This study was carried out to determine the impact of the HACCP control system on the safety of the final products of collagen hydrolysate production. The object of the study was equine connective tissue. Using the FMEA model, established by a three-factor assessment of the risk priority number (RPN), critical control points (CCP) in the processes of hydrolysis, inactivation of the enzyme preparation, drying and storage were identified. For two CCP, measures for continuous monitoring were identified, and critical limits were developed. For CCT 1, the calculation of optimal fermentation processes using a mathematical model for the hydrolysis of raw materials is given. The optimal values of the Neutrase enzyme, providing a maximum content of water-soluble proteins of 55.0 mg/cm², were determined: T=37 °C, dosage 5 Pa/g, t=210 min.

For CCP 2, to avoid protein denaturation during hydrolysis, a critical limit was developed by determining the heat inactivation point and optimum temperature. Experimental analyses show that the inactivation point of the Neutrase enzyme, estimated by the rate of FTN accumulation, which has 20 % at 60 °C, is reached at the 11th minute.

As a result of the study, the effect of enzyme preparations on the safety of collagen hydrolysate was also determined. The result confirms that the Neutrase enzyme preparation had a positive effect on all safety indicators compared to the Trypsin enzyme. The optimal parameters for reducing microbiological indicators, pesticides, antibiotic and toxic metals are: T=40 °C, duration 210 min, dosage of the Neutrase enzyme 5 units/Pa.

The results can be used in collagen hydrolysate production to better ensure the quality and safety of the final product.

**Keywords:** collagen hydrolysate safety, HACCP, CCP, critical limit, FMEA, enzymatic hydrolysis

### 1. Introduction

With increasing globalization, there is a trend towards the production, distribution and consumption of food around the world, which makes food safety a topical issue for the health of the global consumer [1]. Ensuring food safety to protect public health, as well as promoting the economic development of processing industries, remains a major challenge in both developing and developed countries [2].

The food industry worldwide uses the HACCP (Hazard Analysis and Critical Control Points) principles as a preventive approach to food safety. In 2005, the International Organization for Standardization (ISO) issued ISO 22000, which incorporated HACCP into its food safety control system [3].

The HACCP system provides control at all stages of the food chain, at any point of production, storage and sale of products, where hazards may arise. Particular attention is paid to critical control points, where all types of risk can be prevented to an acceptable level as a result of targeted control measures [4].

Collagen is the most common animal protein, accounting for about 30 % of the total. This protein is involved in forming fibrillar and microfibrillar networks of the extracellular matrix and basement membranes. Fibrillar proteins are the main protein components of bones, cartilage, tendons, skin, and other forms of connective tissue [5].

Over the past decades, many reviews have summarized various uses of collagen as a biomaterial due to the important structure-forming function. In the food industry, collagen and its hydrolysate are used to produce food films, various health drinks, as well as a structure former for canned food, etc. [6].
The chemical, physical, biological, immunological or toxicological properties of the collagen material may vary depending on the starting material and treatment [7].

Safety includes biological, chemical and physical factors. The entire technological process cannot be fully controlled, since this requires significant resources. The control must be carried out at certain production stages, which are critical to prevent a hazard or reduce it to an acceptable level. It should be emphasized that the HACCP concept is aimed at preventing possible violations at each stage of foodstuff production (in this case, collagen hydrolysate), rather than detecting low-quality products and rejecting them at the end of the technological cycle. This not only guarantees food safety, but also increases production competitiveness by reducing the cost of control measures and increasing the investment attractiveness of the industry [8].

It is important to keep in mind that the most difficult step in creating a HACCP system is selecting hazardous factors to consider. This is due to a large number of these factors, insufficient awareness of specialists, the expert nature of choosing hazards, as well as chaos in process flowsheets at the enterprise. As a result, it is relevant to study hazard risks, determine CCP and study critical limits for producing a safe product [9]. With the occurrence of a hazard in the CCP, there is a scientifically grounded confidence in the loss of product safety and negative consequences for the production cycle.

When determining critical control points (CCP), GOST R 51705.1-2001 recommends using the “decision tree” method [10]. However, this solution method divides boundaries with certain limitations, which is why the decision tree is inferior to other methods in terms of classification quality.

