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Effect of Sterilization Methods on Collagen Hydrolysate Obtained from Tuna Tendon

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Abstract: Collagen hydrolysates derived from tuna tendons have potential applications in various industries, but sterilization is crucial to ensure their safety. This study investigated the effects of ethylene oxide (EtO), beta radiation, and gamma radiation sterilization methods on the structural and functional properties of collagen hydrolysates using nuclear magnetic resonance (NMR) spectroscopy, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and differential scanning calorimetry (DSC). EtO sterilization caused significant physical and chemical changes in the hydrolysates, as evidenced by the altered appearance and ^{1}H-NMR and ^{13}C-NMR spectra. In contrast, beta and gamma radiation did not induce notable changes in the physical characteristics and NMR spectra. MALDI-TOF MS analysis revealed slight alterations in the molecular weight distribution after sterilization, with beta irradiation causing a minor decrease and gamma irradiation and EtO leading to small increases. DSC analysis showed shifts in the heat absorption peaks after sterilization, indicating changes in the thermal properties. The findings suggest that while all three methods effectively sterilize collagen hydrolysates, EtO causes more significant structural modifications compared to beta and gamma radiation. This study provides valuable insights into the impact of sterilization on collagen hydrolysates, facilitating the selection of appropriate methods for specific applications.

Keywords: sterilization methods; collagen hydrolysate; tuna tendon; spectrometry; peptide mass determination

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1. Introduction

Collagen is a fibrous structural protein found abundantly in the extracellular matrix of various connective tissues, including tendons, ligaments, bones, and skin [1–3]. It plays a crucial role in providing mechanical strength and structural integrity to these tissues. Collagen hydrolysates, obtained through the enzymatic or chemical hydrolysis of collagen, have gained significant attention due to their potential bioactive properties and applications in the food, cosmetic, and pharmaceutical industries [3–7]. However, the sterilization of collagen hydrolysates is a critical step to ensure their safety and prevent microbial contamination, especially for applications in the medical and pharmaceutical fields.

Several sterilization methods, including ethylene oxide (EtO), beta radiation, and gamma radiation, have been employed for the sterilization of collagen-based materials. EtO sterilization is a widely used method that relies on the alkylating properties of ethylene oxide gas to inactivate microorganisms [8]. Beta radiation, which utilizes high-energy electron beams, and gamma radiation, which employs radioactive isotopes like cobalt-60, are also effective sterilization techniques. However, these sterilization methods may potentially alter the structural and functional properties of collagen hydrolysates, necessitating a comprehensive evaluation of their effects [9–11].

Identifying the changes in collagen hydrolysates after sterilization can be performed via many techniques. Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique that can be used for the investigation of chemical structure, enabling the detection of modifications or degradation caused by the sterilization processes [12]. Additionally, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can be employed to analyze the molecular weight distribution in collagen hydrolysates [13]. Differential scanning calorimetry (DSC) is a thermal analysis technique that can provide information about the thermal stability and denaturation behavior of collagen hydrolysates by monitoring the heat flow associated with phase transitions or structural changes, which can reveal alterations in the thermal properties of the hydrolysates caused by the sterilization processes [14]. This study aims to investigate the effects of the ethylene oxide, beta radiation, and gamma radiation sterilization methods on the collagen hydrolysates obtained from tuna tendons, utilizing NMR, MALDI-TOF MS, and DSC analyses. The findings can contribute to a better understanding of the impact of these sterilization methods on the structural and functional properties of collagen hydrolysates.

2. Materials and Methods

2.1. Collagen Hydrolysate from Tuna Tendon

Collagen hydrolysate was extracted from tuna tendons using an ultrasound-assisted method, following a protocol from the College of Maritime Studies and Management, Chiang Mai University [15]. The tendons, sourced as by-products from tuna tails provided by a cannery (Thai Union Group PCL, Samut Sakhon, Thailand), were packed in 1 kg polyethylene bags, frozen, and transported to the laboratory in an insulated box with ice, maintaining a temperature below 7 °C. Once in the lab, the tendons were stored at −18 to −20 °C and used within four months. To prepare the tendons, they were thawed, cut into small pieces, and minced using a Philips HR7627 blender (Bangkok, Thailand). The minced tendons were then soaked in a 100 mmol/L sodium chloride (NaCl, pH 7.5) solution with a tendon-to-solution ratio of 1:10 (w/v) at 4 ± 1 °C for 12 h. After soaking, they were drained, washed with distilled water, and ready for collagen extraction. The pretreated tendons were immersed in a solution of 1% (w/v) pepsin and 0.5 M acetic acid with a tendon-to-solution ratio of 1:10 (w/v). The tendons were allowed to swell for 10 min at 10 ± 1 °C in a cooling bath (Eyela, CA-1115-CE, Tokyo, Japan). Following this, the sample underwent ultrasonication and precipitation, then was dialyzed against distilled water for 24 h using a Spectra/Por dialysis membrane (100 kDa; Thermo Fisher Scientific, Waltham, MA, USA). The resulting dialysate was freeze-dried with a GFD-3H freeze-dryer (Gririanthong, Ratchaburi, Thailand) and stored at −18 to −20 °C as pepsin-soluble, ultrasound-extraction-assisted collagen (PUTC) until needed.

