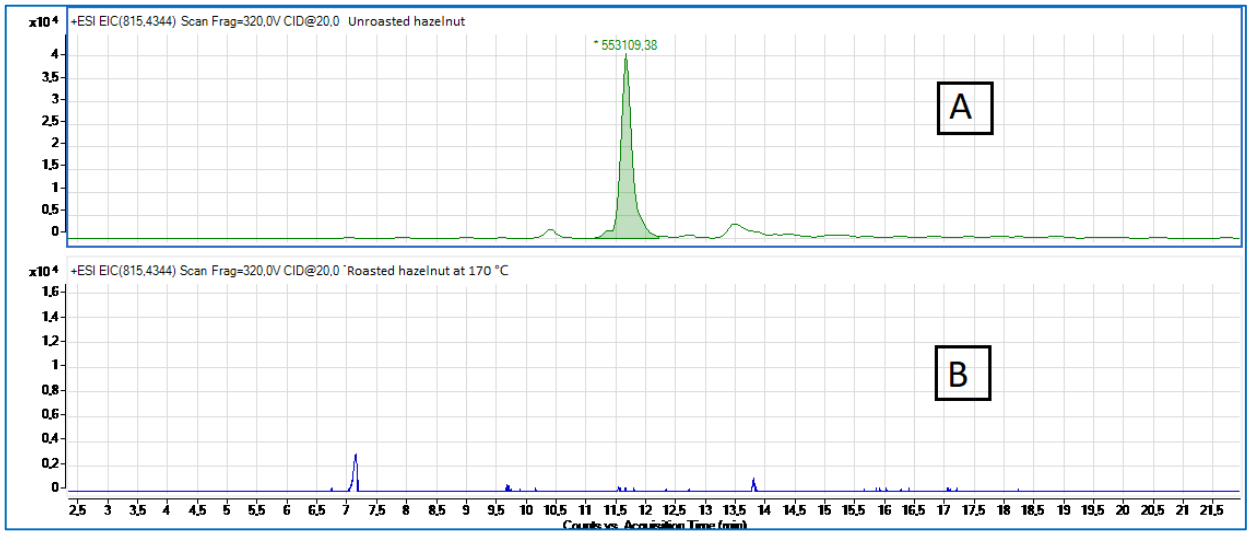

Figure 7. Extracted ion chromatogram of ALPDDVLANAFQISR peptide (A: Unroasted hazelnut, B: Roasted hazelnut at 170°C)

Food processing techniques including fermentation, hydrolysis, heat treatment, high pressure applications and extrusion can cause alterations in the allergic properties of proteins in foods (Cucu et al., 2011; Mills and Mackie 2008; Su et al., 2004; Verhoeckx et al., 2015). However, each protein is affected differently by these processes. Factors such as the type of heat treatment applied, temperature, heat treatment time, pH can lead to alterations in the physiochemical properties of allergen proteins. Ambiguities regarding the effects of heat treatment on the physical and chemical properties of proteins complicate establishing methods for the detection of allergen proteins in particular (Parker et al., 2015). Within the scope of study, two marker peptides maintaining their stability and giving higher intensity after roasting process were selected as markers in order to determine the almond, hazelnut, pistachio, walnut and sesame allergens in food samples, and information on marker peptides are shown in Table 3. Other peptides which didn't give reproducible responses and had low intensities after roasting process were removed from the marker peptide list. The MS/MS spectrum of selected peptides is unique and shows no cross reactivity with other samples according to NCBI-Prot/Swiss-Prot database.