One of the most important aspects of veterinary medicine is the safety of raw meat, processed products and preventing the intake of toxic substances. Analysis of normative and technical documentation (standards, specifications) shows that strict compliance with the requirements of these documents can guarantee the quality and safety of the product (raw materials or semi-finished products) only in a limited part of the process chain. To ensure the absolute safety and quality of products, a systematic approach is needed, that is, continuous control, monitoring and adjustment of relevant parameters throughout the entire process chain. Optimization of the HACCP system using the FMEA model in collagen hydrolysate production ensures the safety and quality of raw materials, identifying critical control points and taking corrective measures. The results of such studies can be used in collagen hydrolysate production to better ensure the quality and safety of the final product.

2. Literature review and problem statement

Any food enterprise, including meat processing, faces a problem of rational use of secondary raw materials and production waste. According to official statistics, in the meat processing industry, livestock processing products for food purposes account for 64 % of live weight, fodder – 12 %; for technical products – 10 %. The remaining 14 % are unclaimed [11].

Hydrolyzed collagen can be obtained from agri-food waste (bones, tendons and skin). Processing these by-products can help reduce the pollution by these types of waste, turning them into a new product with high functional value. Low molecular weight peptides can be used in food systems, for example, collagen hydrolysate drinks show great advantages of easy digestion, high assimilation (about 80 %) and good absorption at the intestinal level. It would be important to develop collagen hydrolysate drinks, given that protein ones will have functional properties for consumption by most people due to zero toxicity and zero allergen [12].

Collagen is widely used in the world medical, food and cosmetology practice, while more than 50,000 tons of collagen are used annually for therapeutic purposes [13].

Loss of collagen in the human body begins at the age of 18–29 years, after 40 years, the human body can lose about 1 % a year, and at about 80 years, collagen production may decrease by 75 % compared to young adults [14].

There are other factors contributing to this, such as free radicals, malnutrition, smoking, alcoholism and disease. The role of collagen in the body is very important, because this protein helps the development of organs, healing of wounds and tissues, restoration of the cornea, gums and scalp. Collagen helps in the repair of bones and blood vessels. Also, this connective tissue is present in bones, tendons, ligaments, hair, skin and muscles [15].

Oral collagen supplements have become popular over the past decades. They are often claimed by consumers as anti-aging products. Oral collagen hydrolysate supplements improve skin physiology and appearance by increasing skin hydration, elasticity, firmness and rejuvenation [16].

Collagen peptides can be used in functional food supplements [17].

Also, in food science, collagen hydrolysate helps to minimize or prevent damage to cells and tissues when stored in a freezer, so it can be used in foods requiring low-temperature storage [18].

The work [19] presents the results of studies on collagen hydrolysate production from cattle tendons. Purification of collagen ballast impurities, targeted modification and sorption capacity of collagen hydrolysate have been investigated. But the issues of product safety remain unresolved.

The work [19] presents the results of studies on biotechnological treatment of collagen-containing raw materials (cattle rumen) to create functional food. It is noted that rumen bio-modification improves its organoepic properties and texture, which becomes juicy, soft and elastic. But the issues of product safety remain unresolved.

There is also the work devoted to the production of meat bread with the replacement of raw meat with a protein product, but not the protein product itself (collagen hydrolysate).

This approach was used in [20], but determining hazards and CCP using a decision tree is considered laborious [21]. All this suggests it advisable to conduct a study on the implementation of HACCP in collagen production using the FMEA model to determine CCP.

However, there is very little information regarding the use of food supplements based on collagen-containing raw materials, reflecting the relationship between production methods, structure and functionality of collagen ingredients and collagen hydrolysate safety. In the study of hazards, risk factors such as biological, physical and chemical were investigated, given that connective protein is part of meat [22].

Risk factors for meat and meat products are not only microbiological risks (food poisoning, spoilage), but also residual amounts of chemicals.
Based on monitoring studies conducted in the EU, the main priority pollutants for meat have been identified and ranked as follows:
- dioxins and dioxin-like polychlorinated biphenyls;
- chloramphenicol and nitrofurans;
- chemical elements – cadmium, lead and mercury.

Dioxins and dioxin-like polychlorinated biphenyls have been identified as having high potential due to their bioaccumulation ability in the food chain, the risk of exceeding limit levels, and toxicological profile. Chloramphenicol and nitrofurans have been rated as having medium risk as the results showed toxicity to humans. The chemical elements – cadmium, lead and mercury – have been identified as having medium risk, taking into account the number of nonconforming results and their toxicological characteristics [24].

A complex arrangement of collagen fibers undergoes continuous support, which requires the catalytic action of enzymes [25].