2.2. Ethylene Oxide Sterilization of Collagen Hydrolysate

Ethylene oxide (EtO) was used to sterilize the collagen hydrolysate in accordance with ISO 11135:2014 [16]. We first sealed the collagen hydrolysate in sterile bags to ensure its purity. We then exposed the samples to 700 mg/L EtO gas with 70% relative humidity for 8 h at 55 °C. Following this exposure, we detoxified the collagen hydrolysate by exposing it to warm air at 45 °C under atmospheric pressure for 48 h. This crucial step removes

all residual EtO, ensuring the safety and sterility of collagen hydrolysate for medical and nutritional use.

2.3. Beta and Gamma Radiation Sterilization of Collagen Hydrolysate

Collagen hydrolysate was sealed in a medicinal pouch and placed in an opaque box for protection from light and contamination. The sample was sterilized with beta and gamma radiation at a minimum of 17.5 kGy.

2.4. Physical Appearances and Image Analysis of Collagen Hydrolysate

Collagen hydrolysate samples were visually photographed before and after sterilization to assess the impact of various sterilization methods on the visual appearance of the material. For each condition, 20 mg of collagen hydrolysate was dissolved in 2 mL of 10% (*w*/*v*) deionized (DI) water to facilitate the evaluation of color changes resulting from the sterilization processes. To quantitatively analyze these color variations, the images were processed by using ImageJ software version 1.54j. The "Histogram" function, located in the "Analyze" menu, was employed to generate histograms of pixel intensity for specific regions of interest within each image. These histograms provide essential data regarding the contrast, brightness, and overall intensity of the collagen hydrolysate samples, enabling a thorough comparison of the color changes induced by the different sterilization techniques. As shown in Figure 1, the region of interest for each sample was carefully selected and enclosed within a yellow frame to ensure consistent and accurate measurements across all samples.

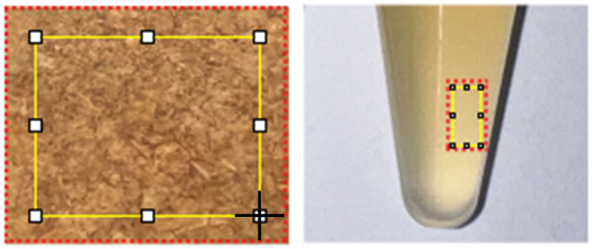


Figure 1. The area (yellow box) of the collagen hydrolysate sample that was used to measure the histogram of the physical appearance image and the red frame was used as the outer edge where the tip of the arrow cross cursor met for measurement accuracy.

2.5. Nuclear Magnetic Resonance Spectroscopy (NMR) Characterization

The structure of collagen hydrolysate before and after sterilization with ethylene oxide, beta, and gamma radiation was determined using ^{1}H-NMR and ^{13}C-NMR techniques. For the analysis, 30 mg of the sample was dissolved at room temperature in 2 mL of deuterium oxide (D_2O) to achieve a 1.5% (*w*/*v*) concentration. The solution was thoroughly mixed until homogeneous and any sediment or dust present was filtered out using a 0.1 µm filter. The prepared samples were then analyzed using a 500 MHz nuclear magnetic resonance spectrometer (NMR) from JEOL, Peabody, MA, USA. Both ^{1}H-NMR and ^{13}C-NMR spectroscopy were employed to obtain detailed insights into the structural changes of the collagen hydrolysate induced by the different sterilization methods.

2.6. Sample Preparation and Generation of MALDI-TOF Mass Spectra

Four samples of collagen hydrolysate were prepared for analysis using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The samples included collagen hydrolysate before and after sterilization with beta radiation, gamma radiation, and ethylene oxide. Each sample was dissolved in 1 mL of methanol and thoroughly mixed by vortex for 5 min at room temperature to ensure homogeneity. Following the preparation, the solution samples were individually subjected to MALDI-TOF analysis to characterize the collagen hydrolysate and assess the impact of different sterilization methods on its composition.

A 0.5 µL volume of each of the solution-sample-containing sterile peptide synthesis (see above) was spotted onto a clean ground steel target plate (Bruker Daltonics, Bremen, Germany) in 8 replicates (for generating a MALDI-TOF MS mass spectrum profile), air-dried at room temperature, and then overlaid with 0.5 µL of the matrix solution (4 mg/mL 4 chloro-α-cyanocinnamic acid in 70% Methanol and 0.1% trifluoroacetic acid. After the matrix solution was air-dried at room temperature, the sample was promptly analyzed using an ultrafleXtreme mass spectrometer and FlexControl software version 3.0 (Bruker Daltonics, Germany), with the following settings: spectrum recording mode, positive linear; mass range (m/z), 700–3500 Da; laser frequency, 60 Hz; ion source-1 voltage, 25 kV; ion source-2 voltage, 24 kV; and lens voltage, 7.0 kV. With the automatic mode and default setting, 50 sets of 500 laser shots were applied in different positions within one sample spot, and the generated mass spectra were accumulated for MALDI-TOF MS analysis. In each experiment, the peptide calibration standard II (Bruker Daltonics, Germany) was used to calibrate the mass spectrometer according to the manufacturer's instructions.

