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ABOUT THE JOURNAL

The journal "Theory and practice of meat processing" is a peer-reviewed scientific journal covering a wide range of issues: formation of the composition and properties of meat raw materials including various methods for raising animals and poultry; the main questions of meat raw material processing, improvement of technologies for meat product manufacture including functional foods, effects of meat and meat product consumption on human health.

The primary objectives of the journal "Theory and practice of meat processing" are to create content for distribution of interdisciplinary and international knowledge in the world scientific community, promote research performed by scientists from Russia and foreign countries, enhance presentation of their scientific achievements in the international arena and highlight promising research directions in the meat industry.

The main tasks of the journal consist in publishing results of theoretical and experimental studies carried out in Russian and foreign organizations, as well as on the authors' personal initiative; bringing together different categories of researchers, university and scientific professionals; creating and maintaining a common space of scientific communication, bridging the gap between publications at the regional, federal and international levels.

The editors strive to expand the pool of writers and welcome new authors.

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- investigation and development of new technologies of meat production
- processes, equipment and apparatus of meat production
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OPTIMIZATION OF PROTEIN-LIPID COMPLEX BY ITS FATTY ACID AND VITAMIN COMPOSITION

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Abstract
The polycomponent protein-lipid compositions are traditionally used in minced meat products to regulate nutritional value, functional, technological and organoleptic characteristics of the finished product. The present article presents the results of research aimed to creation of antioxidant-enriched protein-lipid complex (PLC) with the optimal ratio ω3: ω6 of PUFA. The ratio of lipid component was optimized by linear programming method, where the recommended ratio of ω-6: ω-3 of PUFA as 10: 1 was used as term of limitation. In result of calculations the fatty component was obtained by blending of rendered beef fat with soybean oil and sunflower oil in the following ratio: rendered beef fat — 73%, sunflower oil — 15%, soybean oil — 12%. After that the PLC formulation was optimized by research of influence of the introduced protein complex in amount from 4% to 10% and the fatty component in amount from 40% to 43% on index of shear stress of the PLC. The introduced amount of protein, fat and water is taken in ratio 1:(4–7):(4–7) commonly used in the meat processing industry to form the functional and technological characteristics of the minced meat. In result of experiments the following PLC formulation was adopted, which provides the necessary stable consistency of the mixture: protein complex — 9%, fatty component — 42%, water — 49%. To enrich meat products with vitamins and antioxidants it is proposed to include into PLC an extract obtained with microwave field 800 W from the Daurian rosehips which grows in the Far East region. The obtained PLC has a high water-retaining capacity; it remains stable while heating and it can be stored for five days without any visible signs of deterioration, as the peroxide value remains within the permissible limits. PLC features optimal ratio of ω-6: ω-3 of PUFA, equal to 10:1, and a high value of the total antioxidants equal to 5.4 mg/g.

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Introduction

The creation of qualitatively new food products is one of the key directions of the state policy in sphere of healthy nutrition.

In the meat processing industry a great attention is paid to the technologies of minced meat products with introduction of polycomponent emulsions, suspensions, pastes, etc. For rational use of animal protein and lipids it is possible to introduce into those compounds by—products, for example, by—products of the II category, mechanically deboned poultry meat, poultry skin, pork skin, pork blood and its formed elements, fat raw materials undesirable for their adding in free form into minced meat in significant amount, for example — beef kidney fat, interior fat, abdominal fat and others.

Scientists of the meat processing industry nowadays offer a wide range of formulations of protein—fat complex prepared on the basis of water, plasma or stabilized blood at various ratios of protein, fat and liquid component. A wide diversity of protein preparations with high functional and technological characteristics is offered as sources of protein.

As components of protein—fat emulsions it is proposed to use chicken skin, which contains up to 25% of low—melting fat, as well as cedar nuts cake, containing up to 30% of vegetable fat [1]. To stabilize the properties of minced meat pasty made of chicken liver and mechanically deboned poultry meat, cedar cake, soy isolate and chicken skin were added in a ratio of 2.5: 1: 2.5. The optimal dose of 20% protein—fat emulsion introduction into the pasty recipe was found. It was established that use of a protein—fat emulsion contributes to enrichment of the product with essential substances, provides high consumer properties, and also solves the problem of rational use of raw materials, in particular chicken skin [1].

In order to partially replace pork fat in emulsified meat products, it is proposed to use functional emulsion gel based on chia plant and olive oil. The functional and technological properties, rheological parameters and texture of the developed emulsion gel have been analyzed. It was established that introduction of a functional emulsion gel contributes to increase in degree of gelation, decrease of moisture release and provides positive effect on the color characteristics of the finished product [2].

The work [3] is devoted to research of the functional and technological properties of beef liver and development of a protein—fat emulsion on its basis. When creating the emulsion, the authors used rendered beef fat, milk powder and soy isolate "Pro—Vo". As a result of analysis of the ob-

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tained data it was found that when the content of fat phase in protein—fat emulsion was equal to 48.3%, after heat treatment of this composition its stratification into fractions was observed.

For the preparation of mixtures with a rational ratio of fatty acids ω-6: ω-3 in two—component (10:1, 5:1) and three—component (5:1) mixtures, sunflower, pumpkin, and linseed oils are used. The combination of mixtures of vegetable oils with a rational ratio of fatty acids ω-6: ω-3 was substantiated. The introduction of developed fortified blended vegetable oils and protein—fat emulsions based on them in amount of 15–20% allows balancing the product in terms of fatty acid and vitamin content [4].