The above information indicates that numerous studies are being carried out to develop a technology for producing collagen hydrolysate for functional use. However, taking into account all the data presented, it should be noted that there is a shortage of theoretically sound effective safety solutions in the field of deep processing of collagen-containing raw materials. Therefore, it is advisable to obtain solutions for optimizing the HACCP system using the FMEA method in collagen production for:
- detailed analysis of risks associated with a decrease in product safety and quality during processing;
- determination of critical control points of the process;
- establishment of permissible deviations of operations from the norm;
- timely application of corrective actions aimed at neutralizing adverse effects.

3. The aim and objectives of the study

The aim of the study is to optimize the HACCP safety control system for the production of collagen hydrolysate using the FMEA model.

To achieve the aim, the following objectives were set:
- to determine the CCP of collagen hydrolysate production applying FMEA models using Microsoft Access software;
- to develop a critical limit for each identified CCP and investigate safety indicators of collagen hydrolysate production.

4. Materials and methods for Hazard Analysis and Critical Control Points planning, analysis of critical control points, critical limits and product safety studies

4.1. Research materials

Collagen hydrolysate can be extracted from various sources and connective tissues [26] using various enzymes. To isolate collagen substances, equine connective tissue was taken from the tendon. 100 g of pre-purified, frozen tendons were taken and soaked in alkaline peroxide solution 140 ml (7(1 % NaOH); 60 ml 3(3 %, H₂O₂) v/v) with a 1:5 water duty for 2 hours with constant stirring. The resulting solution was filtered through a metal sieve. The solid residue was repeatedly washed with distilled water until neutral pH. Next, the collagen mass was subjected to enzymatic hydrolysis with a 0.125 % Trypsin and Neutrase solution for 210 min. After the hydrolysis, the enzyme was deactivated, then the solution was decanted passing through a metal sieve. After that, the collagen mass was precipitated with NaCl to a final concentration of 0.9 M. The resulting collagen hydrolysate was poured into metal molds and dried.

To assess the potential of enzyme preparations in the production of collagen substances, Trypsin and Neutrase preparations were used.

To determine the CCP, the risk factors for each process were studied. To solve this problem, the FMEA model was applied. After determining the CCP, studies were carried out to develop a critical limit for each CCP. The critical limit developed for each process should minimize the risk [29].

Safety indicators of collagen hydrolysate production were determined. Based on the results of the studies, conclusions were made about the possibility of using enzyme preparations in the production of equine collagen hydrolysate, as well as their effect on the safety and quality of the finished product.

4.2. Methods of study of critical control points and critical limit in collagen hydrolysate production

To determine the CCP in the production of collagen hydrolysate, the FMEA model using Microsoft Access software (USA) was applied. Process failure mode and effects analysis (FMEA) is the method that aims to improve the process by analyzing potential process nonconformities with a quantitative analysis of the consequences and causes of nonconformities [30].

In the development of the critical limit for each CCP, the following methods were used:
- mass fraction of protein according to the Kjeldahl method using an automated incinerator and distillation apparatus [31];
- modeling and processing of experimental data using Statistica 10 (StatSoft, Inc.);
- pesticide content in the finished products was determined by atomic absorption spectroscopy (AAS) on a “KVANT-Z.ETA-T” electric atomization spectrometer [32];
- microbiological analyses of collagen hydrolysate were carried out by classical methods: sampling and preparing samples for microbiological analyses [34], culturing microorganisms [35].
- organoleptic evaluation of the finished products was carried out in accordance with GOST 9959-91 [36].

5. Results of the study of the critical control point, critical limit and safety indicators of collagen hydrolysate

5.1. Results of determining the critical control point of collagen hydrolysate

Determining the CCP includes a risk hazard assessment. Risk assessment is intended to assess the probability of the presence of the earlier revealed and described hazard in the product and the severity of the consequences of this fact for human health. First of all, information about the product composition is studied. Whether the hazard identified at
Technology and equipment of food production

The first stage of the risk analysis is present in the product, whether it can be present in the components of the product and packaging.

To identify hazards, risks to collagen were studied, given that collagen is the connective tissue of the tendon, the risks related to meat and meat products were investigated [37].