Next, all m/z values for each sample were calculated using Equation (1). The values for each sample group were then compared to the non-sterile group to determine the percentage difference from the control group:

$$\% \text{ Difference from control group } = \frac{B - A}{\left(\frac{B+A}{2}\right)} \times 100 \quad (1)$$

where:

A is the sum of m/z value from the control group or non-sterile;

B represents the sum of m/z from the comparison group (ethylene oxide, beta, and gamma).

2.7. Differential Scanning Calorimetry Analysis

The thermal properties of collagen hydrolysate were evaluated using differential scanning calorimetry (DSC) analysis (Rigaku, Tokyo, Japan) to compare the effects of various sterilization methods, i.e., beta radiation, gamma radiation, and ethylene oxide. Each sample, weighing approximately 2.5 mg, was hermetically sealed in an aluminum pan. The temperature was then increased to 250 °C at a rate of 10 °C/min.

3. Results and Discussion

3.1. Physical Appearances of Collagen Hydrolysate before and after Sterilization

The physical appearances of the collagen hydrolysates were visibly altered after undergoing the various sterilization methods, as shown in Figure 2. Ethylene oxide sterilization caused the collagen hydrolysate to change from a light brown powder to a burnt brown and sticky mass. When dissolved in pure distilled water, the resulting solution appeared dark brown. This transformation can be attributed to the high temperatures experienced during the ethylene oxide sterilization method. In contrast, both beta and gamma radiation sterilization methods did not result in any observable physical changes to the collagen hydrolysate, which maintained its pre-sterilization appearance.

The visual appearances of the samples before and after the sterilization of collagen hydrolysate can be quantitatively analyzed by image histograms from the ImageJ program, as presented in Figure 3. The histogram of the non-sterilized collagen hydrolysate exhibited a normal distribution of pixel intensities, indicating a homogeneous appearance. However, the histogram of the ethylene-oxide-sterilized sample showed a significant shift toward darker pixel intensities, confirming the visual observation of a darkened/burnt zone of the specimen. The histograms of both the beta- and gamma-radiation-sterilized samples almost resembled that of the non-sterilized sample, suggesting minimal impact on the physical appearance of the collagen hydrolysate.

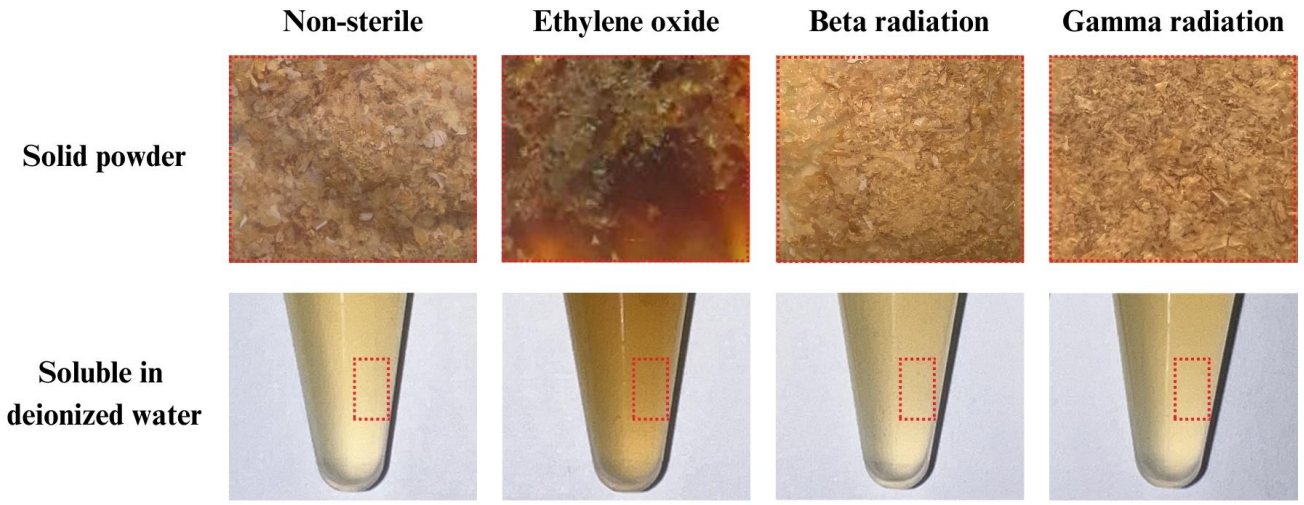


Figure 2. Physical characteristics of collagen hydrolysate after different methods of sterilization image. The red frame was used as the outer edge where the tip of the arrow cross cursor met for measurement accuracy.