Serdaroğlu, M. et al. obtained results of this development, producing and analysis of a double emulsion with extra virgin olive and linseed oil [5]. It was found that introduction of this emulsion in composition of a meat product allows increasing of total protein content, reducing fat content, which contributes to prolongation of shelf life, and improves technological parameters.

The possibility to obtain protein—fat emulsion on the base of animal protein Capremium 95 and powder from pomace of red—fruited rowanberries is considered [6]. The composition of the emulsion was determined as follows: Capremium 95 — from 10.5% to 11.0%; powder obtained from pomace of red—fruited rowanberries — from 6.5% to 7.0%; fatty component — from 19% to 20%. During the research it was found that introduction of a protein—fat emulsion increases the nutritional value and yield of the finished product without reducing the quality properties of the finished product.

Lukin A. A. proposed a partial replacement of meat raw materials with a protein—fat emulsion in amount of 5–10%. The influence of protein—fat emulsion on physicochemical and organoleptic characteristics of finished sausages was considered; the nutritional value of sausages was calculated. Improving of the functional and technological characteristics of the minced meat system with the emulsion was observed [7].

The research of the amino acid composition and the degree of digestibility of control sample and experimental samples of cooked sausages which were cooked with protein—containing emulsion additives is presented in the article [8]. It was found that application of this protein—emulsion additive allows replacing part of the main raw material without deteriorating the amino acid composition. Also the authors note an improvement in quality characteristics of the finished product.

When introducing the fatty component into a product, attention is paid not only to its fatty acid composition, but also to its biological efficiency which can be measured by one of its criteria — i. e. the ratio of polyunsaturated fatty acids.

The possibility of enriching turkey meat with linseed oil was shown. The introduction of this additive made it possible to provide the desired ratio of omega-6: omega-3 in minced meat systems with the help of vegetable oils, providing their nutrient—adequate balance not more than 10 units [9].

The method of blending vegetable oils into optimized fatty acid composition was studied in [10], the fatty acid composition of vegetable oils and soybean fat emulsion were compared. The developed soy—fat emulsions provide the human body with polyunsaturated fatty acids (PUFA) of the omega-6 and omega-3 families in recommended ratio. The emulsion is recommended as a fatty component in pasties of meat, fish and offal.

The possibility of using oil seeds (sesame seeds, sunflower seeds, pumpkin seeds) for production of minced meat semi—finished products was confirmed [11]. The chemical composition of sesame, sunflower and pumpkin seeds was studied. The optimal ratio of plant and animal components in terms of functional, technological and organoleptic properties was obtained. A recipe for chopped semi—finished products was developed.

For young children nutrition the targeted researches were carried out, and canned meat was developed. The chemical composition and biological value of canned food for children made of quail and chicken meat, olive and linseed oil, chicken liver, quail eggs, buckwheat or rice, mineral calcium fortifier obtained from chicken eggshells, salt and water were calculated [12]. It was found that finished products feature high biological value, possess an optimal ratio of omega-6 to omega-3 of polyunsaturated fatty acids, and are a good source of minerals and vitamins.

The use of banana flour and tapioca as a source of natural antioxidants was suggested by the authors in the article [13] in meatballs production. The optimal doses of banana flour and tapioca flour were defined at 5% each. The authors note the high content of phenols and tannins in the studied samples and recommend production of the product as functional.

The possibility of using phytoextracts (rosemary extract in amount of 0.1%, extract of green tea in amount of 0.05%) in production of frozen meat semi—finished products was studied. For uniform distribution in semi—finished products, the extracts were added during the process of the emulsion preparation. It was found that during the entire storage time of the samples (180 days) the researched semi—finished products featured high organoleptic assessment. The values of acid, peroxide and thiobarbituric value in experimental samples were lower than in the control ones, which confirms the slowing down of lipid oxidation processes [14].

The antioxidant activity of water extracts obtained from various parts of plants of lamellar (labiate) family — mint, garden mint, lemon balm and violet basil was confirmed. The extracts can be used as antioxidants in composition of vegetable oils and fat—containing products like minced meat. It was shown that extracts from violet basil have the highest antioxidant activity, the least was observed in garden mint extracts [15].

Researchers consider the optimization of the fatty component in minced meat systems as one of the important ingredients that possess high physiological activity [16, 17, 18, 19].

When creating healthy food products it is assumed to use raw materials of natural origin, which in case of their systematical application have a positive regulatory effect on certain human systems of organs or their functions, thus improving physical health and quality of life. Among the wide range of food products the share of meat products in population diet is quite high. Meat products are a source of basic substances necessary for life of a human body. However the content of substances with antioxidant properties in meat products is low in comparison with vegetable raw materials.

Therefore, research on creation of functional meat products fortified with plant materials rich in antioxidants is relevant.

Thus the relevant direction of research in sphere of creating healthy food products is the optimization of the composition of protein—lipid complexes with high nutritional value. In this regard the purpose of this study was to create a formulation of a protein—lipid complex with the optimal ratio of ω-6: ω-3 of polyunsaturated fatty acids and introduction of phytonutrients rich in antioxidant activity.

Objects and methods

Blended fatty component from rendered beef fat, soybean oil and vegetable oil, extract from Daurian rosehips, protein—lipid complex, and minced meat were taken as the object of research. To achieve the aims set in the research, the studies were run to create a protein—lipid complex containing, firstly, a fatty component with an optimal ratio of ω-6 and ω-3 polyunsaturated fatty acids, and secondly, a plant component with a high content of biologically active substances.

To ensure the optimal ratio of ω-6: ω-3 polyunsaturated fatty acids, it was envisaged to blend the fatty component based on rendered beef fat, to which oils containing ω-6 and ω-3 polyunsaturated fatty acids, such as soybean oil and sunflower oil, were selected.