The FMEA method was adapted taking into account the specifics of collagen hydrolysate production. The difference of the technology of meat products from most production processes of other products lies in the multifactor and multi-causal flow of the processes of ensuring product safety [38]. Given the characteristics of raw materials, it can be stated that many biological and chemical hazards are characteristic of meat products. In particular, hazards may arise in technological operations (introduction, inactivation of enzymes, etc.). Thus, technology analysis is one of the key processes in risk assessment. At the first stage of analysis, it is necessary to determine the scope of the FMEA model. Further, based on various processing techniques [39], the process of collagen hydrolysate production is formalized. When determining the degree of detail, it is necessary to identify the critical technological stages affecting the considered hazard. After a qualitative and quantitative assessment of the dynamics of hazard changes, it is necessary to estimate the contribution of each technological stage in the process of risk realization. For these purposes, a three-factor assessment of the risk priority number (RPN) was used. The risk priority number was calculated using the following formula [39]:

\[
RPN = S \times O \times D,
\]

Where \(RPN\) is the risk priority number; \(S\) is the severity of consequences; \(O\) is the probability of occurrence; \(D\) is detectability.

Collagen analysis was performed based on the FMEA model. First of all, the FMEA analysis of collagen must comply with the basic principles of the FMEA model. It is necessary to combine collagen characteristics using the FMEA model, which mainly include functional requirements for meat products. The modes of possible functional and potential failures, the causes and mechanisms of potential failures, the risk priority number, improvement of the measure and other elements are also considered, as shown in Table 1.

### Criteria for assessing the severity of consequences "S" [39]

<table>
<thead>
<tr>
<th>Description for severity of consequences</th>
<th>Rank value</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hazardous component is present in the product, exceeds standard values, leads to serious health consequences</td>
<td>Fully complies</td>
<td>9–10</td>
</tr>
<tr>
<td>Hazardous component is present in the product and slightly exceeds standard values, which does not lead to serious health consequences</td>
<td>Partially complies</td>
<td>7–8</td>
</tr>
<tr>
<td>Hazardous component in the product is at the limit of the established standards</td>
<td>Partially complies</td>
<td>5–6</td>
</tr>
<tr>
<td>Hazardous component is present in the finished product, but does not exceed the established standard values (if no standards, it does not harm health in estimated concentrations)</td>
<td>Partially complies</td>
<td>3–4</td>
</tr>
<tr>
<td>Hazardous component is not present in the finished product</td>
<td>Fully complies</td>
<td>1–2</td>
</tr>
</tbody>
</table>

The severity index characterizes the impact of the identified hazard on human health. To a greater extent, this parameter indicates the presence of a hazardous substance in the finished product.

To assess the probability of occurrence, a ten-point rating scale was used, presented in Table 2 [39]. To assess these characteristics, it is necessary to use available statistics (or, if not, conduct additional monitoring) for each specific process of collagen hydrolysate production.

### Table 2

<table>
<thead>
<tr>
<th>Criteria for assessing the probability of occurrence &quot;O&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description for the probability of occurrence</td>
</tr>
<tr>
<td>Almost inevitable (O=10–50)</td>
</tr>
<tr>
<td>Occurs over and over (O=5–10)</td>
</tr>
<tr>
<td>Sometimes occurs (O=2–5)</td>
</tr>
<tr>
<td>Relatively rare (O=1–2)</td>
</tr>
<tr>
<td>Very low (O&lt;less than 1)</td>
</tr>
</tbody>
</table>

The third evaluation characteristic is the detectability of a hazard at a specific process stage. For this, a ten-point scale for assessing detectability was used, presented in Table 3 [39].

### Table 3

<table>
<thead>
<tr>
<th>Criterion for assessing detectability &quot;D&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detectability of nonconformity</td>
</tr>
<tr>
<td>Detectability methods are not available</td>
</tr>
<tr>
<td>Detection methods are available, but control is impossible at this stage or the indicator is not regulated by law</td>
</tr>
<tr>
<td>Detection methods are available, but control is not carried out at this stage</td>
</tr>
<tr>
<td>Hazards can be detected at this stage, methods are available</td>
</tr>
<tr>
<td>The stage is designed to detect the hazard, necessary methods are available</td>
</tr>
</tbody>
</table>

Applying these criteria in collagen-containing raw materials and in collagen, it is necessary to detect the identified hazard at a specific production stage.

Ranking of the risk priority number is performed for each stage of collagen hydrolysate production described in Table 4. Using the obtained values, in quantitative terms, it is possible to estimate the contribution of each stage to the probability of realization of the considered risk. It is also possible to identify critical technologies in the implementation and propagation of hazards, using the total value of the risk priority number for several technologies. The information obtained is used at the next stage, when formulating the probabilistic characteristics of the risk.

The occurrence of any kind of potential risk affects product quality, as well as consumer satisfaction and reduces product safety [40].