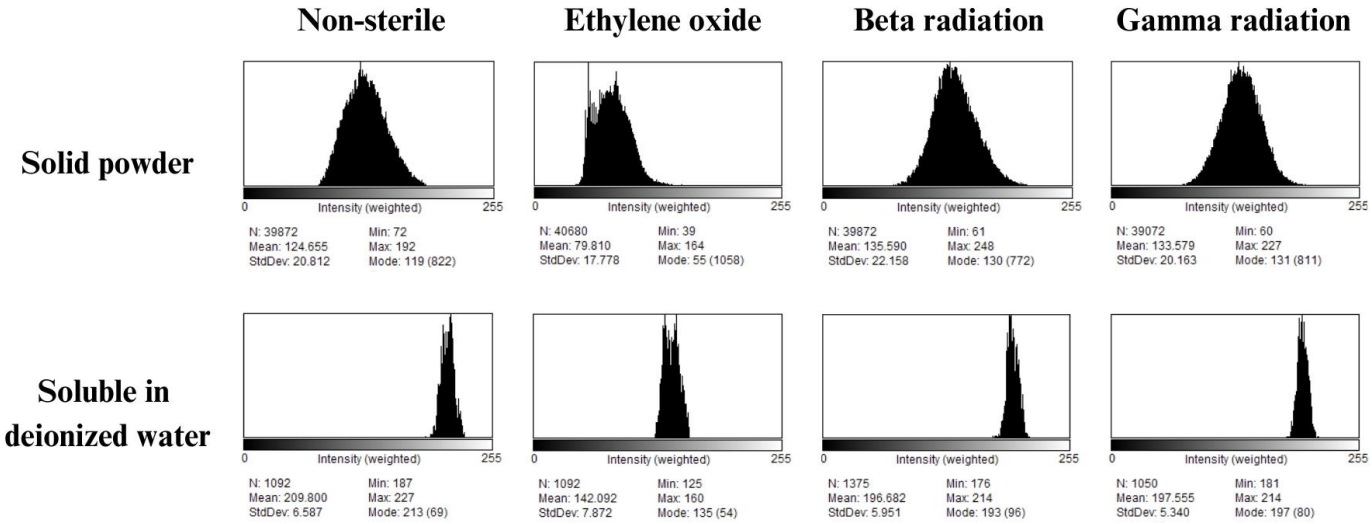


Figure 3. Image histogram of collagen hydrolysate before and after different sterilization methods obtained from ImageJ analysis.

These findings demonstrate that the choice of sterilization method substantially influences the physical appearance of collagen hydrolysate. Ethylene oxide sterilization, likely due to the high temperatures involved, causes significant changes in color and texture, potentially affecting the material's properties and applications. In contrast, the beta and gamma radiation sterilization methods preserve the original physical appearance of the collagen hydrolysates, making them more suitable for maintaining the material's integrity.

3.2. NMR Characterization of the Chemical Structure of Collagen Hydrolysate before and after Sterilization

The ^{1}H-NMR spectral analysis from Figure 4 indicates visible changes in the chemical structure of collagen hydrolysate after sterilization. It was found that sterilization with beta and gamma radiation at a dose of 17.5 kGy did not change the chemical structure of collagen hydrolysate. This means that the energy of beta and gamma particles does not cause molecular fragmentation or change. In contrast, ethylene oxide sterilization showed pronounced changes and new peaks in the 2–3 ppm range, indicating changes in the methylene and methyl groups [17]. This could be caused by breakdown products

or the crosslinking of protein chains, with a slightly more pronounced peak shift. This may indicate chemicals forming between ethylene oxide and amino acids in the collagen hydrolysate [10,18–22].

