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ARTICLE

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Application of LC-MS/MS Coupled with Various Digestion Methods for the Identification of Porcine Gelatin Markers in Confectionery Matrices

Kifayati Rosiyanti Dewi[1], Feri Kusnandar[2]*, Nancy Dewi Yuliana[3], Maya Ismayati[4], Nissa Nurfajrin Solihat[5], Handy Riantana[6], Heryani[7]

Abstract: *Gelatin is a high-risk ingredient in terms of its halal status. Liquid chromatography combined with mass spectrometry (LC-MS/MS) was used to identify the source of gelatin based on marker peptides and proved to deliver higher reliability than other methods. However, the digestion method is essential before LC-MS/MS analysis. This research evaluated different digestion methods against selected porcine gelatin marker peptides and assessed LC-MS/MS sensitivity through adulteration experiments in various mixed matrices. The study involved three digestion methods (conventional, microwave, and ultrasound) before LC-MS/MS analysis to determine the most effective method for detecting marker peptide targets from porcine gelatin. The appropriate method was applied to isolate porcine gelatin peptides in the matrices of bovine gelatin and confectionery products (lozenges, marshmallows, and soft candy) at concentrations of 0.01, 0.1, and 1% (w/w). Relative detection limit values were determined. The results showed that conventional digestion treatment yielded a higher marker peptide detection rate than microwave and ultrasound digestion. Meanwhile, the detection limit of porcine gelatin in bovine gelatin ranged from 0.09 to 0.89%, depending on the marker peptide used, and could be significantly detected at a concentration of 1% in the confectionery product. The marker peptide TGQPGAVGPAGIR exhibited the highest stability, as it was detectable at the lowest concentration across all mixed matrices. The LC-MS/MS method has been proven to afford sensitive results and has the potential to serve as an alternative for detection of halal status.*

Keywords: *confectionery, gelatin, LC-MS/MS, porcine*

e-mail: dkifayati@gmail.com[1], fkusnandar@apps.ipb.ac.id*[2], nancy_dewi@apps.ipb.ac.id[3], maya.ismayati@brin.go.id[4], nissa.nurfajrin@gmail.com[5], handy.riantana@gmail.com[6], heryani@halalmui.org[7]
*Corresponding Author

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[1,2,3]*Department of Food Science and Technology, IPB University, Jl. Kamper, Dramaga, Bogor, 16680, Indonesia*. [1,4,5,6]*Research Center for Biomass and Bioproducts, National Research and Innovation Agency (BRIN), Jl. Raya Bogor KM 14, Cibinong, Bogor, 16911, Indonesia*. [2,3]*Halal Science Center, Jl. Botani Square No. 7, IPB University Baranangsiang Campus, Bogor, 16129, Indonesia*. [6]*Department of Biochemistry, Khon Kaen University, Mueang Khon Kaen District, Khon Kaen, 40002, Thailand*. [7]*The Assessment Institute for Foods, Drugs and Cosmetics, the Indonesian Council of Ulama (LPPOM MUI), Bogor Global Halal Centre, Jl. Pemuda No.5, Tanah Sereal, Sempur, Bogor Tengah, Bogor, 16161, Indonesia.*

1. Introduction

Halal food certification is mandatory according to Indonesian regulations to safeguard Muslim consumers. The Global Islamic Report 2020-2021 indicates that worldwide Muslim consumer expenditure on halal food and beverages reached USD 1.17 trillion in 2019 and is projected to reach USD 1.38 trillion by 2024. Indonesia is a significant market, with its Muslim population comprising 87%, ranking fourth globally in halal food consumption indicators, and reaching USD 144 billion in 2019 (State of the Global Islamic Economy, 2021). The implementation of Law Number 33 of 2014 concerning the Halal Product Guarantee underscores the importance of addressing the halal-haram issue throughout the manufacturing chain until it reaches consumers (Charity, 2017).

Gelatin poses a significant risk regarding its halal status, as it can be derived from haram food materials such as pig bones or from halal animals that were not slaughtered according to halal procedures. Gelatin finds widespread use in the food industry as a thermoreversible emulsifier, foaming agent, and gelling agent (Ali et al., 2015; Rakhmanova et al., 2018; Uddin et al., 2021). The demand for gelatin is particularly high in Indonesia, with import data from Statistics Indonesia indicating a value of 1.19 million kilograms between January and June 2022. Gelatin is commonly derived from pig and cow bones or skins (Ahmed et al., 2020). The gelatin production process can destroy Deoxyribonucleic Acid (DNA) and various post-translational modifications (PTM) in the collagen protein, posing a challenge in tracing the origin of gelatin. Identifying the source of gelatin is crucial for consumers due to food safety concerns, including animal-borne diseases and allergenicity, as well as socio-cultural considerations related to halal compliance. Consequently, there is a growing need for the traceability of gelatin sources (Hermanto et al., 2015).

Confectionery products are prepared from a complex mixture of ingredients. Although the gelatin content in confectionery products is typically low, there exists a significant risk of mislabeling and adulteration due to the widespread availability of porcine gelatin and its lower cost compared to gelatin from bovine or other sources. Incorporating additives, preservatives, and intricate matrices can obscure the presence of protein or nucleic acid analytes in the final product (Gelatin Manufacturers Institute of America, 2019). This emphasizes the need to explore innovative approaches for rapid halal screening without compromising sensitivity and accuracy (Atefi et al., 2021; Hassan et al., 2018; Ng et al., 2021).