Among the plant raw materials, interest was aroused by the fruits of the Daurian rosehip — a wild—growing unpretentious shrub, the fruits of which are rich in antioxidants. The rosehip extract was prepared as follows: the rosehips were carefully sorted by their quality. Then the sorted, full—fledged fruits were thoroughly washed with cold water. Then the fruits were dried at moderate temperatures (no more than 80 °C), bringing down their moisture content to 13–15%. The extract was retrieved with a water—alcohol solution with the help of electromagnetic microwave field with 800 W of capacity.

During the experiment, the following research methods were used. The mass fraction of moisture was determined by the arbitration method, by drying a weighed portion of the sample till its constant weight. Sand was placed in a weighing bottle and dried at a temperature of 103 ± 2 °C for 30 min. Then the weighing bottle with a closed lid was cooled in a desiccator, then weighed on a precision analytical balance. After that the weighed sample (weight was equal to 5 g) was introduced into a weighed weighing bottle with sand with an accuracy of 0.0001 g, 5 cm^3 of ethanol was added and mixed. The bottle was placed in a water bath and heated till disappearing of ethanol odor. Then the test sample was introduced and dried for 2 hours in a drying cabinet ShS-80–01 SPU manufactured by "Smolenskoye SKTB SPU" (Russia) at a temperature of 103 ± 2 °C, then cooled and weighed. The mass fraction of moisture was calculated as difference in the mass of the sample before and after drying. The mass fraction of protein was determined by the Kjeldahl method, based on mineralization of organic matter with subsequent determination of nitrogen content. The sample under consideration was thoroughly crushed and placed on the bottom of a flask with a capacity of 50 cm^3. 1–3 cm^3 of concentrated sulfuric acid, 1 g of a mixture of copper sulfate and potassium sulfate were added. Then the flask was heated until a brown color appeared and cooled down at room temperature. 2–3 cm^3 of hydrogen peroxide solution (mass concentration 30%) was added and heating continued on until colorless mineralizate was obtained. The mineralizate was cooled, poured into a flask with a volume of 250 cm^3, diluted to the mark with distilled water, and stirred. In a volumetric flask (with capacity of 100 cm^3), 5 cm^3 of the obtained mineralizate was diluted to the mark with distilled water. In order to execute the staining reaction, 1 cm^3 of the re—diluted mineralizate was introduced into a test tube, 5 cm^3 of reagent 1 was added (10 g of phenol and 0.05 g of sodium nitroprusside were dissolved in distilled water in a volumetric flask with capacity of 1.000 cm^3 and the volume of the solution was diluted up the mark with distilled water) and 5 cm^3 of reagent # 2 (5 g of sodium hydroxide was dissolved in distilled water in a volumetric flask with capacity of 1.000 cm^3. After cooling the initial solution of sodium hypochlorite was added to achieve its mass concentration of 0.42 g / dm^3 or 0.2 g of sodium dichloroisocyanurate. The volume of the solution was diluted with distilled water up to the mark) and stirred (the control solution was being prepared in parallel). In 30 minutes the optical density of the solutions was determined using a photometer KFK-3–01–30MZ. Main technical characteristics of the device: wavelength range 304.6–990 nm; the emitted spectral interval — no more than 5 nm; range of readings: transmittance — 0.1–100%, optical density — 0–3 B, concentration, concentration units — 0.001–9999; the limit of the permissible value in basic absolute error when measuring spectral ratio of directed transmission is 0.5%; working length of cuvettes — 1.3.5.10.20.30.50.100 mm. Measurements were taken in reference to the control solution.

The mass fraction of fat was determined with the help of Soxhlet extraction device by repeated extraction of fat

with a solvent from a dried test sample. The test sample weighing 3.0 ± 0.0002 g after drying was transferred into a filter paper tube (pre—weighed) and placed in the Soxhlet extractor. The approximate duration of the extraction was 5–6 hours with solvent draining frequency 5–6 times per 1 hour. The quality and completeness of degreasing was checked by drop of the solvent to filter paper. The process was considered complete in absence of a grease stain on the filtering paper after evaporation of the solvent. At the end of extraction, the tube was removed from the extractor, dried and weighed. The amount of fat was determined by the difference between the mass of the tube before and after fat extraction.

The water and fat—retaining capacity was determined from one sample by sequential determination of moisture and fat content separated after stirring the sample for 1 min in a homogenizer, then leaving to rest for 5 minutes, then again stirring for 1 min and leaving for 5 minutes. After that the analyzed protein—fat system was placed in centrifuge tubes and was centrifuged for 10 min at a speed of 3000 rpm. The released moisture with fat was withdrawn and the content of the components was determined. The difference in moisture and fat content in the sample and in the released supernatant liquid was used to determine the water and fat retention capacity. The stability of the protein—lipid complex after heat treatment was determined as follows: a test sample was placed in test tubes, closed and heated up in water bath at 85 °C for 30 minutes, then the samples were immediately placed into refrigerator, kept there for 24 hours at 4 °C, and at this point the amount of released moisture was determined. The ultimate shear stress (ULS) was determined by a penetrometer by immersing of an indenter into the researched sample. The principle of this method is based on determining the depth of an indenter immersion of a certain mass into the analyzed sample. The container for the product was filled with the test sample, the surface was leveled with a spatula or knife, setting its level relative to the zero division of the instrument scale. The scale determined the depth of immersion of the indenter cone into the product.

The total content of antioxidants was determined by the amperometric method with the help of device "Tsvet Yauza-01—AA". This device is designed to determine the total content of antioxidants in food, beverages, dietary supplements and medicinal products, as well as in technical products (the device is manufactured by NPO "Khimavtomatika", Russia).