After calculating the final score for each block, the results were analyzed to assess the risk to food safety. The risk was calculated for each process using FMEA analysis. The risk was calculated as a ratio of three factors: significance of audit criteria for food safety, reflecting the severity of consequences of nonconformities (S), frequency of nonconformities based on audit evidence (O) and detectability of nonconformities (D). Each factor was assigned a value ranging from 1 to 10 and a relative risk index (R) was calculated, presented by a simple equation.


Analysis of hazards in collagen hydrolysate production and CCP determination

<table>
<thead>
<tr>
<th>Process stage</th>
<th>Consequences of possible defects</th>
<th>S</th>
<th>Mechanism of potential defect</th>
<th>O</th>
<th>Current control process</th>
<th>D</th>
<th>RPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acceptance</td>
<td>Tendons are contaminated with hormones, diseases, veterinary drugs, etc.</td>
<td>6</td>
<td>Food may contain hormones, etc. Foreign inclusions in feedstock can injure consumers. Affects the functional state of the body</td>
<td>4</td>
<td>Documentation control, feedstock control upon acceptance Visual inspection, Sanitary condition of the vehicle</td>
<td>6</td>
<td>144</td>
</tr>
<tr>
<td>Washing in running water</td>
<td>Microbes and pathogenic bacteria contaminate meat products</td>
<td>7</td>
<td>Untreated tools, cross-contamination of operators and transfer of microorganisms by employees Biological hazards can lead to product spoilage and food poisoning</td>
<td>5</td>
<td>Avoid workers and customers who have direct contact with meat; implement operational preliminary programs Thorough timely disinfection of tools</td>
<td>4</td>
<td>140</td>
</tr>
<tr>
<td>Grinding</td>
<td>Contamination of equipment with disinfectants and chemicals</td>
<td>5</td>
<td>Excess amounts of these substances can lead to consumer poisoning, violation of the functional state of the body</td>
<td>5</td>
<td>Programs of mandatory preliminary measures for cleaning and sanitation of equipment</td>
<td>5</td>
<td>125</td>
</tr>
<tr>
<td>Collagen hydrolysis</td>
<td>Excessive dosage of hydrolyzing agents</td>
<td>7</td>
<td>Incomplete protein modification, hydrolysate safety during hydrolysis</td>
<td>7</td>
<td>Temperature and time control</td>
<td>5</td>
<td>245</td>
</tr>
<tr>
<td>Enzyme inactivation</td>
<td>Active enzyme, protein denaturation</td>
<td>7</td>
<td>Active enzymes in food can affect gastrointestinal walls</td>
<td>7</td>
<td>Temperature and time control</td>
<td>5</td>
<td>245</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>Biological: yeast, mold, CGB, pathogenic microorganisms, including Salmonella L. monocytogenes S. Aureus</td>
<td>5</td>
<td>Pathogenic microorganisms in the finished product can cause severe intestinal diseases</td>
<td>5</td>
<td>Maintenance and control of humidity, temperature and time</td>
<td>5</td>
<td>64</td>
</tr>
<tr>
<td>Drying</td>
<td>Biological: yeast, mold, CGB, pathogenic microorganisms, including Salmonella L. monocytogenes S. Aureus</td>
<td>6</td>
<td>Pathogenic microorganisms in the finished product can cause severe intestinal diseases</td>
<td>6</td>
<td>Maintenance and control of humidity, temperature and time</td>
<td>5</td>
<td>216</td>
</tr>
<tr>
<td>Packaging</td>
<td>Packaging contamination</td>
<td>6</td>
<td>Packaging materials contain harmful components, as well as mobility and diffusion capacity</td>
<td>5</td>
<td>Strict observance of technical standards and regulations for packaging materials</td>
<td>5</td>
<td>150</td>
</tr>
<tr>
<td>Storage, distribution and transportation</td>
<td>Product spoilage</td>
<td>6</td>
<td>Non-compliance with hygiene characteristics of storage site</td>
<td>6</td>
<td>Strict observance of storage temperature, period and site</td>
<td>6</td>
<td>216</td>
</tr>
</tbody>
</table>

The results of the risk assessment for each process were collected and calculated using the Access software. As can be seen from Table 4, the highest RPN was shown by the hydrolysis process – 294, then the enzyme inactivation process – 245, drying – 216 and storage process – 216. The program automatically detects the RPN and the production process with CCP is detected by the values of the numbers.

According to the results of the FMEA analysis of collagen hydrolysate (Table 4), possible options of risk of collagen hydrolysate with higher RPN values were identified. By combining and analyzing the relationship between these modes and each link, the critical control points were determined:

1) the process of purification of collagen-containing raw materials from ballast impurities and its modification;
2) the process of inactivation of the enzyme preparation;
3) drying process;
4) storage of the finished product.

