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The journal "Theory and practice of meat processing" is an international peer-reviewed scientific journal covering a wide range of meat science issues.

We publish original results of the fundamental and applied research in the field of:

- regulation of feeding rations and keeping conditions of animals (including poultry);
- targeted modification (selection, hybridization, operative manipulation);
- processing of meat raw materials;
- improvement of technologies for meat product manufacture;
- study of effects of meat and meat product consumption on human health;
- rational use of secondary resources of the meat industry and the ecological problems of the industry

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PATHOLOGICAL FEATURES OF THE LUNGS AND LIVER OF PIGLETS UNDER CONDITIONS OF CONSTANT VACCINATION OF LIVESTOCK AGAINST CIRCOVIRUS INFECTION

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Keywords: *piglets, circovirus diseases, vaccine, antibodies, livestock safety, live weight*

Abstract

The pathogenicity of PCV 2 in the body of vaccinated piglets was studied based on the results of pathomorphological changes in the lungs and liver of animals. The work was carried out on commercial piglets vaccinated with the vaccine Ingelvak CircoFLEX (Germany) against circovirus. The work used clinical, zootechnical, enzyme immunoassay and pathomorphological research methods. It has been established that under the conditions of ongoing vaccination of piglets against PCV2, 30.3% of piglets still do not have virus-neutralizing antibodies. The main reason for the culling of animals are circovirus diseases that have respiratory clinical signs, as well as signs of multisystem wasting syndrome, determining the safety of the livestock at the level of 68.05%, the average live weight of 1 head at the moment of its transfer for fattening is 40.44 ± 0.78 kg, and the average daily gain in live weight is 346.00 ± 9.18 g. At autopsy, sick piglets reveal an increase in the lungs and liver, and the signs of inflammation in them, as a result of circulatory disorders, damage to the lymphoid tissue, the development of dystrophic and necrotic changes. The results of the research suggest that in order to increase the efficiency of the formation of post-vaccination immunity, specific medical preparations can be used to stimulate the immune response of the body, as well as to enhance the resistance of the lymphoid tissue of the lungs and liver in animals.

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Introduction

In Russia, like in many countries of the world, pig breeding supplies most of the population's need for meat. The economic efficiency of the industry and the quality of the obtained products are directly related to the level of pigs' health, which depends not only on the technology of their feeding, managing, the genetic potential of their productivity, but also on the prevention of infectious and non-infectious pathologies in them [1–3].

Thus, among viral infections, the enterprises of the industry suffer the greatest economic damage not only from African swine fever (ASF), but also from circovirus diseases (CVD) [4, 5]. For example, in the USA the annual losses from the CVD amount to more than 600 million dollars, in Denmark — the annual losses make about 44 euros per sow.

Russia is no exception. Here from 80 to 100% of pig-breeding enterprises are "not safe" for being exposed to swine circovirus diseases. Chelyabinsk region, as a place of porcine farming, also suffers heavy losses from the porcine circovirus (PCV). This infection emerged and spread around Russia, as well as in other countries of the world, due to purchase of breeding pigs of foreign selection.

The main etiological agent of porcine circovirus diseases is porcine circovirus 2 (PCV2) [4], which not only suppresses the host immune system, but also enhances infection due to the replication of other pathogens (porcine reproductive and respiratory syndrome virus (PRRS), porcine parvovirus, swine influenza virus, swine fever virus) [6]. For example, when diagnosing pneumonia in pigs after weaning, the frequency of co-infection diagnosing is 42–85.4% of detected pneumonia cases [7]; in Chinese pig farms up to 52.4% of pigs are affected by PCV2 and PRRS, including service boars [8].

The complex of clinical signs in circovirus diseases associated with PCV2 includes "post-weaning multisystem wasting syndrome", respiratory diseases symptoms, enteritis, dermatitis, nephropathy, reproductive system issues, etc. [9–11].

At the same time, the most profound clinical signs of CVD are manifested in young pigs after their weaning, during the periods of their nursery and raising [10], thus causing the greatest rate of piglets culling. According to [4, 13, 14], the clinical manifestations of the infection develop due to the depletion of lymphoid tissue and lymphocytes, which proves the immunosuppressive functions of the PCV2, as well as its ability to infect and modulate the properties of a range of immune cells (macrophages, dendritic cells).

The main method of preventing pigs' circovirus diseases is vaccination. Among the prophylactic agents, PCV2 vaccines are the ones of the best-selling vaccines in pig farming [12, 15], as their use reduces mortality, decreases the culling rate of young animals, occurrence of concomitant diseases, and also increases the growth rate of the animals. After vaccination, virus-neutralizing antibodies appear in the body of pigs, and factors of humoral and cellular immunity are activated, thus determining the level of "immunological "protection" [14].

However, despite the ongoing vaccination, the PCV2 virus is constantly found among the vaccinated livestock. For example, in the United States in 2012 the virus was detected in 7.70–8.40% of vaccinated pigs [16], and in 2014–2016 in 11.30–29.00% already [17].

In Russia, the efficiency of vaccination ranges from 73.3–86.70% [18, 19] depending on the vaccine injected.

However, in recent years, studies have stated that before vaccination the virus was already present in the body of almost all animals, including sows [19, 20]. Therefore, at present, the decrease of the vaccination efficiency is caused not so much by the genetic variability of the virus, but to its constant circulation in industrial premises due to its high resistance to the environment.

According to [8, 12], the main reason of failing vaccination is the infection of animals before the vaccination. It happens when the healthy and infected pigs are kept together. Therefore, in the body of PCV2-vaccinated pigs, severe proliferative interstitial pneumonia and liver damage develop [21], that cause a mass mortality among the young animals as well as the decrease in pigs' growth rates and their meat quality.

To study the pathogenicity of PCV-2 in the body of piglets subjected to ongoing vaccination, we studied the specificity of pathomorphological changes in the lungs and liver of animals with a negative test for the presence of virus-neutralizing antibodies and which showed characteristic clinical signs of infection.

Materials and methods

Study design

The research part of the work was approved by the Bioethics Board of the South Ural State Agrarian University (Russia, Chelyabinsk region) and agreed upon with the veterinary service of LLC "Agrofirma Ariant" (Chelyabinsk region, Russia).

The design of the work provided for the formation of an experimental group of suckling piglets (n=3,618) at one of the commercial pig farm complexes of LLC "Agrofirma Ariant" (Chelyabinsk region, Russia).

Piglets on the 21st day of their life were vaccinated with the vaccine *Ingelvak CircoFLEX* (Germany) against circovirus in accordance with the vaccine manufacturer's recommendations.

At age of 23–24th days the piglets were weaned from their mothers and were transferred to a rearing shop, where they were distributed into group cages per 20–25 animals each.

All cages were identical in their design; every cage was equipped with automatic drinkers and feeders, which provided free access to water and feed.

The technology of feeding and managing the animals complied with the recommendations of Genesis.

Evaluation of the efficiency of post-vaccination immunity

Currently the following major methods are used to evaluate the results of vaccination and the efficiency of PCV2 vaccines:

1. Diagnostic method — the method based on determining the amount of virus-neutralizing antibodies.

To count the antibodies, blood was taken from the *vena cava cranialis* from 10% of the animals of the experimental group by random sampling at age of 60–70 days (40–50 days after vaccination). Blood samples were placed into a thermal container and were delivered within 3 hours after sampling to the Chelyabinsk branch of the Central Scientific and Methodological Veterinary Laboratory (Chelyabinsk, Russia). This lab determined the count of the specific antibodies to PCV2 with the help of the enzyme immunoassay method, using the CIRCOSerotest kits ("Vetbiokhim LLC", Russia) in accordance with the user's manual. The positive sample was considered valid if the coefficient of conjugate binding to blood serum antibodies exceeded 20%, and the negative one was less than 20%.

2. Production method — this method is based on the definition of the main production parameters: the safety of the livestock, average live weight (kg) of piglets at the end of the growing period and the average daily gain in live weight (g) for the nursery period.

The rate of livestock safety, i. e. livability (R_{saf}) for the nursery period was calculated by the following formula:

$$R_{saf} = 100\% - R_{mor}, \quad (1)$$

where:

R_{mor} — this is the rate of mortality and death of piglets in the experimental group.

For its calculation, the following formula was used

$$R_{mor} = \frac{P_{dead}}{P_{total}} \times 100, \quad (2)$$

where:

P_{dead} — is the number of dead and dead piglets (heads);

P_{total} — this is the total number of piglets in the experimental group (heads), 100 is the result conversion into a percentage.

The live weight of piglets was determined by the results of their individual weighing on the stationary livestock scales "Elton" (Russia). When calculating the average daily weight gains, we used data on live weight at the beginning and at the end of the nursery period.

Histological studies

The state of health in the experimental group of piglets was daily monitored by veterinary specialists of the pig farm. The piglets with clinical signs of CVD were transferred to the sanitary shop.

To obtain material for pathomorphological studies, from the sick animals we selected the sick piglets with a negative test for the presence of virus-neutralizing antibodies. The selected animals were subjected to euthanasia in the euthanasia unit of the agricultural company's recycling plant, and then their bodies were opened up to obtain biomaterial (lungs, liver).

Pieces of the liver and lungs, 1 cm^3 in size, were fixed with 10% formalin solution for 24 hours, washed with running water for 1 hour, and embedded in paraffin according to the following scheme: 1) sequential dehydration of samples in 70%, 80%, and 96% alcohol for 4 hours in each concentration; 2) overdrying in a mixture of alcohol and chloroform (1:1) for 1 hour, then in chloroform for 2 hours; 3) heating in a mixture of chloroform and paraffin for 1 hour at a temperature of 37 °C; 4) soaking with two volumes of paraffin at 56 °C for 45 minutes and making blocks.

Histological sections 5 μm thick were cut on the MS-2 sledge microtome (Russia). Before staining, the sections were deparaffinized in xylene for 2 minutes, then the xylene was removed with 96% ethanol for 2 minutes and washed with distilled water. For staining, a drop of hematoxylin was applied to the section for 2–3 minutes, washed off with water for 5–10 minutes, a drop of eosin was applied for 1 minute, washed off again with water, dehydrated in two volumes of 96% alcohol for 1 minute each, exposed to final dehydration in 100% alcohol for 1 minute and clarified in xylene for 2 minutes. After that the stained preparation was placed in a balm and covered with a coverslip. To detect lipids in the liver, formalin-fixed tissues were stained with sudan III (cerasine red) without embedding into paraffin. To do this, frozen sections were placed in an alcoholic dye solution for 15–20 minutes, rinsed in 50% alcohol, washed with distilled water, and embedded in glycerol for microscopy.

Sections were microscopically studied at various magnifications and photographed using a Leica DMRXA microscope (Germany) and a Leica DFC290 camera (Germany).

Statistical analysis

Statistical analysis considered the calculation of the mean value of the feature (X) and its standard error (Sx). The analysis was performed with the software "VERSIA". The signification of the differences was set at $p < 0.05$.

Results and discussion

Detection of specific antibodies to PCV2 after vaccination

Analysis of the results of enzyme immunoassay obtained from the Chelyabinsk branch of the Central Scientific and Methodological Veterinary Laboratory (Chelyabinsk, Russia) showed that in 10% of the samples from the experimental group, the share of negative samples amounted to 30.30%. Consequently, no post-vaccination immunity was formed in these animals after the vaccination, which accordingly created the basis for the circulation and reproduction of the virus in their body.

Clinical signs

All piglets euthanized by us were classified as infected with PCV-2, as they had not only a negative test for the presence of virus-neutralizing antibodies after vaccination, but showed the following clinical signs as well:

1. Oppressed behavior, food refusal, weight loss, inanition of the body, pale skin.

2. Shortness of breath, cough, nasal discharge, mucous membranes were either pale or icteric, fever. Some animals featured nervous issues in the form of tremors and paralysis. Inguinal superficial lymph nodes were enlarged.

The combination of these clinical signs proved that the piglets suffered from multisystem wasting syndrome and respiratory diseases.

Parameters of livestock safety and growth of piglets

The safety of piglets in the experimental group during the nursery period was equal to 68.05% (Figure 1). When comparing this parameter with the number of negative samples obtained during detection of specific post-vaccination antibodies to PCV2, it can be stated that the main reason for the culling of the young animals of the sampled group was the development of circovirus infection, as the basis for CVD formation.

The main zootechnical parameters, characterizing the growth rate of piglets during the nursery period, indicated that the average live weight (LW) of 1 head at the moment of its transfer for fattening was 40.44 ± 0.78 kg, and the average daily live weight gain for the considered period was 346.00 ± 9.18 g (Figure 1).

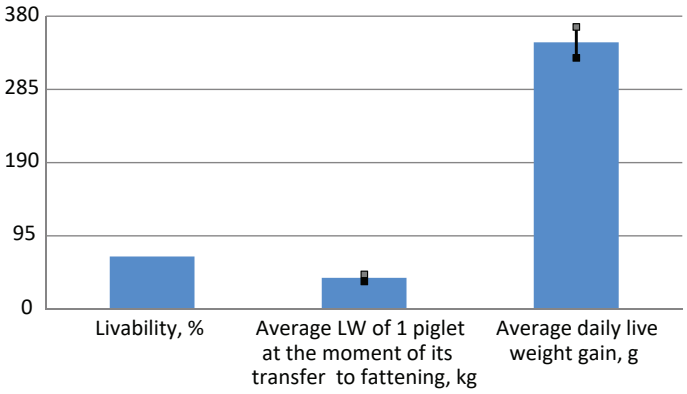

Figure 1. Indicators of safety and productivity of animals

Macroscopic features of the piglets' lungs and liver

At autopsy, the piglets showed an increase in lungs size. They were dark red and had blunt edges; foamy fluid was accumulated in the trachea and bronchial lumens. The texture of the organ was dense or rubberish, but with a well-defined lobation. At the same time, the spaces between the lobules were expanded and filled with a clear liquid. Mucous liquid was released from the small bronchi when pressed. The mucous membrane of the large bronchi was reddened, swollen and covered with plentiful mucus.

Macroscopic evaluation of the liver found an increase in its size and the blunt edges. The parenchyma of the organ was bulging on the incision and the edges of the incision did not converge anymore; the texture was flaccid. The surface of the organ featured a grayish tint, in some places there were yellow spots, the lobulation pattern of was blurred. The liver was easily torn when pressed.

Histological features of the lungs

Histological examination of the lungs in all fields of view showed a pronounced vascular congestion in all veins of any calibers with a clear picture of erythrostasis and erythrocyte thrombi in the capillaries (Figure 2A, 2D). Alternating large areas of dystelectasis and atelectasis, small foci of acute

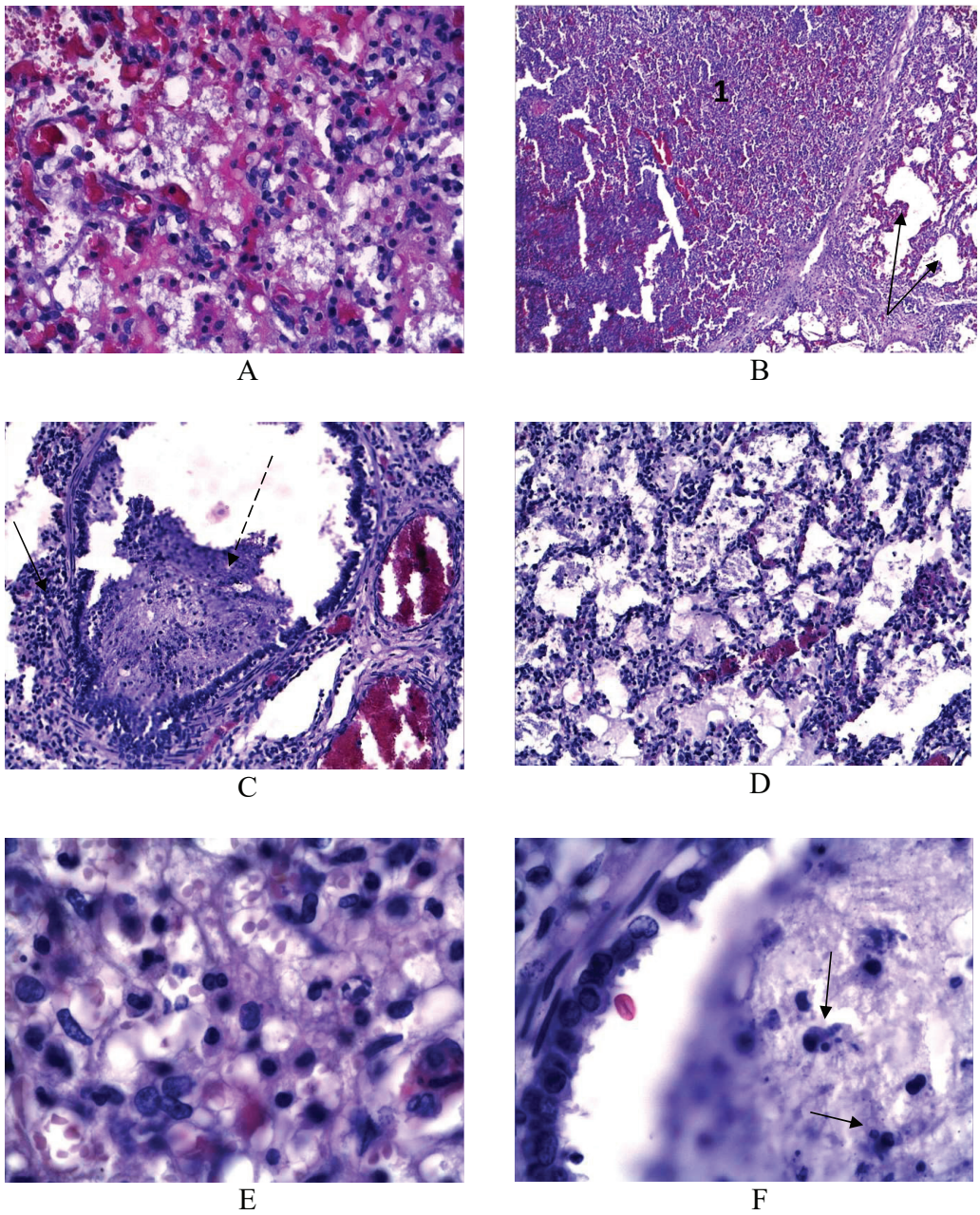

Figure 2. Morphological changes in the lung:

A — paretic plethora of vessels of all calibers with a picture of erythrostasis and erythrocyte thrombi in the capillaries, magnification x200; B — extensive area of atelectasis (1) and small foci of acute emphysema (arrows), magnification x50; C — neutrophil-lymphocytic infiltration of the peribronchial connective tissue (arrow); in the lumen of the bronchus — layers of desquamated epithelium, fine-grained structureless masses with an admixture of cells of the granulocytic series (dashed arrow), UVX200; D — focus of serous pneumonia, uv.x200; E — detail of the picture "D": in the cellular composition of the exudate, lymphocytes, neutrophilic leukocytes, erythrocytes and macrophages are visible, magnification x1000, MI; F — rounded inclusions (arrows) in the cytoplasm of macrophages in the lumen of the bronchus, x1000.
Stained with hematoxylin and eosin.

emphysema (Figure 2B) were found. Bronchial walls of all calibers were infiltrated with granulocytic cells, predominantly neutrophilic leukocytes and lymphocytes with small amount of eosinophilic leukocytes (Figure 2C). The lumen of the bronchi is uneven due to the lungs walls folding. Bronchial epithelium with a large amount of amorphous mucous masses on the surface was partially desquamated. The lumen of the bronchi was filled with layers of desquamated epithelium, with fine-grained, structureless masses with an admixture of a moderate amount of lymphocytes and neutrophilic leukocytes (Figure 2C). The lumen of the alveolar groups was filled with exudate consisting of fine-grained structureless masses and a small amount of granulocytic cells with an admixture of desquamated alveolar epithelial cells, macrophages and erythrocytes (Figure 2D, 2E). At high magnification, small, round, optically dense basophilic inclusions (most likely of a viral nature) were detected in the cytoplasm of macrophages (Figure 2F).

The walls of the alveoli were thickened due to neutrophil-lymphocytic infiltration; the capillaries here were sharply congested, with a peculiar pattern of widespread erythrocyte thrombosis (Figure 2A, 2D).

Histological features of the liver

When examining the microscopic picture of the liver in all fields of view — paretic venous and capillary plethora. The lobules are clearly visible. Cords discomplexation is caused by expansion of the pericapillary spaces (Figure 3A). The walls of the portal tracts are sharply edematous, defibular in structure, with dense neutrophil-lymphocytic infiltration (Figure 3B). Infiltrate cells migrate through the pericapillary spaces deep into the parenchyma (Figure 3D). The central veins are dilated, their walls are swollen, edematous, in the pericapillary spaces, large amounts of loosely piled, and clearly contoured erythrocytes are detected with an admixture of evenly distributed white blood cells well-visible against their background (Figure 3C). Hepatocytes are in state of severe protein vacuolar dystrophy, up to necrobiosis. Their nuclei are of different sizes — from small hyperchromic to enlightened bubble-shaped nuclei. When stained with sudan III, all preparations showed a small amount of fat droplets in the cytoplasm of hepatocytes under the organ capsule (Figure 3E, 3F).

The combination of pathomorphological changes in the lungs and liver of circovirus-infected piglets made it possible to classify these organs as PCV2 targets, since their lymphoid tissue played an important role in formation immune processes nature and direction.

Circovirus diseases are widespread in pigs farming in almost all countries of the world, and Russia is no exception. Meanwhile vaccination is the main way of preventing viral infection [12, 15].

However, continuous ongoing vaccination does not provide complete immune protection of piglets against PCV2, thus leading to losses in the livestock, as well as to replication and transmission of the virus. This was evidenced by both the number of negative samples (30.30%) in the experimental group when virus-neutralizing antibodies were detected in the body of vaccinated animals, by the level of animals livability (68.05%) and by zootechnical parameters (average live weight of a piglet at the moment of its transfer for fattening, its average daily gains in live weight).

Accordingly, the combination of these parameters influenced the economic performance of the enterprise and its profitability. According to [22, 23], one of the reasons for this trend is that PCV2 vaccination can induce the emergence of virus variants that resist the vaccine. In result the overall prevalence of PCV2 positive herds becomes unchanged. At the same time, the use of monovalent PCV2 vaccines can provide protection only against homologous infection, since these vaccines do not provide complete cross protection against other genotypes of PCV2 [24].

However, for vaccination the pig farming company, being an experimental venue, used the vaccine *Ingelvak CircoFLEX* (Germany), which is effective against all strains of PCV-2: PCV-2a, PCV-2b, PCV-2c, PCV-2d. Therefore, it is logical to suggest that a certain part of the piglets before vaccination was either seropositive [19, 20], or maternally derived antibodies circulating in their body, did not allow the formation of the required level of immune protection against the infection [22].

The formation of a healthy livestock of animals and the success of getting a full-fledged immune response when using a vaccine depends on the state of cells and tissues at the possible inlets of infection and the liver, as the liver serves as biological laboratory responsible not only for the disinfection of metabolic products, but also the formation of immunity [25–27].

According to [28] the most common clinical symptoms of circovirus infection in porcine, especially in nursery piglets, are the signs of respiratory diseases, as well as of digestive organs. Therefore, we chose the lungs and liver as the priority internal organs that play a crucial role both in the vital processes of the pigs in the state of the "conditional norm" and in the formation of their immune resistance to the virus.

According to [15], PCV2 in animals affects lymphoid tissue. In the lungs the lymphoid tissue is associated with the bronchi, ensuring the flow of immune reactions.

Our studies have found that in the lungs of circovirus infected pigs, the processes of circulatory disorders and inflammation were observed, which processes were associated with catarrhal inflammation and impaired microcirculation of the body's immunocompetent cells. In the cells of the first line of defense — i. e. lung macrophages — microscopic analysis found the included bodies of basophilic color, which were the result of the assembly and accumulation of circovirus virions. Our observations are consistent with the data [29] as those bodies are a consequence of viremia [30]. Meanwhile it was noted [25] that alveolar epithelial cells in the local area of the lung are the most sensitive to the virus, and start the congestive processes at the local edge of the lung lobule.

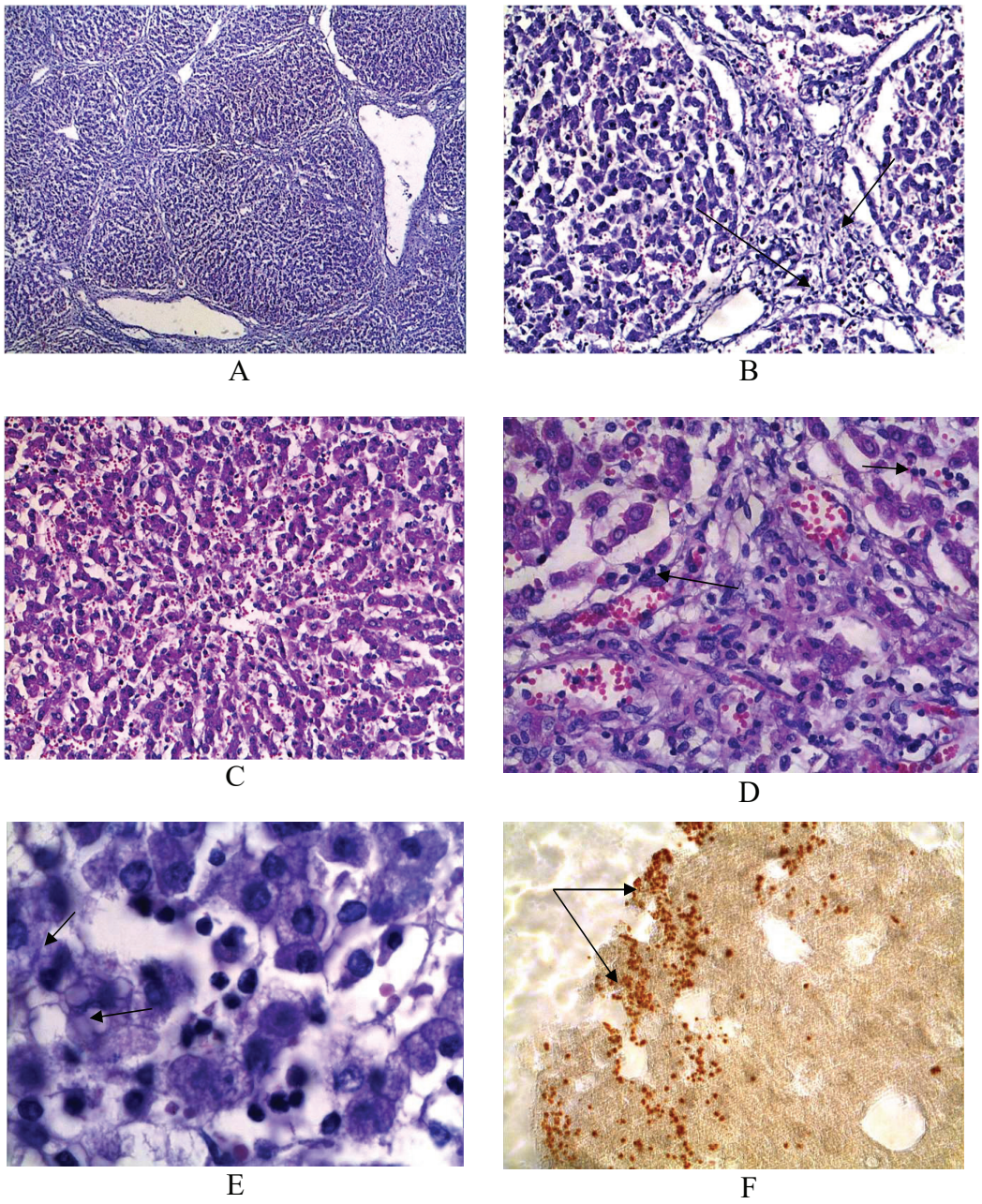

Figure 3. Morphological changes in the liver:
A — a distinct lobed structure, discomplexation of the hepatic cords, uv.x50; B — edema of the portal tract wall with infiltration by cells of the granulocytic series (arrows), magnification x200; C — the center of the lobule: swelling of the pericapillary spaces, a sharp plethora of sinusoids, magnification x200; D — cells of the neutrophilic-lymphocytic infiltrate spread through the expanded pericapillary spaces deep into the parenchyma (arrows), magnification x400; E — severe proteinaceous and hydropic (arrows) dystrophy and polymorphism of hepatocyte nuclei, magnification x1000; F — small accumulations of fat droplets (arrows) in the cytoplasm of hepatocytes under the liver capsule, magnification x100.
Staining: A-E: hematoxylin — eosin, F — sudan III.

The immune response is impossible to form without maintaining the homeostasis of the animal body with the liver [31], as this organ consists not only of hepatocytes but of immune system cells also (for example, macrophages — Kupffer cells, endothelial cells, pit cells, polymorphonuclear leukocytes, T- lymphocytes and B-lymphocytes). Moreover, the liver regulates the level of antigenic load on the animals' organism [32].

According to our studies, the liver of circovirus infected pigs features the processes of circulatory disorders and severe protein-fat degeneration up to hepatocyte necrosis. According to [33] liver damage is a frequent finding in microscopic studies in cases of porcine circovirus infection, and hepatocytes are the target cells for infection and virus replication [11, 34].

Conclusion

According to the results of these studies it was found that under the conditions of ongoing PCV2 vaccination of piglets with the vaccine *Ingelvak CircoFLEX* (Germany), no virus-neutralizing antibodies were detected in 30.3% of the piglets. Meanwhile, some individual piglets with clinical signs of multisystem wasting syndrome and respiratory diseases were constantly found.

This very symptomatic complex is the main reason for rejecting of animals, which reduces the safety of

livestock in the experimental group down to 68.05% and makes the average live weight of 1 piglet around 40.44 ± 0.78 kg (weighed at the moment of transfer to fattening stage), the average daily live weight gain is generally 346.00 ± 9.18 g.

At autopsy, sick piglets showed increased lungs and liver, and the signs of inflammation in these organs. Those problems were caused by circulatory disorders, damages to the lymphoid tissue, and development of dystrophic and necrotic changes.

The results of this study suggest that in order to increase the efficiency of post-vaccination immunity formation, specific medical preparations can be used to stimulate the immune response of an animal body, as well as to stimulate the resistance of the lymphoid tissue of the animal lungs and liver.