Table 3. List of the marker peptides selected for each sample, including the retention time, parent ion mass and product ions mass

Sample	Sequence	Accurate mass (m/z)	Charge state	Retention time	Product ions
Hazelnut (*Corylus avellana* L.)	INTVNSNTLPVLR	720.9121	+ 2	11.283	175.1193 228.1349 484.3248
	VQVVDDNGNTVFDDELR	967.9582	+ 2	12.127	228.1342 327.2020 794.3662
Almond (*Prunus dulcis* L.)	ALPDEVLQNAFR	686.8626	+ 2	13.524	213.0872 342.1299 157.1337
	VQVVNENGDPILNDEVR	955.4829	+ 2	10.579	228.1346 72.0806 327.2020
Pistachio (*Pistacia vera* L.)	LVLVALADVGNSENQLDQYLR	1165.6205	+ 2	14.107	86.0950 213.1578 397.2762
	MQIVSENGESVFDEEIR	991.4637	+ 2	116.33	260.1084 104.0535 373.1922
Sesame (*Sesamum indicum* L.)	ISTINSQTLPILSQLR	892.5181	+ 2	13.325	183.1481 284.1600 413.1473
	SPLAGYTSVIR	582.3124	+ 2	9.408	104.0534 258.0902 428.1689
Walnut (*Juglans regia* L.)	DLPNECGISSQR	659.8064	+ 2	10.395	461.2389 820.3981 270.1504
	GEEMEEMVQSAR	698.2989	+ 2	7.867	155.0791 291.1627 674.0930

There are several studies investigating the effects of heat treatment applications on allergen proteins conducted by different researchers. In the study conducted by Lopez et al. (2012), it was reported that autoclaving at 138°C for 15-30 min caused a decrease in allergen-specific *IgE* levels in hazelnuts and it was concluded that this decrease may be related to the decline in the solubility of the protein. Cabanillas et al. (2014) revealed that pressure and temperature (256 kPa, 138°C) applied to walnuts reduced the binding capacity of IgE for Jug r 4 walnut allergen. On the other hand, in a study carried out on walnuts, Sordet et al. (2009) demonstrated that the allergen Jug r 1 was resistant to heat treatment at 90°C. It was reported that dry roasting had no effect on the IgE binding capacity of pistachio allergenicity depending on temperature, while steam roasting led to a decrease in IgE binding capacity due to temperature-induced protein aggregation (Noorbakhsh et al., 2010). Sealey-Voyksner et al. (2016) stated that the decrease occurring in the signals of allergen peptide sequences in nuts after heat treatment may be due to the alteration in protein structure due to glycosylation. Allergens from lipid transfer proteins or seed storage proteins are more stable to heat treatments. There are present studies indicating that the allergen peptides of Cor a 8, Cor a 9, Cor a 11 in hazelnut maintain their stability after heat treatment in the range of 140-185°C (Dooper et al., 2008; Wigotzki et al., 2000). In almonds, Pru du 1 loses its stability, while Pru du 6, which is an 11S globulin protein, maintains its stability (De leon et al., 2003).

4. Conclusion

The development of reliable methods for the detection of food allergens is very crucial in order to control compliance with the legislation on food labeling and to minimize the risks to allergic consumers. In the study, peptide sequences of allergen proteins in unroasted hazelnut, almond, walnut, pistachio, and sesame were primarily determined. Subsequently, the stability of the peptide sequences against heat treatment was investigated by applying the roasting process at different times and temperatures. Two peptide sequences that maintained stability after roasting process at 170°C for 30 minutes were determined as markers. During the development of allergen detection method, peptides giving higher intensity were preferred. Up to now, various methods have

been developed to detect some food allergens, however, it is still important to develop a rapid method for simultaneous detection of several food allergens in a single analysis. The presented method has a high potential for the further development of additional methods for detection of allergen proteins in complex food matrices.

5. Acknowledgement

This study is a part of a PhD thesis of the first author. The authors would like to thank TAGEM (General Directorate of Agricultural Research and Policies, Türkiye) for financially support this research project and to Dr. Tuğba Er and Ahmet Şahan for assistance with providing hazelnut and almond samples. (Project Number: TAGEM/ HSGYAD/A/18/A3/P1/546).

6. References

Anonymous (2002). AOAC Official Method 990.03. Protein (Crude) in Animal Feed, Combustion Method, Chapter 4, 30-31.