Currently, a sensitive and efficient halal authentication detection system has been developed. Calcium phosphate precipitation and fourier-transform infrared (FTIR) methods have been utilized to differentiate between bovine and porcine gelatin. However, both techniques face challenges when identifying animal sources within mixtures of gelatin and other ingredients (Cebi et al., 2016; Hidaka & Liu, 2003). Enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) methods enable the tracing of animal origins in gelatin. However, these methods still have limitations. DNA degradation during the manufacturing process reduces identification accuracy, and the quality and concentration of gelatin influence their performance (Grundy et al., 2016; Shabani et al., 2015). Liquid chromatography combined with mass spectrometry (LC-MS or LC-MS/MS) has been employed to identify gelatin sources based on marker peptides. This approach has demonstrated greater reliability in tracking gelatin origins than ELISA and PCR-based methods (Grundy et al., 2016). LC-MS/MS allows for the simultaneous use of multiple marker peptides from the same species in sample identification, which gives it an advantage over other techniques and enhances selectivity (Huang et al., 2020; Kleinnijenhuis et al., 2018).

Gelatin protein was extracted from the sample and then digested into peptides. Peptides obtained from trypsin digestion underwent an identification process using bioinformatics tools and were confirmed for their specificity through liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS). Subsequently, the peptides were further analyzed using triple quadrupole mass spectrometry (QQQ-MS) with multiple reaction monitoring (MRM) to determine which peptides were consistently detected in the sample (Sarah et al., 2016). MRM is a modified version of selective reaction monitoring (SRM) that can monitor several SRM transitions for the same or different analytes within a single mass spectrometry (MS) run. MRM can differentiate between highly homologous protein forms and recognize post-translational modifications (PTMs) by tracking multiple transitions in a single run. Therefore, for identifying proteins in complex matrices, MRM is preferred (Gianazza & Banfi, 2018). A targeted proteomic approach was developed and validated for commercial products with gelatin marker peptide as candidate screening (Jumhawan et al., 2019). This method can detect 0.1% adulteration of porcine gelatin in bovine gelatin and vice versa, but it has yet to provide quantitative information in more complex matrices.

The digestion process is a crucial in sample preparation for proteomic analysis with LC-MS/MS. However, the conventional digestion process in proteomic analysis is time-consuming, prompting the development of microwave and ultrasound-assisted digestion methods to expedite the transition from overnight digestion to just a few minutes. An ultrasonic machine is employed to digest gelatin from donkeys, cattle, horses, deer, and pigs dissolved in phosphate-buffered saline (Cai et al., 2021). The study demonstrated that digestion times ranging from 5 to 45 minutes did not significantly affect the number of identified peptide markers. While this method is relatively new, further testing against marker peptides is necessary. Another developed digestion method is microwave-assisted protein enzymatic digestion (MAPED), which has been the focus of several studies emphasizing its speed and efficiency (Juan et al., 2005; Lin et al., 2005; Pramanik et al., 2009). Sun et al. (2006) conducted a study using samples of bovine serum albumin mixed with protein complexes derived from human urine and yeast lysate. The results revealed comparable digestion efficiency for in-solution digestion and superior performance for in-gel digestion compared to conventional methods, which typically require 16 hours or overnight incubation. This method could accelerate protein sample preparation and enzymatic digestion in proteomic studies involving biological and clinical samples. However, the application of microwave digestion specifically for gelatin analysis still needs to be improved.

Detecting halal gelatin in food products using LC-MS/MS has yet to be widely explored. To date, no study has examined the effect of digestion methods and the concentration of porcine gelatin in complex food matrices on the detectability of specific marker peptides. This research gap is significant, as different marker peptides exhibit varying sensitivity levels (Jumhawan et al., 2019). Therefore, this study aimed to assess the effectiveness of different digestion methods in detecting porcine gelatin marker peptides within mixed matrices using LC-MS/MS with the MRM mode. The detectability of each marker peptide was evaluated through adulteration experiments conducted in various mixed matrices, including bovine gelatin and confectionery products. The findings of this study are valuable for determining the relative extent of porcine gelatin contamination in confectionery products and contribute to advancements in halal detection methodologies.

2. Materials and Methods

2.1. Reagent and Instruments

The chemicals used for analysis included sequencing-grade trypsin enzyme (Promega, Madison, WI, USA), gradient-grade acetonitrile, analytical-grade ammonium bicarbonate powder, formic acid (Sigma-Aldrich®, Saint Louis, MO, USA), and water (Ikapharmindo Putramas, Jakarta, Indonesia). The instruments utilized were a centrifuge-5425 (Eppendorf™, Hamburg, Germany), WIS-30 incubator shaker (Witeg Labortechnik, Wertheim, Germany), EM-S105AS microwave oven (Sanyo™, Qingdao, China), WUC-D03H ultrasound machine (Daihan Scientific, Wonju-si, South Korea), and LCMS-6080 (Shimadzu™, Japan).