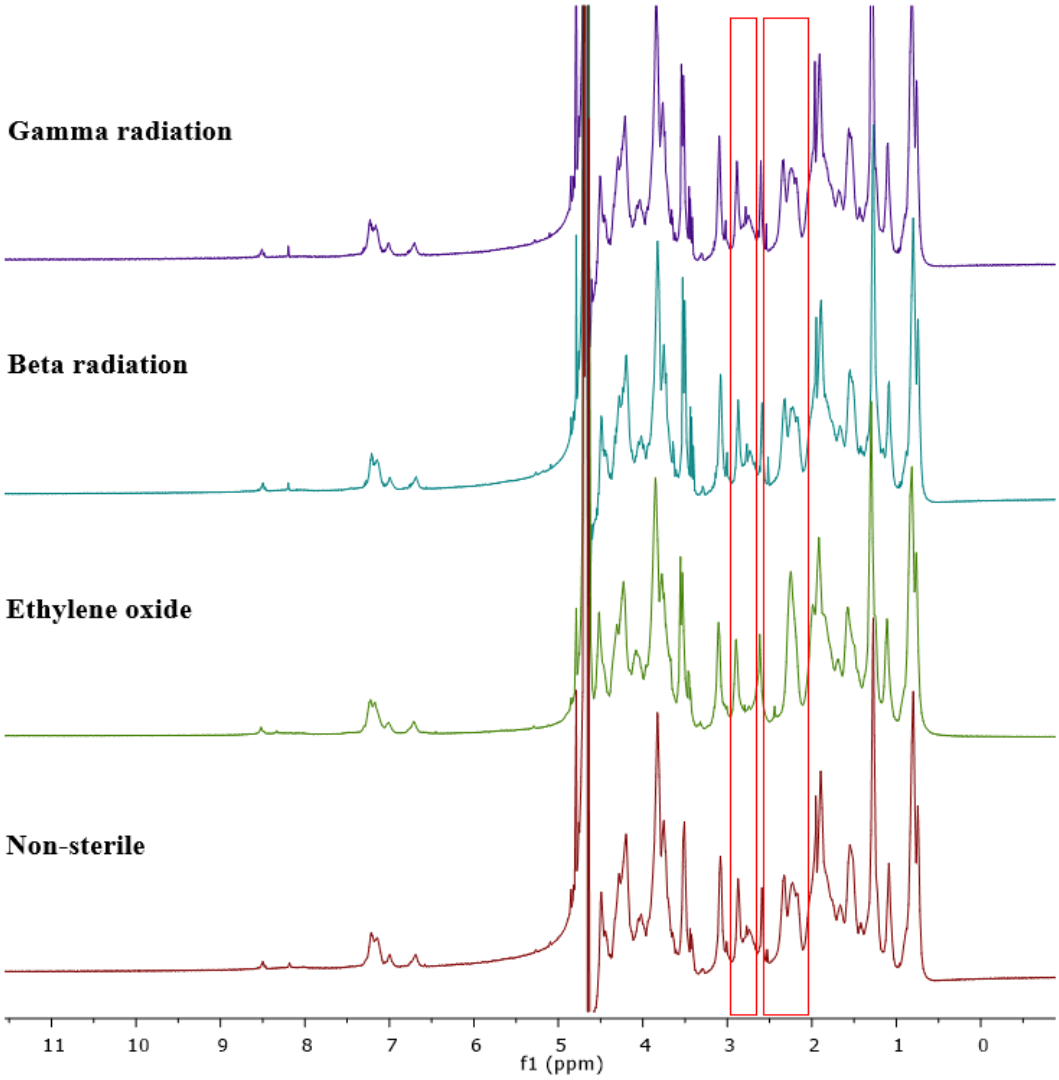


Figure 4. ^{1}H-NMR spectra of the chemical structure of collagen hydrolysate before and after sterilization. The red frame indicats the position of the peak that had shifted and/or changed.

The ^{1}H-NMR technique clarified the effect of sterilization on the chemical structure of collagen hydrolysate. Sterilization with ethylene oxide appears to have a marked effect on the secondary structure of the protein [23]. This can be seen from the change in the chemical shift in the aliphatic region. This includes protons attached to carbon atoms in the protein's backbone and side chains. The use of high heat during sterilization can lead to molecular damage, causing more significant structural changes compared to beta and gamma radiation [20,24,25]. A few small peaks can be observed, indicating the potential to preserve the original collagen structure. These changes in molecular structure can profoundly affect collagen hydrolysate's physical and biological properties, including its solubility, gelling properties, and interactions with cells and tissues [19–21,26]. Therefore, the selection of a sterilization method for protein-based biomaterials has to consider these structural changes to preserve the original characteristics of the material.

The ^{13}C-NMR spectral analysis of collagen hydrolysate after various sterilization methods is shown in Figure 5. It presents a remarkable change in chemical structure; focusing specifically on the regions around 180 ppm and 31–33 ppm for samples exposed

to beta and gamma radiation, it did not show any significant peaks on the spectrum, indicating that the sterilization process did not alter the methylene groups in the aliphatic chains of the collagen and therefore gave rise to the same results as the control group that was not sterilized. This is in contrast to ethylene oxide sterilization. The sample displayed a peak at 180 ppm, indicating oxidative changes, and a peak in the 31–33 ppm range. This illustrates the impact of ethylene oxide sterilization on the structure of collagen hydrolysate, as EtO reacts with NH_2, potentially causing changes in the chemical structure or generating significant heat, which leads to protein aggregation [10,18–22].