The specific gravity of fat was found by calculating the ratio of fat weight (at temperature of 18 °C) to weight of the same volume of water at this temperature. The melting point of the fat mixture was determined by melting of the hardened fat mixture in capillary with recording of the melting temperature. The pour point of the fat mixture was found from the average pour point (temperature of hardening). The optical density of Daurian rosehip fruit extract was determined using a photoelectric photometer KFK-3–01–30MZ. The peroxide value was determined by a method of measuring oxidation of hydroiodic acid with peroxides, followed by titration of released iodine. In a conical flask with a volume of 200 ml a weighed portion of a test sample of 1 g was added, 10 ml of chloroform, 10 ml of glacial acetic acid and 0.6 ml of potassium iodide were added as well. The content of the flask was stirred with rotary movements and left for 3 minutes to sit in a dark place. Then 100 ml of distilled water was poured in, stirred and 1 ml of 1% starch solution was added. Titration was run with 0.01 N sodium thiosulfate solution until the blue color disappeared. The experiment on control sample was carried out in parallel.

Results and discussion

When planning the composition of the protein—lipid complex (PLC) for finely ground meat products, studies were aimed to optimize the composition of PLC in terms of fatty component biological efficiency. The high nutritional and energy value of finished meat products does not prove the high degree and efficiency of their digestion. The biological efficiency of the fatty component, or the ratio of polyunsaturated fatty acids, especially ω-6, ω-3 PUFA play special role in formation of nutritional value of food products.

It is known that animal fats are depleted in PUFAs. Fish oils and vegetable oils are the richest in PUFAs.

In the research the experimental studies were run to increase the efficiency of the fatty component of PLC to ensure the optimal ratio of ω-6: ω-3 polyunsaturated fatty acids. This ratio is recommended for healthy people 1:10, in food products recommended for therapeutic and prophylactic nutrition 1: (2–5).

To achieve a balance of fatty acids the raw beef fat, which has a dense, solid consistency due to its fatty acid composition, is blended with deodorized vegetable oils of low cost and nutritional value.

The disadvantage of natural beef fat is a quite high pour point of 37–41 °C; to reduce this point the sample was heated at a temperature of 85–95 °C, which ensures maximum and rapid defatting of raw materials.

After rendering the pour point of beef fat dropped significantly — by ten degrees almost, and it amounted to 27–31 °C, although it was still quite high. For further reduction of the point vegetable oils were introduced into the blend, as those vegetable oils have a low pour point — from minus 15 °C to minus 19 °C and possess high nutritional value.

To design a protein—lipid complex containing a given ratio of ω-6: ω-3 of polyunsaturated fatty acids equal to 10: 1, first we studied their content in rendered beef fat and some vegetable oils. Analysis of literature data allowed selecting two types of vegetable oils for their blending to beef fat — sunflower and soybean oils, which contain a sufficient amount of ω-6 acids around 5–10 times more than ω-3 acids (Table 1).

Table 1. Composition of fatty components

Fatty acids*	Rendered beef fat, x	Refined sunflower oil, y	Soybean oil, z
Fatty acids (sum)	94.70	95.0	94.90
Saturated:	50.90	11.30	13.90
Capronic	—	—	—
Caprylic	—	—	—
Capric	0.10	—	—
Lauric	0.60	—	—
Myristic	3.40	—	Traces
Pentadecane	0.70	—	—
Palmitic	24.70	6.20	10.30
Margarine	1.40	—	—
Stearic	20.0	4.10	3.50
Arachinic	—	0.30	—
Behenic	—	0.70	Traces
Lignoceric	—	—	—
Monounsaturated:	40.60	23.80	19.80
Myristoleic	1.10	—	—
Palmitoleic	3.0	Traces	—
Oleinovaya	36.50	23.70	19.80
Gadoleic	—	Traces	—
Erukovaya	—	0	—
Polyunsaturated:	3.20	59.80	61.20
Linoleic acid 6	2.50	59.80	50.90
Linolenic 3	0.60	—	10.30
Arachidonic 6	0.10	—	—
Eicosadienoic 3	—	—	—
Docosodienic 6	—	—	—

The content of polyunsaturated fatty acids of the ω-6 and ω-3 groups in the selected fatty components is presented below in Table 2.

Table 2. Content of PUFA groups of ω-6 and ω-3 in fatty components

Fatty acids	Beef fat, x	Sunflower oil, y	Soybean oil, z
PUFA ω-6	2.60	59.80	50.90
Linoleic	2.50	59.80	50.9
Arachidonic	0.10		
PUFA ω-3	0.60	0	10.30
Docosodienic	—	0	0
Linolenic	0.60	0	10.30

Based on content of these fatty acids in the test samples and reference ratio of PUFA of the ω-6 group to PUFA of the ω-3 group as 10: 1, an equation was drawn up where we assume that the mixture contains x kg of beef fat, y kg of sunflower oil, and z kg of soybean oil. In this case the equation haw the following form:

$$\frac{2{,}6x + 59{,}8y + 50{,}9z}{0{,}6x + 10{,}3z} = \frac{10}{1},$$

As a result of simplifying this expression we obtain the following equation

$$x = 17{,}59y - 15{,}32z$$

To solve this equation we admit an arbitrary choice of vegetable oils. In this case we chose: the amount of sunflower oil — from 20 to 25 kg, soybean oil — from 15 to 20 kg, and by solving the equation the amount of beef fat was calculated (Table 3).