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THE EFFECT OF LONG SKIN SOAKING IN THE CALCIUM SOLUTION ON THE QUALITY OF RAMBAK CRACKERS FROM BUFFALO SKIN

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Abstract
Buffalo skin is an underutilized by-product of buffalo slaughter. It is perishable and if it is not processed immediately, it will rot. One of the applications of buffalo skin is processing into skin crackers. The present research was aimed at determining the best duration of buffalo skin soaking in the lime solution with regard to its effect on quality of rambak from buffalo skin. The study used a completely randomized design (CRD) with a factor of treatment time, which consisted of four levels, i.e. A = 76 hours, B = 86 hours, C = 96 hours, D = 106 hours. Each treatment was performed with five replicates, so that 20 samples were obtained. The data were analyzed using the statistical analysis of variance (ANOVA). Parameters observed were the water content, yield, volume expansion, and organoleptic characteristics such as taste, aroma, color, and texture. The results show that different soaking duration (76 hours, 86 hours, 96 hours, and 106 hours) did not have a significant effect on yield, taste, aroma, color, and texture, but significantly affected the volume expansion and water content in rambak from buffalo skin. The best result was achieved with soaking duration of 106 hours; the rambak was characterized by the following parameters: water content 10.83%, yield 57.87%, volume expansion 76.66%, color score 3.51, taste score 3.61, aroma score 3.63 and texture score 3.30.

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Introduction

The skin, the outermost layer of the body in cattle, is a by-product of slaughtering livestock and is obtained after an animal dies and is skinned. The skin of large and small livestock, be it cows, buffaloes, sheep, or goats, has a strong tissue structure, so that it can be used for food and non-food purposes [1]. Skin that has just been removed from the animal's body is called fresh rawhide. This skin is easily damaged when exposed to chemicals, such as strong acids, and strong bases, or microorganisms. The average chemical composition of fresh skin is 64% water, 33% protein, 2% fat, 0.2% minerals, and 0.8% other substances [2]. Fresh skin from livestock slaughter can be directly tanned or further processed, but not all skin becomes the raw material for the tanning industry. The skin that cannot be used in tanning can be directly processed in the form of food products such as rambak crackers.

Crackers are a type of snacks that undergo volume expansion to form a porous product and have a puffed appearance during frying [2], while skin crackers are a by-product of processed animal skins. The raw material for making crackers is fresh skin. Fresh skin intended for producing skin crackers should be thick skin separated from fat and meat [3]. Cracker processing can be done by frying, sand roasting [4], and using a microwave [5].

One of the stages in the manufacture of rambak crackers is the calcification process, which will affect the chemical quality, especially the water and protein content of crackers. Skin that undergoes calcification will have the low water content due to the presence of Ca^{++} ions that enter the tissue so that the cell wall becomes sturdy and water can be pulled out of the cell tissue [6]. Soaking in the 0.15% lime solution for 15 min allowed achieving the expected quality [7]. A higher concentration of the lime solution and longer soaking will lead to high-quality crackers.

Liming greatly influences quality of produced skin crackers. The purpose of the liming process is to remove globular proteins as well as to swell the skin so as to facilitate the subsequent process, especially to improve the physical, chemical and organoleptic qualities of skin crackers [8].

The length of calcification will affect the chemical, physical and organoleptic quality of the resulting skin rambak crackers. The longer the calcification process, the better the process of removing globular proteins and the hair. The calcification process will result in some fat being turned into calcium soap that is insoluble in water, so it will be difficult for water to be absorbed into the skin and this can increase the enlarging capacity of the resulting buffalo skin rambak crackers [9].

Based on this background, further studies are needed for the manufacture of skin rambak crackers in the long process of skin immersion in the lime solution to produce high-quality skin rambak crackers.

Materials and Methods

A part of the buffalo skin used for preparation of crackers was belly, and the selected parts of the skin were intact/not torn, not injured/bruised, without black spots on the skin surface. This skin was dried using a temperature of 60 °C, which is expected to be sufficient to inhibit the microbial growth. The dry skin containing 2.31% — 2.84% of water was obtained after drying.

In this study, the skin from Simeulue buffalo and calcium oxide obtained from Lambaro's traditional market, Aceh Besar, were used. Ingredients used were cooking oil, water, garlic and salt. The tools used in this study were cork, winnower, knife, pan, cutting board, stove, tarpaulin, fried spoon, oil sieve, scales, bucket, porcelain cup, oven, and desiccator.

Research Procedures

The stages of the process of making buffalo skin rambak crackers were as follows:

1. Soaking in water
   Skin soaking was done with clean water for two hours.
2. Calcification
   Skin that underwent soaking in water, was then put in a solution of lime (0.4 kg of lime in 5 L of water for 1 kg of skin). The treatment length was 76 hours, 86 hours, 96 hours, and 106 hours. Stirring was done every five hours.
3. Removal of lime and the hair
   Removal of lime was done by washing the skin using clean water (five liters for each treatment) repeated five times. The hair was removed by scraping using a knife.
4. Boiling
   Boiling of the skin was done in boiling water at a temperature of 90 °C for two hours. Then it was winded.
5. Grinding
   Skin was ground to a size of 3 × 2 cm.
6. Drying I
   Drying was done in sunlight for one day.
7. Seasoning by soaking
   Crackers from drying I were soaked in a seasoning solution (salt 2% and garlic 5%).
8. Drying II
   Second drying was done in sunlight for 2–3 days (until dry).
9. Frying
   Frying I: crackers were put in a frying pan at a temperature of 80 °C for 5 min and watered in a tub for one day. Frying II (temperature 80 °C for 10 min) and frying III (temperature 160 °C until crackers expand perfectly) were performed afterwards.

Research Methods

This study used a factorial complete randomized design (CRD) with a length of calcification consisting of four treatment standards namely A = 76 hours, B = 86 hours, C = 96 hours and D = 106 hours. Each treatment was carried out in five replicates so that 20 samples were obtained.

Analysis Methods

The parameters analyzed included the water content, yield, volume expansion and organoleptic characteristics. To determine the water content, a sample was put into a porcelain dish and dried in an oven for two hours at 110 °C; after drying, the sample was placed in a desiccator and the final weight was measured [10]. The yield determination was carried out by comparing the percentage of crackers produced per unit of skin used [11]. The volume expansion was determined by the ratio of the volume of raw crackers to the volume of fried crackers [12]. For determination of organoleptic characteristics (color, taste, aroma, and texture), the 5-point hedonic scale was used, where: 1 = dislike very much; 2 = dislike; 3 = like slightly; 4 = like; and 5 = like very much. Organoleptic analysis was performed by panelists recruited and trained to carry out specific tasks of organoleptic evaluation [13]. All data presented in the study were analyzed using analysis of variance (ANOVA) with SPSS2010 software. If the results of ANOVA showed a difference in treatment, analysis was continued with the smallest real difference test with a level of 5%.

Results and Discussion

Water content

The water content is an amount of water contained in the material expressed in the percent. The water content is one of the very important characteristics of food, because water can affect the appearance, texture, and taste of food. The water content in food also determines the freshness and durability of foodstuffs. When the water content is high, it is easy for bacteria, mold, and yeast to multiply, so that there will be changes in food [14].

The water content of the resulting skin crackers ranged from 10.96% to 11.36% with an overall average value of 11.16%. The results of the analysis of variance show that the long treatment by skin immersion in the calcium solution did not have a noticeable effect ($P > 0.05$) on the water content of buffalo skin crackers produced.

Table 1. Effect of skin immersion in the calcium solution on the water content of buffalo skin crackers

Length of calcification (hours)	Water content (%)
A = 76	11.04 ± 0.03^{ns}
B = 86	11.26 ± 0.06^{ns}
C = 96	11.36 ± 0.10^{ns}
D = 106	10.96 ± 0.17^{ns}

Note: ns = not significant

Table 1 shows that the highest water content (11.36%) was found in the sample that underwent treatment by skin immersion in the calcium solution for 96 hours, while the lowest water content (10.96%) was in the sample subjected to skin immersion in the calcium solution for 106 hours. This is because the length of skin immersion in the lime solution can enlarge the pores on the skin, so that the water contained in the skin can come out and the water content of the skin will decrease. In the frying process, there will be evaporation of bound water due to an increase in temperature, so air cavities in fried crackers will be formed [15].

From the results of the study, it is clear that there are differences in the water content whereby not all treatments by skin immersion in the calcium solution for a relatively long time resulted in the low water content. It can be seen from Table 1 that skin immersion in the calcium solution with a 96-hour soaking duration led to a higher water content than skin immersion in the calcium solution for 76 hours and 86 hours. It is assumed that unequal levels of skin thickness may cause fluctuations in the water content in the skin. This is confirmed by the opinion of [9], who stated that the water content in each part of the skin is not the same.

An effect of liming time on rambak quality parameters was also observed by other researchers. In [16] Widati et al. compared four treatments with liming duration of 24, 48, 72 and 96 hours and found that the best result was achieved when using 96-hour treatment. The rambak crackers had the protein content 6.10%, water content 0.11%, calcium content 1.88%, volume expansion ratio 372.12%, crispness score 5.38 and taste score 6.89 [16].

The water content of crackers decreases from the beginning to the end of the drying process. One of the studies on drying skin crackers reports that during the 30-hour drying stage, the cracker moisture content dropped from 63.4% to around 10–20% [17]. However, the moisture content during storage is affected by the length of storage. An increase in the water content causes a decrease in crispness of crackers [18].

Yield

Yield is a percentage of products obtained from the comparison of the initial weight and final weight of the material. The yield of buffalo skin crackers was determined by weighing the final product and comparing the obtained weight to the weight of the initial product before the process.

The resulting yield value ranged from 57.87% to 61.23%. The average overall yield value was 59.56%. The results of the analysis of variance show that the long treatment by skin immersion in the calcium solution did not have a noticeable effect ($P > 0.05$) on the yield value of buffalo skin crackers produced. The small yield of skin crackers was affected by cell, tissue and structure types. It is worth noting that each part of the skin has different types of cells, tissues and structures.

Table 2. Effect of skin immersion in the calcium solution on the yield of buffalo skin crackers

Length of calcification (hours)	Yield (%)
A = 76	60.49 ± 1.22^{ns}
B = 86	61.23 ± 2.79^{ns}
C = 96	58.64 ± 0.10^{ns}
D = 106	57.87 ± 0.95^{ns}

Note: ns = not significant

This corresponds to [19], which states that one of the factors that influence the yield of skin crackers is the skin structure or the part of the skin itself, where each layer has the specific structure. The skin structure can be loose, dense, thick, thin, so it could affect the small yield produced.

Volume expansion

Volume expansion of crackers is one of the most important factors for cracker quality because it determines consumer acceptance [20]. Basically the phenomenon of cracker volume expansion is caused by the pressure of steam formed from heating the water in the material so that it urges the structure of the material to form an expanding product. The enlarging capacity of skin crackers ranged from 33.33% to 83.33%. The overall average volume expansion of crackers was 63.33%. The results of the analysis of variance for volume expansion in buffalo skin crackers show that the length of skin immersion in the lime solution had a very real effect ($P < 0.01$) on the enlarging capacity of buffalo skin crackers produced.

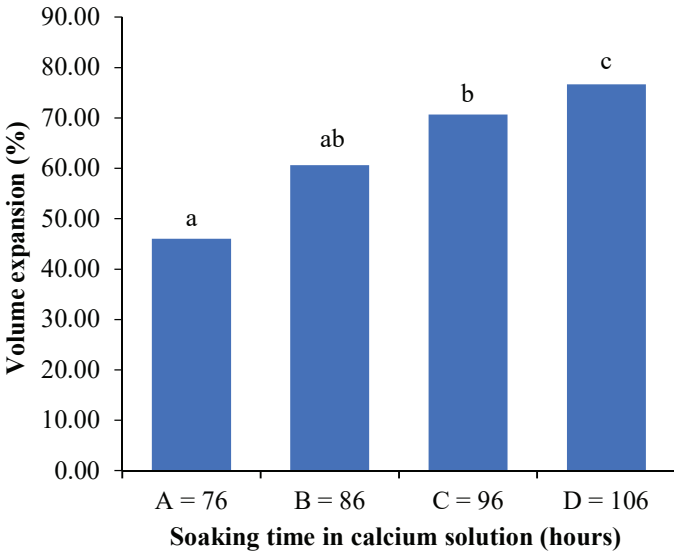

Figure 1. Influence of skin immersion in the calcium solution on the volume expansion of buffalo skin crackers. The letters indicate a significant difference ($P < 0.01$) between treatments

Figure 1 demonstrates that the highest volume expansion (76.66%) was obtained when using the long treatment by skin immersion in the lime solution for 106 hours, the lowest volume expansion (46.00%) was in the sample subjected to this treatment for 76 hours, while the samples treated for 86 hours and 96 hours occupied the intermediate position (60.66% and 70.66%, respectively). The results of the study

show a real effect of the length of skin immersion in the lime solution. The longer the soaking of the skin in the lime solution, the higher the value of the resulting volume expansion. It is assumed that long immersion of the skin in the lime solution can remove water contained in the buffalo skin so that the resulting skin crackers will expand more easily. When crackers expand, cavities will be formed in them. The more cavities formed, the more tenuous the structure, so it will be easier to break them. Thus, the higher the enlarging capacity of rambak crackers, the higher crispness. The mechanism of cracker development resides in the release of water trapped in the gel during calcification [21].

The frying process at high temperature also influenced the quality of the skin crackers in this research. Choe and Min [22] noted that frying is the process of cooking food ingredients using hot fat or oil at high temperatures. Deep frying leads to changes in stability and quality, taste, color and texture of fried food and the nutritional value of fried food [22].

The frying process causes the crackers to expand/develop. The cracker development is caused by the formation of air cavities because of the evaporation of water bound to the cracker structure due to the influence of frying temperature. At the time of boiling the skin, the water will be bound in the collagen protein forming a gel with a very strong bond so that at the time of drying it is difficult to evaporate it, but it can be removed at the time of frying due to the use of high temperatures [23]. The amount of water absorbed during cooking plays a role in conversion collagen into gelatin, and affects the level of the development and crispness of the rambak crackers produced [16].

Organoleptic test

The organoleptic test is also called the sensory test because it is based on sensory stimulation of the five senses such as sight, smell, touch, taste and hearing [24]. The purpose of the hedonic test or the test of preference is to find out the panelists' response to sensory quality characteristics, for example, color, taste, aroma, and texture [13]. Sometimes, the organoleptic test can be more dependent on a type and specifications of ingredients / food products.

Table 3. Average organoleptic scores of buffalo skin rambak crackers depending on the length of immersion in the calcium solution

Soaking time in calcium solution (hours)	Organoleptic scores			
	Color	Taste	Aroma	Texture
A = 76	3.52^{ns}	3.47^{ns}	3.44^{ns}	3.51^{ns}
B = 86	3.55^{ns}	3.55^{ns}	3.51^{ns}	3.38^{ns}
C = 96	3.57^{ns}	3.53^{ns}	3.59^{ns}	3.41^{ns}
D = 106	3.51^{ns}	3.61^{ns}	3.63^{ns}	3.30^{ns}

Note: ns = not significant.
Organoleptic scores: 1) dislike very much, 2) dislike, 3) like slightly, 4) like, and 5) like very much.

The results of the organoleptic evaluation (color, taste, aroma, and texture) demonstrate that the buffalo skin rambak crackers produced using soaking in the calcium solution corresponded to the panelists' acceptance level as shown in Table 3. The panelists showed a slightly higher preference for samples soaked in the calcium solution for 96 hours with a level of acceptance between "like slightly" and "like". It can be seen from Table 3 that most of organoleptic characteristics had scores lower than 4 with the average organoleptic score of 3.5. It is possible to improve the new technology to produce skin crackers with better organoleptic characteristics. Additional formulation ingredients may be necessary.

Color

Consumer preference for food products is also determined by the food color. The food color is influenced by the light absorbed and reflected from the material itself and is also determined by color dimensions [25]. Color is a feature of food products that attracts the most attention of consumers and most quickly gives the impression of being liked or not. The color intensity of the crackers depends on the pigment changes that occur during ripening; the changes are determined by a type of crackers and length of ripening. The process of cooking or processing food can cause the color of foodstuffs or products to become brighter because of the loss of pigment due to the release of cell fluids [26].

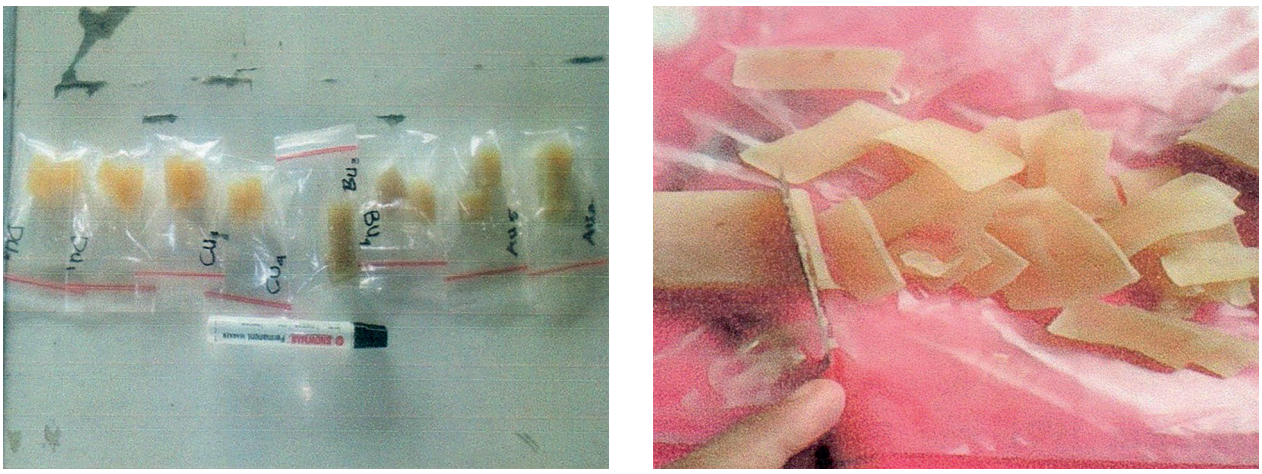

Figure 2. The color of rambak crackers from buffalo skin

The data from the analysis of the influence of long immersion of the buffalo skin in the lime solution on the color of the produced buffalo skin crackers show that the organoleptic scores of the color ranged from 3.51 to 3.57 with the average score equal to 3.54. The results obtained suggest that the long treatment of the buffalo skin by immersion in the lime solution had an unreal effect ($P > 0.05$) on the color of the resulting buffalo skin crackers.

The color of buffalo skin crackers can been seen in Figure 2.

Figure 2 shows that the color of the skin crackers did not differ between treatments. The color of skin crackers produced in this research tend to be bright and there was no dark color in the tested samples. Relatively the same color impression is associated with relatively the same sorting process resulting in relatively the same color changes.

Taste

One of the factors that play an important part in determining the final decision of consumers to accept or reject a product is taste. Even if other evaluation parameters are good, a product with bad taste will be rejected by consumers. The basic tastes include salty, sweet, bitter, and sour [27]. The great role in perceiving these basic tastes is played by taste buds on the tongue in the oral cavity. However, besides the above mentioned basic tastes, some researchers also regard other tastes such as metallic and savory as a result of perception by taste receptors [28].

In this study, the value of the panelists' liking of the taste of buffalo skin crackers ranged from 3.47 to 3.61 with the average score equal to 3.54.

The results of the analysis of variance of organoleptic scores for skin cracker taste show that the length of skin immersion in the lime solution (76, 86, 96 and 106 hours) did not have a noticeable effect ($P > 0.05$) on the organoleptic scores for taste of the resulting buffalo skin crackers.

In addition, skin crackers that were fried using oil had a distinctive flavor of fried skin crackers. The savory taste found in skin crackers can be caused by protein contained in the crackers. During the cooking process, protein will be hydrolyzed into amino acids, namely glutamic acid, which can cause delicious taste.

Aroma

The aroma organoleptic analysis is a sensory analysis that is usually carried out after observing the general appearance of the product. The aroma of a food product can determine the delicacy of the food. Its emergence is caused by the formation of volatile compounds. The aroma that each food emits is different. In addition, different cooking methods will lead to different aromas [29,30].

Based on the sensory test results, it has been found that buffalo skin crackers had a distinctive aroma, which was also influenced by sunlight drying. The value of panelists' liking of aroma of skin crackers ranged from 3.44 to 3.63 with the average score equal to 3.54. The highest panelists' score for aroma of skin crackers (3.63) was in the samples with a 106-hour soaking length, while the lowest score (3.44) was in the samples subjected to immersion for 76 hours.

The results of the analysis of the organoleptic properties of skin crackers demonstrate that the length of skin immersion in the lime solution (76 hours, 86 hours, 96 hours, and 106 hours) did not have a noticeable effect ($P > 0.05$) on the organoleptic scores for aroma of the resulting buffalo skin crackers. This is because skin soaking in the lime solution does not impart aroma that would be strong enough to change the aroma of the resulting skin crackers. However, the results show that the longer skin soaking in the lime solution, the higher the level of panelists' liking of aroma of buffalo skin crackers.

Texture

Texture is a factor that determines a product. Texture assessment aims to determine the panelist's acceptance of the level of elasticity or tenderness of a product that can be assessed using the sense of touch. Food texture testing is an attempt to find the exact texture parameters that are the quality attributes of the food in question, and then determine the most appropriate common terms in the category of parameters accompanied by additional information to state their levels [31].

The organoleptic scores for texture ranged from 3.30 to 3.51. The overall average organoleptic score of buffalo skin cracker texture was 3.40. The score for panelists' liking of the texture of buffalo skin crackers was the highest (3.51) upon using the long treatment by skin immersion in the lime solution for 76 hours, while the lowest panelists' score (3.30) was in the samples treated by skin immersion in the lime solution for 106 hours.

The results of the analysis of organoleptic scores of buffalo skin cracker texture show that the treatment with the lime solution using different calcification periods did not have a noticeable effect ($P > 0.05$) on the organoleptic scores of the texture of buffalo skin crackers. This is because long skin immersion in the lime solution can remove water contained in the skin. The skin has different connective tissues, so the skin will be easily overhauled by lime and cavities contained in the skin will be more easily opened. If the cavities in the skin have opened, then the skin will more easily expand and produce a crisp skin cracker texture, so that the resulting skin crackers will be preferred by panelists. Each panelist's perception of food product taste is different, so the rate of panelist acceptance of skin crackers was not the same. This is supported by [32], which states that all resulting skin cracker products have a level of liking that tends not to be the same for mature products.

Analysis of the chemical characteristics of various commercial animal skin cracker products indicate that the cow-

hide crackers have the best nutritional value compared to other skin crackers. Table 4 demonstrates that the highest crude protein content was in cowhide crackers (56.79%) and buffalo skin crackers (51.45%), while the lowest crude protein content (42.00%) was in the samples of chicken skin crackers [33].

Table 4. Comparison of the chemical characteristics of various commercial animal skin cracker products [33]

	Product category			
	Cowhide crackers	Buffalo skin crackers	Fish skin crackers	Chicken skin crackers
Crude protein content	56.79	51.45	48.00	42.09
Fat content (%)	23.33	33.05	35.13	45.53
Moisture content (%)	1.79	1.95	2.21	2.15
Ash content (%)	3.96	1.25	4.14	3.91
Free fatty acid value (%)	0.41	0.75	2.24	1.79
Thiobarbituric acid value (mg malondialdehyde /kg)	0.96	1.43	1.75	1.97

It can be seen from Table 4 that buffalo skin crackers have a good nutritional value and the nutritional composition of buffalo skin crackers is not much different from that of cowhide crackers.

Conclusion

The results of the study indicate that the length of skin soaking in the calcium solution in the manufacture of buffalo skin crackers had a very real effect ($P < 0.01$) on the enlarging capacity of skin crackers and had no real effect ($P > 0.05$) on yield, organoleptic scores of color, taste, aroma, and texture. Skin immersion in the calcium solution for 106 hours led to the best skin crackers with the following characteristics: moisture content 10.83%, yield 57.87%, expansion volume 76.66%, color score 3.51, taste score 3.61, aroma score 3.63, and texture score 3.30. Although, all organoleptic characteristics had scores lower than 4, it is possible to improve the new technology with appropriate selection of ingredients to produce skin crackers acceptable to all circles of consumers.

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NILE PERCH FISH NUGGETS: PARTIAL REPLACEMENT OF FISH FLESH WITH SESAME HULLS AND SUNROOT — QUALITY ASSESSMENT AND STORAGE STABILITY

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Keywords: *Nile perch, sesame hulls, sunroot, quality properties, sensory evaluation*

Abstract
This study aimed to produce Nile perch fish nuggets by replacing a part of fish flesh with different concentrations of sesame hulls and sunroot to reach the optimal recipe. Chemical, microbiological, and sensory characteristics of nuggets were evaluated during 3 months of frozen storage at –18 °C. According to the obtained data on the chemical composition of raw materials, Nile perch flesh had the highest content of protein (20.21%), sesame hulls contained the highest amount of fat (13.54%), fiber (17.24%) and ash (16.11%), while sunroot tubers had the highest amount of carbohydrates (15.76%). Based on the sensory score, the acceptable replacement ratio for fish nuggets prepared with sunroot (T1) and sesame hulls (T2) was 10% and 7.5%, respectively. Thiobarbituric acid (TBA) analysis at zero time shows that the T1 samples had the minimum value compared to the T2 and control samples. During storage, the TBA levels increased slightly in all samples, but after three months T1 also showed the lowest value. The total plate count (TPC) and psychrophilic bacterial (PSY) count in the samples were affected by the period of frozen storage at –18 °C. The initial TPC and PSY loads were 2.32 and 2.02 log cfu/g for control; 2.24 and 1.72 log cfu/g for T1; 2.30 and 1.47 log cfu/g for T2, respectively. During storage, the values of TPC and Psy slightly decreased. In conclusion, this study succeeded in the replacement of Nile perch fish with sesame hulls and sunroot as new sources to improve the nutritional value and quality characteristics of fish nuggets.

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Introduction

In recent years, the consumption of breaded and battered foods, particularly seafood, fish, poultry, vegetables, and cheese has increased significantly [1,2]. Fish and its products have polyunsaturated fatty acids that are good for health, high-quality proteins that are easy to digest, and other nutrients like minerals and vitamins that are also important for human nutrition [3]. Dietary fiber plays a significant role in the preparation of fish mince-based products due to its fat-binding capacity, water-holding capacity, gelling characteristics, texturizing, viscosity, etc. [4,5]. Koh et al. [6] state that incorporation of the appropriate amount and type of soluble-fiber polysaccharides into a recipe may improve the eating quality of processed fish products by taking advantage of their multidimensional functionality as instrumental/sensory texture modifiers. Accordingly, the addition of a suitable fiber source is sought to enhance the functional qualities of products based on fish mince.

Due to the busy lifestyle of the most urban population, there is an ongoing demand for nutritional, quick to prepare, and appetizing food products. Therefore, it is necessary to produce diverse, value-added, and convenient fish products based on fish mince. Consequently, it is a good raw material for the creation of value-added products, such as cooked products (for example, sausages), as well as mince-based fish nuggets, burgers, fingers and fish balls [7,8,9]. In general, fish does not contain carbohydrates including dietary fibers and different sugars, therefore, the use of plants as a source of carbohydrates (including dietary fiber and various important sugars) in food as functional components is thought to increase the product stability, texture, and nutritional value, as well as reduce total production costs [10,11].

Sesame hulls are a byproduct of sesame seeds (*Sesamum indicum* L.), which include substantial amounts of oxalic acid, calcium, crude fiber, and other minerals. Once the seed is correctly dehulled, the oxalic acid concentration is decreased from approximately 3% to less than 0.25% of the

seed weight [12]. During the oil extraction of sesame seeds and the production of sesame hulls, 12.0% to 13.6% of the initial seed weight is lost, and a large proportion of small, undamaged seeds escape the hulling process [13]. The chemical composition of sesame hulls differs amongst extraction facilities as a result of the various oil extraction procedures. Sunroot belongs to the *Asteraceae* family. Kays and Nottingham [14] reported nearly 100 common names in different languages. The most commonly used English names are Jerusalem artichoke, woodland sunflower, earth apple, and sun-choke. It is considered a good source of carbohydrates (containing a high amount of inulin (8–13%) that is considered a prebiotic). When sunroot is stored for some time, inulin will convert into fructose. Therefore, it has a sweet taste because of fructose [14,15]. Also, sunroot contains a high amount of phenolic compounds such as coumarins, poly acetylenic derivatives and sesquiterpenes with high antioxidant capacity [14]. Sunroot contains 15% carbohydrate, 1 to 2% protein and 80% water, no fat and little starch.

In light of these facts, the purpose of this investigation was to determine the influence of replacing a portion of Nile perch flesh with sesame hulls powder and sunroot puree on the improvement of nutritional quality, cooking properties, technical functionality, and shelf life of Nile perch mince-based nuggets.

Materials and methods

Raw materials

Nile perch flesh (*Lates niloticus*), pepper, cardamom, garlic paste, salt, and lime juice were obtained from a local market in Giza, Egypt. Sunroots were obtained from the Vegetable Department, Faculty of Agriculture, Cairo University, Egypt. Sesame hulls were obtained from Halawa El Rashidi El Mizan factory in 6th of October city, Egypt. All chemicals used for the analysis were bought from Sigma Aldrich Chemical Company, United Kingdom.

Sesame hulls and sunroot preparation

Sesame hulls were oven-dried (Shel-lab, Cornelius, OR, USA) at 50 °C for 24 hours and then ground in an analytical mill (Cole-Parmer, Vernon Hills, IL, USA) to obtain powder with a particle size of 1 mm. Unblemished sunroot tubers were selected, washed, dried with tissue paper, and cut into small cubes. The small cubes were transformed to puree by a mixer (PHILIPS, HR1865–700W, China).

Nile perch fish nuggets preparation

During this study, fish flesh was replaced with different concentrations of sesame hulls (2.5%, 5%, 7.5%, 10%, 15%, and 20%) or sunroot (2.5%, 5%, 10%, and 15%) to reach the optimal recipe. After optimization, the final recipes for Nile perch fish nuggets with sesame hulls and sunroot as well as the control sample were as follows:

- control sample: 100 g minced Nile perch flesh, 1.175 g pepper, 0.055 g cardamom, 4.62 g garlic paste, 1.175 g salt, 0.5ml lime juice;
- Nile perch fish nuggets with 10% of sunroot (T1): 90 g minced Nile perch flesh, 10 g sunroot puree, 1.175 g pepper, 0.055 g cardamom, 4.62 g garlic paste, 1.175 g salt, 0.5 ml lime juice;
- Nile perch fish nuggets with 7.5% of sesame hulls (T2): 92.5 g minced Nile perch flesh, 7.5 g sesame hulls powder, 1.175 g pepper, 0.055 g cardamom, 4.62 g garlic paste, 1.175 g salt, 0.5ml lime juice.

All components for each sample were mixed well, formed into nugget shape and covered by wheat flour then liquid egg, and finally rusk. All samples were stored at –18 °C for further analysis.

Storage conditions

Nile perch fish nugget samples were packed in polypropylene bags and stored at –18 °C for three months. The chemical and microbiological profiles of all samples were determined every month for three months.

Methods of analysis

The moisture and ash content were determined according to Baioumy et al. [16]. The protein and fat content were determined according to AOAC [17]. The pH was measured in the slurry prepared by blending nuggets and distilled water with a 1:2 ratio. A digital pH meter (model 420A, Orion Benchtop pH meter, Allometrics Inc.) was calibrated before use.