Further, for HACCP planning, critical limits for CCP 1, 2 were developed.

5.2. Development of critical limits for critical control points of collagen hydrolysate production for two processes

Fitting CCP were determined, critical limits for CCP for two processes were developed. The critical limit is a criterion that separates acceptable and unacceptable values of the controlled quantity in relation to the above indicator organisms for the quality and safety of collagen hydrolysate [41].

The critical limit for CCP 1 was developed by constructing a mathematical model to determine the optimal hydrolysis time and enzyme dosage for the complete hydrolysis of protein fractions. The criterion for assessing the effect of time was protein accumulation during hydrolysis.

Using the obtained data on the optimal parameters of enzymes, an experiment was carried out on hydrolysate safety during production.

It is known [42] that the restriction of functional properties of collagen-containing raw materials and waste products of processing animal raw materials is associated with the spatial structure, insufficient solubility and high strength of proteins.

The practice of using enzyme preparations shows that not all enzymes are highly active, and when processing meat industry waste, they give the desired effect. This is due to the presence of proteins, which are the most difficult to break down by digestive enzymes, the main of which is collagen. One of the ways to solve this problem is to use proteolytic and collagenolytic enzymes [43].

Enzymes were selected based on the main parameters that directly affect the level of deproteinization of the protein-mineral substrate, inactivation temperature, and
specificity of the effect on the substrate. When developing a technology for isolating a protein hydrolysate by enzymatic hydrolysis, it is necessary to take into account the possible formation rate of peptides having pronounced surface-active properties. The use of enzymes during hydrolysis, depending on their concentration, significantly increases the rate of biochemical processes. When selecting an enzyme, it is also necessary to consider many additional factors: hydrolysis temperature, enzyme concentration in the solution, pH, water duty, etc. [43].

In this regard, the effectiveness of enzyme modification was studied in order to isolate and purify collagen fractions of proteins, as well as to assess production safety. At the next stage, the effects of the Trypsin and Neutrase enzyme preparations on equine connective tissue were investigated.

At the first stage of research, in accordance with the objectives, a critical limit of collagen hydrolysate was developed. To eliminate CCP 1, the critical limit is the reduction of microbiological and safety indicators by hydrolysis with enzyme preparations, investigating the optimal hydrolysis time and dosage.

**Determination of the optimal time and enzyme preparation dosage in the process of collagen hydrolysis.**

The mathematical problem of planning an experiment to determine the optimal dose of enzyme preparations for the hydrolysis of collagen hydrolysate is to solve the equation

\[ y = f(x_1, x_2, ..., x_n), \]

where \( y \) is the resultant, process outcome, i.e. optimization parameter; \( x_i \) is the factors that vary during the experiment.

We considered the regression equation of the quadratic model of the following form

\[ Y_i = \beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2}^2 + \ldots + \beta_n x_{in}^2 + \epsilon_i, \]

where \( \beta_0 \) is the shift, \( \beta_i \) is the linear effect coefficient, \( \beta_2 \) is the quadratic effect coefficient, \( \epsilon_i \) is the random error of the variable \( Y \) in the \( i \)-th observation.

In the process under study, the quadratic response function was chosen: WSP is the protein content, mg/cm²; AEP is the amount of enzyme preparation, Pa/g; \( t \) is the hydrolysis time, min

\[
\text{WSP} = -2.1 + 0.38t + 0.14AEP - 0.03t^2 + 0.02tAEP \quad \text{for Trypsin};
\]

\[
\text{WSP} = -2.09 - 0.49AEP + 0.19t + 0.24AEP^2 + 0.02tAEP \quad \text{for Neutrase}.
\]

As a result of carrying out the process for 210 min with a Trypsin amount of 4 Pa/g, the result was better than the previous ones — in terms of hydrolysis time, the optimal dose of Trypsin is 4 Pa/g (Fig. 1).

![3D surface: WSP – Water-soluble protein content mg/cm² using the Trypsin enzyme](image)

The graph in Fig. 2 shows the response surface of the Neutrase enzyme preparation.

Fig. 2 shows that the optimal values of the Neutrase enzyme are 5 Pa/g with a hydrolysis time of 210 min, providing a maximum content of water-soluble proteins of 55.0 mg/cm².