2.2. Sample Preparation

2.2.1. Digestion Method Experiment

Commercially pure porcine gelatin was utilized as the reference sample in this study. A total of five mg of the sample was added to 0.6 mL of cold 50 mM ammonium bicarbonate buffer solution. The mixture was thoroughly vortexed until no sample residue remained on the tube wall and was then subjected to centrifugation. The solution was heated at 37°C and 150 rpm for 15–20 minutes. Approximately 6 µL of trypsin enzyme (1 µg/µL in ammonium bicarbonate buffer) was added to achieve a trypsin-to-sample ratio of 1:100. The solution was vortexed again and underwent centrifugation. Next, the solution was subjected to three different digestion treatments: conventional digestion (Jumhawan et al., 2019), microwave-assisted digestion (Sun et al., 2006), and ultrasound-assisted digestion (Cai et al., 2021) with slight modifications. The digestion conditions for each method were adjusted to approximate conventional digestion temperatures, utilizing the available instruments and their corresponding power and time settings in our laboratory. For conventional digestion, the sample solution was incubated overnight at 37°C for 15 hours with continuous shaking at 150 rpm in an incubator shaker. Microwave digestion was performed using a domestic microwave oven, with a container of water placed alongside the sample vial to absorb excess microwave radiation. The microwave oven was set to 400 W for 1 minute. Ultrasound-assisted digestion involves the use of an ultrasound machine (output power: 290 W) for 20 minutes. After digestion, the solution was vortexed again, followed by a 5–minute centrifugation at 12,000 rpm to separate the mixture. The supernatant containing the digested peptides was collected for subsequent LC-MS/MS analysis. The step-by-step procedure for sample preparation is illustrated in Figure 1.

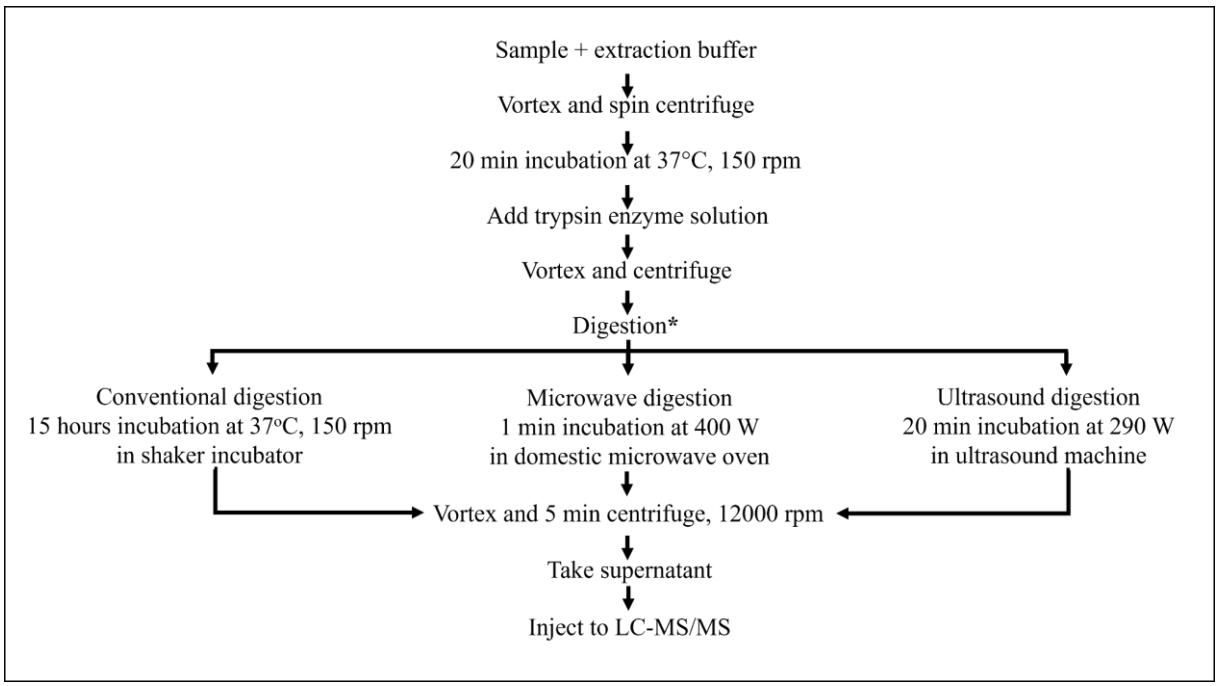


Figure 1. The procedure for sample preparation used for the subsequent experiment.