The water holding capacity (WHC) of the samples was determined using the filter paper press technique according to Daum-Thunberg et al. [18]. A fish nugget sample (0.3 g) was put under ashless filter paper (Whatman, No. 41) and pressed for 10 min using 1 kg weight. Two zones were formed on the filter paper and their surface areas were measured by a planimeter. The outer zone resulted from the water separated from the pressed tissues thus indicating the WHC value and the internal zone was due to the fish pressing indicating the plasticity. WHC was calculated by subtracting the area of the internal zone from that of the outer zone. Data were presented as cm^2.

Thiobarbituric acid (TBA) values of fish nuggets were measured according to Sallam et al. [19] with a slight modification. One milliliter of the homogenized sample was mixed with 2 ml of the stock solution (prepared as follows: 0.37% TBA, 15% TCA, and 0.25 N HCL were slowly heated to 75 °C in a water bath to facilitate the dissolution of thiobarbituric acid). After that, the mixture was heated in a boiling water bath for 15 min to develop pink color. After cooling with tap water and centrifuging at 2000 rpm for 15 min, the absorbance was measured at 532 nm (Unico UV-2000, Dayton, NJ, USA). The TBA value was expressed as mg malonaldehyde/kg of nuggets.

Cooking loss (%) of nugget samples (40 g) was determined by their weighing before and after the cooking process by deep-fat frying. Samples were removed, drained, lightly blotted, and weighed. Cooking loss (%) was calculated as weight loss due to cooking divided by the initial

weight of raw samples. The calculation was carried out as% on a wet weight basis. Oil uptake of nugget samples was determined by weighing before and after the frying process with the use of immersion of these samples in the oil. Oil uptake% was calculated by the following equation:

$$\text{Oil uptake\%} = \frac{\text{weight after frying} - \text{weight before frying}}{\text{weight before frying}} \times 100 \quad (1)$$

The color values [L* (lightness), a* (redness), and b*(yellowness)] of each sample were measured using a colorimeter (Model CR-200; Konica Minolta, Japan) as described by Abedelmaksoud et al. [20].

The total plate and psychrophilic bacterial counts were determined as follows: 1 ml of each sample was transferred into 9 ml of the 0.1% peptone water or 0.85% physiological saline solution (sterile) and homogenized with a blender for 2 min. From the 10^{-1} dilution, other decimal dilutions were prepared. Plate count agar was used as a medium, and plates were incubated at 35 °C for 48 ± 2 h for the total plate count [21] and in the refrigerator at 5 ± 2 °C for 10 days for the psychrophilic plate count [22].

Sensory evaluation of nuggets included color, odor, flavor, texture, and overall acceptability. Samples were evaluated using a 10-point hedonic scale to establish the optimal recipe for producing fish nuggets with high eating quality attributes. The panelists were asked to evaluate the quality characteristics of the samples based on the following criteria: 0–2 = extremely dislike, 3–4 = slightly dislike, 5–6 = average, 7–8 = like considerably, and 9–10 = outstanding.

Using analysis of variance (ANOVA) XLSTAT software version 2014, 5.03 (Addinsoft, New York, NY, USA) in three repetitions, experimental results were evaluated and expressed as the mean ± standard error of the mean. Differences between sample means with a *p-value* ≤ 0.05 were considered to be significant.

Results and discussion

Chemical composition of Nile perch flesh, sesame hulls and sunroot

Table 1 indicates the chemical composition of Nile perch flesh, sesame hulls powder and sunroot puree. According to the results, the sample of Nile perch flesh had the highest protein content (20.21%) followed by sesame hulls (10.52%) and sunroot (2.10%).

Table 1. Chemical composition of Nile perch flesh, sesame hulls and sunroot (g/100g sample)

Parameters %	Nile perch flesh	Sesame hulls	Sunroot
Crude protein	20.21 ± 0.21^a	10.52 ± 0.32^b	2.10 ± 0.17^c
Crude fat	0.54 ± 0.16^b	13.54 ± 0.23^a	0.22 ± 0.15^c
Crude fiber	—	17.24 ± 0.22^a	3.24 ± 0.12^b
Ash	0.61 ± 0.19^c	16.11 ± 0.23^a	0.8 ± 0.13^b
Total carbohydrates	—	12.53 ± 0.21^b	15.76 ± 0.32^a
Moisture	78.64 ± 0.33^b	47.3 ± 0.36^c	81.12 ± 0.41^a

The letters (a, b, and c) represent the statistically significant changes between treatments (p ≤ 0.05).

The fat content was the highest in sesame hulls (13.54%) followed by Nile perch (0.54%) and sunroot (0.22%). The sesame hull sample showed the highest crude fiber and ash content (17.24% and 16.11%) compared to the sunroot (3.24% and 0.80%) and Nile perch flesh (0% and 0.61%) samples, respectively. There were no carbohydrates in Nile perch flesh, while the content of carbohydrates in sunroot and sesame hulls was 15.76% and 12.53%, respectively. Table 1 also shows that the moisture content was high in both sunroot (81.12%) and Nile perch flesh (78.64%) samples compared to sesame hulls (47.3%).

In this respect, Okeyo et al. [23] reported that the protein, lipid, ash and moisture contents in Nile perch edible tissue varied between 19.8 and 17.7%; 0.59 and 0.63%; 0.55 and 0.63%; 78.5 and 79.5%, respectively. Also, the chemical composition values (protein, lipid, fiber and ash contents) of sunroot agree with Kays and Nottingham [14] and those of sesame hulls agree with Nikolakakis et al [23] and Bonos et al. [24].

Sensory evaluation of Nile perch fish nuggets with different concentrations of sunroot and sesame hulls

The obtained results presented in Table 2 and Figure 1 show the sensory score (aroma, texture, color, and overall acceptability) of Nile perch fish nugget samples at different concentrations of sesame hulls (2.5%, 5%, 7.5%, 10%, 15%

Table 2. Sensory evaluation of the Nile perch fish nuggets prepared with different concentrations of sunroot and sesame hulls

Sample	%	Color	Texture	Taste	Odor	Overall Acceptability
Control	—	8.8 ± 0.21^a	8.7 ± 0.12^a	8.7 ± 0.16^a	8.7 ± 0.22^a	8.7 ± 0.25^a
Sunroot	2.5	8.8 ± 0.23^a	8.7 ± 0.25^a	8.7 ± 0.12^a	8.7 ± 0.27^a	8.7 ± 0.25^a
	5	8.8 ± 0.21^a	8.6 ± 0.29^a	8.6 ± 0.27^a	8.6 ± 0.22^a	8.6 ± 0.13^a
	10	8.6 ± 0.23^a	8.6 ± 0.26^a	8.5 ± 0.15^a	8.6 ± 0.28^a	8.6 ± 0.11^a
	15	7.7 ± 0.22^b	7.5 ± 0.16^b	6.5 ± 0.45^b	7.8 ± 0.22^b	7.1 ± 0.26^b
Sesame hulls	2.5	8.2 ± 0.24^a	8.0 ± 0.28^a	8.2 ± 0.24^a	8.2 ± 0.23^a	8.2 ± 0.14^a
	5	8.1 ± 0.26^a	7.9 ± 0.16^a	8.1 ± 0.23^a	8.2 ± 0.21^a	8.2 ± 0.21^a
	7.5	8.0 ± 0.15^a	7.8 ± 0.24^a	8.0 ± 0.27^a	8.1 ± 0.24^a	8.2 ± 0.23^a
	10	7.3 ± 0.19^b	7.1 ± 0.12^b	7.4 ± 0.15^b	7.5 ± 0.10^b	7.3 ± 0.12^b
	15	6.5 ± 0.25^c	6.7 ± 0.21^c	6.5 ± 0.27^c	6.6 ± 0.29^c	6.7 ± 0.14^c
	20	5.8 ± 0.23^d	5.6 ± 0.24^d	5.8 ± 0.28^d	6.4 ± 0.26^c	5.7 ± 0.25^d

The letters (a, b, and c) represent the statistically significant differences between treatments (p ≤ 0.05).

and 20%) and sunroot (2.5%, 5%, 10% and 15%) compared to the control sample. The results show that the sensory score of fish nuggets with sunroot was acceptable up to the 10% replacement ratio, and beyond this ratio, a big change in texture and taste, as well as overall acceptability of fish nuggets, was observed.

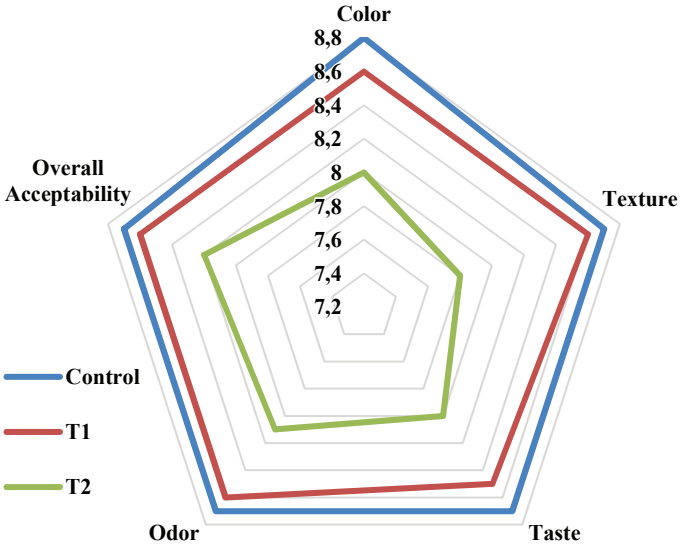

Figure 1. Sensory evaluation of Nile perch fish nuggets with the best concentrations of sunroot (10%) (T1) and sesame hulls (7.5%) (T2)

On the other hand, regarding the sensory evaluation of fish nuggets with sesame hulls, the acceptable replacement ratio reached 7.5%. This percent (7.5%) of sesame hulls was less than the level of fish replacement with sunroot because a higher percentage led to more changes in texture and taste that were rejected by the test panel. Based on the sensory evaluation, 10% of sunroot and 7.5% of sesame hulls were considered to be the best concentrations in the recipes of fish nuggets (Table 2 and Figure 1).

Physical, chemical and microbiological profiles of Nile perch fish nuggets prepared with 10% of sunroot and 7.5% of sesame hulls

Table 3 and Figure 2 show the physical, chemical and microbiological profiles of Nile perch fish nuggets with 10% of sunroot (T1) and 7.5% of sesame hulls (T2) compared to the control sample. The results show an increase in the ash content in T2, which was attributed to the addition of sesame hulls with the high ash content (see Table 1), and no significant changes in T1 compared to the control sample (1.35%). A decrease in the protein content was observed in T1 (17.12%), while there was no significant decrease in T2 (18.32%) compared to the control sample (18.90%). This decrease may be due to the replacement of Nile perch flesh with sunroot and sesame hulls, which have a lower content of protein than Nile perch flesh. An increase in the total carbohydrate content was recorded in the T1 and T2 samples compared to the control, while no significant differences were found between T1 and T2. This increase in carbohydrates is attributed to the addition of sunroot and sesame hulls, both of which are considered a good source of carbohydrates (as shown in Table 1). A significant decrease in the fat content was recorded in T1 (0.52%), while it increased in T2 (1.77%) compared to the control sample (0.56%). This increase in the fat content for T2 is attributed to the addition of sesame hulls, which have the high fat content (see Table 1).

Table 3. Physical and chemical profiles of Nile perch fish nuggets with 10% of sunroot and 7.5% of sesame hulls

Parameter	Control	T1	T2
Ash, %	1.35 ± 0.21^{b}	1.51 ± 0.12^{b}	3.10 ± 0.23^{a}
Protein, %	18.90 ± 0.35^{a}	17.12 ± 0.14^{b}	18.32 ± 0.13^{a}
Total Carbohydrates, %	1.11 ± 0.15^{b}	4.1 ± 0.17^{a}	4.31 ± 0.16^{a}
Fat, %	0.56 ± 0.18^{b}	0.52 ± 0.17^{b}	1.77 ± 0.14^{a}
Moisture, %	78.12 ± 0.32^{b}	76.72 ± 0.41^{a}	72.50 ± 0.23^{c}
pH	6.52 ± 0.14^{a}	6.64 ± 0.28^{a}	6.26 ± 0.27^{b}

T1 (with 10% of sunroot); T2 (with 7.5% of sesame hulls); the letters (a, b, and c) represent the statistically significant differences between treatments ($p \leq 0.05$).

The water holding capacity (WHC) is an important factor that expresses the ability of protein to bind water. According to the data presented in Figure 2, the WHC of the control, T1 and T2 samples was 1.9, 2 and 2.3 cm^2, respectively. The difference between the samples was attributed to a higher percent of protein in the control sample compared to T1 and T2. On the other hand, the difference between the control and samples T1 and T2 is not big, thus this result is good considering the cost of using plant sources to replace fish.

A significant decrease in oil uptake (%) was observed in T1 (1.65%) and T2 (1.68%) compared to the control sample (1.86%), while an increase in cooking loss (%) was found in both T1 (18.25%) and T2 (17.75%) compared to the control sample (16.6%).

Table 4 shows an effect of storage time (for 3 months at –18 °C) on TBA, WHC, color values (L*, a* and b*) and microbial load (total plate count and psychrophilic bacterial count) of Nile perch fish nuggets with 10% of sunroot (T1) and 7.5% of sesame hulls (T2) as well as the control sample. An increase in TBA, WHC, a* and b* values, and a decrease in L*, TPC and PSY values with storage time were observed for all samples (control, T1 and T2). Lipid oxidation is a significant component in the deterioration of frozen fish and fishery products, since it severely affects protein functioning and causes discoloration, off-odor, and off-taste [26]. TBA is the secondary lipid oxidation product that was measured each month during the storage period (3 months). The results are presented in Table 4. The lower TBA value was observed in T1 (0.294 mg malonaldehyde/kg) compared with the control (0.327 mg malonaldehyde/kg) and T2 (0.405 mg malonaldehyde/kg) samples ($p < 0.05$). Moreover, an increase in the TBA values was detected as the storage duration was extended ($p < 0.05$). At the beginning of storage, the TBA values for the T1, control, and T2 samples were 0.224 (mg malonaldehyde/kg), 0.241 (mg malonaldehyde/kg), and 0.337 (mg malonaldehyde/kg), respectively. TBA is a crucial

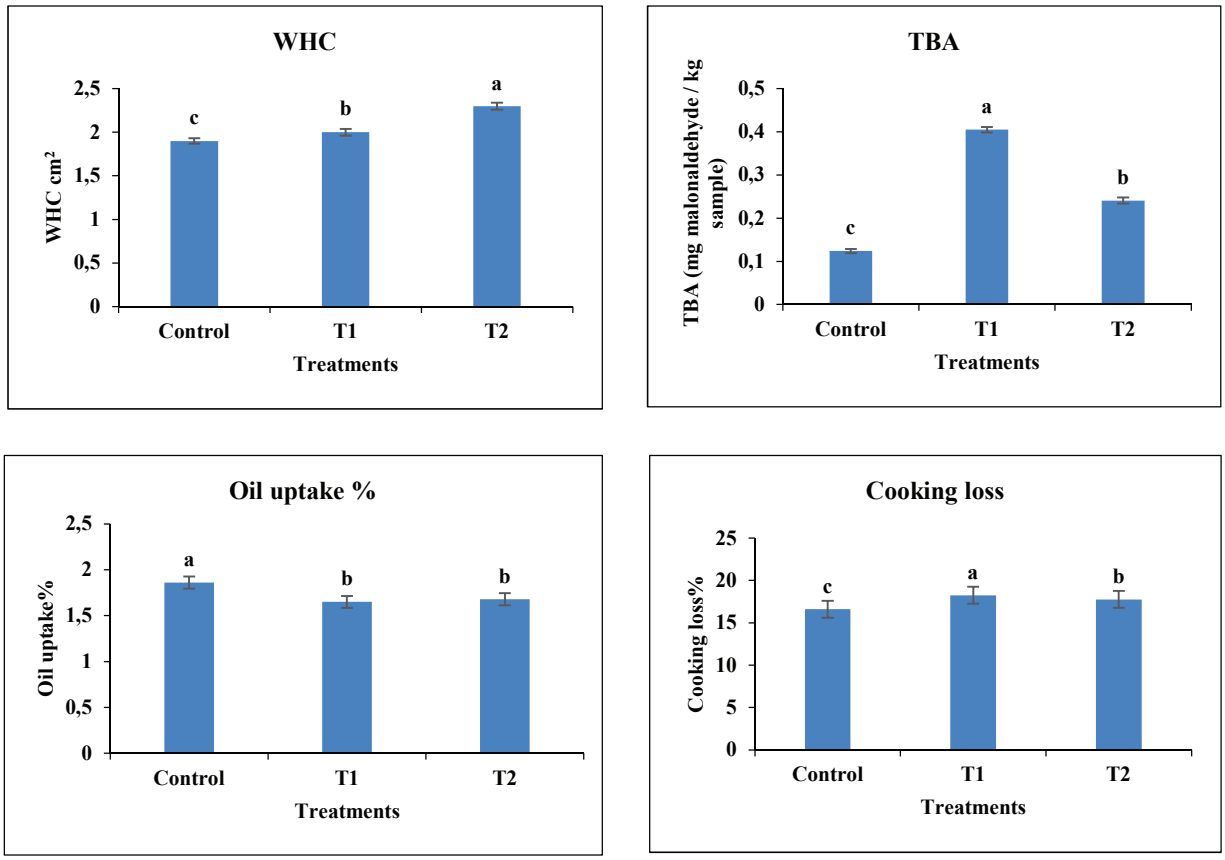

Figure 2. Water-holding capacity (WHC), thiobarbituric acid (TBA), oil uptake and cooking loss of Nile perch fish nuggets with the best concentration of sunroot (10%) (T1) and sesame hulls (7.5%) (T2) compared to the control; the letters (a, b, and c) represent the statistically significant differences between treatments ($p \leq 0.05$)

Table 4. Effect of storage time (three months at –18 °C) on some quality parameters of Nile perch fish nuggets prepared with 10% of sunroot and 7.5% of sesame hulls

Time (Month)	Treatment	TBA (mg malonaldehyde/kg)	WHC ($cm^{2)}$	L*	a*	b*	TPC (log cfu/g)	PSY (log cfu/g)
0	Control	0.241 ± 0.01	1.9 ± 0.1	63.03 ± 0.34	3.58 ± 0.15	20.72 ± 0.35	2.32 ± 0.17	2.02 ± 0.09
	T1	0.224 ± 0.01	2.0 ± 0.1	67.61 ± 0.32	4.34 ± 0.18	20.21 ± 0.33	2.24 ± 0.10	1.72 ± 0.06
	T2	0.337 ± 0.01	2.3 ± 0.1	68.79 ± 0.35	3.94 ± 0.13	19.22 ± 0.30	2.30 ± 0.02	1.47 ± 0.04
1	Control	0.267 ± 0.01	2.1 ± 0.1	58.66 ± 0.26	3.73 ± 0.11	20.77 ± 0.17	2.28 ± 0.05	1.99 ± 0.07
	T1	0.243 ± 0.01	2.1 ± 0.1	63.65 ± 0.22	4.74 ± 0.12	22.44 ± 0.24	2.16 ± 0.06	1.56 ± 0.1
	T2	0.356 ± 0.01	2.4 ± 0.1	67.55 ± 0.31	3.93 ± 0.15	19.81 ± 0.25	2.18 ± 0.09	1.59 ± 0.01
2	Control	0.289 ± 0.01	2.2 ± 0.1	56.78 ± 0.28	4.16 ± 0.12	20.84 ± 0.19	2.27 ± 0.06	1.89 ± 0.11
	T1	0.262 ± 0.01	2.2 ± 0.1	55.92 ± 0.23	5.74 ± 0.16	22.59 ± 0.32	1.95 ± 0.04	1.45 ± 0.09
	T2	0.372 ± 0.01	2.5 ± 0.1	66.78 ± 0.21	3.96 ± 0.12	20.15 ± 0.17	2.02 ± 0.07	1.38 ± 0.08
3	Control	0.327 ± 0.01	2.5 ± 0.1	54.28 ± 0.32	4.21 ± 0.13	21.90 ± 0.15	2.25 ± 0.04	1.81 ± 0.05
	T1	0.294 ± 0.01	2.4 ± 0.1	52.67 ± 0.34	5.53 ± 0.17	20.68 ± 0.22	1.82 ± 0.05	1.23 ± 0.09
	T2	0.405 ± 0.01	2.7 ± 0.1	65.85 ± 0.30	4.06 ± 0.15	20.96 ± 0.18	1.89 ± 0.07	1.28 ± 0.1

* T1 (with 10% of sunroot); T2 (with 7.5% of sesame hulls); TBA: thiobarbituric acid; WHC: water-holding capacity; TPC: total plate count; Psy: psychrophilic bacteria

measure of the quality of fish and fish products. It can be concluded that the sunroot addition in T1 decreased the TBA value due to removing a considerable amount of lipids.

Tokur et al. [27] likewise found an increase in the TBA value of tilapia (*Oreochromis niloticus*) fish burger after eight months. In addition, similar patterns were seen in the TBA values of the Nile tilapia fish burger, tilapia fish cutlet [9], grass carp fish cutlet and fish finger [28], and Nile tilapia nuggets [29]. The rise in TBA may be attributable to the availability of oxygen for oxidation, which is linked to the mechanical chopping of fish meat or the mixing of ingredients [27] or to the packaging. The TBA value is an indicator of lipid oxidation in fish products with the generation of aldehydes and carbonyl-containing compounds. Günşen et al. [30] stated that the TBA value should not be higher than 5 mg malonaldehyde/kg in good quality products. Nevertheless, rancidity had been identified when TBA values exceeded 4 mg malonaldehyde/kg [30]. Also, Connell [31] reported that rancidity develops in fish when TBA levels are above 1–2 mg malonaldehyde/kg. According to Egyptian requirements, the allowed upper limit of TBA as an index of fish quality during storage of fish is

4.5 mg malondialdehyde/kg fish meat [32]. It is evident from the data that none of the investigated samples exceeded the limits indicated above. An increase in the WHC values was recorded for the control, T1 and T2 samples with pass of storage time. An increase for the control, T1 and T2 was 31.6%, 20.0% and 17.4%, respectively. In general, the microbial load of fish leads to its more rapid spoilage compared to other muscle foods like meat and chicken, and this spoilage is largely bacterial in nature. Therefore, effective preservation methods should prevent the microbiological decomposition of fish without diminishing its quality or nutritional value. To test the microbiological safety of the products, the total bacterial count and psychrophilic count of the control, T1 and T2 samples were examined microbiologically each month during frozen storage at –18 °C.

Table 4 shows the total plate count (TPC) and psychrophilic bacterial (PSY) count of the control, T1 and T2 samples as affected by storage time at –18 °C. The initial TPC and PSY loads were 2.32 and 2.02 log cfu/g for control; 2.24 and 1.72 log cfu/g for T1; 2.30 and 1.47 log cfu/g for T2, respectively. During frozen storage, the levels of TPC in the control, T1, and T2 samples changed. The TPC and PSY values in the control, T1 and T2 samples showed the downward trend to the values of 2.25 and 1.81; 1.82 and 1.23; 1.89 and 1.28 log cfu/g, respectively, at the end of 3-month storage. A decrease in TPC and Psy may be attributable to bacterial cell damage brought on by the ice crystal growth [33]. Freezing causes a reduction in bacterial count, which continues to fall in the majority of cases throughout frozen storage [34]. The permissible recommended limit of TPC by EOS in chilled fish is 10^6 cfu/g= 6 log cfu/g [35]. Our results after storage time of 3 months at –18 °C did not exceed the recommended limits.

Conclusion

In summary, for innovative utilization of Nile perch fish, we have successfully prepared Nile perch fish nuggets by replacing a part of fish flesh with sesame hulls or sunroot and stored for 3 months at –18 °C. Nile perch flesh had the highest protein content (20.21%), while sesame hulls were rich in fat (13.54%), crude fiber (17.24%) and ash (16.11%). The results show that the sensory score of fish nuggets with sunroot was acceptable up to the 10% replacement ratio (T1). When this ratio was exceeded, a big change in texture and taste, as well as the overall acceptability of fish nuggets, was observed. On the other hand, regarding the sensory evaluation of fish nuggets with sesame hulls, the replacement ratio reached 7.5% (T2). In conclusion, this study suggests that it is possible to develop an alternative ready-to-eat product from fish by replacing a part of fish flesh with sesame hulls or sunroot with an appropriate amount of ingredients in the formulation.

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EFFECT OF ORGANICALLY BOUND IODINE IN CATTLE FEED ON HEALTH INDICATORS

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Abstract
Currently, the problem of iodine deficiency is actual in the world, which may cause a large number of diseases and disorders. The problem of iodine deficiency for humans may be partly solved by enriching agricultural products with iodine, i.e. by providing animals with an increased intake of iodine during their growth. Theoretically, the most effective way to use iodine is the form bound to tyrosine, since diiodotyrosine has been proven to be a thyroxine precursor. Taking it into account, a supplement was developed containing iodine organically bound to tyrosine and histidine. In this work, we studied the effect of this supplement introduced into the diets of cattle on biochemical parameters of animal blood. In the test group, which received the supplement with organically bound iodine, an improvement in nitrogen metabolism was noted compared to the control group. This was most clearly demonstrated by the content of urea, since in the test group, its content decreased by ≈15 percentage points, and by the content of creatinine, since its increase in the test group was more than 20 percentage points. Differences in the parameters of carbohydrate and lipid metabolism between treatments were also noted, as in the blood of animals from the test group, the content of cholesterol, triglycerides, phospholipids, glucose and malondialdehyde was lower than in the control group. In mineral metabolism and morphological parameters, there was no significant difference between treatments. Among the indicators of pigment and hormone metabolism, it is important to note the reduced content of cortisol in the blood of animals from the test group. Its level was lower by ≈17.23 percentage points compared to the control group. A decrease in cortisol levels indicated a lower stress load in the test group. In general, studies have shown that the use of a feed supplement containing organically bound iodine has a positive effect on the metabolism of animals.

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Introduction

To maintain health and immunity, the human body needs a wide range of microelements, macronutrients and vitamins. Currently, the conventional lifestyle does not allow proper monitoring the diet. To a greater extent, this applies to residents of large industrial cities, where it is especially important to maintain immunity due to poor ecological conditions. Many people do not think about microelement and macronutrient deficiencies in their diet until such a deficiency leads to serious illness. It is also worth noting the large-scale nature of commodity production, the main goal of which is not the quality of products, but the profit from their sale. That's why manufacturers reduce the price of production technology.

Iodine is one of the microelements essential for humans. In the body of animals and humans, iodine is part of the thyroid hormones produced by the thyroid gland, i.e. thyroxine and triiodothyronine. These hormones play an important role in the development, growth and metabolism of the body [1]. According to WHO, the average daily intake of iodine for the humans is 120 to 150 micrograms. At the same time, it should be noted that natural analogues of the studied supplement have not been studied and there are no statistical data on their reference intake in both humans and animals.

Lack of iodine leads to a large number of diseases, including cretinism, thyroid disease, hypothyroidism. Even a small deficiency of iodine leads to fatigue, laziness, headaches, depressed mood, causes nervousness and grumpiness, reduces intelligence and weakens memory. One of the most important tasks that must be solved with the elimination of iodine deficiency is a healthy generation of people and procreation. In general, iodine deficiency diseases are one of the most common non-communicable diseases. According to WHO, more than 2 billion people are iodine deficient [2].

Often, the problem of iodine deficiency is tried to be solved with dietary supplements and iodized salt. But iodine in the inorganic form is more difficult to absorb by the body than iodine in the organic form (for example, associated with the amino acid tyrosine in the form of mono- and diiodotyrosines). Therefore, the most relevant direction for enriching the diet of people with iodine is the enrichment of food with the organic form of iodine, i.e. iodine covalently bound to the amino acid.

The best options in this case are either consumption of products with already high content of bound iodine, or production of plants and animals using iodine, so it is consumed by humans in its natural form.

More and more works are currently focused on this approach. Enrichment of feed with iodine during animal maintenance increases the amount of iodine in the final raw materials, which improves product quality and increases attractiveness to consumers. Moreover, it has a positive effect on the health of animals themselves due to the same need for iodine like in humans.

For example, lettuce was grown with the addition of iodine to the soil in an inorganic form of potassium iodate and in an organic form bound to salicylic acid [3]. As a result, it was found that in the leaves of lettuce grown with the addition of iodine to the soil, it accumulated more than in the control samples without soil enrichment with iodine. It has also been found that organic iodine accumulates better than inorganic iodine.

Significantly increased iodine amount in the leaves and fruits of tomato was demonstrated [4]. Similarly to the above work [3], the enrichment was carried out with potassium iodate. In addition to potassium iodate, potassium iodide was used, as well as their mixtures with salicylic acid. The results showed that the use of potassium iodate had the best effect on the nutritional value of tomatoes. It is not recommended to use these clathrates directly in human nutrition due to their toxicity and negative effects on mucous membranes.

Animal breeders are also engaged in similar work. The most suitable direction in enrichment of products with an organic form of iodine through feeding them to animals is the dairy industry.

Milk itself is a rich source of iodine [5]. This is also why it is important to add iodine to the diet of dairy cows in order to supply them with iodine that goes into milk. But before considering the issue of cattle diet enrichment with iodine, it should be considered in other areas of livestock breeding.

Works focused on animal diet enrichment with iodine have been carried out for a relatively long time. So, the effect of iodine on the health of goats, both castrated and uncastrated, was studied [6]. In addition to a positive effect on health, iodine supplementation has been shown to improve seminal properties, i.e. sperm/ejaculate volume, motility, concentration and count.

Russian scientists are also working to enrich the diet of small cattle, in particular sheep, with essential elements. According to published studies, in test groups of buck lambs that received additional iodine, selenium and silicon in their diet, higher parameters of live weight gain were noted. In one of the test groups, greater chest girth and greater height were noted. An 18.8% higher content of free amino acids compared to the control group was noted. The meat contained more protein and less fat [7].

The results of the above work [7] showed the effect of enriched diets in the production of small cattle on the fatty acid composition, activity of lipolytic enzymes, and lipid oxidation process. A significant dependence of the fatty acid composition of lamb meat on the content of microelements in the supplement (in particular, iodine and selenium) was shown. In test samples, a more balanced fatty acid composition was noted with near optimal ω6/ω3 ratios.

Further studies in this direction [7] showed that the use of supplements containing iodine in a natural bound form led to an increased iodine content in the thyroid gland of test groups by 5–6 times compared to the control group. In this case, iodine accumulated in the thyroid gland in an organic form of iodotyrosine. This clearly shows the effectiveness of supplements containing iodine in organically bound form.

In addition to the described benefits of enriching the diet of buck lambs with essential elements, the meat obtained from such animals contains more iodine, which makes it a functional product with more beneficial properties for humans. According to the above work [7], iodine content varied in the range of 54.6 to 61.5 μg/kg.