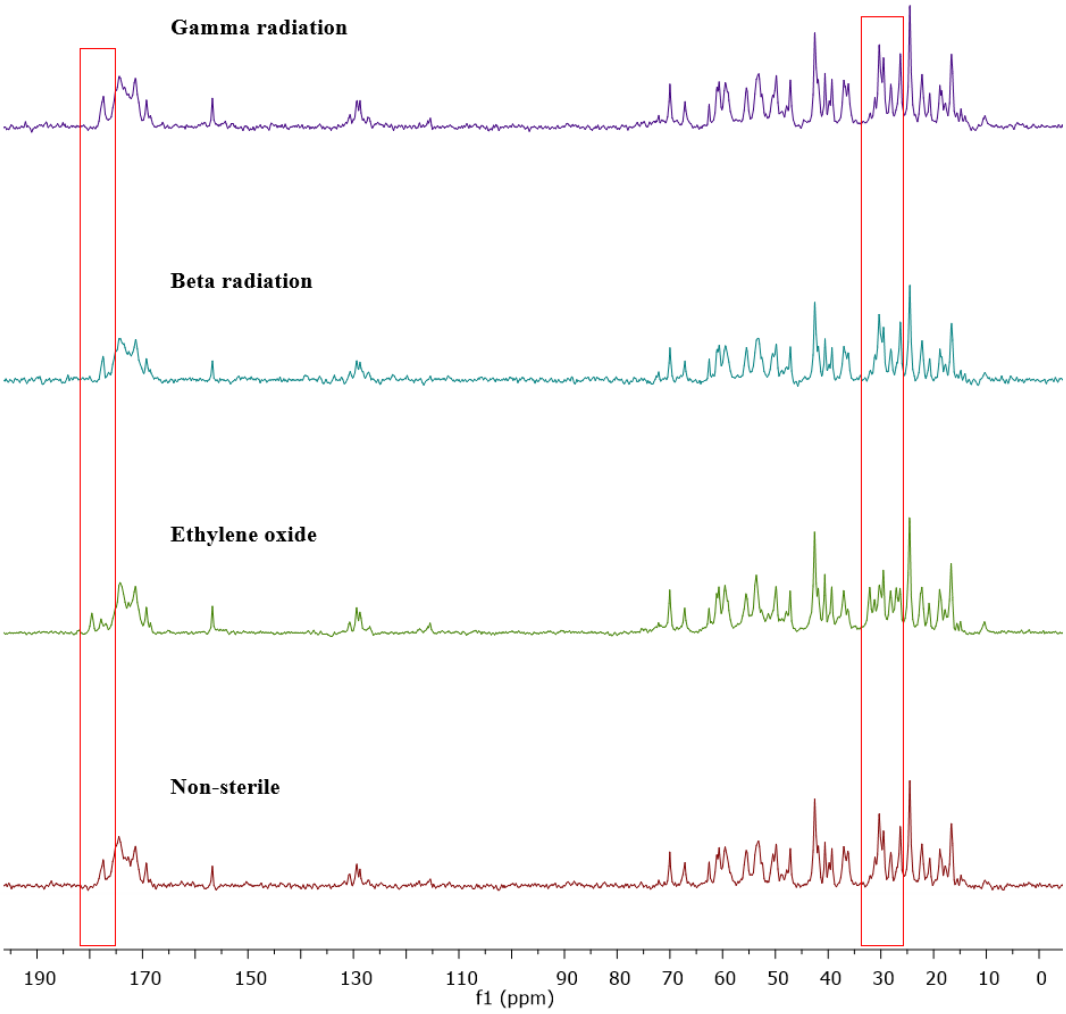


Figure 5. The spectral results of ^{13}C-NMR technique for examining the chemical structure of collagen hydrolysates before and after sterilization. The red frame indicats the position of the peak that had shifted and/or changed.

3.3. MALDI-TOF-MS Characterization of the Molecular Weight of Collagen Hydrolysate before and after Sterilization

MALDI-TOF-MS was employed to investigate the potential changes in the structure and composition of the collagen hydrolysate that were induced by different sterilization processes. Figure 6 shows the mass spectra of collagen hydrolysate when using MALDI-TOF mass spectrometry techniques to examine the molecular weight of the collagen hydrolysate before and after sterilization. The sums of the m/z values of collagen hydrolysate before and after sterilization using various techniques are listed in Table 1.