2.2.2. Adulterated Product Samples

Adulterated samples were created to simulate the adulteration of gelatin and gelatin-based products commonly found in the market. Adulterated gelatin was prepared by mixing 0.01%, 0.1%, and 1% porcine gelatin into bovine gelatin. Similarly, adulterated confectioneries (soft candy, marshmallow, and lozenges) were crafted using bovine gelatin, with the addition of porcine gelatin at concentrations of 0.01%, 0.1%, and 1% of the total product weight (w/w). Bovine gelatin and bovine gelatin-based confectioneries were also prepared as controls. Soft candies were prepared by heating a gelatin and water mixture at approximately 70℃ for 10–15 minutes, ensuring complete dissolution and the absence of lumps. Sugar and flavor essence were added and stirred until dissolved. The resulting solution was poured into molds and left at room temperature overnight (Graboski et al., 2018). Marshmallows were made by heating a water and gelatin mixture to 70℃ for 5 minutes. In another bowl, sugar, glucose syrup, and water were combined and heated to 112℃ for 5 minutes. The next step involved mixing the sugar and gelatin solutions using a hand mixer for 10 minutes at high speed. The mixture was then molded and left to set overnight at room temperature. Before cutting, the marshmallows were coated with powdered sugar and cornstarch to prevent sticking (Arizona et al., 2021). Lozenges were made by combining gelatin and water, setting it aside for 10 minutes. The mixture was heated at around 70℃ for 10–15 minutes until fully dissolved. Glycerin was added, stirred, and reheated for 5 minutes. Next, simplex syrup was added and stirred. After heating, the mixed solution was allowed to cool for 5 minutes. Then, the mixed solution was combined with methylparaben and mint essence and stirred. The solution was poured into jelly molds and left overnight (Aryani et al., 2015). The prepared adulterated samples were subsequently analyzed by LC-MS/MS using the previously optimized digestion method.

2.3. LC-MS/MS Analysis

LC-MS analysis was conducted following the method established by Jumhawan et al. (2019). Chromatographic separation was accomplished using a Phenomenex Aeris™ 1.7 µm PEPTIDE XB-C18 100Å column (150 × 2.1 mm I.D., part number 00F-4506-AN). The mobile phase for the separation consisted of 0.1% formic acid in water for phase A and 0.1% formic acid in acetonitrile for phase B. The gradient elution process was fine-tuned based on the parameters specified in Table 1.

The flow rate was set at 0.3 mL per minute. The injection volume was 1 µL. Oven temperature 40°C. MS analysis used MRM-positive mode for six porcine-specific peptides. The ionization mode uses heated ESI. The heat block temperature was set at 400°C, the DL temperature at 250°C, the interface temperature at 300°C. Nebulizing gas used N_2 with a flow rate of 3 L per minute, drying gas used N_2 at a flow rate of 10 L per minute, heating gas uses zero air with a flow rate of 10 L per minute. Six porcine gelatin marker peptides as detection targets were listed in Table 2. All experiments are performed in duplicate. Data viewing and analysis is performed using LabSolutions Browser software.

Table 1. Liquid Chromatography Mobile Phase Gradient Elution

Time (minute)	Mobile phase B (%)
0.0 (Start)	5
2.0	5
15.0	25
15.2	50
16.0	50
16.2	5
19.0	Stop

Table 2. List of Porcine Gelatin Marker Peptides (Jumhawan et al., 2019)

Peptides	Sequences	UniProt Accession	Protein	AA Position	Precusor Ion	Product Ion
PGa	GYPGNPGPAG AAGAPGPQGA VGPAGK	A0A1S7J1Y9	Alpha-2 chain of type I porcine collagen	949-974	1103.05++	881.5, 372.2, 850.95
PGb	QGPSGPSGER	A0A1S7J210	Alpha-1 chain of type I porcine collagen	987-996	486.25++	786.35, 689.3, 602.3, 545.25
PGc	GETGPSGPAG PTGAR	A0A1S7J210	Alpha-1 chain of type I porcine collagen	784-798	656.3++	967.5, 870.45, 783.4, 726.4
PGd	GETGPAGPAG PVGPVGAR	A0A1S7J210	Alpha-1 chain of type I porcine collagen	1,069-1,086	773.9++	1034.55, 977.55, 880.5, 809.45, 752.45, 499.3
PGe	TGETGASGPP GFAGEK	A0A1S7J1Y9	Alpha-2 chain of type I porcine collagen	831-846	731.85++	1017.5, 946.45, 802.4, 705.35
PGf	TGQPGAVGPA GIR	A0A1S7J1Y9	Alpha-2 chain of type I porcine collagen	1,068-1,080	590.85++	894.5, 797.45, 740.45, 669.4, 513.3

3. Results and Discussion

3.1. Digestion Experiment

Protein digestion is a pivotal step within bottom-up proteomic methodologies, particularly when analyzing gelatin through LC-MS/MS. In intricate matrices, proteins are enzymatically broken down into peptides through proteolytic digestion, with trypsin being the enzyme of choice (Xie et al., 2011). Conventionally, the digestion process in proteomic analysis necessitates an overnight duration (Qi et al., 2019). However, cutting-edge techniques like microwave and ultrasound have been explored to expedite this process as alternatives to the more time-consuming traditional digestion methods that often exceed 12 hours. This study focuses on assessing the detectability of specific peptide markers utilizing various digestion techniques, an area that has not been extensively explored in previous investigations. The results of the MRM analysis for six porcine marker peptides across the three digestion experiments are presented in Table 3.