When enriching the diet of cattle with microelements, in addition to the benefits described, milk enriched with iodine obtained from lactating cows is of great interest. This is confirmed by the results of work, where iodine in the form of iodate and iodide was added to the diet of cows [8]. But, when enriching the diet of animals with iodine, especially in inorganic form, it is important not to exceed the reference levels required for the body [9]. Concerning the milk enriched in this way, the products of its processing will also have a high content of iodine. What was shown in the relevant work [10].

In terms of biology, the enrichment of animal diet with an organic form of iodine will be more favorable for them. As such sources, either complex supplements containing iodine in the form of mono- and diiodotyrosines, or natural sources of iodine in organic form, may be used.

In addition to obtaining better quality raw materials as a result of enriching the diet of cattle with essential elements, it is necessary during lactation. A well-known scientific fact: if a heifer does not meet daily weight gain parameters, then high production characteristics cannot be expected from her. High-yielding cows with a daily milk yield of 30–35 kg excretes from the body about 1000 g of protein, 1500 g of lactose, 1000 to 1200 g of fat per day, which is ensured by a high-intensity metabolism. The realization of such a genetically defined productivity potential is determined by the full-value feeding and depends 50% on metabolizable energy, 25% on protein and 25% on minerals and vitamins. Moreover, in the nutrition of such cows, a catalyst for such high metabolic processes should be presented, which is the basis of the hormonal background of the animal, i.e. covalently bound iodine [11].

The high milk yield leads to a weakening of natural defense mechanisms, immunity, failures in metabolism mechanisms and, as a result, reproductive system diseases, udder diseases, musculoskeletal system disorders and a

long recovery period after calving (open period). It is no coincidence that 25% of culls occur at the peak of milk production in the first 60 days of lactation. Often the situation is aggravated by violations in feeding organization, especially during the critical period, i.e. three weeks before calving and in the first three weeks after it, which leads to a negative balance of energy in the body. In the last decade, the average tenure of cows in the herd has decreased to 2.9 to 3.7 lactations, and the payback period for one animal is about 4 to 4.5 lactations. For this reason, annual losses amount to hundreds billion rubles. So, for example, in the USA, 25% to 40% of cows are culled annually; the world average is 35%. Reasons for culling cows in the US are: reproductive dysfunction (27%), mastitis and other udder diseases (27%), lameness/limb injuries (16%), low productivity (19%), aggressiveness (1%), and other reasons (10%). According to foreign data, the total culling of cows should not exceed 25% to 28% [12]. In Russia, due to the lack of clear up-to-date statistics, this parameter is about 32% to 37%, and there is also no well-defined state policy aimed at preserving the dairy herd and its production characteristics.

Unbalanced diets and poor-quality feed are the main causes of metabolic disorders. Its consequences are an increase in barrenness, birth of a weak offspring, decrease in resistance to diseases, decrease in live weight and milk production, deterioration in milk quality, which ultimately leads to premature culling of animals.

With respect to lactating cow diet enrichment with selenium and iodine, Russian scientists received the RU2769978 C1 patent. As a result of patented supplement using, it was possible to increase milk yield and improve biochemical parameters of lactating cows' blood [13].

Another Russian preparation is based on iodine bond to the complex protein, i.e. casein. It was shown that the use of Iodocasein in the diet of cows with hypothyroidism completely restores the concentration of thyroid hormones [14]. The results of the studies showed that the use of Iodocasein in test groups helped to achieve a decrease in milk production by only 2.3%, while in the control group this parameter ranged from 3.3% to 11.8% [15]. Moreover, Iodocasein helped to increase the fat content in milk.

In the mineral supply of high-yielding cows, iodine is of particular importance. With its deficiency in cows, the estrus cycle is disturbed, impregnation capacity and fertility decrease, fetal resorption, miscarriages in the early stages of pregnancy, abortions, retention of the placenta are observed. The birth of dead or non-viable offspring with a goiter (thick neck) is possible. Milk productivity and fat content are reduced. Weight gain in young animals is reduced. This may mainly be due to the content of the inorganic form of iodine in the blood and milk. With an excess of the inorganic form of iodine, which is primarily due to the use of premixes and various supplements to eliminate IDD in calves, there is a decrease in live weight gain and milk production, an increase in feed costs per production unit. Moreover, the existing supplements based on inorganic forms of iodine (iodides, iodates, clathrates and others) are known not to produce the desired result, and negative dynamics are increasingly observed.

Depending on the species, age and physiological state of the animals, the need for iodine ranges from 0.2 to 1.4 mg per 1 kg of feed dry matter. The need for iodine in non-milking cow is 5.4 to 10.7 mg/animal/day depending on the live weight and planned milk yield. The need for iodine in lactating cows with a live weight of 600 kg is 8.9 to 27.7 mg/animal/day depending on the daily milk yield (16 to 40 kg). According to the nutrient reference intake for cows (USA), the need for iodine per 1 kg of dry matter in non-milking cows is 0.33 mg, and per 1 kg of dry matter in lactating cows it is 0.45 mg. It is necessary to point out the fact that these reference values in scientific work were based on the use of inorganic matter, and did not imply other forms similar to natural ones, while the covalently bound form of iodine is much more effective and biologically available for a living organism.

With an iodine content in milk of 30 μg/L, a high-yielding cow excretes about 1 mg of iodine daily. Therefore, when consuming 18 kg of feed dry matter with apparent absorption of iodine of 80% and iodine concentration in feed of 0.1 mg, a cow receives ≈1.4 mg of iodine per day per 1 kg of feed dry matter. However, this amount of iodine does not guarantee that the lower limit of requirements is met, and therefore it is still recommended to give lactating animals 0.4 mg of iodine per 1 kg of feed dry matter. When feeding cruciferous plants, this dose should be increased to 0.8 mg per 1 kg of feed dry matter.

Due to the fact that iodine deficiency is most often detected mineral deficiency, it is recommended to increase its reference intake to 1.3 mg/kg [16]. At the same time, its toxicity and effect on the general condition of the animal's body are not taken into account at all.

In summer period, high-yielding cows eat with feed 28% to 41% of recommended iodine amount. The physiological role of iodine is associated with its participation in the formation of the thyroid hormone thyroxine. Deficiency of correct bound iodine leads to reduced fertility of cows, poor digestibility of dietary nutrients, low milk production and reduced fat content in milk. This is due to several factors, such as a decrease in the total amount of iodine in soils, a decrease in the quality of bacteria organizing iodine on plant roots, a lack of proper education for people who make decisions about how and what to feed, which leads to the absence of covalently bound iodine in feed and premixes.

Recently, interest in iodine-containing supplements has increased significantly, which is associated with an increase in the need for iodine by animals in intensive livestock production, as well as with the deterioration in the general condition of animals throughout the country.

Therefore, it is necessary to reconsider not only the sources of iodine intake in the nutrition of high-yielding cows, but also develop its bioavailable chemical compound.

Such a compound in the present study is the Carbon-Iodine (C–I) formed by a covalently polar bond. To designate the compound, the introduction of the term CI is proposed, and the method of enrichment with this form of bound iodine is CI technology. To solve the problem, a group of Russian specialists with the support of the Research and Testing Center of the Federal State Budgetary Scientific Institution "V. M. Gorbatov Federal Research Center for Food Systems" of the Russian Academy of Sciences developed a supplement based on a covalently bound form of iodine (containing the CI component in its structure) in a protein matrix, and produced on the basis of iodized milk protein, which is a mixture of whey proteins containing 2.5% of iodine atoms covalently bound to them. Iodine is in the tyrosine or histidine amino acid molecule and has a positive valence, due to which it is bioavailable. Due to the covalent bond between iodine and proteins, the supplement has a high stability when heated up to 300 °C, resistance to light and heat during long-term storage, which guarantees iodine content in the production of various premixes and starter feeds for all types of animals. It is especially important in the early stages of animal growth, when the main physical and chemical indicators of future performance are formed.

Based on the above, the aim of this work was to study the effect of adding organically bound iodine to the diet of lactating cows on biochemical parameters of their blood.

Objects and methods

The experiment was carried out at the Zybino farm, Federal State Unitary Enterprise Research Farm "Klenovo-Chegodaevo" of the Federal State Budgetary Scientific Institution "All-Russian Research Institute of Animal Husbandry named after L. K. Ernst", with 2 groups of white-and-black cattle (10 animals in each) during the summer pasture period. The experiment was carried out only on lactating cows. In one group of cows, the supplement was added to the main diet (test group). 100 g of supplement contains: 25 g of crude protein (protein and non-protein nitrogenous substances), 65 g of glucose, 0.7 g of covalently bound iodine, ash elements (≈2%) and moisture (≈7.3%).

The cows that participated in the experiment arrived at the Zybino farm from the Dubrovitsy farm in March. As a result, they suffered transport stress and, before the start of the experiment, were in a state of adaptation to new technological, hygienic and zootechnical conditions, in particular, to the maintenance conditions (shortened stalls), the change from triple to double milking, the new composition and nutritional value of the diet, as well as grazing together with the Zybino cow herd (social stress). The calculation of cows' need for iodine was made on the basis of recommendations by the All-Russian Research Institute of Animal Husbandry [11] and for lactating cows was 0.45 mg of iodine per 1 kg of diet dry matter. Thus, the daily requirement of a lactating cow is approximately 8 to 10 mg of iodine. For feeding test and control animals, the same diet based was used [11]. The control group received iodine in the form of potassium iodide, and the test group received iodine in the form of a developed supplement. Based on the needs of cows for iodine and its content in the supplement, cows in the test group were given 1.3 g of the developed supplement mixed with bran per animal per day. The supplement was added manually to the feed of each cow individually during their feeding. Thus, one animal received ≈9.1 mg of iodine per day. Cows in the control group were given 2 tablets each containing 6 mg of potassium iodide. The tablets were pre-crushed, after which they were added manually to the feed also in a mixture with bran to each cow individually during their feeding. Thus, one animal received ≈9.0 mg of iodine per day.

The experiment lasted for 100 days, except for the period of adaptation to new maintenance conditions. At the end of the experiment, material for determining the following indicators was taken from the animals:

— morpho-hematological blood parameters: (leukocytes, erythrocytes, hemoglobin, hematocrit), color index on the ABC VET analyzer (Horiba, ABZ, France);
— biochemical parameters of serum (plasma): (total protein, albumin, globulin, urea, uric acid, creatinine, malondialdehyde, cholesterol, phospholipids, triglycerides, NEFA, glucose, total bilirubin, ALT, AST, phosphorus, calcium, iron, magnesium, chlorides, alkaline phosphatase) on the Chem Well automatic biochemistry analyzer (Awareness Technology, USA);
— iodine by volt-amperometry method according to GOST R52689–20061;
— thyroxine by enzyme immunoassay;
— cortisol by enzyme immunoassay.

Statistical data processing was carried out using the Statistica software ver. 10.0.1011 (StatSoft). For statistical processing, Mann-Whitney U-test was used. At $p \leq 0.01$, the results were considered statistically significant; at $p \geq 0.05$, the results were considered statistically insignificant.

Results and discussion

When assessing the state of nitrogenous substances metabolism in the body of cows, great importance is attached to the content of total protein in the serum, as well as albumins, globulins, their ratio, urea and creatinine.

In cows fed a diet containing CI supplement, the content of total protein ($p \leq 0.01$) in serum was higher by 3.9% with a lower (by 3.4%) content of albumin ($p \leq 0.01$) and higher (by 7.9%) content of globulin ($p \leq 0.01$). As a result, their protein index was lower and amounted to 0.46 versus 0.52 in the control group. This ratio characterizes the level of protein metabolism and its direction associated with the increased need of the body for amino acids and energy. These parameters did not go beyond the physiological reference values, i.e. the content of total protein in serum

[1] GOST R52689–2006 "Foods. Anodic stripping voltammetric method of iodine mass concentration determination". Retrieved https://docs.cntd.ru/document/1200051514/titles Accessed December 27, 2022. (In Russian)

of 72.00 to 86.00 g/L, and albumin content in serum of 27.00 to 43.00 g/L [17–19].

Table 1. Parameters of nitrogen metabolism in the blood of cows, (n = 3)

Parameters	Treatments		Test to control ratio	Test vs. control difference
	Control group	Test group	%	±
Total protein, g/L	85.64 ± 2.50	89.0 ± 2.99	103.9	+3.36
Albumin, g/L	29.2 ± 0.78	28.2 ± 1.33	96.6	–1.0
Globulin, g/L	56.4 ± 2.03	60.8 ± 2.39	107.9	+4.4
A/G	0.52 ± 0.02	0.46 ± 0.03	—	–0.06
Urea, mmol/L	4.81 ± 0.29	4.12 ± 0.34	85.6	-0.69
ALT, IU/L	22.79 ± 2.15	17.67 ± 1.87	77.53	–5.12
AST, IU/L	78.6 ± 0.19	68.99 ± 4.45	87.77	–9.61
De Ritis ratio	3.83 ± 0.43	4.08 ± 0.44	106.53	0.25
Uric acid, mmol/L	123.4 ± 15.79	98.2 ± 5.76	79.5	–25.2
Creatinine, μmol/L	87.8 ± 13.13	106.3 ± 6.98	121.2	+18.6

Urea is formed in the liver during the neutralization of ammonia. It quite accurately reflects the concentration of ammonia in the rumen, the level and quality of dietary protein. In these studies, the level of urea in the serum of the test group was lower by 14.4% ($p \le 0.01$), which was ensured by a well-balanced diet in terms of nutrients and, accordingly, good work of the rumen microbiome during the breakdown of protein and a decrease in ammonia transfer into the blood. The level of urea in the blood of animals that received the experimental supplement as a part of the diet also indicated normal liver function. Other researchers also report this [20, 21].

According to the researches, to assess the functional state of the liver in cows, it is recommended to determine the activity of AST and ALT in the blood, and for accurate differentiation, it is recommended to calculate their ratio (De Ritis ratio) [22, 23]. In the blood of cows from both groups, a high activity of transamination enzymes was noted, i.e. aspartate aminotransferase (AST, EC2.6.1.1) and alanine aminotransferase (ALT, EC2.6.1.2). Parameters of ALT ($p \le 0.01$) and AST ($p \le 0.01$) activity in the blood of animals from the test group decreased by ≈22.47 and ≈12.23 percentage points, respectively. The increase in the De Ritis ratio in the test group compared to the control group is insignificant due to the decrease in ALT and AST in the test group. These values were above the reference limits. In this case, the De Ritis ratio in animals from the control group was 3.83, and in the test group it was 4.08. Beliaeva et al. [23] report the values of this ratio at the level of 2.58 to 3.08. Miller et al. [24] gives the De Ritis ratio for healthy cows as 3.56. The values of this parameter obtained in this work are comparable with the data of these researchers and, in this case, indicate a high metabolic rate to ensure lactation.

Creatinine is a product of protein metabolism and its level in the blood depends both on the intensity of metabolism and on its excretion by the kidneys. In these studies, creatinine content ($p \le 0.01$) in the serum of cows receiving the new supplement was higher by 18.6 μmol/L than in the control group. This may be due to the intensive protein and energy metabolism necessary for the intensive synthesis of milk components. In general, this indicator was within the physiological reference values, i.e. 55.8 to 176.8 μmol/L [25].

The content of creatinine in the serum of test and control cows is consistent with the content of uric acid, a product of the metabolism of nucleic acid purine bases, which reflects the degree of body cells decay. In cows from the test group, the content of uric acid ($p \le 0.01$) was lower by 20.5% and amounted to 98.2 mmol/L compared to 123.4 mmol/L in the control group.

The use of supplement in the diet of animals in a state of homeostasis led to micro-shifts in it. In nitrogen metabolism, these micro-shifts were manifested by an increase in the amount of total protein, globulins, and creatinine concentration, a decrease in the amount of urea and uric acid, reduced activity of AST, ALT, and a higher AST/ALT ratio. The amount of total protein, albumin, uric acid, urea, and creatinine in animals from both groups did not exceed normal values [21]. The values of biochemical parameters obtained as a result of this work are consistent with those given in previous studies [19, 26, 27].

In addition to indicators of nitrogen metabolism, blood obtained from test and control cows was examined for parameters of carbohydrate and lipid metabolism. The results are shown in Table 2.

Table 2. Parameters of carbohydrate and lipid metabolism in the blood of cows, (n = 3)

Parameters	Treatments	
	Control group	Test group
Triglycerides*, mmol/L	0.24 ± 0.03	0.22 ± 0.018
Cholesterol, mmol/L	5.44 ± 0.46	4.25 ± 0.39
NEFA*, mmol/L	0.41 ± 0.06	0.44 ± 0.05
Cholesterol/NEFA ratio	0.09 ± 0.03	0.13 ± 0.02
Phospholipids, mmol/L	3.12 ± 0.38	2.57 ± 0.17
Glucose, mmol/L	4.46 ± 0.14	3.49 ± 0.20
Malondialdehyde, μmol/L	1.98 ± 0.24	1.60 ± 0.14

* $p \ge 0.05$

Of the studied metabolism indicators, the most striking changes were noted in the phospholipids ($p \le 0.01$), i.e. their decrease in the blood of animals from the test group relative to the control group was ≈1.2 times. There was also a decrease in the amount of cholesterol ($p \le 0.01$) in their blood by ≈1.3 times relative to the control animals. In these cows, the glucose content decreased ($p \le 0.01$) relative to the control group and amounted to ≈21.75 percentage points. Cholesterol/NEFA ratio in the test group increased by ≈42.20 percentage points. The amount of malondialdehyde decreased ($p \le 0.01$) by ≈1.18 times in the test group.

The decreased cholesterol level in the blood in animals from the test group was due to its use in the synthesis of milk components, and decrease in glucose was because it

provides the body with energy. This is also evidenced by a higher cholesterol/NEFA ratio and a lower level of phospholipids.

Lactation is the most difficult period for cows. During it, the likelihood of developing oxidative stress in these animals is high. It may cause metabolic shifts [28, 29]. However, a decrease in the amount of malondialdehyde in the blood of cows that received supplement indicated that the processes of lipid peroxidation were occurring normally.

Studies on the content of microelements, macronutrients, and alkaline phosphatase in serum showed the following results (Table 3).

Table 3. Parameters of mineral metabolism in the blood of cows, (n = 3)

Parameters	Treatments	
	Control group	Test group
Calcium*, mmol/L	2.68 ± 0.05	2.59 ± 0.08
Phosphorus**, mmol/L	2.43 ± 0.08	2.41 ± 0.16
Alkaline phosphatase activity*, IU/L	108.65 ± 23.80	91.60 ± 6.14
Phosphorus/calcium ratio	0.90 ± 0.02	0.93 ± 0.04
Iron*, μmol/L	17.65 ± 0.78	17.82 ± 0.85
Magnesium*, mmol/L	0.79 ± 0.06	0.99 ± 0.13
Chlorides*, mmol/L	94.12 ± 1.12	94.60 ± 2.15
Iodine*, μmol/L*	0.123 ± 0.010	0.156 ± 0.0025

* $p \leq 0.01$, ** $p \geq 0.05$

For most indicators of mineral metabolism in the blood obtained from animals in both groups, no difference was found. All of them were within the normal values. The greatest differences between the test and control groups were found in the content of magnesium. In animals from the test group, its amount increased by ≈25.32 percentage points relative to control. This fact should be regarded as positive one. This is due to the fact that this microelement plays a significant role in protein, mineral and fat metabolism, digestion in rumen, as well as acts as an activator for protein synthesis, promotes the formation of enzymes and maintains acid-base balance, and has a positive effect on milk production and milk quality [30–33].

It was found that iodine content was higher in the blood of cows from the test group. The increase was ≈26.83 percentage points relative to control. This difference was significant ($p < 0.05$, $n = 3$), indicating a positive effect of the diet containing the studied supplement.

The parameters characterizing the functional state of the liver and endocrine glands were also studied in the blood. The results are shown in Table 4.

Table 4. Parameters of pigment and hormone metabolism in the blood of cows, (n = 3)

Parameters	Treatments	
	Control group	Test group
Total bilirubin, μmol/L	7.65 ± 0.95	7.61 ± 0.48
Cortisol, nmol/L	15.44 ± 5.50	12.78 ± 3.64
Thyroxine, nmol/L	5.04 ± 1.71	6.99 ± 1.69
Thyroxine/cortisol ratio	0.32	0.54

To analyze the excretory capacity and the pigment metabolism by the liver, the amount of total bilirubin in the blood of cows was determined ($p \leq 0.01$). Its level in the blood of cows from both groups was the same. According to Korochkina et al. [22], determination of the bilirubin amount in the blood is necessary to assess the liver function and the intensity of hemolytic processes in the body. Total bilirubin is the end product of hemoglobin breakdown. Its concentration may be increased in the blood when liver is damaged. According to the latest scientific data, normal total bilirubin is 3.4 to 17.1 μmol/L [21]. However, higher values of this indicator for cows were also established, i.e. 8.6 to 18.2 μmol/L [34]. In some works, reference value for this metabolite is stated at the level of 0.5 to 10 μmol/L [24, 28]. Bilirubin values in the blood obtained in this work are comparable with the data of these studies, and correspond to the reference, thus indicating normal liver function. It was found that in the blood of animals from the test group, the level of cortisol ($p \leq 0.01$) decreased by ≈17.23 percentage points, which indicates that the animals in the test group experienced less stress. Therefore, the raw materials obtained from them will have the best organoleptic properties. In the test group, the content of thyroxine ($p \leq 0.01$) increased by ≈38.69 percentage points. The main function of this hormone is the activation of metabolic processes, which is carried out through the stimulation of protein synthesis. Therefore, its increased amount in the blood of animals treated with an iodine supplement and characterized by an intensive protein metabolism is quite reasonable. The values of thyroxin established in the experiment are comparable with those given by other researchers [26, 35]. A decrease in cortisol and an increase in thyroxine led to a decrease in the thyroxine/cortisol ratio.

Blood tests for morphological parameters did not show a big difference between the treatments (Table 5).

Table 5. Morphological parameters in the blood of cows, (n = 3)

Parameters	Treatments	
	Control group	Test group
Leukocytes*, 10^9/L	8.46 ± 0.66	9.03 ± 1.11
Erythrocytes*, 10^{12}/L	8.36 ± 0.23	8.00 ± 0.35
Hemoglobin*, g/L	100.9 ± 2.83	96.3 ± 2.03
Hematocrit*,%	40.5 ± 1.13	38.5 ± 0.95
Color indicator**	0.37 ± 0.01	0.36 ± 0.01

* $p \leq 0.01$, ** $p \geq 0.05$

In addition to the positive effect on health parameters, feeding cows with a diet containing the supplement with CI component had a positive effect on the composition of milk and some of its technological properties, such as milk fat content, lactose content, and dry matter content. The use of the supplement also had a positive effect on the content of somatic cells in milk, and on its thermal stability.

Conclusion

In the studies conducted, it was found that the diet containing organic or covalently bound iodine (CI component) had a positive effect on the homeostasis of cows, which was manifested by an intensive metabolism of nitrogenous substances, i.e. an increase in the amount of total protein, concentration of globulins, creatinine, a decrease in the amount of urea, uric acid, reduced activity of AST, ALT and a higher AST/ALT ratio. In carbohydrate and lipid metabolism, a decrease in blood cholesterol levels was noted in animals from the test group, due to its use in the synthesis of milk components and glucose to provide the body with energy. The intensity of this metabolism was also evidenced by an increased cholesterol/NEFA ratio and normal processes of lipid peroxidation. The established parameters of mineral metabolism in cows from the test group as expressed in increased content of magnesium in the blood and significantly increased iodine level in the blood and milk, confirmed the effectiveness of the diet with the new supplement. The relationship of the above facts allows to conclude that it had an anabolic effect on the metabolism in the body in general, and hormones, liver pigment in particular. All these facts ultimately contributed to an increased milk productivity in animals.

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HYDROLYSATE OF OVALBUMIN: PRODUCTION AND EVALUATION OF FUNCTIONAL PROPERTIES OF PEPTIDES

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Keywords: *ovalbumin, pepsin, trypsin, hydrolysate, fractionation, antioxidant activity, functional and technological parameters*

Abstract

Chicken eggs proteins and their derivatives, like protein hydrolysates, peptides and amino acids, possess high nutritional value and provide a wide range of biological activity. They serve as sources for development of functional ingredients that draw the attention of specialists in the food production and biomedical industries, as well as the livestock feed industry. Enzymatic hydrolysis of proteins is a popular process for obtaining bioactive peptides with multifunctional properties. The purpose of this study is to obtain a hydrolysate of ovalbumin with a high degree of hydrolysis and to determine its functional and technological parameters. The research presents a two-stage scheme of ovalbumin hydrolysis with the help of pepsin and trypsin which provide high degree of hydrolysis (82–83%). The fractional composition of the hydrolysate is determined. The fractional composition is represented by three main fractions (high, medium and low molecular weight). The summarized antioxidant activity (SAA) of the hydrolysate is considered within the dynamics of the hydrolysis process. The highest SAA value was noted after 2 hours, and it amounted to 170.23 mg/l; at the end of hydrolysis the SAA value was equal to 114.31 mg/l. When analyzing the SAA, it was found that the main contribution to the summarized antioxidant activity of the ovalbumin hydrolysate is made by peptides of the medium molecular fraction. The microfiltration process, used in the research, made it possible to separate high-molecular compounds, which led to an increase in the SAA of the hydrolysate to 189.9 mg/l. The main functional and technological parameters of the hydrolysate are determined in this research. The comprehensive study of the biological activity and functional characteristics of egg protein hydrolysates and their peptides provides a theoretical basis for expanding the range of functional ingredients obtained from food proteins and for replenishing the range of functional foods.

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Introduction

Proteins are an important macronutrient that provides catalytic, structural, regulatory, receptor, energy, transport, protective and respiratory functions in the human body. In addition, some dietary proteins may provide a positive effect on body functions and/or human health with the help of bioactive peptides release. Recently the close attention of scientists around the world has been attracted to the production and analysis of bioactive peptides, necessary for production of functional ingredients [1]. As a rule, bioactive peptides are oligopeptides that are included in the sequence of a protein molecule and that can be released in result of enzymatic hydrolysis, microbial fermentation or digestion in the gastrointestinal tract. Peptides with a low molecular weight are easily digested by the body, are available for nutrition of people with various disorders and diseases of the digestive system, and do not cause allergic reactions [2,3].

Studies have shown that protein hydrolysates (peptides) feature a wide range of biological activities. They possess immunomodulatory, anticancer, antihypertensive, antioxidant, anti-inflammatory, antilipidemic, osteoprotective, antimicrobial, and other useful properties [2]. In addition to biological activity, food-derived protein hydrolysates (peptides) have different physicochemical properties, including good solubility, ability of lipid binding, foaming and emulsification, etc., depending on their composition, sequence and length, which makes them quite demandable for their inclusion into the composition of food products [4].

For the recent years the demand for bioactive protein hydrolysates, obtained from natural sources in the food industry, has dramatically increased. Those researches are valuable due to opportunity to use the bioactive peptides as food additives (to increase the biological and nutritional

value of the product; the development of functional foods). In addition, consumers are increasingly aware that proper nutrition (i.e. not only balanced, but also functional) is directly related to their physical well-being and contributes to the prevention of various diseases [5,6].

Among various food sources of animal origin, eggs, in particular chicken eggs, are considered as inexpensive food products, but at the same time rich in nutrients, with a well-balanced composition of essential amino acids [7]. Eggs contain many biologically active compounds with antimicrobial, immunomodulatory, antioxidant, anticarcinogenic, hypotensive, and other properties [8].

In addition, chicken eggs are one of the most popular foods that are consumed all over the world. An egg contains an average of 13% proteins, which consist of several functionally active proteins: ovalbumin (about 54%), ovotransferrin (12–13%), lysozyme (3.4–3.5%), ovomucoid, ovomucin (1.5–3.5%), ovoglobulins (2%). Egg protein is considered a vital source for obtaining bioactive protein hydrolysates and peptides with multifunctional properties and potential application in healthy nutrition [9,10].

Since Fujita et al. [11] in 1995 identified the first bioactive peptide derived from egg protein (ovokynin — Phe-Arg-Ala-Asp-His-Pro-Phe-Leu), the interest to research and development of new bioactive peptides from this source has been rising vigorously. Hydrolysates and peptides have been obtained from various protein-rich egg structures, like egg white, egg yolk and eggshell [11].

In the review article, written by Moreno-Fernández et al. [11], the researcher presents data obtained from numerous researches devoted to the production and evaluation of the activity of enzymatic hydrolysates and peptides, prepared from egg white. These peptides have a hypoallergenic effect, and also exhibited antioxidant, antihypertensive, anti-inflammatory, hypocholesterolemic, and antidiabetic activities.

In the work of Venkatachalam et al. [12] reported data on enzymatic hydrolysis and production of bioactive hydrolysates/peptides from egg protein using various proteases (alcalase, ficin, protamex, and neutralase). The authors studied their influence on the parameters like the efficiency of hydrolysis, foaming and free radicals inactivation ability.

Although the isolated egg protein fractions (ovalbumin, lysozyme, ovotransferin) have been used in researches as a substrate for enzymatic hydrolysis and the production of bioactive peptides, more often the hydrolysis process is carried out using whole egg white using enzymes such as alcalase, flavorzyme, pepsin, bromelain, trypsin, α-chymotrypsin, papain, etc. [13].

It has been noted that peptides obtained from egg protein as a result of in vitro digestion "imitation" are resistant to in vivo digestive enzymes, which is a great advantage when they are delivered orally (as part of food products) [11].

Thus, the interest in technologies for the enzymatic modification of food proteins, including egg protein, remains extremely high.

The article by Zhamsaranova et al. [14] describes the production of an enzymatic hydrolysate of soy protein, while gel filtration was used to chromatograph it, and the summarized antioxidant activity of the obtained three main fractions (high, medium, and low molecular weight) was determined. The research of the authors B. A. Bolkhonova et al. is devoted to the selection of operable parameters for obtaining peptides of an egg white [15].

The purpose of this research is to obtain a hydrolysate of ovalbumin with a high degree of hydrolysis and to determine its functional and technological parameters.

Objects and methods

To obtain a hydrolysate, ovalbumin (Igreca SAS, France) and proteolytic enzymes pepsin (Reakhim, Russia), trypsin (Spofa, Czech Republic) were used as an object of study.

At the first stage, one-stage hydrolysis of ovalbumin was run separately with pepsin and trypsin. A 1% protein solution was prepared. Pepsin-aided hydrolysis was run in 0.1 M HCl (pH = 1.6) at a temperature of 39 °C for 1 to 5 hours after the introduction of the enzyme. Trypsin-aided hydrolysis was run in 0.1 M NaHCO3 (pH = 7.8) also at 39 °C for 1 to 5 hours after the introduction of the enzyme. For both enzymes the researchers used the different enzyme-substrate ratios: 1:30, 1:20, and 1:10.