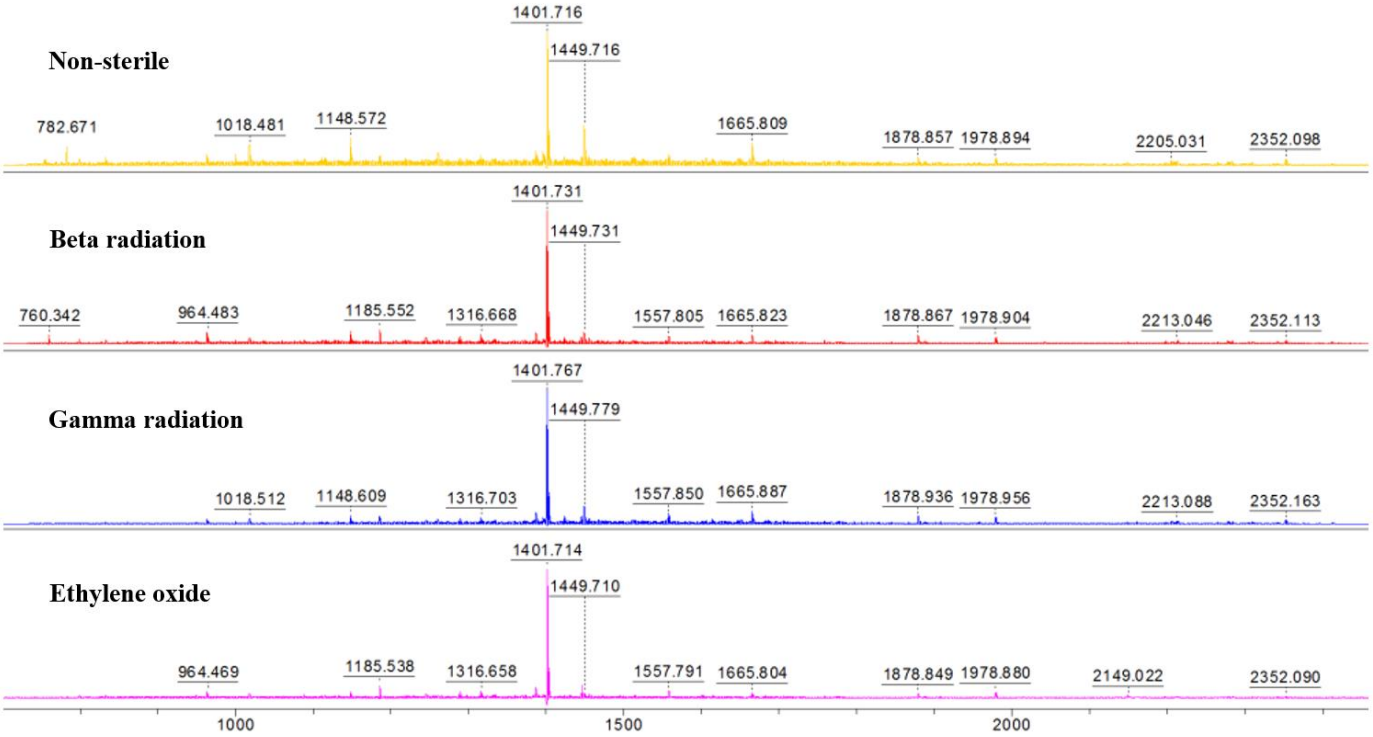


Figure 6. Mass spectra of the MALDI-TOF mass spectrometry technique for examining the molecular weight of collagen hydrolysate before and after sterilization.

Table 1. The sums of the m/z values of collagen hydrolysate before and after sterilization using various techniques.

Sample	m/z	% Difference from the control group
Non-sterile	782.671 + 1018.481 + 1148.572 + 1401.716 + 1449.716 + 1665.809 + 1878.857 + 1978.894 + 2205.031 + 2352.098 = 15,881.845	
Beta	760.342 + 964.483 + 1185.552 + 1316.668 + 1401.731 + 1449.731 + 1557.805 + 1665.823 + 1878.867 + 1978.904 + 2213.046 + 2352.113 = 15,725.065	−0.987%
Gamma	1018.512 + 1148.609 + 1316.703 + 1401.767 + 1449.779 + 1557.850 + 1665.887 + 1878.936 + 1978.956 + 2213.088 + 2352.163 = 15,981.250	+0.626%
Ethylene oxide	964.469 + 1185.538 + 1316.658 + 1401.714 + 1449.710 + 1557.791 + 1665.804 + 1878.849 + 1978.880 + 2149.022 + 2352.090 = 15,900.525	+0.118%

The molecular weight profiles of collagen hydrolysate from tuna tendon were analyzed using MALDI-TOF mass spectrometry and the results are shown in Figure 6, which reveals the effects of various sterilization methods on molecular structure. The mass-to-charge ratio (m/z) was used as an indicator of approximate molecular weight [27]. While initial analysis showed a maximum peak at 1401 Da across all sample groups, in-depth analysis using Equation (1) to calculate the percentage difference in total molecular weight for each sample group compared to the control or non-sterilized group in Table 1 demonstrated subtle changes. Beta radiation resulted in a 0.987% decrease in the total molecular weight, indicating partial degradation of amino acids in some protein structures [10,28,29]. Conversely, gamma radiation and ethylene oxide treatment slightly increased the total molecular weight by 0.626% and 0.118%, respectively, possibly due to crosslinking, molecular aggregation, or residual EtO particles [10,19–22,28–30]. These minor alterations suggest that the backbone structure of collagen hydrolysate was not significantly affected by the sterilization processes in this study. However, to confirm safety for medical applications or consumption, additional cytotoxicity testing is necessary. Furthermore, if radiation

sterilization methods are used, consideration should be given to reducing the radiation dosage to minimize impacts on molecular structure while being mindful of the potential reductions in antimicrobial efficacy. Further research into the relationship between radiation dosage and sterilization efficiency is crucial to determining an optimal balance. This study demonstrates that while sterilization methods do affect the molecular structure of collagen hydrolysate to some extent, these impacts are limited and may not significantly compromise the material's biological properties.