The outcomes of the MRM analysis demonstrated that all porcine marker peptides were successfully detected using conventional digestion. In ultrasound digestion, MRM analysis identified five marker peptides, while microwave digestion detected two marker peptides out of the total six porcine marker peptides targeted for detection. Notably, the PGf marker peptide was detectable across all digestion methods. Several factors influence the rate of digestion, including protein structure (Šlechtová et al., 2015). One critical aspect is the positioning of the peptide within the protein chain,

affecting the ease of cleavage. Trypsin, being a highly specific protease, cleaves peptide bonds at the C-terminal side of lysine (K) or arginine (R) residues, except when followed by proline (P). It is worth noting that trypsin prefers arginine cleavage over lysine cleavage due to the variance in bond strength (Temple et al., 2006). This preference is attributed to the presence of arginine residues flanking both sides of the cleavage site within PGf peptides, which also fall within the ideal length range (6 and 20–25 amino acids) for mass spectrometry (MS) detection (Kleinnijenhuis et al., 2018). Microwave-assisted digestion yielded the production of two marker peptides, PGa and PGf. This indicates that the microwave method may not be optimally suited for quantitative analysis during sample preparation. While microwave heating theoretically accelerates enzymatic reactions, our study observed a significantly lower number of detected peptide markers than conventional and ultrasound digestion methods. This divergence likely arises from the higher rate of enzymatic reaction within the microwave method. Despite its shorter duration, this method may not allow for the complete unfolding of protein polypeptide chains. For future research endeavors, it is advisable to incorporate reducing agents such as dithiothreitol before conducting microwave digestion (Rivera-Albarran & Ray, 2020). This approach could aid in reducing disulfide bonds that contribute to protein folding, thereby facilitating the unfolding of protein chains. By doing so, the trypsin enzyme would gain improved access to maximum substrate cleavage sites, potentially enhancing the efficacy of the microwave digestion process.

The viability of a digestion method can be assessed based on the number of markers detected using the MRM mode. Zhang et al. (2019) discovered that microwave digestion yielded an equivalent count of detectable target marker peptides as conventional techniques. However, upon examining the total peak areas from the chromatograms of peptides generated through the respective digestion methods, it was observed that the peak area resulting from conventional digestion was notably higher than those obtained from ultrasound and microwave digestion (Figure 2). Conventional digestion, carried out at 37°C for several hours, affords the enzyme ample time to recognize binding sites on the protein substrate, thereby promoting precise cleavage (Pramanik et al., 2009). In contrast, the total peak area of ultrasound digestion was smaller than that of conventional digestion and the peak area of microwave digestion to ultrasound digestion. Although digestion efficiency is typically evaluated by calculating the ratio between the peak area of a specific peptide and an internal standard (Doneanu et al., 2012), this study did not employ any internal standards. Consequently, the digestion method that yielded the highest number of marker peptides and the greatest peak area detected via the MRM mode was considered the most effective. Hence, conventional digestion methods were selected for further research.

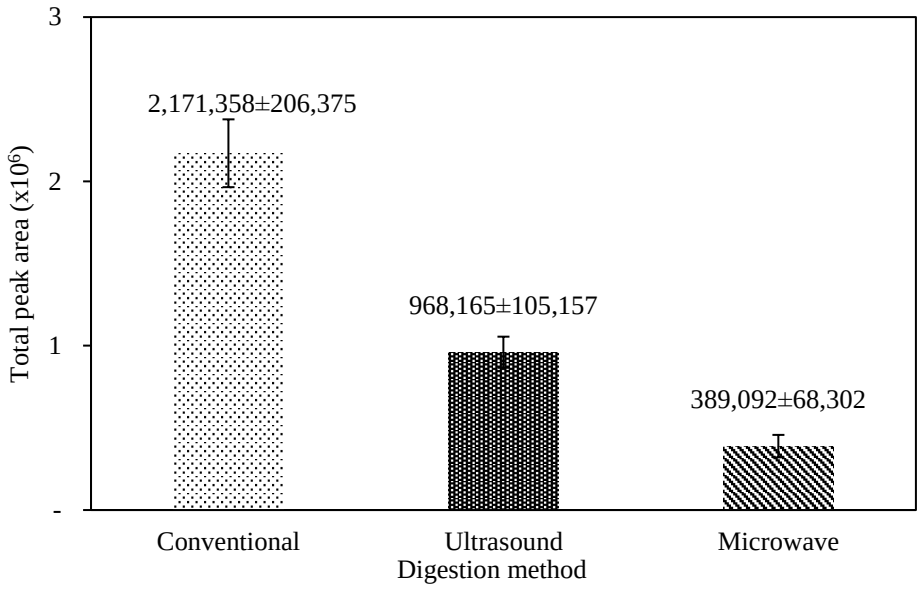


Figure 2. Total peak area of all marker peptides from each digestion process.

Table 3. Detection of Porcine Gelatin Marker Peptides and Their Respective Peak Areas

Marker Peptide (Peak area in AuC unit)	Digestion Method		
	Conventional	Microwave	Ultrasound
PGa	23,289±2,415	5,872±71	15,962±1,418
PGb	628,448±72,287	n.d	42,920±11,573
PGc	19,523±2,768	n.d	1,657±639
PGd	95,346±7,192	n.d	4,868±2,626
PGe	10,339±2,839	n.d	n.d
PGf	1,394,415±129,383	381,212±66,953	902,759±93,015
Number of detected marker peptides	6/6	2/6	5/6