Table 3 shows the results of calculations of obtained variants of the blended fat mixture. To select the most optimal option the indicator of the average pour point and the average melting point of the obtained fat mixtures were investigated (Figure 1).

Table 3. Variants of recipes for fat mixture

# of blend	Beef fat, x		Sunflower oil, y		Soybean oil, z		Total, %
	kg	%	kg	%	kg	%	%
1	45.4	53.1615925	20	23.4192037	20	23.4192037	100
2	78.31	66.1905164	21	17.7499789	19	16.0595047	100
3	111.22	73.5484724	22	14.5483402	18	11.9031874	100
4	144.13	78.2762179	23	12.4911747	17	9.2326074	100
5	177.04	81.5702175	24	11.0578695	16	7.37191301	100
6	209.95	83.9967994	25	10.0020004	15	6.00120024	100

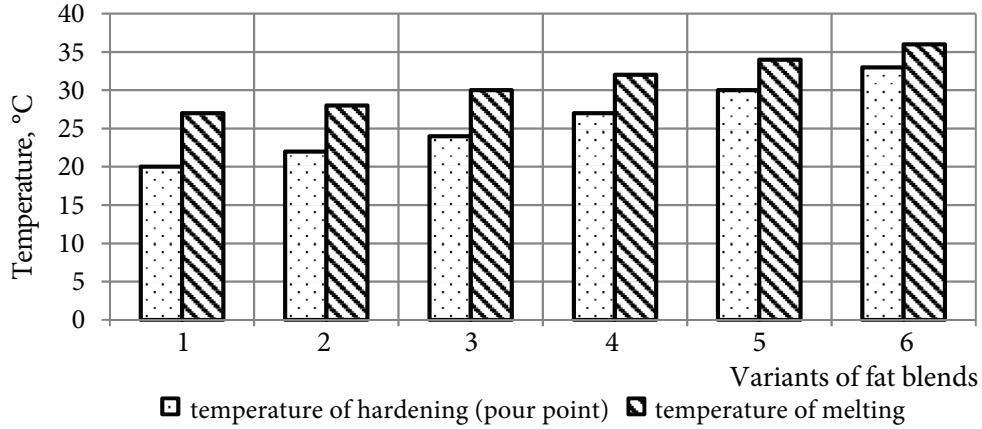

Figure 1. Average values of melting and pour points for fat mixtures

The data in Figure 1 indicate that the average values of the pouring and melting temperatures of the obtained variants of fat mixtures vary due to different ratios of fatty components, including hard—melting beef fat, albeit it was in melted state. From the data obtained it is obvious that in options 4–6 the considered values increase, that's why option 3 was chosen which provides pour point is about 25 °C — this is favorable from the point of view of organoleptic properties. The optimal composition of fat mixture is as follows: rendered beef fat — 73%, sunflower oil — 15%, soybean oil — 12%.

The physicochemical parameters of obtained fat mixture were determined, the results are presented below in Table 4.

Table 4. Physical and chemical parameters of the fat mixture

Specific gravity at 18 °C, kg/m³	Melting point, °C	Pour point, °C	Ratio ω-6: ω-3
934.2	30–33	21–24	10:1

In order to create a protein—lipid complex with high functional and technological properties, the additive Skinprot A105 based on pork skin was chosen as a protein component. This choice is explained by results of tests of the proposed preparations. The proposed preparation, which is a finely ground cream—colored powder, remains stable after heat treatment, and it is recommended for use in minced meat and fish products. The formulation of the protein—lipid complex was improved by studying the influence of amount of the introduced protein preparation which varies from 4% to 10%, and introduction of the fatty component within limits from 40% to 43%, on ultimate shear stress value of PLC. The introduced protein preparation was limited in its amount according to the authors' information [20] that the critical concentration necessary to create gel—like structure in protein preparations varies within 5.5% to 9%. The amount of the added fatty component was taken based on the ratio of the protein preparation: fat: water as 1:(4–7):(4–7) commonly accepted in the meat industry to form the necessary functional and technological properties of minced meat products. The data obtained are presented below in Figure 2.

The data presented above in Figure 2 show that increase of injected protein preparation amount up to 9% promotes an increase in ultimate shear stress value, beyond certain point the rate of increase in ultimate shear stress value decreases.

As a result of the analysis of obtained data, the formulation of the protein—lipid complex was obtained, which provides the necessary stable structure and consistency of the mixture: protein preparation = 9%, fatty component = 42%, water for hydration = 49%. Vegetable raw materials are used to enrich meat products with essential micronutrients. We proposed to include into PLC composition a plant component — this is extract from the fruit of Daurian rosehip, which grows in the regions of Transbaikal and in the Far East region. The results of earlier studies of the vitamin composition of the Daurian rosehips showed that vitamin C in them reaches 1250 mg%, β— carotene — 6.71 mg%, vitamin E — 170.25 mg%, flavonoids — 6.8–15.6%, organic acids — 2.0–2.4% [21, 22, 23].