At the second stage of research, a two-stage process of protein hydrolysis was used. The first stage of hydrolysis of ovalbumin (pepsin-aided hydrolysis) was run in 0.1M HCl (pH = 1.6) for 5 hours. During the second stage of hydrolysis (trypsin-aided hydrolysis for 3 hours), the pH of the reaction mixture was changed to 7.8 by adding 10% NaOH.

The content of total nitrogen was determined with the help of Nessler's reagent according to GPM.1.7.2.0027.151, the content of amine nitrogen in non-hydrolyzed raw materials and the resulting hydrolysates was determined by the method of formal titration (Sorens method) according to GPM.1.2.3.0022.152.

The degree of hydrolysis (DoH) of the protein was calculated by the following formula:

$$DoH = \left(\frac{N_{AA} - N_{AAo}}{N_{OA} - N_{AAo}}\right) \times 100\%, \quad (1)$$

where

N_{OA} is the content of total nitrogen,%;

N_{AAo} is the content of amine nitrogen in non-hydrolyzed raw material,%;

N_{AA} is the content of amine nitrogen in the hydrolysate after hydrolysis for a certain period of time, %.

[1] GPM.1.7.2.0027.15 "Determination of total nitrogen with Nessler's reagent in immuno-biological medications" Retrieved from https://pharmacopoeia.ru/wp-content/uploads/2016/09/GPM.1.7.2.0027.15-Opredelenie-obshhego-azota-s-reaktivom-Nesslera-v-immunobiologicheskih-lekarstvennyh-preparatah.pdf Accessed January 24, 2023. (In Russian)

[2] GPM.1.2.3.0022.15 "Determination of amine nitrogen by formol and iodometric titration methods" Retrieved from https://pharmacopoeia.ru/wp-content/uploads/2016/10/GPM.1.2.3.0022.15-Opredelenie-aminnogo-azota-metodami-formolnogo-i-jodometricheskogo-titrovaniya.pdf Accessed January 24, 2023. (In Russian)

The parameters of enzymatic hydrolysis were optimized with Mathcad 15 software. In order to find the maximum value of the response function and the corresponding values of the factors, a standard procedure was run to find the maximum of the function of two variables in a limited domain of definition. To obtain the value of the desirability function from the values of the response function, the Harrington formula was used [16].

The obtained hydrolysate was separated into peptide fractions by gel filtration on the G-55 molselect [14]. The hydrolysate was fractionated on a 1×25 cm column filled with molselect, while the gel height in the column was equal to 20 cm. The amount of exposed sample was 1 cm^3. Distilled water was used as the eluent. After applying the hydrolysate to the column, the hudrolysate was eluted in a stream of water at a rate of 35 cm^3/h. Sampling was carried out at rate of 1 cm^3. The absorbance of the eluate was determined at a wavelength of 210 nm on a Cary 300 spectrophotometer (Varian Optical Spectroscopy Instruments, Australia). Each selected fraction was additionally analyzed to determine the content of peptides by the method of Warburg and Christian [17], after that the percent content of each fraction was calculated in relation to the total number of components.

The macromolecular compounds in the hydrolysate composition were separated out by microfiltration through commercial filters Sartorius Minisart™ NML Syringe Filters, Sterile, with a pore diameter of 0.2 μm.

The summarized antioxidant activity (SAA) of the peptides was assessed by the amperometric method on the "Tsvet-Yauza-01-AA" antioxidant activity analyzer (Khimavtomatika, Russia) according to GOST R54037–2010[3]. The concept of the amperometric method is to measure the electric current that occurs during the oxidation of the tested substance (or mixture of substances) on the surface of the live electrode at a certain potential, and to compare the obtained signal with the signal of the standard (quercetin) under the same measurement conditions. The parameter is characterized as the summarized content of water-soluble antioxidants in the analyzed sample (mg/dm^3 or mg/l).

Solubility was determined according to GPM.1.2.1.0005.15[4], the viscosity of the solution — with a liquid capillary viscometer (glass capillary viscometer PVZh-2, Ekokhim, Russia) according to GPM.1.2.1.0015.15[5], foaming and foam stability were determined according to GOST 23409.26–78[6].

pH was determined by the potentiometric method using ph-009 (measurement range from 0 to 14, error ±0.1 pH, Kelilong, China) according to GOST 32892–2014[7], density was determined by the areometric method (AON-1 hydrometer, Khimlaborpribor, Russia) according to GOST R54758–2011[8].

The mass fraction of moisture and the mass fraction of solids were determined according to GOST 54607.4–2015[9].

Appearance, color, smell and taste were determined according to GOST R ISO 22935–2–2011[10].

The experimental data were processed by calculation of mean values (M), with standard error of the mean (m), and parametric evaluation criterion (Student's t-test). The results were considered significant when the threshold of differences significance was reached ($p \leq 0.05$)

Results and discussion

Ovalbumin was enzymatically converted with the proteolytic enzymes pepsin and trypsin, which are available on the Russian market and are relatively inexpensive. In addition, the application of these proteases imitates (simulates) the natural process of protein hydrolysis in the human body, which makes it possible to use the resulting hydrolysates orally (as part of food products) [18].

As noted above, at the first stage of the research, the conditions for one-stage enzymatic hydrolysis of ovalbumin were arranged separately by pepsin and by trypsin. During the study, after 1, 3 and 5 hours of hydrolysis, aliquots of the hydrolysate were sampled and analyzed to determine content of amine nitrogen. The content of total nitrogen and amine nitrogen was preliminarily determined in a sample of non-hydrolyzed raw materials. The degree of protein hydrolysis (SH) was calculated according to formula 1.

The results of a one-step process of ovalbumin hydrolysis, assessed by its degree (DoH) by the enzymes used (pepsin and trypsin), are presented below, respectively, in Figures 1 and 2.

From the data presented above in the Figure 1, it follows that in all variants of ovalbumin pepsin-aided hydrolysis, a significant increase in hydrolysis degree was observed. The highest efficiency was noted after 5 hours in the second variant of hydrolysis (ESR ratio 1:20) and DoH was 70.4 ± 0.21% ($p \leq 0.05$).

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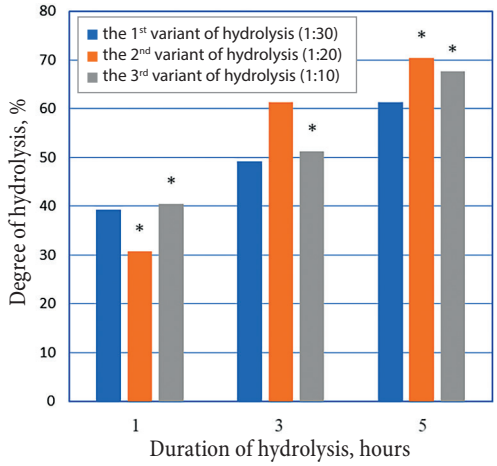
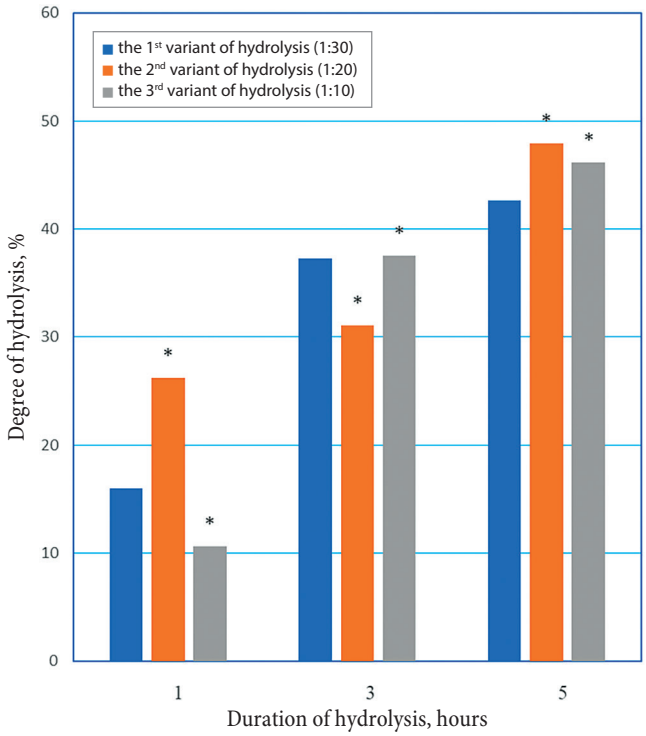


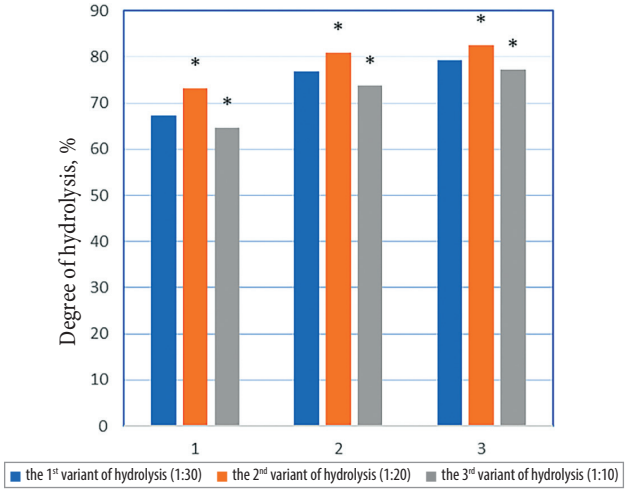
* significant differences between hydrolysis variants ($p \leq 0.05$).

Figure 1. Dynamics of the degree of ovalbumin hydrolysis by pepsin



* significant differences between hydrolysis variants ($p \leq 0.05$).

Figure 2. Dynamics of the degree of ovalbumin hydrolysis by trypsin



* significant differences between hydrolysis variants ($p \leq 0.05$).

Figure 3. Dynamics of the degree of ovalbumin hydrolysis by trypsin in a two-stage process

As can be seen from the Figure 2, in all variants of hydrolysis of ovalbumin with trypsin, an increase in its degree was also observed. The highest efficiency was noted for the second hydrolysis variant after 5 hours (ESR1:20) and DoH amounted to $47.9 \pm 0.49\%$ ($p \leq 0.05$).

At the second stage of research, in order to obtain a higher degree of protein splitting, a two-stage hydrolysis process was used, in which the enzyme productions pepsin and trypsin were introduced sequentially. The variant with the simultaneous introduction of enzymes was not considered, because the conditions for their action (pH values) are different. In a two-stage process, hydrolysis was at first run with pepsin for 5 hours at ESR1:20 (Figure 1, variant 2), after that trypsin was added (at ESR equal to 1:30, 1:20 and 1:10) and hydrolysis continued for another 3 hours. The obtained data on the degree of hydrolysis (DoH) of ovalbumin with trypsin in a two-stage process are shown below in the Figure 3.

From the data presented in the Figure 3, it follows that the two-stage hydrolysis process (first stage was run with pepsin at ESR equal to 1:20 for 5 hours, and then with trypsin at ESR equal to 1:20 for 3 hours) contributed to an increase in degree of hydrolysis up to the maximum value — $82.56 \pm 0.66\%$ ($p \leq 0.05$).

This hydrolysate can be characterized as a "deep" hydrolysate, i.e. it features a high degree of protein splitting. It can be used to produce the functional hypoallergenic food products [19].

Based on the obtained data, factorial experiments using mathematical methods were run to optimize the enzymatic hydrolysis of ovalbumin.

Main parameters for optimization:

- Duration of hydrolysis
- Enzyme-substrate ratio (ESR)

Results of a two-factor experiment

Based on the planned levels (Table 1 and Table 2), a matrix of a two-factor experiment was drawn up, according to which 9 experiments were run. When compiling the structure of the matrix, it was taken into consideration that during all experiments, each level of any factor occurs once along with each level of any other factors.

Table 1. Levels of the analyzed factors

Factor	Level		
	1	2	3
X1, Duration, hours	1	3	5
X2, Enzyme-substrate ratio	1:10	1:20	1:30

Table 2. The levels of the analyzed factors in a two-stage process: at first — pepsin-aided hydrolysis for 5 hours (at ESR1:20), and then with trypsin for 3 hours

Factor	Level		
	1	2	3
X1, Duration of trypsin hydrolysis, hours	1	2	3
X2, Enzyme-substrate ratio	1:10	1:20	1:30

Results of the regression analysis of the response function

To obtain the response function, multivariate non-linear regression was performed with the help of the second-order polynomial in *Statistica 10* software, and the equations 2–4 were obtained.

Response function that characterizes the degree of pepsin-aided hydrolysis of ovalbumin is as follows:

$$Y(x_1, x_2) = 0.259x_1^2 - 331.167x_2^2 + 5.317x_1 + \\ + 685.713x_2 - 350.96 \quad (2)$$

Response function that characterizes the degree of trypsin-aided hydrolysis of ovalbumin is as follows:

$$Y(x_1, x_2) = 0.355x_1^2 - 301.457x_2^2 + 4.122x_1 + \\ + 671.575x_2 - 389.572 \quad (3)$$

Response function that characterizes the degree of two-stage hydrolysis of ovalbumin by pepsin and trypsin:

$$Y(x_1, x_2) = -2.468x_1^2 - 713.1x_2^2 + 34.541x_1 + \\ + 1011.57x_2 - 987.56 \quad (4)$$

An assessment of the quality of regression equations is presented below in the Table 3.

Table 3. Evaluation of the regression levels quality

Quality parameters of the regression equation	Parameters of pepsin-aided hydrolysis	Parameters of trypsin-aided hydrolysis	Parameters of two-stage hydrolysis
Multiple Correlation Index	R = 0.981	R = 0.973	R = 0.9922
Determination coefficient	$R^2 = 0.986$	$R^2 = 0.944$	$R^2 = 0.985$
Fisher's criterion	F = 37.72 p < 0.002	F = 31.56 p < 0.002	F = 63.46 p < 0.001

From the parameters given in the Table 3, it follows that the obtained regression equations feature high accuracy and statistical reliability.

Results of solving the extremal problem

In order to find the maximum value of the response function and the corresponding factors values, the standard procedure for determination of the maximum of two variables function in a limited domain of definition was run. When solving the extremal problem, the following solution was obtained (Table 4).

Table 4. Solution of extremal problems

Enzyme	The value of factors at the maximum point	The value of the response function at the maximum point
Pepsin	$X_1 = 7$ ч, $X_2 = 1{:}22$	Y = 77.631%
Trypsin	$X_1 = 7$ ч, $X_2 = 1{:}30$	Y = 79.92%
Pepsin (5 hours, ESR1:20) + trypsin	$X_1 = 3$ ч, $X_2 = 1{:}19$	Y = 88.275%

The influence of technological parameters on the degree of ovalbumin hydrolysis by enzyme preparations can be assessed by contours of "desirability" depending on the values of these factors (duration of the process and ESR) (Figures 4–6).

In our case, the higher the value of the desirability function, the higher the degree of hydrolysis (the value of Y response function at the maximum point, Table 4). At the same time, the range of values of the desirability function, equal to 0.63–0.37, is considered an acceptable and sufficient level [16].

Thus, in the course of processing of the experimental data, the factors contributing to the intensification of the enzymatic conversion of ovalbumin and the optimal parameters of the process were identified. For a one-stage process of pepsin-aided hydrolysis, the duration was 7 hours, enzyme-substrate ratio was equal to 1:22. For a one-stage process of trypsin-aided hydrolysis, the duration of hydrolysis was also equal to 7 hours, while enzyme-substrate ratio was equal to 1:30.

For a two-stage hydrolysis process, first pepsin-aided hydrolysis lasted for 5 hours at an enzyme-substrate ratio of 1:20, then with trypsin it lasted for 3 hours at an enzyme-substrate ratio of — 1:19.

As noted earlier, peptides of protein hydrolysates possess a whole range of biological properties, including antioxidant ones [2]. The study showed that peptides manifest their antioxidant properties in several ways — that is inhibition of free radicals, chelation of metal ions of variable valence, inactivation of reactive oxygen intermediates or reduction of hydroperoxides [18].

During the study, the dynamics of changes in the summarized antioxidant activity (SAA) during the hydrolysis of ovalbumin was determined. Sampling was carried out every hour for 8 hours long. The SAA of the non-hydro-

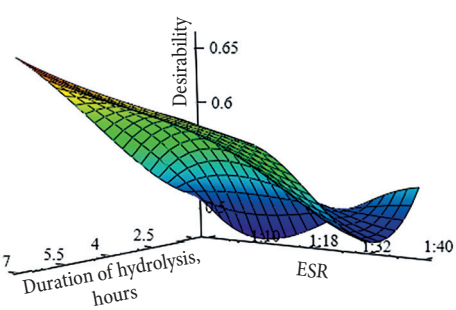


Figure 4. Contour of desirability of degree of pepsin-aided hydrolysis of ovalbumin

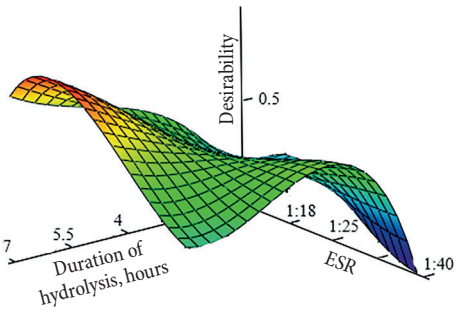


Figure 5. Contour of desirability of degree of trypsin-aided hydrolysis of ovalbumin

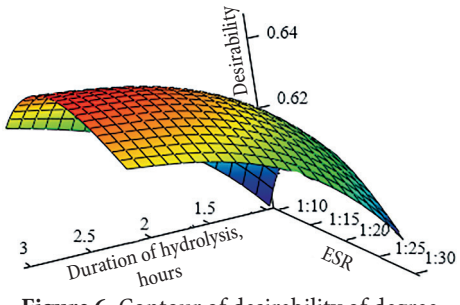


Figure 6. Contour of desirability of degree of two-stage hydrolysis of ovalbumin (at first with pepsin, then with trypsin)

lyzed protein was preliminarily determined. The data obtained is presented below in the Figure 7.

From the obtained results, presented above in the Figure 7, it follows that the dynamics of changes in SAA depended on the duration of the hydrolysis process and showed a wavy pattern. The maximum SAA value was noted after 2 hours of hydrolysis and amounted to 170.23 mg/l, which was almost 10 times higher than SAA before the beginning of hydrolysis (0 hours) ($p \leq 0.05$). Then, the SAA index decreased to 59.03 mg/l by the 5th hour, and then increased again almost 2 times ($p \leq 0.05$) an hour later, remaining almost at the same level until the end of the hydrolysis process. At the end of hydrolysis, the SAA value was equal to 114.32 mg/L. The obtained results prove that peptides with high antioxidant activity were formed during the ovalbumin hydrolysis.

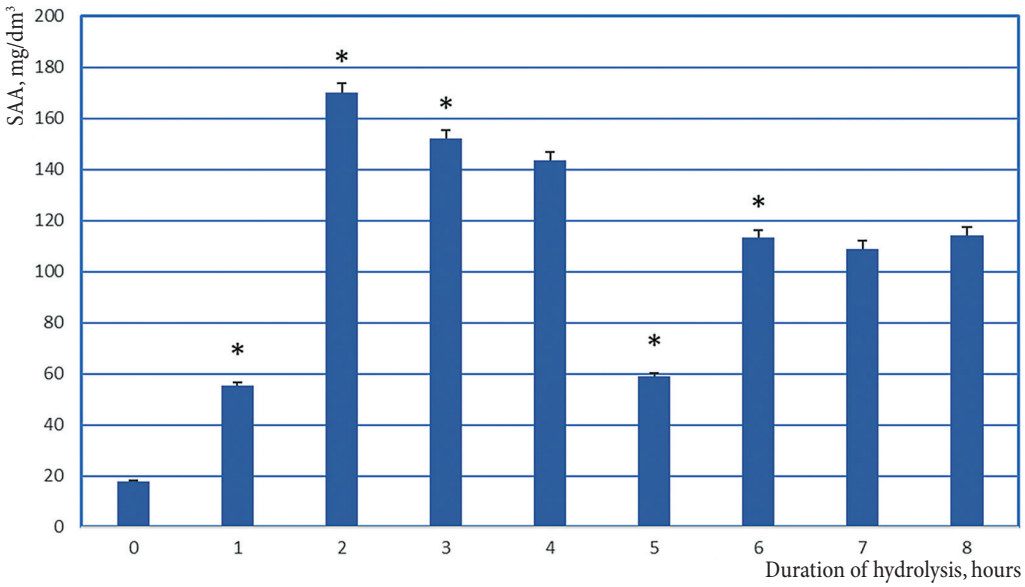
According to the literature, ovalbumin makes up 54% of proteins of an egg white and most of the identified antioxidant peptides are derived from it. Thus, ovalbumin is a rich source of antioxidant peptides that can be released under the action of gastrointestinal proteases [20, 21].

As follows from the literature data, most biologically active antioxidant peptides derived from dietary proteins consist of 2 to 20 amino acid residues, although some longer peptides have also been described [22].

Therefore, further in the research the fractional composition of the hydrolysate obtained after 8-hours two-staged hydrolysis process was studied with the help of gel chromatography method and the antioxidant activity of the obtained fractions was found.

Fractionation data are presented below in the Figure 8, from which it follows that as a result of egg white gel chromatography, the sample was divided into three main fractions (conditionally to the fraction of high molecular weight (I), medium molecular weight (II) and low molecular weight (III)):

1) Fraction I (11–20 vials) — eluate volume amounted to 10 ml;
2) Fraction II (21–33 vials) — eluate volume amounted to 13 ml;
3) Fraction III (34–80 vials) — eluate volume amounted to 47 ml.



* significantly significant differences in SAA subject to hours of hydrolysis ($p \leq 0.05$).

Figure 7. Dynamics of changes in the summarized antioxidant activity of egg hydrolysate peptides

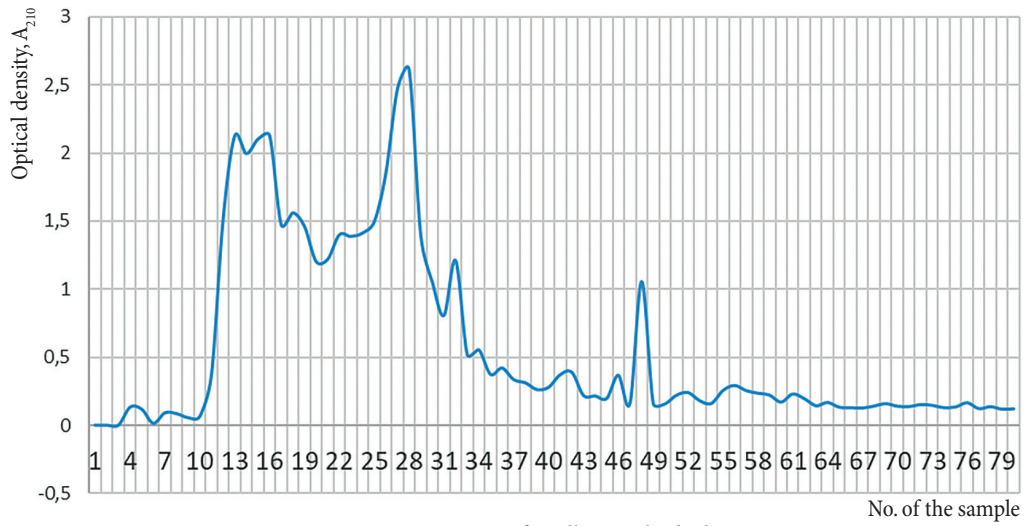


Figure 8. Fractionation of ovalbumin hydrolysate

The protein concentration in the hydrolysate was equal to 7.54 mg/mL. The protein concentration in the fractions was also 0.27 mg/mL in fraction I, 0.3 mg/mL in fraction II, and 0.02 mg/mL in fraction III, respectively. Thus, the proportion of proteins in fraction I was equal to 2.7 mg (35.8% protein), fraction II — 3.9 mg (51.7% protein), and fraction III — 0.94 mg (12.5% protein).

The summarized antioxidant activity of the three fractions at the same protein concentrations was comparatively assessed. The results of the assessment are presented in the Table 5 below.

Table 5. Results of antioxidant activity of peptide fractions

No. of the sample	No. of the fraction	Concentration of protein in the fraction, mg/ml	Summarized antioxidant activity, mg/l
1	I	0.27	11.2 ± 0.21
2	II	0.27	12.28 ± 0.23
3	II	0.3	13.54 ± 0.25*1
4	I	0.02	0.9 ± 0.02*1,2,3
5	II	0.02	4.9 ± 0.10*1,2,3,4
6	III	0.02	3.0 ± 0.06*1,2,3,4,5

*1,2,3,4,5 — significantly significant differences in reference to, respectively, the 1,2,3,4,5 sample ($p \leq 0.05$)

As it follows from the data in the Table 5, at equal protein concentrations the fraction II (medium molecular weight) (the samples 2, 3, and 5) featured the highest SAA. Thus, we can conclude that the main contribution to the antioxidant activity of ovalbumin hydrolysate is made by peptides of the average molecular weight fraction.

To separate macromolecular compounds from the hydrolysate obtained in result of high degree hydrolysis of protein (82.56%) and SAA equal to 114.32 mg/l, the hydrolysate was microfiltrated. This process also made it possible to increase the SAA to 189.9 mg/l.

Literature data testify that the identification and definition of peptides responsible for antioxidant activity is of great theoretical and practical interest. The authors Benedé et al. [23] showed that pepsin-aided 3-hours proteolysis of egg white at pH 2 and at temperature of 37 °C with an ESR ratio of 1:100 made it possible to obtain a peptide fraction with a molecular weight below 3 kDa, which featured an antioxidant activity 3 times greater than that of the native egg white. Four ovalbumin peptides were found whose antioxidant activity was caused by the presence of tyrosine at the N-terminus.

In addition to amino acid sequence, the molecular weight of peptides can also influence their antioxidant activity. Akbarian et al. [18] showed that the maximum antioxidant activity of hydrolyzed corn gluten protein was observed within the range from 0.5 to 1.5 kDa. In addition, it was found that the higher the degree of hydrolysis, the lower the antioxidant activity of the peptides. This process is associated with their further splitting to free amino acids with significantly lower antioxidant activity.

In further studies, the functional and technological parameters of the obtained egg hydrolysate were determined in comparison with ovalbumin solution. Summary data are presented below in the Table 6.

Table 6. Functional and technological parameters of ovalbumin hydrolysate

№	Parameter	Ovalbumin solution	Hydrolyzed ovalbumin
1	Appearance	Homogeneous liquid	Homogeneous liquid
2	Color	Creamy white	Milk transparent
3	Flavor	With a specific flavor	Tasteless
4	Smell	Weak specific odor	Odorless
5	Dry matter content,%	2.0 ± 0.1	2.0 ± 0.1
6	Moisture contents,%	98.0 ± 1.9	98.0 ± 1.9
7	Density, g/ml	0.999 ± 0.001	0.999 ± 0.001
8	pH	6.8 ± 007	7.8 ± 0,1
9	Protein content, mg/ml	3.85 ± 0.04	3.85 ± 0.04
10	Degree of hydrolysis,%	—	82.56 ± 0.66*
11	Solubility,%	100.0	100.0
12	Viscosity, mP	4.47 ± 0.04	4.08 ± 0.04*
13	Foaming ability,%	50.0 ± 0.50	100.0 ± 0.10*
14	Foam stability, min.	130.0 ± 2.6	65. 0 ± 1.3*
15	Summarized antioxidant activity, mg/l	17.78 ± 0.52	189.9 ± 3.79*

* reliably significant differences in the hydrolysate compared to the parameters of ovalbumin solution ($p \leq 0.05$)

As follows from the data in the Table 6, the hydrolysate of ovalbumin compared with its solution shows a row of differences. First of all, it features a high degree of protein hydrolysis (the 10th parameter) and SAA, which is 10.7 times higher than this parameter of the solution (the 15th parameter). The hydrolysate has a lower viscosity (by 8.7% less compared to the ovalbumin solution), 2 times higher foaming ability and 2 times lower foam stability; odorless and tasteless. These parameters will be taken into consideration when using the hydrolysate as an ingredient in a functional food products.

The work of Chinese researchers Chen et al. (2012–2018) showed that the functional properties of egg powder can be modified by enzymatic conversion. They have developed a whole series of egg protein powders (gel-like, foamy, emulsion, instant powder, etc.). A number of works by these authors are devoted to the production of egg protein hydrolysates and the study of peptides. Various enzymes were used to obtain such hydrolysates as papain [24,25], trypsin [26], pepsin [27], and other proteases [28]. The authors found that the functional properties of hydrolysates and peptides are directly affected by the type of enzyme, its concentration, pH, and duration of hydrolysis time. The obtained hydrolysates have high biological activity (in particular, antioxidant)

and improved functional characteristics (emulsification, foaming ability, solubility, etc.). From these hydrolysates, antioxidant peptides were obtained, purified and identified. Two purified peptides were synthesized [28]. In addition, the authors studied the influence of ultrafiltration [26] and types of drying (lyophilization, spray drying) [25] on the functional and technological parameters of hydrolysates.

Conclusions

Thus, a hydrolysate with a high degree of hydrolysis (82.56%) was obtained by a two-stage process of enzymatic conversion of ovalbumin using mathematical methods for planning an experiment. The optimal parameters of this process were determined: first, pepsin-aided hydrolysis lasted for 5 hours at an ESR of 1:20, and then trypsin-aided hydrolysis took over for 3 hours at an ESR of 1:19.

The SAA of the hydrolysate was determined, and the function of this parameter on the duration of the hydrolysis process was determined. This dependence had wavy pattern. The highest SAA value was recorded after 2 hours of hydrolysis and amounted to 170.23 mg/l. After 8 hours of hydrolysis, the SAA value was equal to 114.31 mg/l.

Fractionation of the ovalbumin hydrolysate allowed its separating into 3 main fractions (of high, medium and low molecular weight respectively). The peptides of the medium molecular fraction of hydrolysate showed the highest SAA values.

The microfiltration process made it possible to partially remove macromolecular compounds from the hydrolysate, which led to an increase of SAA value up to 189.9 mg/l.

The main functional and technological parameters of ovalbumin hydrolysate were determined.

Ovalbumin hydrolysate can be recommended as a component of functional hypoallergenic and antioxidant food products.

This way, the comprehensive study of the biological activity and functional characteristics of egg protein hydrolysates and their peptides provides a considerable theoretical basis for expanding the range of functional food ingredients derived from food proteins and thus expanding the assortment of functional food products.