In recent years, numerous researchers have developed and applied alternative energy sources to enhance the catalysis of proteomic reactions, specifically through ultrasound-assisted and microwave-assisted digestion. However, as of now, there is no literature reporting the routine utilization of microwave or ultrasound digestion methods for sample preparation. The choice of domestic microwave ovens is rooted in their easy accessibility. The principal challenge associated with domestic microwave oven usage revolves around reproducibility and temperature control, given that temperatures can escalate rapidly within seconds. Due to variations observed in methods within the existing literature, it is recommended to restrict the temperature not to exceed 60°C to prevent potential damage to the trypsin enzyme. A common practice among researchers is to include a water-filled beaker in the microwave to absorb excess heat energy while ensuring consistent sample placement within the microwave for enhanced repeatability (Lill et al., 2007). As for the ultrasound digestion method, the instruments used in several publications were probes, baths, and sonoreactors (Shin et al., 2011), which probes were usually rarely available in many labs (Cai et al., 2021) as well as sonoreactors. Ultrasound-based digestion methods usually do not specify the temperature used. Notably, the ultrasound method is more inclined towards inducing partial digestion than the completed digestion achievable through the conventional method (Cheng et al., 2012). The unsatisfactory outcomes observed in this study may be attributed to potential limitations inherent to ultrasound and microwave methods. In these techniques, the digestion process may not be optimal, thus resulting in inadequate marker peptide production, crucial constituents for target analysis. While the conventional digestion method maintains its status as the superior approach, this discovery underscores the viability of the ultrasound digestion method for qualitative analysis, even in situations where specific markers like PGe are not the primary focus of analysis.

3.2. Detection of Porcine Gelatin Markers in Adulterated Samples

Complex matrices have the ability to hide the presence of proteins, including peptides. Porcine gelatin was introduced into various matrices, and subsequent analysis was conducted using MRM, to assess the availability of each marker peptide. As such, this study aimed to simulate the adulteration of bovine gelatin with different concentrations of porcine gelatin. The outcomes of the MRM analysis are presented in Table 4.

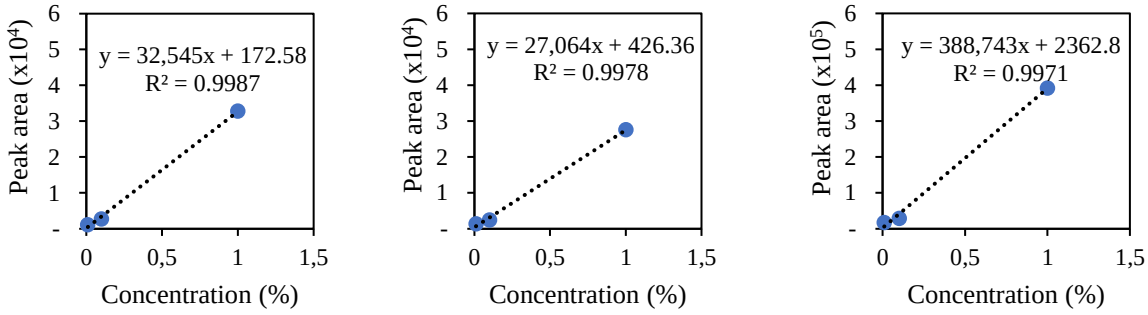
Table 4. Detection of Porcine Gelatin Marker Peptides in Bovine Gelatin and Their Respective Peak Areas

Marker Peptide (Peak area in AuC unit)	Concentration		
	0.01%	0.1%	1%
PGa	n.d	1,807±284	11,644±284
PGb	1,355±71	2,409±426	27,557±4,472
PGc	603±142	853±355	10,941±1,136
PGd	1,105±142	2,760±1,491	32,779±2,058
PGe	904±426	402±0	2,329±114
PGf	17,114±1,775	29,287±745	392,193±4,167
Number of detected marker peptides	5/6	6/6	6/6

In the bovine gelatin matrix, all porcine gelatin markers were detectable at a concentration of 0.01%, except for PGa. The coefficient of determination for all marker peptides exceeded 0.99, except for PGe, where it was 0.8. Based on these coefficients, the top three peptides were PGd, PGb, and PGf, exhibiting a correlation between porcine gelatin concentration and the peak area of each respective marker peptide (Figure 3). With the coefficient of the determination being satisfactory for each marker peptide, the determination of the limit of detection (LOD) value for each marker was calculated using

the standard deviation method and the formula below (Prandi et al., 2019; Sha et al., 2020). The linear regression equation and the relative LOD value for each peptide marker are presented in Table 5. The relative LOD value for detecting porcine gelatin in bovine gelatin ranged from 0.09% to 0.89%.

$$LOD = 3.3 \times \frac{\text{Standard deviation}}{\text{Slope}} \quad (1)$$



(a) (b) (c)

Figure 3. Correlation of porcine gelatin concentration in bovine gelatin with the peak area marker peptides. (a) PGd; (b) PGb; (c) PGf.

Table 5. The Linear Regression Equation and the LOD Value of Porcine Gelatin Markers in Bovine Gelatin Matrix

Marker Peptide	Linear regression equation	LOD (%)
PGb	y=27,064x+426.36	0.12
PGc	y=10,753x+153.39	0.16
PGd	y=32,545x+172.58	0.09
PGe	y=1,723.6x+573.58	0.89
PGf	y=388,743x+2,362.8	0.14

To estimate the unknown concentration of gelatin-based products in food systems, a high R2 value linear regression or calibration equation based on the relationship between the peak area of marker peptides (y) and gelatin content (x) can be utilized (Huang et al., 2020; Sha et al., 2020; Yang et al., 2018). The area under the chromatographic elution profile of the identified peptides can be compared between samples for relative quantitation (Xie et al., 2011). Some published works determine the detection limit based on a signal-to-noise ratio of 3 (Jumhawan et al., 2019; Pan et al., 2018). However, this approach can present challenges when the noise ion is too high or too low, as in cases where the background noise is zero, resulting in an infinite signal-to-noise ratio (Wells et al., 2011). Hence, the standard deviation method based on peak area can be an alternative for determining the LOD (Evard et al., 2016a, 2016b).