To increase the efficiency of the extraction process of biologically active substances of rosehips, microwave field with a power of 800 watts was used. It is known that the electromagnetic microwave field enhances the rate of substances extraction. To determine the efficiency of the extraction process, the values of the optical density of the control sample (not processed with the microwave field) and experimental sample (processed with the microwave

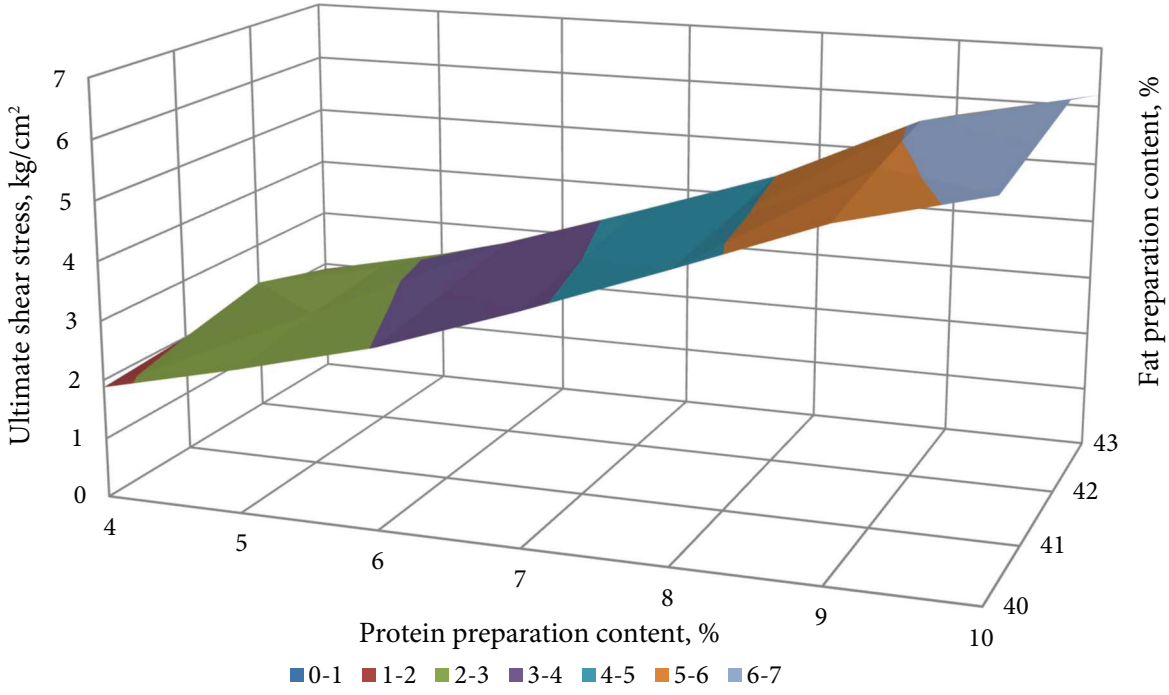

Figure 2. Optimization of the formulation of the protein—lipid complex

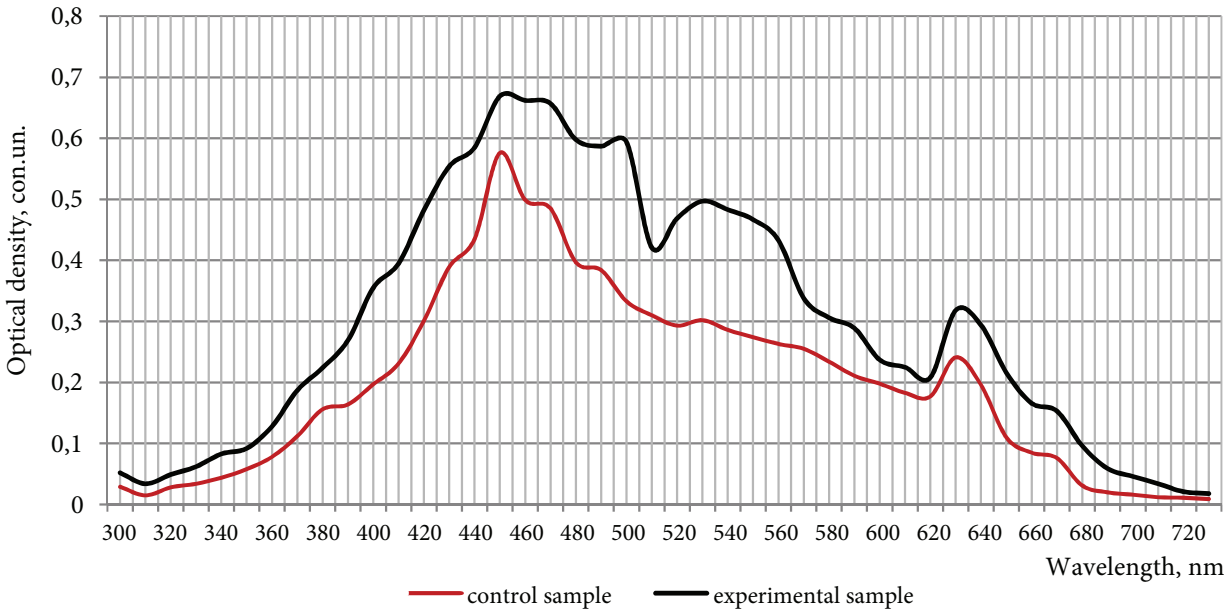

Figure 3. Optical density of rosehip extracts

field) water—alcohol extracts of the Daurian rosehips were compared (Figure 3). A water—alcohol solution is adopted as an extractant agent based on its high extracting capacity, its availability and low cost. The extraction modes were determined experimentally, the ratio of the extractant and the extracted object was chosen as 5:1 based on the analysis of the accepted recommendations for production of plant extracts.

The data in Figure 3 show the occurrence of peak optical density within the wavelength range of 440–500 nm peculiar for anthocyanins, which possess high antioxidant properties. A higher value of optical density was noted in the experimental sample, which was obtained using a microwave field, in this period compared to the control sample, which confirms the higher degree of extraction of biologically active substances.