3.4. Characterization of Collagen Hydrolysate before and after Sterilization by DSC

The examination of the thermal properties of collagen hydrolysate using DSC, as depicted in Figure 7, revealed the distinct characteristics of its heat absorption profile. The DSC thermogram displayed a heat absorption peak within the 30–90 °C range. Notably, the heat absorption peak for collagen hydrolysate remains at 40 °C both before and after sterilization with ethylene oxide. However, when collagen hydrolysate is sterilized using beta and gamma radiation, a peak is shifted to approximately 50 °C. This temperature shift corresponds to the findings in prior studies [31], suggesting that the highest peak temperature indicates the material's melting point. In the temperature range 175–200 °C, an endothermic peak was observed after sterilization, which resulted in the peak shifting to higher temperatures. The group sterilized with ethylene oxide had a peak at 185 °C, the group sterilized with beta radiation had a peak at 205 °C, and finally, the group sterilized with gamma radiation had a peak at 196 °C. These peaks are caused by the degradation of polypeptide chains and the evaporation of residual water and/or tightly bound water. The different sterilization methods may cause changes in the peak position compared to the original. Both beta and gamma irradiation can lead to the formation of radicals, which can cause the oxidation and aggregation of proteins. Ethylene oxide sterilization, which uses high temperatures, may result in the melting of collagen hydrolysate and, upon cooling, the formation of densely packed crystals with a higher melting point. These factors contribute to the requirement of higher temperatures for the degradation of polypeptide chains. Additionally, these peaks suggest a correlation with the material's water solubility.

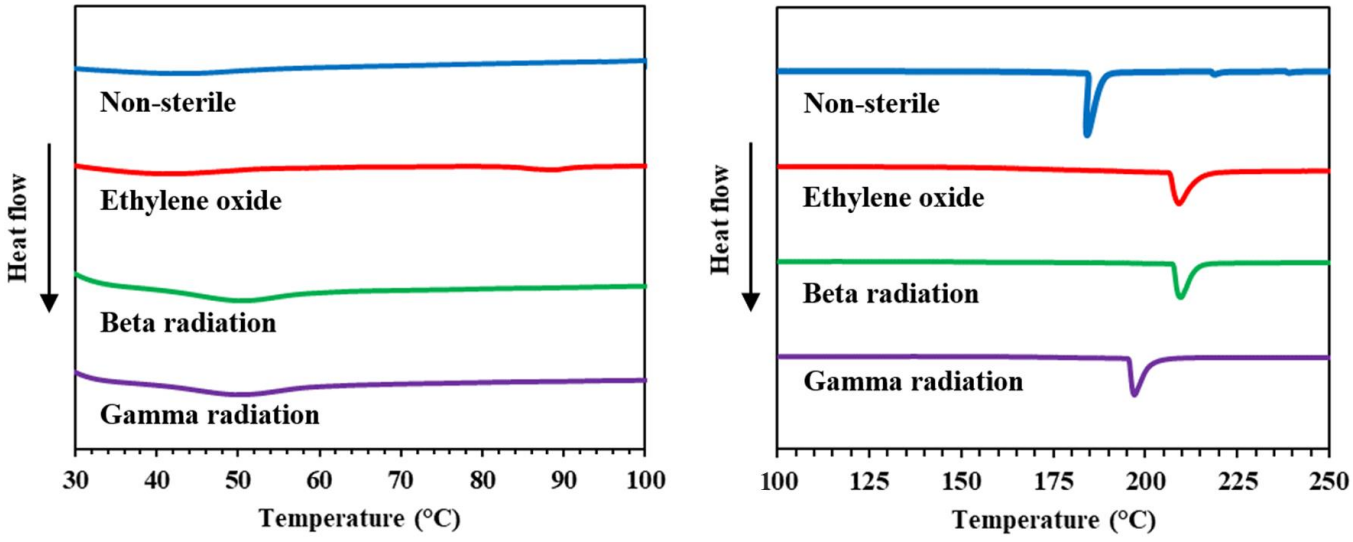


Figure 7. DSC thermograms of collagen hydrolysate samples before sterilization and after sterilization.

4. Conclusions

From the results, it can be concluded that EtO causes significant physical and chemical changes, while beta and gamma irradiation have less impact on the protein structure. This finding has implications for the development of sterilized collagen hydrolysates and other protein-based materials for applications such as their use in biomaterials and drug delivery systems, suggesting that beta and gamma irradiation should be used to minimize

protein structure modifications. However, this study has limitations, such as the use of alternative measurement tools. Furthermore, future research should include cell toxicity testing and biocompatibility evaluation in animal models to ensure safety and efficacy before consumption or use, as even small changes in structure or chemical composition may adversely affect the biological properties of these materials.

Author Contributions: Conceptualization, K.P. and N.T.; Data curation, K.P. and N.T.; Formal analysis, K.P., P.J., S.B. and N.T.; Funding acquisition, S.W., P.R. and N.T.; Investigation, K.P., P.J. and N.T.; Methodology, P.J. and N.T.; Project administration, S.W. and N.T.; Resources, N.S., S.W. and T.T.; Software, P.J. and N.T.; Supervision, S.B., S.W., K.J., P.R., T.T. and N.T.; Validation, K.P., P.J., N.S., S.B., S.W., K.J., P.R., T.T. and N.T.; Visualization, K.P. and P.J.; Writing—original draft, K.P. and N.T.; Writing—review and editing, K.P., S.B. and N.T. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflicts of interest.

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