Porcine gelatin can be readily detected even in the lowest concentrations within bovine gelatin. However, food products containing gelatin, such as confectioneries, are widely distributed in the market, and people are more likely to encounter these products than raw gelatin. Therefore, the detection of porcine gelatin in more complex food matrices, such as confectionery, was investigated. National Agency of Drug and Food Control of the Republic of Indonesia stated that confectionery items like soft candy, marshmallows, and lozenges were selected for analysis as they represent gelatin-containing confectionery products. The outcomes of MRM analysis for porcine gelatin marker peptides in various confectionery products are outlined in Table 6.

Porcine gelatin marker peptides were not identified in the control confectioneries based on bovine gelatin. At a concentration of 0.01% porcine gelatin in all products, only the PGf marker peptide was detectable. For the 0.1% concentration, the range of detected marker peptides spanned from one to three markers, specifically PGb, PGf, and PGd. When the porcine gelatin concentration was raised to 1% in all products, four markers were detected: PGb, PGc, PGd, and PGf. PGf consistently demonstrated detection across all concentrations and products, displaying a coefficient of determination exceeding 0.99. As a result, relative LOD was determined based on the PGf marker peptide. As elucidated earlier, the variation in marker peptide detectability could be attributed to their distinct positions within the protein chain. Moreover, the presence of a sugar matrix may obscure certain signals originating from the porcine marker peptide.

The linear regression equation and the relative LOD value of the PGf marker in confectionery products are presented in Table 7. The LOD, determined based on the PGf marker peptide, ranged from 0.01 to 0.09%. Notably, the relative LOD value of the PGf porcine gelatin marker peptide in the bovine gelatin matrix exceeded that in the confectionery matrix. This discrepancy arises from the substantial disparity in bovine gelatin quantities between the two matrices. Flaudrops et al. (2015) and Jumhawan et al. (2019) have highlighted that bovine gelatin peptides can mask signals originating from porcine gelatin. It is important to note that there may be more accurate approaches than relying on a single marker for LOD determination, given the variance in sensitivity among individual peptides. Therefore, the detection of porcine gelatin can be confidently achieved at a concentration of 1% within confectionery products, as over 50% of the target marker peptides can be detected. Moreover, extending the analysis to concentrations beyond the aforementioned, the authors evaluated 5% porcine gelatin within all confectionery matrices. Consequently, five markers, excluding PGa, were detected, namely PGb, PGc, PGd, PGe, and PGf.

Table 6. Detection of Porcine Gelatin Marker Peptides in Confectionery Products and Their Respective Peak Areas

Marker Peptide (Peak area in AuC unit)	Concentration	Product		
		Soft candy	Marshmallow	Lozenges
PGa	0.01%	n.d	n.d	n.d
	0.1%	n.d	n.d	n.d
	1%	n.d	n.d	n.d
PGb	0.01%	n.d	n.d	n.d
	0,1%	1,154±355	n.d	653±71
	1%	23,743±2,342	8,080±1,916	10,993±4,331
PGc	0.01%	n.d	n.d	n.d
	0.1%	n.d	n.d	n.d
	1%	4,819±568	1,456±354	2,861±213
PGd	0.01%	n.d	n.d	n.d
	0.1%	1,155±71	n.d	n.d
	1%	12,098±1,916	6,926±1,277	8,935±5,396
PGe	0.01%	n.d	n.d	n.d
	0.1%	n.d	n.d	n.d
	1%	n.d	n.d	n.d
PGf	0.01%	603±284	1.154±781	1,004±284
	0.1%	8,633±4,826	3,765±1,348	8,613±1,732
	1%	84,037±6,250	52,104±6,817	65,611±356
Number of detected marker peptides	0.01%	1/6	1/6	1/6
	0.1%	3/6	1/6	2/6
	1%	4/6	4/6	4/6

Table 7. The Linear Regression Equation and The LOD Value of PGf Porcine Gelatin Marker in Confectionery matrices

Confectionery matrices	Linear regression equation	LOD (%)
Soft candy	y=84,076x-17.722	0.01
Marshmallow	y=52,375x-371.31	0.09
Lozenges	y=64,477x+1,219	0.07