Anonymous (2017). European Union Commission Notice C 428/1 of 13 July 2017. Commission Notice of 13 July 2017 relating to the provision of information on substances or products causing allergies or intolerances as listed in Annex II to Regulation (EU) No 1169/2011 of the European Parliament and of the Council on the provision of [Internet]. Vol. (2017/C 42, Official Journal of the European Union. 2017. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:OJ.C_.2017.428.01.0001.01.ENG. (Erişim tarihi: 08.02.2020).

Anonymous (2017). Türk Gıda Kodeksi Etiketleme ve Tüketicileri Bilgilendirme Yönetmeliği. https://www.resmigazete.gov.tr/eskiler/2017/01/20170126M1-6.htm. (Erişim tarihi: 15.03.2020).

Ansari, P., Stoppacher, N. and Baumgartner, S. (2012). Marker peptide selection for the determination of hazelnut by LC-MS/MS and occurrence in other nuts. *Analytical and Bioanalytical Chemistry*, 402 (8), 2607–2615.

Boo, C.C., Parker, C.H. and Jackson, L.S. (2018). A Targeted LC-MS/MS Method for the Simultaneous Detection and Quantitation of Egg, Milk, and Peanut Allergens in Sugar Cookies. *Journal of AOAC International*, 101(1), 108–117.

Breiteneder, H. and Radauer, C. (2004). A classification of plant food allergens. *Journal of Allergy and Clinical Immunology*, 113(5), 821–831.

Cabanillas B., Maleki S.J., Rodríguez J., Cheng H., Teuber S.S., Wallowitz M.L. and Crespo J.F. (2014). Allergenic properties and differential response of walnut subjected to processing treatments. *Food Chemistry,* 157, 141–147.

Calinoiu, L.F., Vodnar D.C. and Socaciu, C. (2013). The Reactivity and Allergenic Potential of Hazelnut Peptides. Bulletin of University of Agricultural Sciences and Veterinary Medicine. Cluj-Napoca: *Food Science and Technology*, 70(1), 25–32.

Carrera, M., Cañas, B. and Gallardo, J.M. (2018). Advanced proteomics and systems biology applied to study food allergy. *Current Opinion in Food Science*, 22, 9–16.

Chassaigne, H., Nørgaard, J.V. and Hengel, A.J. (2007). Proteomics-based approach to detect and identify major allergens in processed peanuts by capillary LC-Q-TOF (MS/MS). *Journal of Agricultural and Food Chemistry*, 55(11), 4461–4473.

Costa, J., Mafra, I., Carrapatoso, I. and Oliveira, M.B. (2016). Hazelnut Allergens: Molecular Characterization, Detection, and Clinical Relevance. *Critical reviews in food science and nutrition*, *56*(15), 2579–2605. https://doi.org/10.1080/10408398.2013.826173

Cucu, T., Platteau, C., Taverniers, I., Devreese, B., De Loose, M., and De Meulenaer, B. (2011). ELISA detection of hazelnut proteins: effect of protein glycation in the presence or absence of wheat proteins. *Food Additives & Contaminants: Part A*, *28*(1), 1-10.

De Angelis, E., Bavaro, S.L., Monaci, L. and Pilolli, R. (2018). Effects of the Varietal Diversity and the Thermal Treatment on the Protein Profile of Peanuts and Hazelnuts. *Journal of Food Quality*, 1–10.

De leon, M.P., Glaspole, I.N., Drew, A.C., Rolland, J.M., O'hehır, RE. and Suphioglu, C. (2003). Immunological analysis of allergenic cross-reactivity between peanut and tree nuts. *Clinical & Experimental Allergy*, 33, 1273–1280.

Dhondalay, G.K., Rael, E., Acharya, S., Zhang, W., Sampath, V., Galli, S.J., … and Andorf, S. (2018). Food allergy and omics. *Journal of Allergy and Clinical Immunology,*141(1), 20–29.

Dooper, M. B. W., Plassen, C., Holden, L., Moen, L., Namork, E. and Egaas, E. (2008). Antibody binding to hazelnut (*Corylus avellana*) proteins: the effects of extraction procedure and hazelnut

source. *Food & Agricultural Immunology*, 19(3), 229–240.