Having determined the optimal hydrolysis time and dosage, the microbiological parameters of collagen hydrolysate during hydrolysis were assessed. For this, enzymatic hydrolysis was carried out for 210 min at a temperature of 30 °C and 40 °C. To determine the product safety, the optimal temperature and time of hydrolysis were studied. In the process of hydrolysis, the safety indicators: microbiological, antibiotics, toxic elements, pesticides were measured every 30 min for compliance with standard documentation of TRCU 034/2013 Technical Regulations of the Customs Union on safety of meat and meat products.

The results of changes in the microbiological parameters of the studied samples during hydrolysis with the enzyme preparations Trypsin 4 units/Pa and Neutrase 5 units/Pa at 30 °C and 40 °C are shown in Table 5.

As a result of the studies, it was found that in the collagen hydrolysate sample at a temperature of 30 °C for 210 min with the Trypsin enzyme preparation, the TVC value showed 1.5*10⁴, yeast 1.43*10⁴. The results of hydrolysis with the Neutrase enzyme preparation for 210 min showed the TVC value of 1.5*10⁴, yeast 1.3*10³. At the same time, at 40 °C, in the hydrolysate sample using the Trypsin enzyme for 210 min, TVC showed 1.2*10⁴, yeast 1.5*10³. TVC during hydrolysis with the Neutrase enzyme preparation reached 1.0*10⁴. Meanwhile, the yeast level was 0.9*10³, which is normal and gives a better result than the Trypsin enzyme.

The quantitative change in chloramphenicol during hydrolysis is shown in Fig. 3.
The amount of chloramphenicol reached acceptable levels during hydrolysis with the Neutrase enzyme preparation at 40 °C. Table 6 shows changes in pesticide indices. According to the pesticide indices at 30 °C and 40 °C, during the hydrolysis with the Neutrase enzyme preparation for 210 min at 40 °C, the amount of HCH (α, β, γ) isomer and DDT and metabolites decreased to normal compared to hydrolysis at 30 °C.

Table 7 shows the data on toxic elements during hydrolysis. As a result of the studies, it was found (Table 7) that the level of toxic metals decreases during hydrolysis. The safety results of the samples given above comply with the safety requirements established by CU TR 021/2011 for all indicators. This shows that the studied sample of collagen hydrolysate using the Neutrase enzyme preparation showed optimal characteristics of product safety.

Table 5

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>Hydrolysis time, min</th>
<th>Microbiological index, CFU/g</th>
<th>Yeast, CFU/g</th>
</tr>
</thead>
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Fig. 2. Response surface describing the effect of preparation amount and hydrolysis time on protein content using the Neutrase enzyme preparation.

Fig. 3. Changes in chloramphenicol amount during hydrolysis, mg/kg.
The obtained result showed that the use of the Neutrase enzyme preparation effectively affects collagen modification, thereby confirming that the enzyme has high proteolytic activity in the hydrolysis of equine connective tissues. Neu-
trase also satisfactorily affects product safety. However, the optimal dosage of the Trypsin enzyme preparation is 1 units/Pa less than for Neutrase, but according to the results of the study, during the hydrolysis with the Neutrase enzyme preparation, optimally affected all product safety parame-
ters, reducing them to a minimum.

**Determination of optimal parameters for enzyme inactivation.**

Enzymes can be used in the food industry only if they are completely inactivated in the finished product, as they can affect gastrointestinal walls, which is unacceptable [45].

But when heated in an aqueous medium (up to 63–64 °C), collagen structure is deformed, the filaments bend, and their length is reduced to the original value. Heat exposure results in denaturation – violation of the bonds holding collagen in the native conformation, as well as partial hydrolytic decom-
position at the site of peptide bonds. Such collagen is called cooked. Cooked collagen (or gelatin) irreversibly loses its native physicochemical properties [46].

At above the optimum temperature, enzyme activity is sharply decreased, which is explained by enzyme protein denaturation. When the temperature drops below the optimum, enzyme activity decreases, and at temperatures below zero, enzyme activity is completely lost, but under these conditions the structure of the enzyme is not destroyed. Therefore, as the temperature rises, the enzyme completely stops its activity [47].

The duration of heat inactivation of the enzyme was determined based on the analysis of temperature effect on the activity of the Neutrase enzyme, estimated by the rate of FTN (formol-titrated nitrogen) accumulation during the inactiva-
tion process. The results of the experiment are shown in Fig. 4. A decrease in the rate of accumulation of hydroly-
sis products (protein) indicates enzyme inactivation. At a minimum temperature of 55 °C, the inactivation point of Neutrase is reached within 12 min, and at a maximum temperature of 60 °C, complete inactivation of Neutrase is reached within 11 min from the start of the process. At the 12th minute, protein accumulation remains at the same level as at the 11th minute, this shows that the enzyme inactivation point is reached at the 11th minute of the inactivation process (Fig. 4). Upon reaching temperatures of 60 °C, the activity of the enzyme preparation slows down and then completely stops, which leads to changes in the structure of the active center during thermal denaturation, as a result, the enzyme loses its effect on the substrate.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Trypsin</th>
<th>Neutrase</th>
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<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>Hydrolysis time, min</th>
<th>Toxic elements, mg/kg</th>
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<tr>
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</table>
Thus, the optimal time of enzyme inactivation in the hydrolysis of equine connective tissue is 11 min at 60 °C.