Analysis of the organoleptic characteristics of the infusion showed that the solution is transparent, red—brown in color, has a slightly tart taste due to tannins, but has no bitterness. A high value of summary antioxidants in the extract was noted, it was equal to 131.8 mg / g. At the next stage the experiment was run to optimize the dose of the rosehip extract added. It was recommended to introduce from 4% to 8% of extract at the expense of share of water, added at the stage of preparation and thorough stirring of the mixture components. The resulting mixture was kept for 3–4 hours and the ultimate shear stress was measured (Figure 4).

Analysis of the data in Figure 4 showed that in case of adding 4% of extract, the value of the ultimate shear stress (USS) was 6.22 kg/cm^2. When the applied dose of the extract increased to 5%, the USS value remained at the same level (6.24 kg/cm^2). Further increase of dose to 6% leaded to a decrease in the USS value to 6.15 kg / cm^2, and then a more intensive decrease was observed at dose of 7% — to 5.84 kg/cm^2, at 8% — to 5.42 kg/cm^2. The decrease of USS value of the protein—lipid complex along with an increase in the content of rosehip extract was possibly related to the acidic medium of the extract, which could affect the formation of the spatial structure of PLC.

Further the sample was analyzed for its organoleptic properties with the help of external examination of the PLC with added rosehip extract. It was found that the introduction of the extract in amount of more than 6% causes a color change to purple and, with a further increase, to violet. Due to this regularity a dose of 6% of the rosehip extract was taken into the PLC composition. The enriched PLC recipe was adopted in the following ratio of components: Skinprot A105 additive 9%, Daurian rosehip extract 6%, lipid mixture 42%, water 43%.

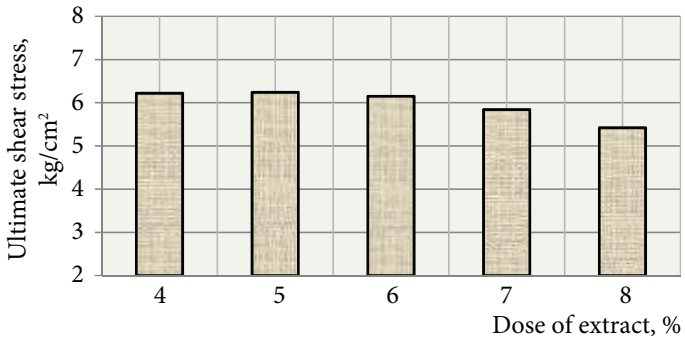

Figure 4. Influence of the dose of extract added into the PLC composition on the ultimate shear stress value

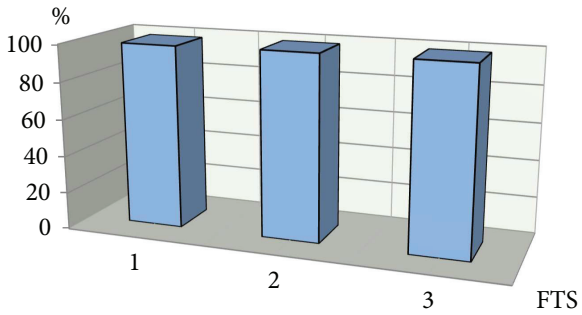

Figure 5. Functional and technological characteristics PLC
1 — water—retaining capacity; 2 — fat—retaining capacity;
3 — stability of system

The functional and technological characteristics of PLC were studied, which showed the absence of moisture and fat losses in the samples under consideration after heat treatment and after additional exposure (test for stability) (Figure 5).

High value of water— and fat—holding capacity of the protein—lipid complex was noted. One hundred percent stability of the complex was found, which was defined in the following way — the protein—lipid complex was placed in test tubes, closed and heated in a water bath to 85 °C for 30 minutes, then the samples were immediately placed in a refrigerator, kept there for 24 hours at 4 °C, and its water—holding capacity was reviewed.

An important characteristic of a food product is the preservation of its quality during storage, which can be determined organoleptically as well as chemically — the preservation is tested by peroxide value, which shows the degree of development of oxidative processes of lipids in the product. We studied the storability of PLC without added extract — control sample, and studied the one with the rosehip extract — experimental sample. The change of peroxide value of the protein—lipid complex during five days of storage at a temperature of (2–4) °C was studied (Figure 6).

The obtained data showed that in the studied samples at the initial point the peroxide value was 0.02 mmol O_2/kg. While in the samples kept for 24 hours a difference between the control sample and experimental sample is noticeable, and from 48 hours a sharp increase in the peroxide value can be noted in the control sample, i. e. up to 1.31 mmol O_2/kg, which proves the continuity of lipid peroxidation in fat—containing system and the further dynamics of a sharp increase of studied parameter is maintained throughout the entire 120 hours of the experiment. If we consider the experimental sample then it can be noted that the curve of values is stable, the increase in the value occurs rather slowly on the 96th day. For instance the peroxide value is only 0.72 mmol O_2/kg. The rosehip extract rich in antioxidants such as vitamin C, anthocyanins, flavonoids, carotene and others, contributes to interruption of the chain of chemical oxidative reactions of lipid fatty acids in the experimental sample.

On the basis of the experiments, a technological scheme for the production of PLC was developed, which includes the hydration of the protein preparation with water, then introduction of Daurian rosehips extract, and at the end of the process the lipid mixture in parts are added with thorough stirring. PLC looks like a well—emulsified pink disperse system. PLC with Daurian rosehip extract can be used in minced meat products, for example, minced semi—finished products.

According to the developed scheme the PLC was developed with extract of Daurian rosehip and the qualitative values were studied, which are presented in Table 5.