Jayasena, S., Smits, M., Fiechter, D., de Jong, A., Nordlee, J., Baumert, J., … and Koppelman, S.J. (2015). Comparison of six commercial ELISA kits for their specificity and sensitivity in detecting different major peanut allergens. *Journal of Agricultural and Food Chemistry*, *63*(6), 1849–1855. https://doi.org/10.1021/jf504741t

Jiménez-Saiz, R., Benedé, S., Molina, E. and López-Expósito, I. (2015). Effect of processing technologies on the allergenicity of food products. *Critical Reviews in Food Science and Nutrition*, *55*(13), 1902–1917. https://doi.org/10.1080/10408398.2012.736435

Johansson, S.G., Hourihane, J.O., Bousquet, J., Bruijnzeel-Koomen, C., Dreborg, S., Haahtela, T., and Wüthrich, B., EAACI (the European Academy of Allergology and Cinical Immunology) nomenclature task force. (2001). A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force. *Allergy*, 56(9), 813–824.

Korte, R. and Brockmeyer, J. (2016). MRM3-based LC-MS multi-method for the detection and quantification of nut allergens. *Analytical and Bioanalytical Chemistry*, 408(27), 7845–7855.

Korte, R., Lepski, S. and Brockmeyer, J. (2016). Comprehensive peptide marker identification for the detection of multiple nut allergens using a non-targeted LC–HRMS multi-method. *Analytical and Bioanalytical Chemistry*, 408(12), 3059.

Köksel, H., Köroğlu, D. and Popping B. (2011). Food Allergens and EU Regulations. 7th Food Engineering Congress, 24-26th November 2011, Ankara.

Kruger, N. (2009). The bradford method for protein quantitation, ed: Walker J.M., *Springer Protocols Handbooks*, Humana Press, Totowa, NJ, Pp, 17-24

Liew, W.K., Williamson, E. and Tang, M.L.K. (2009). Anaphylaxis fatalities and admissions in Australia, *The Journal of Allergy and Clinical Immunology, 123,* pp. 434–442.

Lopez, E., Cuadrado, C., Burbano, C., Jiménez, M.A., Rodríguez, J. and Crespo, J.F. (2012). Effects of autoclaving and high pressure on allergenicity of hazelnut proteins. *Journal of Clinical Bioinformatics*, 2(1), 12.

Ma, X., Ge, Y., Zhang, J., Huang, W., Han, J., Chen, Y., … and Sun, J. (2020). Comprehensive quantification of sesame allergens in processed food using liquid chromatography-tandem mass spectrometry. *Food Control*, 107, 106744.

Meyer, R., Wright, K., Vieira, M.C., Chong, K.W., Chatchatee, P. and Vlieg-Boerstra, B. J. (2019). International survey on growth indices and impacting factors in children with food allergies. *Journal of Human Nutrition and Dietetics*, 32(2), 175–184.

Mills, E.N. and Mackie, A.R. (2008). The impact of processing on allergenicity of food. *Current Opinion in Allergy and Clinical Immunology*, 8(3), 249–253.

Nitride, C., Nørgaard, J., Omar, J., Emons, H., Esteso, M.M. and O'Connor, G. (2019). An assessment of the impact of extraction and digestion protocols on multiplexed targeted protein quantification by mass spectrometry for egg and milk allergens. *Analytical and Bioanalytical Chemistry*, 411(16), 3463–3475.

Noorbakhsh, R., Mortazavi, S.A., Sankian, M., Shahidi, F., Maleki, S.J., Nasiraii, L.R., … and Varasteh, A. (2010). Influence of processing on the allergenic properties of pistachio nut assessed in vitro. *Journal of Agricultural and Food Chemistry*, 58(18), 10231–10235.

Onwude, D.I., Hashim, N., Janius, R., Abdan, K., Chen, G. and Oladejo, A.O. (2017). Nonthermal hybrid drying of fruits and vegetables: A review of current technologies. *Innovative Food Science and Emerging Technologies*, (43), 223–238.