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METHODOLOGY OF ADIPOSE TISSUE TYPE DETECTION IN MAMMALS

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Abstract
Nowadays, an interest in studying the composition, properties and functions of adipose tissue (AT) is growing among researchers, which is conditioned by its important role in the normal functioning of the body. Due to different types of adipose tissue (AT) in mammals (white, beige, brown and pink) and different physiological tasks performed by each type of AT, rapid, correct and effective detection of an AT type is highly topical. Methods used today are labor consuming and in the case of NMR and CT expensive, which limits possibilities of scientists. In this connection, the aim of this research was to develop a methodological approach allowing rapid and effective detection of an adipose tissue type. A methodology was formed based on the concept, formalized requirements for the method, step-wise structure of investigations and interpretation of results. The concept is based on differences in the structure of the adipose cell (adipocyte) of different AT types. The method is based on extraction of heme containing proteins. To this end, solvents and parameters of extraction that facilitate their better extraction have been chosen. An AT type has been determined by the total content of iron contained in the cytochrome fragment. Our own modification was selected. This modification includes preliminary mincing of a sample with the ice-cold TES buffer (pH 8.5) in a ratio of 1:5 (g: mL), homogenization at 9,000 rpm for 2 min with the following centrifugation at 10,000 g and 4 °C for 15 min. Effectiveness of the proposed method was confirmed by the histological and electrophoretic analyses. Therefore, the new methodology of identification and differentiation of adipocytes was proposed for rapid and effective detection of an adipose tissue type.

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Introduction

Adipose tissue (AT) is a type of connective tissue, which cells are filled with a fat droplet [1]. Adipose tissue is distributed throughout the whole body and plays a key role in energy homeostasis of the body as a reservoir of lipids. In addition, adipose tissue produces and releases various pro-inflammatory and anti-inflammatory factors, including adipokines (leptin, adiponectin, resistin and visfatin), as well as cytokines and chemokines, monocyte chemoattractant protein-1 and others [2]. Distribution of adipose tissue in mammals depends on genetic and ecological factors, while its lipid composition strongly depends on a biological species, diet, climate, and so on.

Historically, mammalian adipose tissue was divided into two types: white adipose tissue (WAT) and brown adipose tissue (BAT) based on their visible difference in color as well as their different physiological functions. WAT represents an overwhelming majority of AT in the body. It consists of many types of cells, but adipocytes are prevalent. White AT is a place of energy storage, while the main role of brown adipose tissue is thermogenesis, especially in small mammals and newborn humans [2]. Anatomically WAT is present in two main depots: subcutaneous and visceral around internal organs [3,4]. White adipose tissue specializes in processing fatty acids and triglycerides, and is critically important for energy storage, endocrine communication and insulin sensitivity [4,5]. The other function of WAT is mechanical protection of muscles and internal organs, as well as maintenance of the body temperature [6]. For example, WAT acts as a shock absorber, ensuring padding at different anatomical sites, while omental WAT is one of the visceral depots of adipose tissue, surrounding and protecting inner organs from physical injuries [7]. WAT is also known as an endocrine organ, especially visceral AT producing adipokines, which take part in metabolism or transport of lipids, immune system, blood pressure regulation, blood coagulability, glycemia homeostasis, angiogenesis, and so on [6, 8].

Brown AT is the main place of thermoregulatory non-shivering thermogenesis [9]. BAT plays an endocrine role releasing endocrine factors in the conditions of thermogenic activation, which affect the glucose level, insulin tolerance and sensitivity [10]. Brown AT is easily found both in infant and adult mammals; however, with aging, it is gradually replaced by WAT and an amount of BAT becomes rudimental in later life [3]. BAT contains abundant mitochondria; free fatty acids serve as substrates for lipid oxidation and potent activators of mitochondrial uncoupling protein 1 (UCP1) — the key transmembrane protein, which catalyzes heat generation at the mitochondrial level [11]. UCP1 is the only member that is capable to transfer protons through the mitochondrial inner membrane of brown adipocytes, uncouples respiration and ATP synthesis, and therefore, causes dissipation of energy as heat, and also stimulates the high level of fatty acid oxidation.

Thus, one of the main differences between white and brown AT is the mechanism of ensuring thermogenesis. BAT cells effectively oxidize glucose and fatty acids. With that, a large amount of heat is released and insignificant part of energy is accumulated as ATP (adenosine triphosphate). Also, because of the high content of mitochondria and ability to increase the cellular metabolic rate, BAT generates heat due to chemical energy [12]. White adipocytes are located throughout the whole body and store triglycerides as an energy buffer in large monolayer lipid droplets. During fasting and upon reduced insulin levels, the rate of fatty acid oxidation is doubled and WAT acts as the main energy source [13].

Unlike white adipocytes, brown adipocytes have abundant mitochondria, which cytochromes contain heme iron determining the brown color of tissue. For cytochromes, the catalytic cycle is typical, which is accompanied by transition of electrons upon a change in iron valence. The decisive role of the heme group consists in the fact that it converts atomic oxygen into the reactive form, which is responsible for all reactions occurring in the catalytic cycle.

Brown-like adipocytes can appear in WAT. They are a result of WAT "browning". Today, scientists assign this adipose tissue to a third type of AT and call it beige AT (brite — brown-white) [14–16]. It was noticed initially that beige AT appears as a response to cold exposure; however, other factors such as diet, physical activity, pre- and probiotics, pharmaceutical and plant substances and so on can cause "beigeing" or "browning" of WAT [17]. Beige AT is similar to BAT in terms of functions; it contains a large number of mitochondria and has high thermogenicity. Beige adipocytes can secrete certain factors that affect the function and systemic metabolism of WAT [18]. Beige AT plays a key role in adaptive thermogenesis [19], which depends on the presence of UCP1 [20].

It is interesting that today five types of adipocytes, five types of AT, are distinguished in the mammalian body. Besides white, beige and brown adipocytes, there are yellow adipocytes that are present in marrow and pink adipocytes revealed in tissues of the mammary gland during pregnancy and lactation [21].

General classification of adipose tissue is based on the AT color, which corresponds to the lipid content, mitochondrial density and vascularization (formation of blood vessels). White adipocytes contain one lipid droplet that occupies about 90% of the cell space. BAT is highly vascularized, and brown adipocytes contain a large number of mitochondria; the lipid droplet is smaller and is represented by multiple vacuoles. Beige adipocytes show characteristics of both brown and white adipose cells; the content of mitochondria is higher than that in white adipocytes and a lipid droplet is not single but bigger than in brown adipocytes [17].

At present, an interest in studying the composition, properties and functions of all AT types is growing among scientists, which is conditioned by an important role of adipose tissue in the normal functioning of the body and absence of diseases. For example, Price et al. [22] studied the functions of white and brown AT, as well as the role of microRNA in regulation of differentiation of these ATs. The authors established that induction of thermogenesis in BAT or WAT leads to an increase in the energy expenditure and prevents the development of obesity and metabolic dysfunction caused by diabetes. Adipose tissue, its properties, function, quantity and distribution in the body have been widely studied in medicine and the food industry.

In medicine, methods for AT detection that characterize its total content in the body, quantity and a degree of the development of the certain tissue type are used. One of the methods is X-ray densitometry based on generation of radiation with stable energy by an X-ray source. Due to attenuation of low-energy radiation, the highest energy level (R-volume) is determined and compared to the calibration curve of the known R-volumes. As a result, the density of soft tissues is assessed and the quantity of AT is determined. The most precise methods of diagnostics used in medicine are computed tomography (CT) and nuclear magnetic resonance imaging (NMR imaging). The CT method is based on the clear difference in X-radiation obtained from bone and adipose tissue as well as tissue that is free from fat. Using a computer processor, visual information about the character of ionizing X-radiation is obtained in a form of cross-sections [23]. In addition, nuclear magnetic resonance (NMR) is used as an additional method for assessing different AT types. NMR is based on interaction between charges of hydrogenic atoms (protons), which are present in all biological tissues. The other method that is also frequently used to study AT is histological analysis, which is applied mainly in the field of the technology development and production of meat and meat products to predict technological 'behavior' of adipose tissue and detect a ratio of all AT types [24].

These methods are time consuming and in the case of NMR and CT expensive, which restricts researchers in terms of rapid and available detection of an AT type. The aim of this research was to develop a methodological approach allowing effective detection of an adipose tissue type within a short time. The proposed method was based on the selection of solvents and extraction parameters that allow extraction and detection of the concentration of total iron contained in the fragments of cytochromes, which presence in a large quantity is characteristic of brown adipose tissue.

Objects and methods

The objects of the research were AT samples: subcutaneous and visceral white AT (WAT) and interscapular brown AT (BAT) from laboratory Wistar rats at the age of 6 or 18 months obtained from the nursery for laboratory animals "Rappolovo" of the Ministry of Science and Higher Education of the RF (Vsevolozhsky district, Leningrad Oblast, Rappolovo). Animal keeping and all manipulations with them were carried out in a compliance with Order of the Ministry of Health of the RF No. 267 of 19.06.2003 "On approval of rules of laboratory practice" and Council Directive 86/609/EEC. The experiment was approved by the bioethical commission of the V. M. Gorbatov Federal Research Center for Food Systems of RAS (protocol No. 01/2019 of 09.05.2019).

To develop the methodology, solvents and parameters of extraction were selected that facilitated better extraction of heme containing proteins, which was assessed using measurement of total amount of iron. The correctness of the proposed method was confirmed by the histological and electrophoretic analyses of AT and extracts, respectively.

Extraction of adipose tissue

The obtained adipose tissue samples were washed in the phosphate buffered saline (PBS10X, ChemCruz, USA), sponged up with filter paper and then minced into small pieces using surgical scissors. The extractants used in this study were: 1%, 2.5%, 5% and 10% acetic acid, 0.3 M acetate buffer (pH 3.6), TES buffer (pH 8.5), containing 30 mmol/L Tris (AppliChem, Germany), 1 mmol/L EDTA (AppliChem, Germany) and 0.25 mol/L saccharose and 2% protease inhibitors (Iniprol, France). The minced AT samples were mixed with the extractant in a ratio of 1:5 (g: mL) in the case of the acetate buffer, TES buffer, and 1:5 and 1:10 (g: mL) in the case of acetic acid. Extraction of target substances with the acetate buffer and acetic acid was carried out by homogenization using a portable homogenizer S10 (Stegler, China) at 8,000 rpm for 1–2 min and then at 9,000 rpm for 2–3 min. The obtained homogenates were centrifuged at 4 °C and 14,000 g for 5, 10 or 15 min (Eppendorf, Germany).

Extraction of proteins from adipose tissue with the TES buffer was performed according to the method [25] or with slight changes. Minced adipose tissue (~ 150 mg) was mixed with the ice-cold TES buffer (700 µL) (pH 8.5). Then the mixture was stirred in cold conditions at 2,800 rpm for 1 h obtaining the extract "TES-1" or homogenized at 9,000 rpm ("TES-2"). Homogenates were centrifuged at 10,000 rpm and 4 °C for 15 min.

Determination of the total iron concentration

The concentration of total iron was determined in all fresh extracts on a biochemistry analyzer BioChem FC-360 (HTI, USA) using a ready-to-use reagent kit (HTI, USA). The results were expressed in nmol iron/g adipose tissue (nmol/g).

Electrophoretic analysis

In the protein subfraction obtained by adipose tissue extraction with the TES buffer (TES-1 and TES-2), 10% acetic acid and the acetate buffer (1 g AT per 5 mL extractant), most of extracted proteins and enzymes were studied by gel electrophoresis [26]. The protein buffer was added to the supernatant in a ratio of 1:1. To prepare the protein buffer, 1 mL 10% sodium dodecyl sulfate (SDS), 250 µL concentrated β- mercaptoethanol, 625 µL 0.5 M tris -HCl, 1.5 g urea were introduced into Eppendorf-type tubes, bromophenol blue was added until the dark color and the volume was brought to 5 mL with water. After that, the samples were heated in a boiling water bath for 5 min. The samples under study were transferred to a well of the gel in an amount of 8 µL.

To carry out vertical gel electrophoresis, a chamber "VE-10" (Helicon, Russia) was used. It was filled with 12.5% polyacrylamide gel, over which 6% gel was poured and wells were made in it to introduce samples. The solution containing 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS was used as a buffer. Electrophoresis was carried out with the following parameters: the first 30 min — 60 V and then at 120 V until the dye (bromophenol blue) front reached the lower edge of the gel plates. Proteins were stained using Coomassie G-250 in the solution of the following composition: 10% acetic acid, 25% isopropanol, 0.05% Coomassie G-250. To remove unbound dye, 10% acetic acid was used.

To carry out computed densitometry, one-dimensional electropherograms in a wet state were used. Their full digital images were obtained using a Bio-5000 Plus scanner (Serva, Germany) in a mode of 600 ppi 2D-RGB. The obtained digital images of polyacrylamide gels were analyzed using ImageJ®, publicly accessible software for processing digital images developed at the National Institutes of Health (NIH, USA) [27]. Background subtraction and 8-bit format were chosen for image analysis to improve the band intensity and reveal differences between the samples under study. Within the framework of the study, protein spots were compared by optical density, and the fold change index, an excess of which by more than two units is generally considered statistically significant difference, was calculated.

Histological analysis

The fat samples were fixed in the 10% neutral buffered formalin solution (BioVitrum, Russia) for 72 hour at 22 ± 2 °C. Preparation of sections with a thickness of 12 μm was performed on a cryostat MIKROM–HM525 (Thermo Scientific, USA). The obtained sections were mounted on Menzel-Glaser slides (Thermo Scientific, USA), stained with Ehrlich's hematoxylin and 1% aqueous-alcoholic solution of eosin (BioVitrum, Russia) and embedded in glycerin-gelatin by the conventional method [28]. The histological preparations were studied using an Axio Imager A1 light microscope (Carl Zeiss, Germany) and the image analysis system AxioVision 4.7.1.0 (Carl Zeiss, Germany). No less than three sections were made for each sample. A diameter of adipocytes was measured for 100 cells in each section in the interactive mode with an accuracy of ± 0.1 μm.

Statistical analysis

In this work, the screening study for designing a method was carried out. For results, therefore, an error of measuring instruments was taken into account. Statistical data analysis was carried out using MS Excel (Microsoft, USA). The results are presented as mean ± SD. Differences were considered significant and a relationship between parameters was acknowledged at a probability level of not higher than 0.05.

Results and discussion

During the experiment, extraction of three AT samples from rats at the age of about 5 months was carried out with 10% acetic acid in a ratio of 1:5 (g: mL). Separation of extracts at the maximum rate of centrifugation (14,000 rpm) for 5 min was impossible. In this connection, the time of centrifugation was increased up to 10 min and then up to 15 min. However, an increase in time did not allow taking average sample of the supernatant of subcutaneous AT. The concentrations of total iron were determined in the samples of visceral and interscapular AT, which were 46.2 and 655.0 nmol/g, respectively.

Upon extraction of AT from animals at the age of about 18 months with 10% acetic acid in a ratio of 1:10 (g: mL), separation of all AT types into fractions was achieved. Iron concentrations (C_{Fe}) for visceral, interscapular and subcutaneous AT were 41.8 nmol/g, 105.3 nmol/g and 34.1 nmol/g, respectively. C_{Fe} for subcutaneous and visceral AT differed insignificantly, while the values for interscapular AT were 3.1 and 2.5 times higher, respectively, than the corresponding values in subcutaneous and visceral AT. It was noticed that upon extraction of interscapular AT in a ratio of 1:5 (g: mL), the iron concentration decreased by about 6.2 times compared to extraction in a ratio of 1:10 (g: mL). It can be assumed that this observation is associated with the animal age as brown tissue is gradually replaced with white AT with age, which is confirmed by Choe S. S. [3].

To check the expediency of using acetic acid, an experiment was carried out with the reduced concentration of acetic acid. Upon extraction of AT from animals at the age of about 18 months with 5%, 2.5% and 1% acetic acid in a ratio of 1:5 (g: mL), separation of homogenates of subcutaneous AT was also impossible. The iron concentration was determined in the samples of visceral and interscapular AT. The results are presented in Table 1.

Table 1. Iron concentration in AT extracts with 1%, 2.5% and 5% acetic acid

$C_{CH3COOH}$, %	C_{Fe}, nmol/g AT	
	Visceral AT	Interscapular AT
5	29.05 ± 1.45	156.55 ± 7.8
2.5	50.00 ± 2.5	144.55 ± 7.22
1	26.95 ± 1.34	207.70 ± 10.38

It is evident from Table 1 that the iron concentration in visceral AT was approximately the same when using 5% and 1% acetic acid, while with 2.5% acetic acid the iron concentration increased up to 50 nmol/g AT. In the case of interscapular AT, the maximum iron concentration was found in the extract obtained upon extraction with 1% acetic acid. It was observed that the iron concentration in interscapular AT exceeded the corresponding values in visceral tissue when using any acid concentration. For example, the iron concentration in the extracts of interscapular AT was 7.7, 2.9, 5.4 and 2.5 times higher, respectively, when using 1, 2.5, 5 and 10% acetic acid as an extractant.

During the experiment, extraction of three AT samples from rats at the age of about 18 months was performed with the 0.3 M acetate buffer in a ratio of 1:5 (g:mL). In the homogenates of all AT types, the average sample of supernatant was taken. The iron concentrations in the extracts of visceral, subcutaneous and interscapular AT were 36.55 nmol/g, 14.15 nmol/g and 79.15 nmol/g, respectively. A diagram was built (Figure 1) for visual comparison of the obtained data with the corresponding values of the iron content in the extracts obtained with the use of 10% acetic acid.

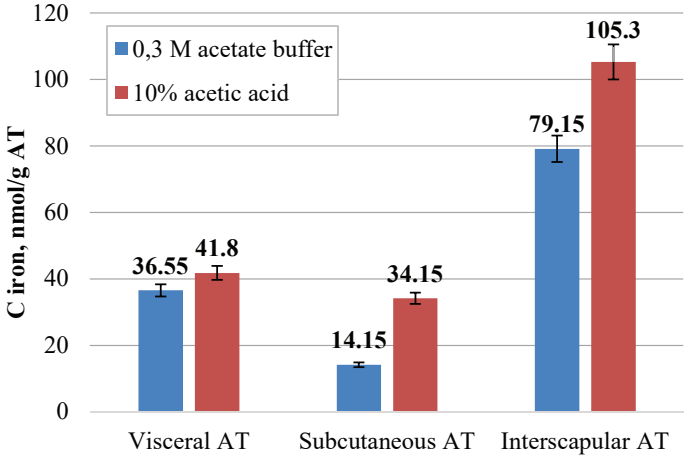


Figure 1. Content of total iron in the AT extracts, when using the acetate buffer and 10% acetic acid

It can be seen from Figure 1 that the iron content in visceral AT differed only by 5.25 nmol/g AT, when using the acetate buffer or acetic acid as an extractant, which was considered an insignificant difference. It was noted that the total iron concentration in the extracts of subcutaneous and interscapular AT obtained using the acetate buffer were lower by 19.95 and 26.15 nmol/g, respectively, compared to extraction with 10% acetic acid. The data obtained allow suggesting that the acetate buffer is a less promising extractant of heme containing proteins from adipose tissue compared to 10% acetic acid. However, its use enables more effective separation of AT extracts into fractions irrespective of the AT type and location.

Comparative analysis of extraction effectiveness of heme containing proteins from adipose tissue by the selected extraction methods was carried out. These methods included: 10% acetic acid and acetate buffer in a ratio of 1:5 (g: mL) and the use of TES buffer in a ratio of 1:5 (g: mL) without and with homogenization (TES-1 and TES-2, respectively) (Figure 2).

It is evident from Figure 2 that all selected extractants enabled taking the average sample of the supernatant. However, in the case of subcutaneous AT, the average sample was extremely small when 10% acetic acid was used, which did not allow performing a large number of experiments. It was noticed that in the case of TES-1, the upper layer was characterized by heterogeneity and the color of the extract of interscapular AT was duller compared to the color in the case of TES-2, which in turn was identical to the color obtained when using the acetate buffer. The extract of interscapular AT (BAT) with 10% acetic acid was characterized by browner color compared to other samples.

In the AT extracts obtained by the selected methods for extraction of heme containing proteins, the iron concentration was determined (Table 3) and the proteomic analysis by one-dimensional electrophoresis was carried out (Figure 3).

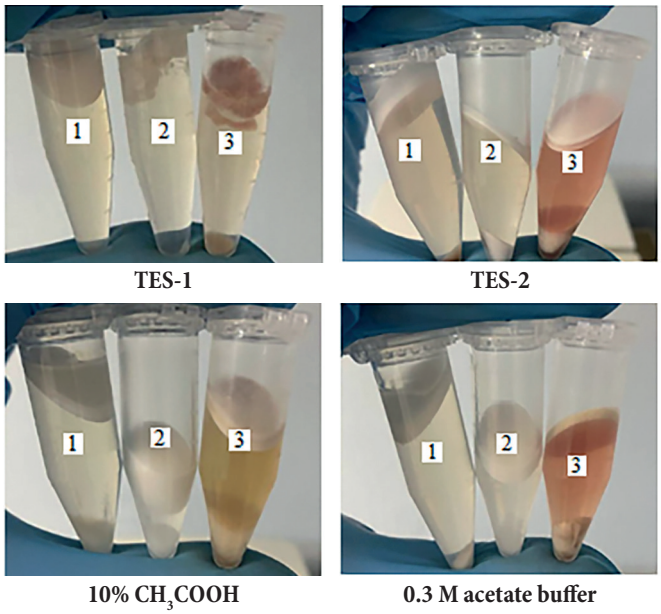


Figure 2. Results of the separation of AT homogenates using different extractants, where: **1** — visceral white AT; **2** — subcutaneous white AT; **3** — interscapular brown AT

Table 3. Iron concentration in AT extracts

AT extracts	C_{Fe}, nmol/g AT		
	Interscapular AT	Visceral AT	Subcutaneous AT
TES-1	109.15 ± 5.45	57.2 ± 2.86	59.45 ± 2.97
TES-2	675.2 ± 33.70	129.8 ± 6.49	138.95 ± 6.90
10% acetic acid	346.5 ± 17.32	105 ± 5.25	115 ± 5.75
0.3M acetate buffer	192.5 ± 9.62	24.55 ± 1.23	27.85 ± 1.39

It was noted that C_{Fe} in all types of interscapular AT extracts exceeded the corresponding values for visceral and subcutaneous AT. The iron concentrations in the extracts of visceral and subcutaneous AT significantly differed between the extractant types; however, they were close to each other. The maximum iron concentration was obtained for interscapular AT upon TES-2 extraction, and it significantly decreased in the row: TES-2, acetic acid, acetate buffer and TES-1.

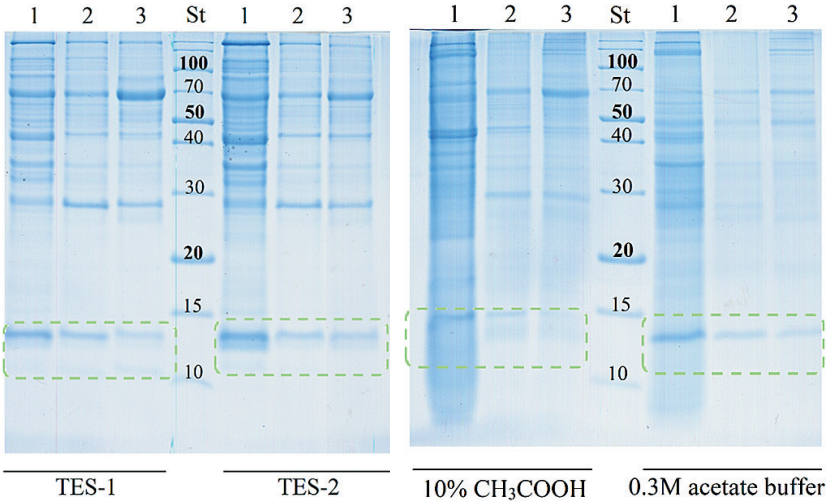


Figure 3. One-dimensional electropherogram of adipose tissue extracts, where **St** — standard of molecular weights: 250, 150, 100, 70, 50, 40, 30, 20, 15 and 10 kDa (from top to bottom), **1** — interscapular AT; **2** — visceral AT; **3** — subcutaneous AT

In the course of processing of the obtained electropherogram images, a wide spectrum of compounds in a molecular weight range from 10 kDa and higher was revealed; with that, the highest number of protein fractions was found in interscapular AT. Using the UniProt database [29], the presence of target iron containing proteins with a molecular weight of less than 15 kDa was assumed: cytochrome c, testis-specific — 11.7 kDa [30] and cytochrome c, somatic — 11.6 kDa [31]. Several significant differences in the optical density of the target protein fractions with MW from 10 to 15 kDa (marked with the green color in Figure 3) were recorded using the program complex ImageJ. The values obtained are given in Table 4. The comparative analysis in certain regions of molecular weights between different samples was carried out.

Table 4. Results of the densitometry analysis of protein fractions in the AT extracts

Samples	Optical density of target protein fraction, a.u.		
	Interscapular AT	Visceral AT	Subcutaneous AT
TES –1	3,918.4 ± 190.4	2,276.0 ± 113.1	1,247.1 ± 56.0
TES –2	5,156.0* ± 211.4	1,708.6 ± 83.3	1,119.9 ± 5.9
10% acetic acid	1,711.0 ± 61.8	947.4 ± 46.5	93.0 ± 2.2
0.3M acetate buffer	3,479.7* ± 172.6	1,308.9 ± 65.0	674.1 ± 26.8

* statistically significant difference according to Fold > 2

A large difference was revealed based on the densitometry analysis of the density of AT protein fractions. Interscapular AT was characterized by maximum values, which significantly exceeded the corresponding values for visceral and subcutaneous AT. Thus, the best variant for revealing target protein fractions with MW from 10 to 15 kDa is a variant of extraction using the TES buffer. For example, the optical density of the target fraction was 5,156.0 a.u. in interscapular AT, 1,708.6 a.u. in visceral AT and 1,119.9 a.u. in subcutaneous AT, when using extraction with the TES-2 buffer.

As a result of the performed histological investigations of the samples from rats at the age of 18 months, the photographs of the microstructure were obtained (Figure 4).

Large adipocytes with a diameter of 67.32 ± 8.13 and 75.46 ± 9.25 μm, respectively, were revealed in subcutaneous and visceral adipose tissue. The revealed adipocytes were typical of white adipose tissue and contained a single large lipid droplet and the nucleus shifted to periphery of the cell. The samples of the interscapular adipose tissue consisted of small brown adipocytes with a diameter of 20.51 ± 2.58 μm with multilocular lipid droplets inside; the nucleus was located both at the periphery of the cell and in its central part. The results obtained correspond to [32,33] regarding characteristics of white and brown adipocytes.

During the experiments, it was established that the optimal method for extraction of heme containing proteins from AT is the TES buffer with homogenization and the following centrifugation. The method makes it possible to distinguish rapidly and effectively brown AT from white AT by the concentration of heme containing proteins in it.

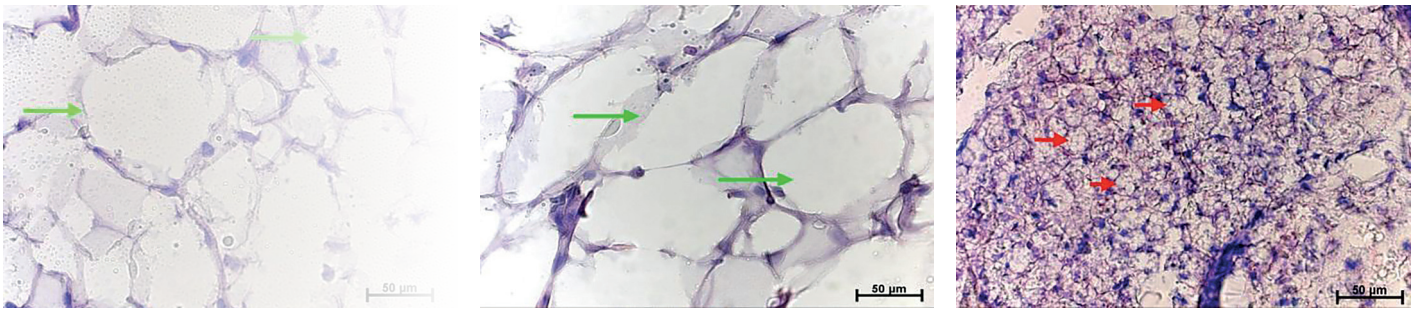
The method is easy to reproduce and it does not require special training of personnel. A biochemical blood analyzer available in a specialized laboratory is used. In future, the method can be made easier and cheaper, if necessary. In addition, a possibility of its use in identification of other AT types can be studied.

Conclusion

In the course of work, the methodology of AT identification and differentiation by types was formulated. The basis of the concept is the knowledge about the structure of adipocytes of white, brown and beige tissues, different content of iron containing proteins (cytochromes) in them as well as other information. The main requirements for the method of investigation were determined: rapidity, ease of use, minimization of hard-to-get reagents, wide accessibility of equipment being used, absence of the need for highly qualified researchers, ease of interpretation of results, possibility of metrological support.

The original investigation method has been proposed. The method consists in preliminary mincing of a sample with the ice-cold TES buffer (pH 8.5) with the protease inhibitor in a ratio of 1:5 (g/mL), homogenization at 9,000 rpm for 2 min and subsequent centrifugation at 10,000 g and 4 °C for 15 min. Certainty of detection of AT types was confirmed by the standardized histological method of analysis as well as by one-dimensional electrophoresis.

In conclusion, different AT types have been studied in detail, the effective methodology for detection of types of mammalian adipose tissue has been proposed.



Subcutaneous AT (WAT) Visceral AT (WAT) Interscapular AT (BAT)

Figure 4. Microstructure of the adipose tissue samples. Staining with hematoxylin and eosin. Scale bar 50 μm. The green arrow indicates white adipocytes, the red arrow indicates brown adipocytes

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ASSESSMENT OF CAUSES AND CONSEQUENCES OF FOOD AND AGRICULTURAL RAW MATERIAL LOSS AND OPPORTUNITIES FOR ITS REDUCTION

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Keywords: *food loss, waste, sustainable development goals, SDG 12.3, national strategies, food supply chain, animal husbandry, processed products, meat industry*

Abstract

The article summarizes and systematizes the causes and consequences of food loss and waste. The uncontrolled accumulation and inefficient management of loss and waste is a serious problem of food security in many countries. Moreover, the world annually loses or wastes about a third of food produced while natural resources are inefficiently used. A significant number of definitions and terms in the field of food loss and waste cause international inconsistency in management systems. So, systematization of terms and research methods for food loss and waste has been carried out. The review analyzes the stages of food supply chain, i.e. the production of agricultural raw materials, their collection, processing, transportation and consumption, where the largest loss and waste occur. Furthermore, environmental, social and other problems associated with the accumulation of loss and waste are considered. Prevention of food loss and waste has been shown to be a potential strategy to improve the balance of food supply and demand and is essential for improving food security while reducing environmental impact and providing economic benefits to various participants in the food supply chain. Separately, the actions taken by the Russian Federation in the field of waste management and reduction of food loss are considered. Attention is drawn to the fact that the difficulties in solving these problems are associated with insufficient progress in legislation and low funding of relevant research projects. Possible approaches for simulation and optimization of actions to support specific strategies to reduce loss and waste are considered, taking into account the following factors: improving the management of various inventories; developing a clear strategy for planning and allocating resources; forming the supply chain based on variability (uncertainty) of customer demand and supply. Knowledge comparison and integration, as well as optimization of food supply chain management are essential for development of strategies and decisions about where and how to focus efforts to most effectively ensure the reduction of loss and waste at each stage of food supply chain. The main decisions and practices for FLW prevention and management have been systematized.