Numerous studies have currently examined the presence of porcine gelatin in mixed samples using either LC-MS or other analytical instruments. Grundy et al. (2016) analyzed various porcine gelatin concentrations (0% to 4.6%) in chicken exudate. Their novel LC-MS/MS approach successfully identified the presence of porcine gelatin with high sensitivity at a level of 1%. However, PCR and ELISA methods proved insufficient to detect porcine gelatin until concentrations reached approximately 4.6%. Yang et al. (2018) detected gelatin adulteration using an HRMS method that initiates with bioinformatic and proteomic analyses to identify target peptides. The findings of their study indicate that this method can detect contamination of porcine gelatin up to a concentration of 0.1%. Flaudrops et al. (2015) differentiated between animal sources in raw meat versus processed meat and gelatin versus gelatin-containing products using MALDI-TOF-MS. Their study identified ten specific peaks corresponding to porcine gelatin and thirteen specific peaks for bovine gelatin. Detectability of porcine

gelatin in bovine gelatin was achieved at concentrations as low as 20%. Below this concentration, the signal from bovine gelatin was observed to mask the presence of porcine gelatin. Yilmaz et al. (2013) analyzed combinations of porcine and bovine gelatin in 90:10, 50:50, and 10:90, and conducted triplicate analyses. The outcome revealed that porcine gelatin was still detectable in a ratio of 10:90 when combined with bovine gelatin. Kleinnijenhuis et al. (2018) comprehensively validated the LC-MS/MS method for detecting gelatin sources, utilizing the internally stable isotope-labeled standard (SIL IS). The smallest quantification limit for detecting porcine gelatin in bovine gelatin and vice versa was determined to be 0.05%.

In MRM mode, quantifying peptides in complex gelatin samples often requires multiple transitions. However, monitoring the single most abundant transition can simplify the method, allowing for the monitoring of peptides from specific proteins (Guo et al., 2017). Figure 4 illustrates the chromatograms of the most stable marker peptide, PGf, and its product ions (m/z 894.500 and 669.400). When using the same amount (1%) of porcine gelatin, the intensity of PGf in the bovine gelatin matrix (Figure 4a) was higher than in the confectionery matrices (Figure 4b, 4c, and 4d). The decrease in ion intensity (ion suppression) between gelatin and confectionery matrices is attributed to the matrix effect. The presence of non-volatile macromolecules (such as sugar in this case) can hinder the target analyte's ability to enter the gas phase, leading to increased droplet viscosity and surface tension at the ESI interface (Panuwet et al., 2016; Zhou et al., 2017). MRM mode enables precise quantification over a broad dynamic range, and the low resolution of the Q1 and Q3 mass analyzer scans can generate interference signals from complex matrices (Huang et al., 2020; Kleinnijenhuis et al., 2018; Sha et al., 2020). Throughout the analysis, the authors were mindful of this food matrix effect, and to evaluate the method's marker detectability for routine analysis, the food matrix was intentionally retained and not removed.

Protein and peptide identification in food matrices presents a challenging task, as both the production process and the complexity of the food matrix can significantly influence the outcomes (Montowska & Spychaj, 2018). The detection sensitivity of each marker peptide varies depending on the food processing conditions. For semiquantitative purposes, selecting the most stable peptide is essential (Jumhawan et al., 2019). It is unsuitable to identify and quantify gelatin using marker peptides with unstable response values (Huang et al., 2020). Nevertheless, the food matrix's impact should be considered before applying this method to measure the target gelatin content in gelatin-based products.

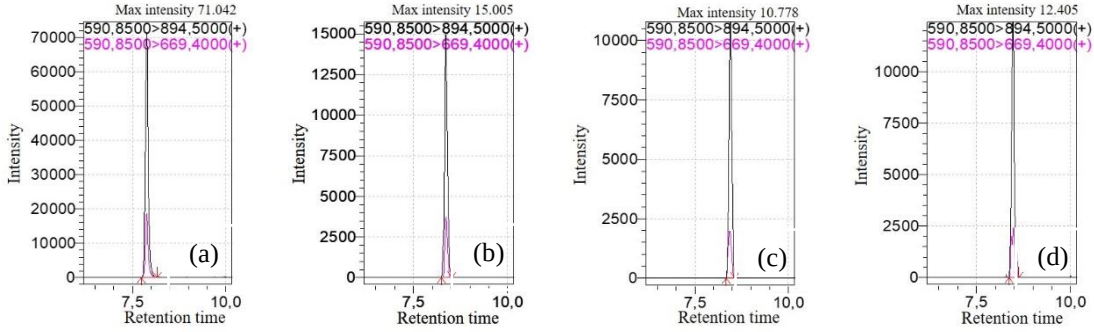


Figure 4. The PGf chromatograms of 1% porcine gelatin in: (a) bovine gelatin; (b) bovine soft candy; (c) bovine marshmallow; (d) bovine lozenges.

4. Conclusion

LC-MS/MS utilizing the MRM mode has proven its effectiveness in detecting and quantifying relatively low levels of porcine gelatin in various products. However, the conventional digestion treatment remains the preferred method for sample preparation in LC-MS/MS analysis, particularly for quantitative purposes. This preference stems from its capability to yield a more significant number of detected target peptides than alternative digestion treatments. Among the assessed marker peptides, the consistently stable PGf peptide (TGQPGAVGPAGIR) was reliably detected in all adulterated samples, even at the lowest tested concentration. The relative LOD for porcine gelatin in bovine gelatin, determined based on each marker peptide, ranged from 0.09% to 0.89%. Within confectionery matrices, the LOD spanned from 0.01% to 0.09%, with assured detection at a concentration of 1%. These study findings are anticipated to provide a valuable reference point for the routine halal analysis of confectionery products, particularly in accurately detecting and estimating porcine gelatin at minimal concentrations. In future

research, substantial potential exists for addressing the matrix effect and elevating result sensitivity, thereby opening avenues for further advancements in this field.

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