Parker, C.H., Khuda, S.E., Pereira, M., Ross, M.M., Fu, T.J., Fan, X., … and Jackson, L.S. (2015). Multi-allergen Quantitation and the Impact of Thermal Treatment in Industry-Processed Baked Goods by ELISA and Liquid Chromatography-Tandem Mass Spectrometry. *Journal of Agricultural and Food Chemistry*, 63(49), 10669–10680.

Pilolli, R., Van Poucke, C., De Angelis, E., Nitride, C., de Loose, M., Gillard, N. and Monaci, L. (2021). Discovery based high resolution MS/MS analysis for selection of allergen markers in chocolate and broth powder matrices. *Food Chemistry*, 343.

Prado, M., Ortea, I., Vial, S., Rivas, J., Calo-Mata, P. and Barros-Velázquez, J. (2016). Advanced DNA- and protein-based methods for the detection and investigation of food allergens. *Critical Reviews in Food Science and Nutrition*, 56(15), 2511–2542. https://doi.org/10.1080/10408398.2013.873767.

Pushpa, B.P., Bhat, G.S. and Jayaprakasha, H.M. (2018). Effect of heat treatment and enzymatic hydrolysis on reduction in allergenicity of milk proteins. *Indian Journal of Nutrition,* 55(2), 156–165.

Sancho, A. I. and Mills, E. N. (2010). Proteomic approaches for qualitative and quantitative characterisation of food allergens. *Regulatory Toxicology and Pharmacology RTP*, *58*(3), 42-46. https://doi.org/10.1016/j.yrtph.2010.08.026

Sealey-Voyksner, J., Zweigenbaum, J. and Voyksner, R. (2016). Discovery of highly conserved unique peanut and tree nut peptides by LC-MS/MS for multi-allergen detection. *Food Chemistry,* 194, 201–211.

Sicherer, S. H. and Sampson, H. A. (2010). Food allergy. *The Journal of Allergy and Clinical Immunology, 125*(2), 116–125.

Sordet, C., Culerrier, R., Granier, C., Rance, F., Didier, A., Barre, A. and Rouge, P. (2009). Expression of Jug r 1, the 2S albumin allergen from walnut (*Juglans regia*), as a correctly folded and functional recombinant protein. *Peptides*, 30, 1213–1221.

Su, M., Venkatachalam, M., Teuber, S.S., Roux, K.H. and Sathe, S.K. (2004). Impact of γ-irradiation and thermal processing on the antigenicity of almond, cashew nut and walnut proteins, *Journal of the Science of Food and Agriculture,* 84, 1119–1125.

Sze-Tao, K.W.C. and Sathe, S.K. (2000). Walnuts (*Juglans regia* L): proximate composition, protein solubility, protein amino acid composition and protein *in vitro* digestibility. *Journal of the Science of Food and Agriculture,* 80, 1393-1401.

Van Boxtel, E.L., Gruppen, H., Koppelman, S.J. and van den Broek, L.A.M. (2008). Heat denaturation of Brazil nut allergen Ber e 1 in relation to food processing. *Food Chemistry*, 110(4), 904–908.

Verhoeckx, K., Vissers, Y.M., Baumert, J.L., Faludi, R., Feys, M., Flanagan, S., … and Kimber, I. (2015). Food processing and allergenicity. *Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association*, 80, 223–240.

Wigotzki, M., Steinhart, H. and Paschke, A. (2000). Influence of Varieties, Storage and Heat Treatment on IgE-Binding Proteins in Hazelnuts (*Corylus avellana*). *Food and Agricultural Immunology*, 12(3), 217.

Yao, M., Xu, Q., Luo, Y., Shi, J. and Li, Z. (2015). Study on reducing antigenic response and IgE-binding inhibitions of four milk proteins of *Lactobacillus casei* 1134. *Journal of the Science of Food and Agriculture,* 95(6), 1303–1312.