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Introduction

According to the UN Food and Agriculture Organization (FAO), by 2050, the world population will reach almost 10 billion people, which will require an increase in food production by at least 70% [1].

Today, the world produces enough and even excess food to meet the needs of the entire population of the planet. According to an analysis of data from the FAO Statistical Yearbook [2], about a third of food produced for human consumption is lost or wasted worldwide, which is about 1.3 billion tons per year [3]. In terms of calories, global food loss and waste (hereinafter referred to as FLW) account for approximately 24% of all food produced, which is equivalent to 614 kcal/person/day [3].

At the same time, according to the UN Food and Agriculture Organization (FAO), more than 821 million people suffer from hunger in different regions of the planet [4].

The growth in the production of food raw materials and foodstuffs is accompanied by an increase in FLW levels. This issue has now become a major restriction for global sustainability, with adverse impacts on food security, natural resources and human health. Therefore, the reduction of FLW is one of the important goals for the world community [5, 6].

The FLW issue was most comprehensively discussed at a United Nations special event in 2015. As a result, UN member countries adopted 17 Sustainable Development Goals (hereinafter referred to as SDGs) for the period up to 2030. These activities are aimed at the elimination of poverty, hunger and improving life quality of the population. One specific target is to halve food waste and loss by 2030 (SDG 12.3). Already now, the adoption of the SDGs

has made it possible for different states to form a general idea of the existing problems in relation to FLW, determine approaches to their solution and introduce several key concepts in this area [7].

There are two main challenges for achieving SDG 12.3. First, there is no reliable global measurement of FLW. Second, the reduction of FLW is not a goal in itself, but a means to establish responsible consumption and production in world practice. This implies a reduction in the economic, environmental and social costs associated with the nutrition of the population, while improving food security [8].

In this regard, the Voluntary Code of Conduct for Food Loss and Waste Reduction developed by FAO should be mentioned. This set of rules aims to achieve a reduction in FLW based on internationally agreed principles and standards of responsible practice [9].

Today, most countries of the world are working on global strategies to reduce FLW, which take into account food waste at each stage of the food chain, combining economic interests with maintaining the natural balance of the ecological system and sustainable environmental management [10,11,12].

Systematization of terms and methods for FLW research

The scientific community has formed a glossary of unified terms and definitions, which makes it possible to appeal to the conceptual framework in the field of sustainable development goals in relation to the triad of "food security and nutrition", "sustainable food systems" and "food loss and waste". However, there are different approaches to separating the concepts of "food loss" and "food waste". What remains unchanged is that the concept of food loss refers to the initial stages of the agri-food value chain, i.e. the growing of cultivated plant and the primary products obtained from their processing. As for food waste, they appear at subsequent stages of this chain, i.e. in retail and during consumption [2,13,14].

Despite the existing glossary, various participants use many of their own definitions and terms for food systems [15]. Because of this inconsistency, there are international nonconcurrences in loss and waste monitoring systems. According to some experts [16], differences in the definition of FLW arise when taking into account such different factors as:

- actual or estimated FLW levels;
- main types of FLW based on their qualitative or quantitative assessments;
- the use of FLW for human consumption or the lack of such possibility;
- application of "edibility" and "nutritional value" criteria to FLW;
- time spent when using FLW (pre-harvest, ready-to-harvest, post-harvest activities);
- prospects for using FLW to improve environmental, social, and food security.

In this regard, it is reasonable referring to the approaches to these issues from the FAO Global Initiative on Food Loss and Waste Reduction [17] and the FAO Technical Platform on the Measurement and Reduction of Food Loss and Waste [18]. According to these organizations, the characteristic of food loss is a decrease in food quantity or quality as a result of activities by food suppliers, excluding retailers, food service providers and consumers. Food waste is the reduction in food quantity or quality as a result of the decisions and actions by retailers, food service providers and consumers.

The significant differences between food loss and food waste are not only conceptually significant. This is also important for entrepreneurs, whose actions affect the appearance of loss and waste. In addition, this circumstance implies the choice of ways to both reduce FLW levels and further use them. In particular, when estimating the amount of FLW, it should be taken into account that uneaten food may be processed into by-products that also have economic value. If these products are redirected to other uses, such as animal feed, they will not be considered as quantitative loss or waste, despite the possible reduction in their economic value. In addition, when calculating FLW amount, it is not recommended to take into account inedible parts of products [19].

The Food Loss and Waste Accounting and Reporting Standard [20] (hereinafter referred to as the FLW Standard) was developed by the Food Loss and Waste Protocol Partners. The standard consists of ten steps, starting with defining the purpose of the loss assessment and ending with the establishment of a way to monitor the relevant processes. The FLW Standard provides guidance on methods for quantifying FLW, including their direct measurement, as well as analysis of their composition, mass balance calculations, and other actions. This Standard may be applied at an individual enterprise and at the country level in order to determine the location and extent of FLW formation and how they can be used to reduce environmental impact [20].

In addition, indicators are being developed at the international level to simplify the process of monitoring the FLW level. The indicator recommended for use at the national level is the rate of food loss and waste per capita in a given country. It is expressed in kg/person/year and consists, in turn, of two indicators. The first one covers loss from the agricultural farm to the distribution points of the agri-food chain (food loss index). The second one describes the loss that occurs at the stage from trade enterprises to households (food waste index) [21].

At the level of the European Union, the FUSIONS (Food Use for Social Innovation by Optimizing Waste Prevention Strategies) project [22] implemented within the framework of the Seventh Framework Program of the European Commission in 2012–2016 should be noted. This comprehensive research project is dedicated to developing a method to measure and prevent food loss and waste in

the EU countries. The FUSIONS program does not separate edible and non-edible food parts, but covers the entire flow of resources removed from the supply chain.

This project considers redistribution as part of the food supply chain up to the point of consumption. According to FUSIONS, waste refers to food that is still fit for consumption but does not meet established criteria for sale. Such foodstuffs include seasonal products, excess inventory, products that are mislabeled or damaged during transportation. The results of the project show that there is no one universal method that allows obtaining representative data. Therefore, to achieve representativeness, it is recommended to use several research methods simultaneously. A proclamation has been issued to all organizations that collect data on food waste to do so in accordance with the FUSIONS guidelines. This is necessary in order to ensure the comparability of data obtained at all stages of food supply chain in the EU countries [23].

Russian experts believe that the further formation of a conceptual framework in the field of minimizing food loss will make it possible to develop a number of harmonized regulatory documents that will help ensure Russia's food security. Thus, saving of food resources by reducing the FLW arising in the technological cycle is an important way in solving economic, social and environmental problems [24].

Analysis of measures to reduce FLW, taking into account the interests of participants in the value chain

When considering the issue of FLW reduction from the point of view of the entire value chain sustainability, it is important to take into account the interests of its main participants. Some of them may lose and others may benefit from appropriate loss reduction measures. A comprehensive consideration of interests is necessary to determine the possibility of sharing financial and other costs between participants. Therefore, when choosing methods to reduce FLW, it is necessary to identify the economic and behavioral factors that led to the occurrence of loss and waste.

To date, there is insufficient information to understand in detail the socio-economic consequences of food and agricultural raw material loss. Therefore, there has been a long debate about the extent to which the investment in reducing FLW outweighs the cost of developing measures for this purpose. In this regard, the question is relevant: what "loss" is acceptable [3].

On the one hand, in order to achieve significant results in reducing FLW, it is necessary to identify the benefits for each of the participants in this process. For example, a supplier may receive additional economic benefits, and a trade enterprise may increase its reputation in the eyes of consumers. The physical availability of food may improve the diet and well-being of consumers. In addition, the reduction of FLW may improve food and environmental security at the state level [25,26].

On the other hand, it should be taken into account that the reduction of FLW cannot bring benefits to all participants in this process. Among others, most vulnerable elements of the value chain are farmers, other small producers and food processors. For them, both the positive and negative consequences of the FLW reduction are more noticeable [4]. Thus, farmers, other producers and consumers may be interested in reducing FLW in order to increase their welfare. However, the existing incentives for realizing such an interest are often not enough. The benefits of reducing FLW do not always justify the money or time invested in them. For example, individuals may face some institutional barriers: credit restrictions or a lack of information about possible actions. At the same time, the government sector is almost always interested in reducing FLW, since successful results in this area help to increase the food security of vulnerable population groups and reduce environmental damage [19].

According to experts, in the world, on average 30% of all food produced, or 1.3 billion tons per year, is lost or misused [2]. Figure 1 shows the loss of food products at the main stages of production, i.e. from growing to sale [27].

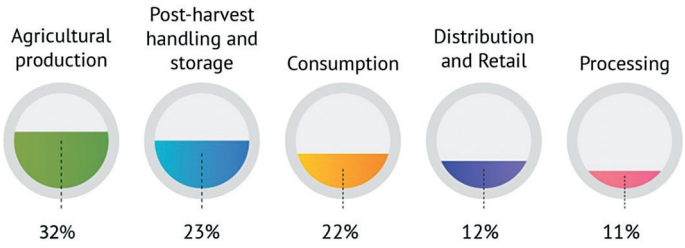

Figure 1. Food loss at the main stages of production (%) [27]

As for the type of food items that are lost all over the world, the statistics depend on whether FLW is measured in calories or in weight. In terms of calories, cereals make up the largest share of the global FLW (53%), followed by roots and tubers (14%), fruits and vegetables (13%). Meat makes up a relatively small share, though not in terms of environmental impact, i.e. 7%. However, on a weight basis, fruits and vegetables are the largest source of FLW (44%), followed by roots and tubers (20%). If we estimate the level of FLW for each commodity production, then 20% to 22% of the total volume of cereals produced is lost, compared to 39% to 44% of fruits and vegetables, 33% of roots and tubers, and 24% of seafood [3].

Figure 2 shows the specific loss for certain types of food products [28].

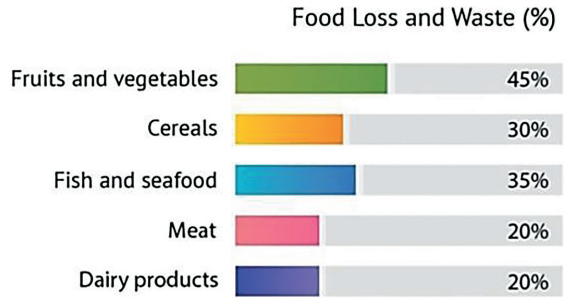

Figure 2. Food loss and waste by food type (%) [28]

Food loss and waste is highly dependent on the specific conditions and local situation in a given country or culture [15].

Environmental issues in the formation of FLW

In a modern world, there is a clear depletion of natural resources, which requires increased environmental control and a more environmental friendly approach, taking into account the labor costs of producing agricultural products and food, organizing supply chains, as well as preparing the land, using fertilizers, and other costs associated with agricultural production [3,29].

Thus, 173 to 250 km^3 of water per year is spent on the production of lost and wasted food products. Food crop production uses approximately 24% of total fresh water resources (27 m^3/person/year) and one fifth of fertilizers (4.3 kg/person/year) [30]. The area of arable land used to grow unused food is 198 million hectares per year, which is one fifth of the arable land used worldwide for food crops, or an area of Mexico size [3]. Reducing FLW will help to decrease the use of land, water and nutrients, as well as external negative impacts on the environment [12].

At the same time, due to the formation of unclaimed products, a significant part of the economic and natural resources spent on its production (labor, water, energy, soil, and others) is wasted, many of which are limited. Global food loss is estimated at a level of $7.5 trillion annually [4].

Efforts to reduce food loss and waste aimed at more rational consumption and production of food may play an important role in ensuring food and environmental security, contribute to solving economic and social problems, and increase the sustainability of value chains both at the regional, federal, and international level.

Most often, the solution to the problem of providing the population with food is seen in one way, i.e. increasing the production volumes by improving the technical means of agriculture and the processing industry. Some business experts think about this situation as about an insoluble contradiction between environmental requirements and real production. Therefore, there is a critical attitude towards the position of the environmental groups. All their proposals are thought to be aimed only at a sharp reduction in the volume of industrial activity. But such an opinion is not sufficiently substantiated. The presence of different approaches to the choice of methods and directions in economic development is quite a normal. However, this circumstance does not prevent the wide use of the achievements of scientific and technological progress for environmental purposes. Thus, increasing production volumes while reducing the level of negative impact are not opposed to each other [31].

For example, let's consider the meat industry in Russia. The production activity of almost all enterprises in this industry has a negative impact on the environment. First of all, this concerns emissions and discharges of pollutants, as well as waste disposal. The volume of this impact varies significantly and depends on the capacity of enterprises, as well as on the technologies and equipment used at the main stages of the production process. Thus, the degree of equipment of the meat industry with modern technical means directly affects not only the quality of products, but also the level of negative environmental impact [32].

In Russia, there are a number of regulations in the field of environmental management. These documents provide, in particular, financial and other forms of liability for violations of a number of requirements and rules. For example, the Code of Administrative Offenses of the Russian Federation[1] introduces penalties for non-compliance with environmental requirements when managing livestock, production and consumption waste. The need for strict regulation of this area of activity is beyond doubt. However, to improve production efficiency, reduce FLW and implement a set of other measures within the framework of the environmental paradigm, this is clearly not enough. Thus, to solve this problem, we cannot limit ourselves to coercive and fiscal measures. It should be cost-effective for entrepreneurs to use scientific developments that reduce the level of environmental impact and FLW. In this regard, it is important to determine which factors will be most effective. Thus, a clear incentive message is needed to implement industrial modernization based on resource-saving technologies.

Obviously, the environmental impact becomes higher the closer to the consumption stage FLW are generated. This is largely due to the process of FLW accumulation at the previous stages of the supply chain. Practice shows that, leaving the production chain, food, as a rule, ends up in landfills, where, under anaerobic conditions of decomposition, gas is released from organic waste, most of which is methane [33].

A similar situation occurs with the generation of waste in animal husbandry. Unfortunately, in some rural areas of Russia, you can still see tons of organic animal waste accumulated near farms. At best, they end up in nearby fields as fertilizer without any transformation. At the same time, many European countries have long had laws prohibiting the use of manure without its appropriate processing [34]. Although manure is a valuable organic fertilizer, however, if it is used improperly, there is a significant negative impact on the environment. Manure becomes especially dangerous if more than 100 tons per day accumulate in one place [35].

Practice shows that it is more difficult for many existing enterprises to move to a new technological level, since the construction of treatment facilities is comparable to the construction of a new farm. Therefore, the area surrounding the old livestock buildings, as a rule, is much polluted. Practice shows that the transition to

[1] Federal Law No. 195-FZ of December 30, 2001 (in the edit December 29, 2022). "The Codex of Administrative Offences of the Russian Federation" Retrieved from https://docs.cntd.ru/document/901807667 Accessed December 27, 2022. (In Russian)

modern technologies requires highly qualified personnel. Its training requires not only a long time, but also additional financial resources [35].

Characteristics of activities concerning FLW in Russia

In a world practice, various approaches and methods are used to solve existing problems related to FLW. In Russia, specialized measures are also being taken to reduce their volume, including in agricultural production, in particular, animal husbandry, and the food industry.

The country has relatively high rates of food loss at several stages of agricultural production and processing. This is due not only to adverse weather conditions, crop pests and animal diseases. Technological and organizational factors also influence, including insufficient technical equipment of agricultural producers [27].

As for the levels of food waste, in Russia they are comparable to those in Europe and North America [28]. The high-risk group, where the loss of food products or raw materials reaches more than 20%, includes the stage of raising livestock and poultry for meat production and the stage of fishing for fisheries, as well as the sale of crop products [4].

According to the data for 2021 of the Federal State Statistics Service of the Russian Federation[2], there is significant food loss in the country at all stages, i.e. from production to sale (Figure 3).

Figure 3. Product loss (thousand tons)

The reason for the formation of significant level of FLW in Russia is a consequence of the existing problems in the production and marketing relationships within the food sector of the agro-industrial complex.

These long-term problems are due to insufficient progress at the legislative level, as well as in research and production activities. This conclusion is supported by several documents. For example, the Strategy for the Development of the Food and Processing Industry of the Russian Federation[3] is focused on the weak material and technical base of many enterprises in this industry. Thus, poor infrastructure for the storage, transportation and refrigeration of perishable raw materials and food does not allow for the complex processing of raw materials and the creation of optimal conditions for their storage. This situation leads to additional product loss and a decrease in its quality.

Therefore, the main solution to this problem is the modernization of equipment and an increase in the extent of raw material processing by involving secondary resources in the economic circulation. These measures will significantly increase the yield of finished products per unit of processed raw materials.

In turn, the Doctrine of Food Security of the Russian Federation[4] also draws attention to the need to develop and implement technical and technological modernization programs in the production of agricultural products, raw materials and food to reduce loss.

In many regions of Russia, a comprehensive infrastructure for the storage and processing of agricultural products has not yet been formed, which causes the need for its rapid sale at reduced prices. In addition, manufacturers often face the problem of low demand for their product. Sufficient storage and refrigeration facilities allows for more flexible planning of supplies at the best market price during periods of high demand and acute stock-out of fresh products [36,37].

Thus, for the successful intensification of the existing process for internal processing of agricultural products, it is necessary to increase raw material storage and refrigeration capacities [38,39], as well as intensify the development of new technologies to reduce loss of agricultural products and increase their shelf life, which will be available to producers even in the most remote regions of Russia [40].

Unlike many countries focused on stimulating waste prevention activities, Russia's economic and environmental policy is more focused on reducing the negative impact of already generated waste and only partial recycling. However, there are norms in Russian legislation declaring a different approach. For example, the Federal Law "About Production and Consumption Waste"[5] indicates the priority areas of state policy in the field of waste management in the following sequence:

[2] Rosstat. Agriculture, hunting and forestry (Balances of food resources). Retrieved from https: https://rosstat.gov.ru/enterprise_economy. Accessed December 27, 2022. (In Russian)

[3] Government Decree of the Russian Federation dated April 17, 2012 No 559-p "Strategy for the development of the food and processing industry of the Russian Federation for the period up to 2020" (as amended on 13 January 13, 2017) Retrieved from https://docs.cntd.ru/document/902343994 Accessed December 27, 2022. (In Russian)

[4] Decree of the President of the Russian Federation No. 20 of January 21, 2020 "The Doctrine of Food Security of the Russian Federation" Retrieved from https://docs.cntd.ru/document/564161398 Accessed December 27, 2022. (In Russian)

[5] Federal Law No. 89-FZ of June 24, 1998 (in the edit December 19, 2022). "About production and consumption waste" Retrieved from https://docs.cntd.ru/document/901711591 Accessed December 27, 2022. (In Russian)

— maximal use of raw materials;
— prevention of waste generation;
— lowering of waste generation and reduction of waste hazard class in the sources of their formation.

Based on the available indicators [41], the food waste amount in the composition of municipal solid waste in Russia is about 35%, which is about 17 million tons per year. Almost the entire food waste (about 90%) ends up in landfills. The specified food waste amount emits about 2.4 million tons of methane. Other gases emitted include ammonia and hydrogen sulfide. Thus, reducing FLW will not only reduce the negative impact on water and land, but will also provide a positive effect in the economics by saving money on the environmental restoration.

The main types of meat raw material loss and the conditions for its formation

According to the studies on the assessment of food loss, at each stage of the value chain in meat livestock husbandry of the Russian Federation [4], it was revealed that the maximum loss of more than 20% to 30% forms the stage of livestock and poultry growing. At the stage of meat raw material processing, loss amounts to 5%; during transportation and storage, loss amounts up to 15%, while during sales it is up to 5%.

The systematization of data on FLW in the EU made it possible to estimate the loss for slaughter animals and poultry along the entire production chain, i.e. from growing to meat consumption, which are presented in Figure 4 [42].

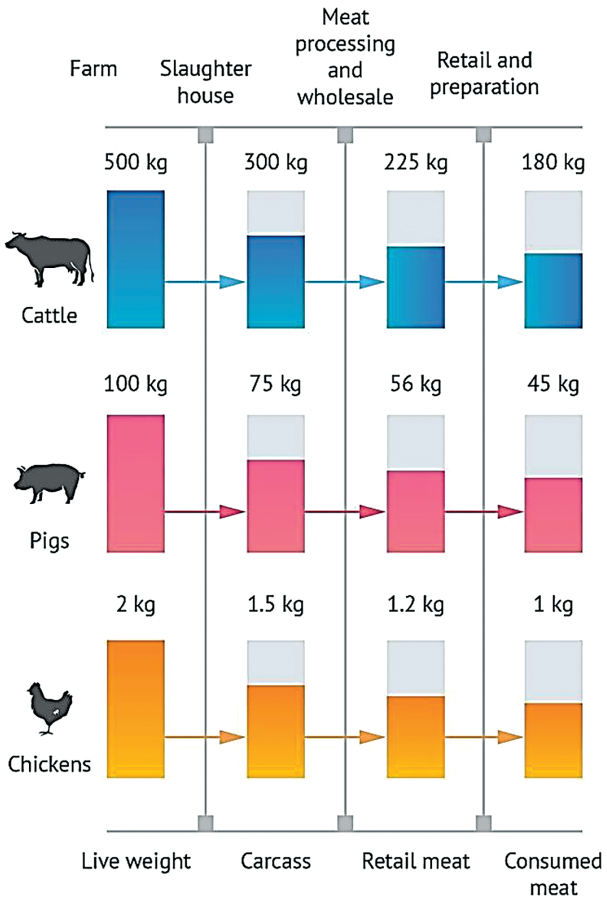

Figure 4. Meat loss (kg)

According to Rosprirodnadzor the data formed on the basis of 2-TP form (waste) of "Information on the generation, processing, disposal, neutralization, transportation and location of production and consumption waste", in the Russian Federation, animal husbandry (meat and dairy) waste amounted to 36.53 million tons in 2017, of which the majority is manure (cattle, pig, horse, etc.), i.e. 68.9%. The key difference between the meat industry and other branches of agriculture is the living nature of the primary raw materials, which imposes certain restrictions on the technological process, since the physical and mental condition of the animal before slaughter directly affects the quality of food products [43].

The main types of loss and the reasons for its occurrence include:

- a significant reduction in the live weight of livestock due to the imperfection of vehicles and pre-slaughter handling of animals;
- veterinary rejection of meat and by-products associated with livestock diseases;
- waste from animal slaughter and carcass butchering;
- low-quality primary processing of livestock;
- decrease in the yield due to the low level of technological support for the processing of raw materials and the sale of by-products (the use of obsolete technologies and equipment);
- defective products in case of violation of technological conditions;
- shrinkage of meat during cooling, freezing and thawing.

Thus, a significant part of loss in meat production is due to the unsatisfactory condition of animals before slaughter, i.e. physical injuries, mental stress, transportation conditions, as well as insufficient use of full-cycle technologies in the processing of raw materials of animal origin.

Also, attention should be paid to meat loss in the process of refrigeration, which is currently still quite high. At the same time, according to some experts, the current industry norms for acceptable loss in this area are maximum permissible and stressful for most enterprises, and it is rather difficult to fully meet them due to the multifactorial nature of the phenomenon. Moreover, there is often excessive loss of meat raw materials and finished products for certain types of food, technological and storage processes, which predetermines the need for special measures to reduce this loss [44].

In this regard, it is worth noting that modern methods and means of controlling temperature conditions in a continuous cold chain of meat and meat products turnover make it possible to ensure the effectiveness of managing these processes at various stages. Temperature measurement and control systems should be aimed at continuous monitoring of the temperature parameters of the environment and the product itself when organizing the processes of its production, storage, transportation and sale. Constant monitoring of temperature regimes allows building an optimal cold chain from producer to consumer,

which reduces risks, maintains the quality and safety of food products sold. Currently, there are opportunities to use various temperature measurement technologies in the implementation of logistical clustering in the supply chain of meat and meat products [45,46].

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Possible solutions and practices for FLW prevention and management

Today, most countries of the world implement various initiatives to prevent or reduce FLW, aimed at creating a circular economics, rational use of natural resources, preserving the environment and natural biodiversity, developing sustainable ways of producing, selling and consuming food. The implementation of these strategies should be tailored to the region, with particular attention to local infrastructure, energy, markets and education (i.e. knowledge at all levels from supplier to consumer) [11,12].

To minimize food loss in most countries, the following approaches are used at the government level: consumer education, optimization of post-harvest technologies, increasing the cost of waste disposal, developing partnerships between the private and government sectors to jointly reduce food waste and share responsibility, etc., as well as an approach to modeling and optimizing FLW to support strategies taking into account factors such as inventory management, processing costs, planning and distribution strategies, taking into account the demand from a particular client, etc., since supply chain building is a complex decision-making process in volatility/uncertainty of supply and demand, etc. [47].

Urbanization is one of the causes associated with a large amount of food waste, as it has led to the lengthening of food supply chains to meet the food needs of the population. Therefore, it is important to improve the methods and conditions of storage, transportation and sale in order to avoid loss and waste due to an increase in the distance between the place of production and the place of final consumption [11].

Thus, an important role is assigned to the improvement of logistics in order to reduce FLW. Optimizing supply chain management may help to reduce food waste.

Logistics, as an effective concept for managing material and related financial and information flows, is becoming more and more in demand in the sectors of the agro-industrial complex. The organization of resource supply for agricultural producers and the promotion of their products to the market on the basis of new logistics principles have significant economic, social and environmental effects. Thus, logistics management becomes one of the optimal tools for solving the problem of reducing food loss and waste (FLW), as well as better consumer service.

Such management is the activity of the enterprises that form a well-functioning system of resource supply. In turn, this system is based on the implementation of concentration, distribution and movement of various types of resources and services along optimal pathways to end consumers located in certain territories [48].

According to PLOS Medicine research, up to 80% of the product turnover on the Russian market is occupied by products of transnational corporations (TNCs). Therefore, the issue of storage and intensification of internal processing, as well as reducing the total loss of agricultural products during transportation is acute [38].

Analysis and systematization of research in the field of development and implementation of effective logistics approaches shows their relevance for reducing food loss both at the global and regional levels.

So, the work of Magalhaes et al. [49] used interpretive structural modeling to identify 14 causes of food loss and waste generation in fruit and vegetable supply chains, which were divided into seven levels of influence. The causes of FLW related to logistics were identified, which have a significant impact on the rest causes. The risks of cold chains in pandemics, adaptive strategies for their mitigations and logistical tracking systems, in particular, using various information technologies, are described too [50].

In addition, the nature and causes of FLW along the chain differ in different regions of the world. Evaluation of the results from 24 interviews with key participants in Barcelona made it possible to systematize the causes of food waste in this region of Spain along the entire food supply chain [51]. The results of this study show the great interest of regional participants in the problem of food waste generation and provide a complete map for the causes of their formation according to their level (micro, meso, macro) and their nature (technological, economic and business management, regulatory and policy, appreciation and enhancement). In the article [52], a survey of 47 Belgian enterprises obtained results showing that processing is by far the most important cause of food waste. While transport, changeovers, production interruptions, human error and product exposure at this stage often result in significant or excessive waste, the causes of food loss during packaging, before or after production have less impact. The work [53] is devoted to the analysis of the location of participants and objects of production, transformation, commercial-

ization and distribution, formation of the configuration of the supply chain for perishable foods. It is shown that the improvement in the logistics chain leads to a significant reduction in the loss of perishable food products on the example of Colombia.

In Russia, the existing system of supplies and transportation of agricultural products from the field to the storage and producer needs to be revised. As for agriculture in Russia as a whole, to ensure its dynamic development, the following is necessary [54]:

- connection to the public railway infrastructure with existing terminals and ones under construction, taking into account the capacity of roads adjacent to port and land railway terminals;
- reduction of infrastructure and logistics costs within the vertical supply chain by developing capacities that ensure the storage and transshipment of agricultural products, and reducing the total costs of its shipment, transshipment and transportation by road, railway and water transport;
- implementation of an effective state tariff policy for the transportation of agricultural products by railway, water and road transport;
- improving the efficiency of supply chain management through the use of a more efficient procurement and inventory management model, as well as through process optimization (integrated planning, sales and operations planning);
- reducing loss in the production of agricultural products by reducing equipment downtime (improving aftersales service);
- reducing the cost of logistics operations through the optimization of logistics routes, warehouse network modeling, reengineering of warehouse processes.

Thus, the use of an effective logistics approach in the agro-industrial complex will make it possible to use resources appropriately, reduce time and financial costs at the stage of product delivery from producer to consumer.

The concept of sustainable supply chain management not only helps enterprises to integrate the principles of corporate social responsibility into their activities, but also increases their ability to achieve efficiency in logistics, resource use and FLW reduction [55]. Also, sustainable supply chain management helps to achieve the UN Sustainable Development Goals, in particular the concept of a triple result, which focuses on integrated economic, social and environmental well-being [56].

For perishable foods, such as raw meats and meat products, systematic process and temperature management throughout production, processing and distribution is important to ensure their quality and safety, as well as minimizing waste at all stages of the food supply chain (FSC). This allows food industry operators and technology companies to determine which combination of technologies best fits a given food supply chain and reduces food waste at minimal cost [57].

In recent years, the traceability-based management of cold FSCs has become a popular concept [58]. Traceability requires identifying and recording processing information for all batches of a product in a given process, defined as traceable resource units (TRUs), and exchanging this information as a product (or TRU) moves through the supply chain [58]. Traceability is achieved through the implementation of an information system that includes a wide range of methods and technologies to reduce food loss due to various measures, including accurate stock counting, real-time monitoring of the environment and product conditions, informing about the history and quality of products, and product distribution planning based on expiration date [57].

Spoilage and expiration date on packaging are important causes of food waste, while a significant amount of food is wasted if a sample from a batch does not meet established quality level, resulting in the destruction of the entire product batch. This food loss may be reduced by monitoring food quality throughout the supply chain and using this information effectively. The causes of food loss and waste in middle- and high-income countries are mainly related to consumer behavior, as well as a lack of coordination among the various actors in the supply chain. At these stages of the supply chain (consumer and retail), quality control is difficult to achieve. Appropriate nondestructive methods for quality control of individual products are not available, since most of the methods are too complex or require expensive and complex equipment or materials [59].

Successful action to prevent food loss may be based on the introduction of new packaging technologies that improve the quality, freshness and safety of food or provide a longer shelf life.

Thus, intelligent packaging makes it possible to control the quality of food products throughout the supply chain, as well as at those stages that were previously not subject to control. Various types of intelligent packaging are packaging systems that monitor the condition of packaged foods throughout their life cycle and communicate information related to the quality or safety of the packaged product. The intelligent packaging may contain sensors and/or indicators that monitor product quality or environmental conditions. The information from these sensors must be translated into a meaningful quality indicator that will be passed on to some or all participants in the chain [60]. To do this, the measured signals may be converted by mathematical models into values that reflect the quality of the food product [59].