Taking into account all the data of the study: risk factor, critical control points, critical limits for CCP 1 and CCP 2, it is necessary to draw up a HACCP plan for collagen hydrolysate production.

6. Discussion of the results of HACCP planning for collagen production: CCP studies and critical limit development

The results of the CCP study applying the FMEA model using Microsoft Access software, compared to the traditional method of solving problems, showed (Table 4) that it is a fairly simple model that makes it possible to effectively affect the quality and safety of objects. The model is also highly efficient in creating competitive products in a short time and significantly saves time and money.

By applying the FMEA model using Microsoft Access software to optimize the HACCP system, the process CCP can be determined in the shortest possible time, which is one of the principles of HACCP planning.

After determining the CCP, a critical limit for two processes was developed.

Critical limits that were developed and scientifically validated are suitable to ensure adequate control of the measures selected for the CCP. Thus, the critical limits meet the “measurable” criteria. In this case, the critical limit is chemical and physical.

The developed critical limits can be used in practical production for the following purposes:

1. Critical limit 1 of the hydrolysis process (with the Neutrase enzyme preparation for 210 min at $T=40 \degree C$, dosage 5 Pa/unit) – by controlling this process, the enterprise controls microbial growth in case of non-compliance with temperature and time regimes, as well as enzyme effect on connective protein modification.

2. Critical limit 2 of the inactivation process at the end of the fermentation process (Fig. 4) (Neutrase enzyme at 60 °C, inactivation time 11 min) is completely inactivated.

Enzymes can be used in the food industry only if they are completely inactivated in the finished product, since active enzymes can affect gastrointestinal walls, which is unacceptable.

It was found that the developed critical limit is sufficient to prevent, eliminate or reduce the identified hazard to an acceptable level.

According to the study of safety during the hydrolysis process, it was found that the Neutrase enzyme preparation had a positive effect on all safety indicators, including toxic metals. This can be explained by the fact that the process of substrate modification can release metal ions. This enzyme has antioxidant properties and displaces toxic metals. Playing the role of antimetabolites, they form stable compounds with metabolites, inactivating them or accelerating the processes of their catabolism.

The analysis shows that the optimal parameters for reducing microbiological indicators, pesticides (HCH ($\alpha$, $\beta$, $\gamma$) isomers, DDT and metabolites), antibiotic (chloramphenicol) and toxic metals (Pb, As, Cd) are: $T=40 \degree C$, duration 210 min, dosage of the Neutrase enzyme preparation 5 units/Pa.

Prospects for further research are: comprehensive quality assessment of collagen hydrolysate; HACCP planning and developing critical limits for the remaining two CCP 3, CCP 4 (drying and storage of the finished product).

7. Conclusions

1. By optimizing the HACCP system using the FMEA method in collagen production, hazards (physical, chemical and biological) arising in collagen hydrolysate production were identified. After identifying hazards using the FMEA model, established by a three-factor assessment of the risk priority number (RPN), CCP in the processes of hydrolysis, inactivation of the enzyme preparation, drying and storage of the product were identified.

2. Based on the identified CCP, critical limits for CCP 1, 2 were developed:

   - 1 – critical limit of the hydrolysis process with the Neutrase enzyme preparation for 210 min at $T=40 \degree C$, dosage 5 unit./Pa;

   - 2 – critical limit of the inactivation process – inactivation of the Neutrase enzyme at 60 °C for 11 min.

The effect of the enzyme preparation on the microbiological parameters was determined. In the production of collagen hydrolysate, the Neutrase enzyme preparation showed the best result compared to Trypsin. The results of determining the safety indicators show the sanitary and hygienic safety of the product. The developed critical limit is recommended for inclusion in the HACCP plan for collagen production to ensure the quality and safety of the final product.


34. ISO 21527-1:2008. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of yeasts and moulds. Part 1: Colony count technique in products with water activity greater than 0.95. doi: http://doi.org/10.3403/30151265