Currently, the world is exploring the possibility of an integrated approach to increasing the value of FLW as a part of the circular economics development as a solution for waste management [61,62].

The sustainable development of the circular bioeconomics concept is possible only with the introduction of advanced technologies for the valorization of food waste.

Increasing the value of food waste opens up new horizons for economic growth, turning waste into raw materials for biological processes that allow the synthesis of bioproducts from a biological source in a closed cycle. Decomposing food waste to a negative level using advanced technology converts food waste into bio-based products such as biologically active compounds (antioxidants, pigments, polysaccharides, polyphenols, etc.), biofuels (biodiesel, biomethane, biohydrogen), and bioplastics.

FLW may be a useful contribution to the circular economics [62] as a source of bioenergy (biodiesel, biomethane, biohydrogen), and for the production of bio-based products such as biologically active compounds (antioxidants, pigments, polysaccharides, polyphenols, etc.). Agribusiness waste may be used to produce bio-based products, such as bioplastics (food packaging), whose production volume reached almost 1 million tons in 2020, which is almost half of the entire bioplastics market [63].

In a modern world, a linear model of natural resources use ("take — use — throw away") has developed based on the assumption that these resources are abundant, accessible, easy to use and cheap. Solving the issues of efficient use of natural resources, which make it possible to derive economic and environmental benefits from their use, is an important component of the currently formed vision for sustainable development of the world economics. To implement tasks in the field of resource conservation for their efficient use, the transition to a circular economics is of great importance.

The basis of the circular economics is formed by closed supply chains, which are supply chains that ensure the maximization of added value throughout the entire life cycle of a product with dynamic recovery of values and volumes within relatively long time intervals [64]. Ideally, the formation of closed supply chains should lead to the observance of the zero waste principle.

Conclusion

FLW amount, as an example of the extremely wasteful use of food and natural resources, is not only a problem for ensuring global food security and increasing life quality for the world's population.

There is a general consensus on the fact that reducing FLW is a great opportunity to improve food security, ensure the sustainability of food systems and avoid economic costs throughout the food supply chain. But there are significant gaps in knowledge and research on FLW. For example, there is no reliable evidence base for estimating food waste worldwide. In order to effectively assess and optimize strategies and decisions to reduce FLW, the lack of reliable and consistent data and inconsistencies in FLW definitions and measurement systems need to be corrected. Moreover, detailed information about where in the food chain, for which products and in which regions the greatest loss forms must be available, as well as the extent and causes of this problem.

At present time, for most countries it has become obvious that lowering FLW contributes to a more careful use of the planet's resources. Efforts to reduce food loss and waste, which are aimed at more sustainable consumption and production of food, may play a particularly important role in ensuring food and environmental security, solving economic and social problems, increasing the sustainability of value chains both at the regional, federal and international levels.

More comprehensive and integrated approaches should lead to future research on preventing FLW as a part of the circular economics, especially in relation to the socio-economic and environmental impacts of FLW reduction strategies at all stages of the food supply chain in various regional contexts and development conditions considering, among other factors, infrastructure, energy, markets and education.

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WATER-HOLDING AND WATER-BINDING CAPACITY OF MEAT AND METHODS OF ITS DETERMINATION

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Keywords: *water-holding capacity, water-binding capacity, meat proteins, forms of water-to-meat bonds, methods of WHC determination, gravity, pressure, centrifugation*

Abstract
*The considered topic is of great interest for researchers and practitioners engaged in the development of technology and the production of meat food. In the review, the authors focus on theoretical aspects of the meat capacity to bind and hold water. The characteristic of the forms and strength of the water-to-meat bonds is given. The influence of the structural elements of muscle tissue on the meat capacity to bind and hold water is shown. The different opinions of the specialists are given in regard to the terms "water-binding" (WBC) and "water-holding" (WHC) capacity of meat, and the authors of this research expressed their own opinion. Basing on the analysis of publications, a characteristic is given to forms of water-to-meat bonds strength from the point of view of technological practice: there is tightly bound water, loosely bound available water (**immobilized**), and loosely bound excessive water (**free**). The article summarizes the material on the methods of determination of water-holding (water-binding) capacity of meat. It is shown that up to date there is no unequivocal answer about the choice of WHC determination method. To define WHC, it is recommended to subtract the WBC value obtained by one of the gravimetric methods from the value of the indicator obtained by one of the methods of external pressure. This problem requires further research, discussion of issues and methods for determination of water-holding capacity.*

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Introduction

The capacity of muscle tissue to hold or to lose water underlies all modern technologies of meat food production. Understanding the mechanisms of water supply and water maintenance allows direct regulating the functional and technological properties of raw meat materials in order to achieve the desired result. In meat and meat products water is the most important component that provides a significant impact on the organoleptic, structural-mechanical properties of raw materials, quality and storability of the ready-to-consume food products.

Modern knowledge about water-holding capacity of meat is based on fundamental researches of Hamm [1], Offer et al. [2], and Honikel [3,4]. However, the essence of water binding in the meat is not yet completely clear till nowadays.

In available publications there are differences in the approaches to the terms "water-binding" and "water-holding" capacity. So Hamm [1], Forelle et.al. [5], Honikel [3] believe that water-binding capacity is more related to potential capacity of raw meat to bind water, while Klima et.al. [6], Naveau et.al. [7] interpret the term "water-binding capacity" as the capacity of the food product exposed to heat treatment to bind and hold water. According to the authors there is a rather close correlation between these two indicators. Water-holding (water-binding) capacity of meat is understood as the difference between the water content in the sample before and after any processing (maturation, pressure, heat treatment, etc.). Pospic et.al. [8] consider the abbreviation of WHC as capacity of raw meat to hold water, and WBC — as capacity of thermally processed meat to bind water.

In domestic researches the term water-holding capacity, as a rule, is construed as capacity of native proteins (raw meat) to bind water, while water-binding capacity is construed as the amount of water bound by the food product, exposed to thermal processing [9,10].

According to the authors of this article, the concept of water-holding shall be understood as the capacity of native proteins in raw meat to bind water through peptide bonds and hydrophilic lateral groups of residues of protein macromolecules amino acids capable to hold the opposite-charged ions and water dipoles. In the long chains of fibrillar proteins (collagen) the terminal

groups create chemical bonds between the chains. While that the terminal groups form a three-dimensional spatial structure, which retains and immobilizes water inside the spatial lattice, thus contributing to the swelling of proteins. Polypeptide chains of globular proteins are rolled up in such a way that hydrophobic centers are oriented inwards the globe, and hydrophilic centers are located on its surface.

When meat is heated, denaturation and coagulation of myofibrillar proteins occurs, which leads to the loss of their biological specificity. In this case, the term water-binding capacity should be understood as the amount of water bound by the structure of the product exposed to heat treatment. Nevertheless, an analysis of the results of forms research and methods of retaining of water within the meat structure proves the presence of water not bound to the structural elements of cell, but still held in its structure. Consequently, the concept of "water-binding capacity" is also applicable to assess the properties of raw meat not exposed to heat treatment. Therefore, before discussing the issue of water holding, it is appropriate to consider the morphological structure of muscle tissue.

Characterization of water bonds in meat

It is known that the main structural element of muscle tissue is muscle fiber, which surface is covered with a sarcolemma. Inside the fiber there are myofibrils which occupy 60–65% of the cell volume. The muscle fibers are separated by layers of connective tissue — endomysium, which is connected to the sarcolemma. A group of muscle fibers forms the primary muscle bundle, surrounded by coat of connective tissue — perimysium. Primary muscle bundles are combined into the secondary bundles, to tertiary bundles and bundles of a higher order, which all together form the muscle. The muscle is surrounded by a coat — epimysium or fascia.

From publications [11,12] it is known that the meat contains approximately 85% of water. This water is part of myofibrils, localized between myofibrils and connective tissue coat — endomysium, between the muscle fibers bundles and the surrounding connective-tissue coat, and between the individual bundles of muscle fibers. The remaining 15% are located in extracellular space. Hamm [13] found that various components of muscle tissue are capable to hold water in various degrees. The water held by myofibrillar proteins accounts for about 50%, by sarcoplasmic proteins — about 3%, and non-protein components of the sarcoplasm hold about 47% of water.

According to the electrostatic theory of Hamm [14], the amount of bound water is determined by the "clean" charge of proteins which repels the adjacent groups of protein molecules with negative charge; also the amount of bound water is determined by swelling of myofibrils and partial destruction of actomyosin complexes, which increases the water-binding capacity of meat.

The water, which is part of the undecomposed meat tissues, is heterogeneous in its physical and chemical properties, and its role is not the homogenous also. There are two forms of water in meat — bound and free. Bound water, according to Collins et al. [15] is mainly bound by polar groups of protein macromolecules, which is explained by the special structure of the water molecule itself. Such water is characterized by a number of specific properties: it has smaller volume, freezes at a lower temperature, chemically inert and is unable to dissolve substances. The water like this accounts for about 0.1% of the total water content in meat.

Water molecules have capacity to bind to each other with hydrogen bonds. Although these bonds are weak, they are very numerous, thus they together provide a significant impact on the structures which they get bound to. N-region of one water molecule, with negative charge, is attracted by the positive area of the other water molecule.

Much larger amount of water (from 5% to 10% of the total content) is in a less organized form and is less tightly bound with protein molecules [16,17]. Due to the presence of hydrophilic centers of proteins of the electrostatic field, water dipoles are oriented around them, forming this way adsorptionally bound water. Depending on the size of the charge, hydrophilic centers are able to hold from 2 to 4 water dipoles, not counting the water of diffusion layers. The force of interaction between active protein groups and water molecules depends on the distance between them, as well as the availability of the group itself in the molecule structure. The water dipoles, which are located close to the polar group, are bound with it quite firmly with the help of van-der-vials forces. When the dipole of water is removed from the polar group, the force of their interaction weakens. The number of subsequent layers can be as many as several dozen, forming a solvate coat around hydrophilic colloids and protein molecules in general. In the last layers, water molecules can move from the outer layer to hydrate layer and vice versa, forming this way the so-called "diffusion layer" [16].

Unlike polar groups, non-polar groups of protein macromolecules repel polar water molecules, creating an arched structure around a non-polar group.

According to Zayas [17], some amino acids possess the capacity to bind water. For example, aspargin and glutamic acids can bind from 4 up to 7 water molecules. Myosin protein also has a high capacity to bind water due to its being rich in these amino acids.

The forms of adsorption binding of water are divided into two types: binding of water with charged protein groups — ion adsorption, and binding of water with uncharged groups — molecular adsorption.

Water molecules bound and held by polar groups of proteins contribute to the preservation of the spatial structure of the protein macromolecules and make up **tightly bound water.** The share of tightly bound water is limited

by the number of cross-line actomyosine bridges and the strength of the relationship between actin and myosin and z-lines. Moreover, according to Clark et al. [18] the so-called costameres prevent the swelling of myofibrils. The costamers are the structural and functional components of the transversus stripe (barred) muscle tissue that connect the muscles with the cell membrane (sarcolemma) and provide a structural base. These bonds between the adjacent myofibrils and cell membrane consist of several proteins, which include desmin, philamin, sinamine, dystrophin, talin and vinculin [8]. Therefore, it can be assumed that water-binding capacity also depends on the structure of muscle tissue.

Water, located outside this adsorption layer formed by the electrostatic interaction by Pospiech et.al. [8], is considered as unbound, although it is immobilized in the structure of the muscles and also determines the water-binding capacity of meat.

Free water is not bound with protein and serves as a solvent for organic substances and minerals. This water freezes at a temperature of about 0 °C and drips out from the tissue easily. Free water is held in capillaries, the space between the proteins and inside them. This water is loosely held in meat and its volume depends on the size of the capillary space between myofibrils.

Offer et al. [16] proposed an alternative hypothesis of the formation of water-binding capacity of proteins (osmotic theory). According to this theory the uneven distribution of ions in the aqueous phase and on the surface of the actomyosin lattice creates an osmotic force that draws water into the system. The factor of swelling is limited by the cross bridges in actomyosin lattice.

The theories stated above of the formation of water-binding capacity were considered and discussed in numerous publications, including the research of this capacity with the help of nuclear-magnetic resonance. However, some issues regarding the theoretical side of this problem have always remained unsolved.

Honikel [19] defines five ways to bind water with proteins:

— extremely strong binding of water with proteins by electrical bonds;
— binding of water with polar groups of actin and myosin;
— immobilization of water in myofibrils structure, depending on pH value;
— immobilization of water in sarcoplasmic space (relatively freely mobile water);
— retention of extracellular water in capillary spaces ("drip losses").

Change of water-holding capacity during autolysis

The processing of animal tissues after slaughter and their transforming into a food product is accompanied with a row of physical-chemical and biochemical changes in muscle fibers, which result to WHC decrease. The reasons of this phenomenon, generally, are related with loosely reversible changes in the state of the protein complex of muscle tissue.

Before the onset of postmortem stiffening, the meat has a high water-holding capacity [16,19], which is explained by the low concentration of hydrogen ions and lack of bonds between actin and myosin due to the high level of ATP. As a result of the enzymatic splitting of ATP and build-up of lactic acid over the next 12–24 hours of autolysis, myosin and actin threads interact among each other and form of cross bridges. This interaction forms actomyosin, and water-holding capacity of meat drops down [2].

Bertram et.al. [20] showed in his researches that more water is contained inside the myofibrils of the I-strip than in A-strip, which is denser in terms of protein. As the myofibrils get shorter during autolysis and postmortem stiffening occurs, the volume of the I-strip area is reduced. According to the research [21], a decrease of myofibrils volume in this area together with their shrinkage leads to the displacement of water from the myofibrillar structure into the interfibrillar intracellular space and, ultimately, away from the muscles. Decrease of the medium pH brings the charge of proteins to the isoelectric state, which also helps to reduce water-holding capacity.

It is necessary to keep in mind that post-slaughet processes in muscle tissue are accompanied by the oxidation of myofibrillar proteins along with the transformation of some amino acids, including histidine, to carbonyl derivatives [22,23]. While that the intra- and/or inter-proteins disulfide transverse bonds [24] are formed. Since tissue proteolytic enzymes of calpain contain in their active centers both histidine and SH-groups of the residues of cysteine, Lametsch et.al. [25], Rowe et.al. [26] believe that these enzymes can be inacted in result of oxidation. Thus, oxidative changes inhibit proteins proteolysis and reduce the functional properties of meat, including its water-holding capacity.

After a certain time, the postmortem stiffening gradually resolves. It contributes to increase of water-holding capacity of proteins due to relaxation of muscle fibers, but not dissociation of actomyosin, and also as result of proteolytic changes in protein macromolecules, which helps increase the amount of available protein groups capable to bind water [12]. While the subsequent development of proteolysis, the number of active groups of proteins capable of binding water increases, however, according to Ke [11], this does not cause any significant increase of water-holding capacity.

So, summarizing the above material, it can be stated that the water in the composition of meat is more or less closely related to the muscle proteins. The water can be localized inside the cell, and in the intercellular space, as well as in micro- and macrocapillaries. The strength of water binding in the structure of the muscle cell according to [5] is determined by the method of its binding. Most of the researchers share the same opinion that the quantity and

condition of electrostatically bound water are not subject to changes during technological processing, including autolysis.

The water, localized around the polar groups of proteins, is quite tightly bound to them. However, as it removes from the centers of binding, the strength of interaction weakens. It can be assumed that part of the adsorptionally bound water is loosely susceptible to technological influences and is more involved in stabilization of protein macromolecules. Part of the adsorptionally bound water removed from hydrophilic areas of proteins is more subject to change due to the formation of actomyosin and subsequent contractions of myofibril sarcomeres. In this sense, this water can be considered rather immobilized than bound. However, being subjected to certain technological processes, like brine treatment, water displaced into the interfibrillar space will turn to bound state.

The water held by myofibrils significantly depends on the conditions within the cell environment and, above all, on the pH level. The state of the water significantly depends on the conformation of protein macromolecules and the number of available hydrophilic centers. Taking into account the high lability of muscle proteins in response to environment conditions changes, water immobilized in protein structures should be considered more likely bound than held. However, with certain technological influences, for example, with the addition of sodium chloride, this part of the water will easily go into a held state.

The water of the sarcoplasm and capillaries can be considered as mobile water, held by the structural elements of muscle tissue, i.e.: sarcolemma or the walls of the capillaries. This water is easily lost in the form of drip losses, dripping fluid, or evaporation from the meat surface. Nevertheless, as well as water associated into the structural elements of muscle tissue, this freely movable water can be transformed into a bound state.

Based on the above, the water should be considered as **held** water, when it is retained by electrical bonds and adsorption interactions. The term **bound (immobilized)** water should be applied to a part of the water in the adsorptionally formed diffuse layer, which is **loosely bound** within the myofibrils structure in proportion to its distance from the polar groups of these proteins and other binding centers, for example, from hydrophobic ones. The water of the sarcoplasmic space and water, bound by osmotic pressure within the capillaries system, should also be considered as bound water (immobilized water). This suggestion is proven by studies [21], which confirm that water loss in the form of droplets of meat juice during the cooling of carcasses occur as the water is released from myofibrils, its movement from the intracellular space to the extracellular space, and, as a result, the release of liquid on the surface of meat (so, it means that water losses occur due to immobilized water).

Huff-Lonergan et.al. [12], Pulanne et al. [27] in technological practice distinguish the following forms of water-to-meat bonds: tightly bound, loosely bound, available (immobilized) and loosely bound excessive (free) water in the composition of meat, while their shares account for 5%, 15% and 85% of them from the total value.

Using this approach, it can be assumed that the capacity of muscle fabric to interact with water is formed due to its capacity to hold water (WHC) and capacity to bind or immobilize it (WBC). To determine the aggregate capacity of muscle tissue to bind (WBC) and hold (WHC) water, probably it's necessary to introduce an additional term, for example: "Water-binding potential" or "capacity of muscle tissue for water interaction". To resolve the conflict of terms, it is also possible to use the term "water-binding capacity" of muscle tissue as a more general one, including the assessment of both held and bound water in the analyzed objects. In foreign literature, the term "WBC" is also used more often as a more general one, and it applies, more often, to assess the general capacity of meat to bind and hold water than in reference to some specific methods and forms of water binding within the meat.

Methods of water-holding and water-binding capacity determination

Currently, to determine water-holding (WHC) and water-binding (WBC) capacity of meat and meat products, many methods are used — from the simple ones, like mere pressing, to the original methods — with the help of nuclear magnetic resonance. All known methods are based on determination of the loss of water in conditions of gravity, under applied pressure, including determination of drip loss of meat exposed to centrifugation. In some methods WHC is indirectly assessed through the reverse parameter, when the amount of meat juice loss (drip losses) is measured. Drip loss includes exudate or water released on the surface of meat during its exposure.

All methods can be divided into gravimetric methods, methods with external pressure, and adsorption methods according to Honikel at al. [28]. It should be kept in mind that it is not possible to determine the WHC in absolute units of measurement, because each of the methods is used within the framework of specific tasks, and the results obtained by different methods are loosely comparable.

Gravimetric methods

This group includes methods for measuring the meat weight loss due to separation of free water from the meat (dripping) at temperatures from 1 to 5 °C for 48–72 hours, sometimes for 18–92 hours [29]. These methods are very conditions-sensitive, but require a lot of time (from one to several days). These methods include the measurement of water losses by the bag (bag-BM) "DL", proposed by Honikel [4], the method using filter paper (FPW) described by Kauffman et al. [30], Rassmussen et al. [31], including the storing of meat samples in a container for collecting of released water, which method is called the EZ-Driploss

method ("EZ"). This method is recommended by the Danish Meat Research Institute (DMRI) for routine lab researches [32].

For WHC determination by the gravimetric method with the bag ("DL"), a sample of muscle tissue weighing 40–100 g after fat trimming is weighed, hung on the thread and is placed in a hermetic plastic bag to prevent loss of water caused by evaporation so that the meat does not contact with any of the walls of the bag. After exposure for 48–72 hours at a temperature of 0 to 4 °C, it is weighed for the second time and the loss of the tissue liquid is determined by the difference of the sample weight before and after exposure [29].

Honkel [19], Abdalhai et al. [33] offer to determine water-binding capacity of meat on the samples of 40–50 g and a size of 30 × 60 × 25 mm, placed in a hermetic container and exposed for 48 hours at 4 °C. Water losses are calculated as the difference in the mass of the sample before and after hanging as a percentage from the initial mass. WHC is expressed as a percentage of water content in meat.

In addition to this method, several gravimetric methods are proposed [30] also, such as "EZ-DRip Loss", "method of the tray" and the Danish "dripping pipe method".

The EZ-Drip Loss method is similar to the "DL" method, but it uses cylindrical samples of muscle tissue weighing 5–10 g. The result is measured, as a rule, after 24 hours of exposure. According to the EZ-Drip Loss method, a 25-mm cork drill is used along the muscle fibers to cut out a sample weighing about 10 g of a cylindrical shape with a diameter of 25 mm and 25 mm long. The sample is weighed and placed in a suspended state in a special container "EZ" to collect tissue fluid. The container is closed with a lid to avoid loss of water due to evaporation. According to the method procedure the samples are exposed for 24 hours at 4–6 °C, after that the sample is re-weighed. Before each weighing, the surface of the samples is carefully wiped with a paper towel. Water losses are expressed as a percentage in relation to the initial mass of the sample.

$$DripLoss = \frac{W_1 - W_2}{W_3 - W_2} \times 100, \%$$

where

W_1 — is weight of container with liquid,
W_2 — is weight of empty container,
W_3 — is weight of container with meat.

To keep accuracy of the method Filho et al. [34] recommend to expose pork samples for 48 hours. Kilgannon et al. [35] offer to expose beef samples for 72 hours, and lamb samples — for 96 hours, according to Holman [36]. Measurement of water losses in the form of drip losses enables to assess water-binding capacity of meat quite accurately, but in practice it is still a difficult and time-consuming task.

Methods using external pressure

The first method for determination of water-binding capacity of meat was published by Child et al. in 1934 [37]. This is the pressing method (FP PM), which was subsequently improved by Grau and Hamm [38]. Currently several modifications of this method have been proposed. In Russia, the method was modified by Vovininskaya V. P. et al. [40]. The method is based on determination of the amount of separated water, determined by the area of the wet spot on filter paper left after pressing the meat sample of 0.3 g with a load of 1 kg for 10 minutes.

Joo [40] recommends this method for determination of meat water-holding capacity. According to this method a sample of meat is placed between two pre-weighed plexiglass plates with a size of 60x60 mm and filter paper with a certain absorbent capacity. Then a load of 2.5 kg is applied and the sample is exposed to pressure for 5 minutes. After the separation of water, the compressed meat sample is taken out, wet filter paper with two plastic films is quickly weighed and the amount of water is recorded. The obtained value serves as determination of water-holding capacity.

This group of methods includes also centrifugation method [41]. This method is based on determination of the amount of water, pressed out of minced meat or meat samples under the influence of centrifugal force. The method is recommended for determination of the WHC of intact (not crushed) muscle tissue, provided that the samples are not destroyed and deformed [42,43]. A sample of 10 g is centrifuged at a rotation speed of 3000 rpm for 15 minutes, using graduated centrifugal test tubes with a mesh. Samples are weighed before and after centrifugation. The mass of dry substances contained in the liquid separated by the centrifugation is added to the mass of the sample after centrifugation. To calculate the amount of bound water, it is necessary to have data on the total content of water in the examined sample.

The method of high-speed centrifugation proposed by Hermanson et al. [42], is used to determine the share of loosely bound water in meat. Samples from 1 to 20 g are centrifuged at centrifugal force rate 5000 × G and 40,000 × g. The amount of water released is determined by weighing the separated water or by weighing the sample before and after centrifugation.

Methods of adsorbtion

Adsorption methods are based on application of adsorbing materials, like filter paper, cotton-viscose material, gypsum, clay. The method is based on the effect of absorption of unbound water from muscle tissue by the adsorbing material. According to the Chan et al. [44] pre-dried and weighed adsorbing material (for example, filter paper) is pressed to the surface of the meat sample and in 3 seconds is weighed again. The amount of absorbed water is calculated as the difference between these two weighings. However, the filter paper method is not

suitable for determination of the WBC in meat samples with high content of fat.

The method of filter paper weighing [30] (FPW) is that pre-weighed filter paper with a diameter of 45 mm is pressed to the surface of the sample, held for 10–20 minutes, and water losses are determined by the difference in weight of filter paper before and after the exposure.

The method proposed by Walukonis et.al. [45] for pork, uses a cotton-gauze material (tampon) weighing about 3 g. This tampon is inserted into the PC muscle through the subcutaneous fat layer. This material is inserted through an incision cut in shape of "+" to a depth of about 2.4 inches in a strictly defined place (for example, in the area of the 12th or 9th rib) and held there for 15 minutes. According to the authors, the exposure for 45 minutes shows the best correlation between adsorption and the loss of meat juice. The value of the adsorption (WHC) is calculated as the difference between the final mass of a cotton-gauze material with absorbed exudate and the initial mass of a dry tampon. This method involves a fairly accurate and quick assessment of the WHC in the early post-slaughter period.

Hofmann [46] proposed a method of capillary volumeter based on application of capillary forces to muscle tissue. The method consists in placing a plaster plate on the surface of intact muscle tissue for 30–120 seconds, while loosely bound water is absorbed into the porous material, and the air displaced from the capillaries under the action of meat juice enters a V-shaped calibrated capillary glass tube with a dyed liquid. The volume of displaced air, determined by fluid displacement, equals to the volume of released water and inversely proportional to WHC of muscle tissue.

Non-traditional methods of WHC determination

The development of technological progress and computerization of scientific research has made it possible to improve the methods of WHC determination.

Method of electrical conductivity

Lee et.al. [47] conducted studies of the electrical conductivity of meat with the help of conductivity analyzer to predict the water-binding capacity of pork. The authors found that PSE meat features higher electrical conductivity than normal meat and DFD meat. According to the authors the higher electrical conductivity of PSE meat is caused by the low water-binding capacity, which leads to losses of liquid and substances dissolved there. The results of this study have shown that electrical conductivity can be used as a possible parameter for assessing the water-binding capacity of meat. Measuring the electrical conductivity in 24 hours after slaughter allows assessing the water-holding capacity of meat.

Method of nuclear magnetic resonance

The method of nuclear magnetic resonance is widely used in various studies and can be used to determine the WHC. Determination by NMR method of relaxation time for water, immobilized in the pores and capillaries of different sizes, allows measuring the relative "freedom" of water molecules movement in the magnetic field, which, in its turn, indirectly allows assessing the level of the WHC.

NMR method proposed by Abdullah et.al. [48] is that the meat sample weighing from 1 to 5 g is evenly distributed in the test tube, brought to setpoint temperature, and then scanned in a NMR-spectrometer several hundred times to obtain the average value of the relaxation time of water molecules in the sample. The measured relaxation time is the time necessary for the nuclei of the water molecules (protons, deutrons or oxygen atom) to return to their first energy level after excitement. According to Bertram et.al. [49] this method correlates quite well with known gravimetric and adsorption methods for the WHC determination.

Method of microwave spectroscopy

The authors [48] used a new method of microwave spectroscopy for assessment of meat juice losses (WHC) and compared the obtained results with the widely used EZ-Driploss method [31]. The principle of using microwave sensors is based on the interaction of electromagnetic waves with the examined sample. When the sample is subjected to electromagnetic irradiation, it changes the speed of signal, weakens or reflects it back. According to this method, the tested sample is placed in the center of the microwave resonator of polyethylene material, and exposed to microwave energy. Depending on the frequency, size and properties of the material inside the waveguide, electric and magnetic fields may take various forms, like transverse electric field and transverse magnetic field. Depending on the resonance mode and frequency, the distribution of electric and magnetic fields will change, thus affecting the interaction between the tested sample and the electromagnetic field inside the cavity. The microwave cavity is connected to the vector network analyzer and a special interface to collect spectral data. The spectra difference will determine the amount of the released water.

Video image method

A modern method is proposed [50] that simplifies the measurement of water-holding capacity of meat by pressing via filter paper. It includes new parameters and a new measuring instrument. In the proposed device "WHC-trend instrument", a video camera is installed above the meat sample compression system, which analyzes the video image by measuring the area formed by 250 mg of homogenized meat. The measurement starts from the beginning of the process, and then the images are taken every 15 seconds for 10 minutes after pressing the sample with a force of 500 N. A dynamic measurement of fluid release over time was obtained, which was called "WHCtrend".

The method has been tested on various types of meat and can be available for fast determination of the water-binding capacity of meat.

Infrared spectroscopy method

The method is based on determination of the reflective coefficient of the meat surface depending on water content. The method measures the difference between the composition of light beam from the light source in the device and the light beam reflected from the tested sample after its exposure to electromagnetic radiation in the near infrared region from 700 to 3000 nm. The light reflected from the sample is converted into units of absorbed light, which quantifies the chemical composition of the meat sample [49].

The method of color differences

According to some researchers, the correlation between the color of meat and water-binding capacity is quite complex. However, Bendall J. R. et.al. [21] believe that to detect PSE meat, it is possible to use the lightness index L, and to determine WHC it is possible to use the reflective coefficient of the sample. According to Swatland et.al. [51], reflective coefficient of the sample correlates with the violet and red spectrum. This correlation can be applied to determine WHC. Joo et.al. [52] make claim that lightness (L) is the best indicator for predicting water-binding capacity.

Conclusion

As the scientific research to determine the WHC, the methods of sample pressing on filter paper and centrifugation are most widely used. However, it must be taken into account that not all methods for the WHC determining are suitable for some particular case and application, as inappropriate method can lead to erroneous conclusions. This means that for assessment of meat capacity to bind and hold water, a special consideration should be given to the selection of a method for each specific case. For instance, it's necessary to have regard to the spatial structure of the sample — whether it is minced meat or undestroyed muscle tissue, it's also necessary to consider the depth of technological processing — whether it is raw meat or cooked meat. The strength properties of the tested sample, the magnitude and duration of the applied load will also affect the obtained results. Nevertheless, the use of any method makes it possible to trace changes in WHC and WBC in the dynamics of some process, for example, autolysis of meat or during its exposure to brine treatment, during mechanical or enzymatic processing.

It should be borne in mind that the presented methods determine different forms of water retention and therefore the results obtained by different methods are not comparable with each other. For example: gravimetric methods make it possible to estimate WBC rather than WHC, while methods of pressing and centrifugation determine the summarized WBC and WHC. To determine the WHC only, apparently, it's necessary to subtract the WBC value obtained by one of the gravimetric methods from the value obtained by one of the methods that use external pressure.

Therefore, for each scientific research it is necessary to choose an appropriate method for determining the capacity of meat to bind or hold water, which is most suitable for the specific goals, objectives and the object under research.

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