Table 5. Composition and properties of enriched PLC

Parameter	Value	Parameter	Value
Contents		Antioxidants, total, mg/g	5.4
Moisture, %	43.27 ± 0.6	Ratio ω-6: ω-3	10:1
Protein,%	8.19 ± 0.2	Stability in heat processing, %	100.0
Fat,%	42.98 ± 0.7	Peroxide value in 120 hours, mmol O/kg of fat	0.94 ± 0.1
Vitamin C, g%	102.3 ± 0.3	Vitamin E, mg%	18.3 ± 0.2

As shown by the results of studies of qualitative indicators (Table 5), PLC has high hydrophilic properties, is stable when heated, can be stored for five days without visible signs of deterioration, and the peroxide value remains within the permissible limits. PLC features optimal ratio of ω-6: ω-3 of PUFA equal to 10:1, availability of vitamins C and E, and high value of the total content of antioxidants equal to 5.4 mg/g.

Conclusion

In result of the experiment the formulation of antioxidant—enriched protein—lipid complex with optimal ω3:

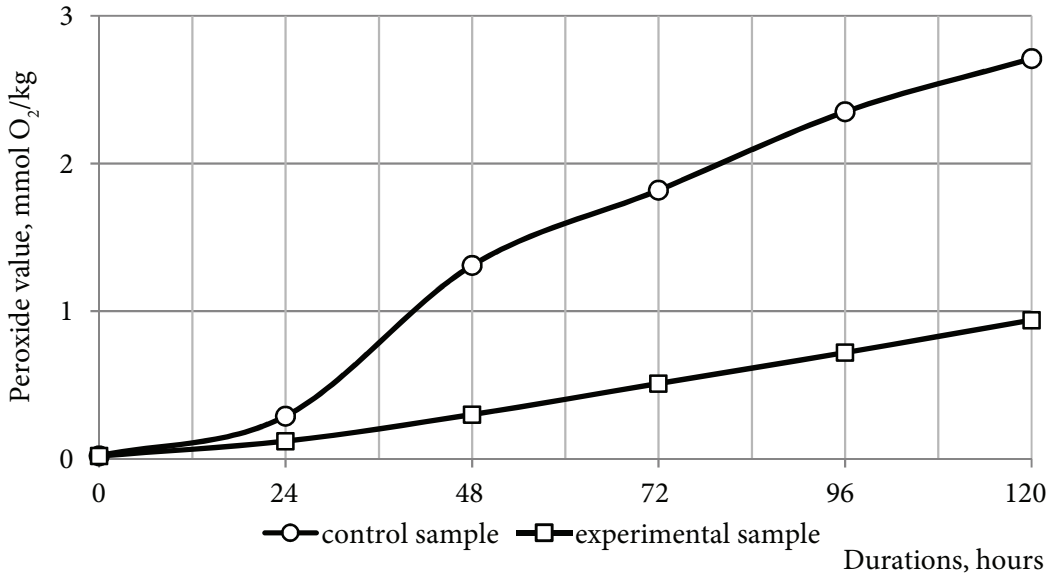

Figure 6. Peroxide values in PLC during storage

ω6 ratio of polyunsaturated fatty acids was optimized. The fatty component was prepared by blending of rendered beef fat with soybean and sunflower oils in the ratio: rendered beef fat 73%, sunflower oil — 15%, soybean oil — 12%. To enrich the protein—lipid complex with vegetative antioxidants the water—alcoholic extract of rosehips is introduced into the composition. The rosehip extract was obtained using a microwave field. The formulation of the protein—lipid complex was adopted, which provides the necessary stable consistency of the mixture: protein preparation — 9%, fatty component — 42%, water for hydration — 49%. Research results have shown that PLC possesses high water—retaining capacity, it is stable when heated, it can be stored for five days without visible signs of deterioration, since the peroxide value remains within the permissible limits. The protein—lipid complex features the optimal ratio of ω-6: ω-3 PUFA equal to 10: 1, availability of vitamins E and C and high value of total antioxidants equal to 5.4 mg/g.

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METHODICAL APPROACH FOR DETERMINATION OF THE HETEROCYCLIC AROMATIC AMINES IN MEAT PRODUCTS USING HPLC–MS/MS

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Keywords: *food safety, carcinogens, PhIP, MeIQx, chromatography*

Abstract
Heterocyclic aromatic amines (HAA) are formed in foods of animal origin during the Maillard reaction due to the high creatine and creatinine contents. HAA have carcinogenic and mutagenic effects. HAA content is not standardized in the Russian Federation and the Customs Union territory. However, in the EU countries, comprehensive monitoring studies are carried out on the HAA contents and effect on the human body. Due to constant expansion of the list of controlled contaminants in food products, analytical laboratories need to develop methods for determining HAA in food items. As a result of the research, a method for HAA determination was developed using high-performance liquid chromatography with mass spectrometry in the mode of specified reaction monitoring. Comparative tests of the two methods for sample preparation were carried out. The advantages and disadvantages of sample preparation approaches were substantiated. The existing SPE conditions were optimized, which made it possible to concentrate trace amounts of MeIQx and PhIP and to dispose of substances suppressing analyte ionization. The estimation of method accuracy and specificity was carried out. The degree of ionization suppression by the matrix for MeIQx and PhIP analytes was determined. The degree of HAA extraction was empirically established. For biological samples of animal origin, it was up to 90.9% for MeIQx and up to 89.4% for PhIP. It is shown that, in accordance with the developed methodology, HAA may be determined with an accuracy of 96.15 to 98.4% at the levels of 5 to 20 ng/g. The limit of quantification of the target substances was 3 ng/g.

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Introduction

Heat treatment of food products leads to the formation and/or accumulation of new compounds. Often, such compounds are not useful and, moreover, may also harm human health due to their mutagenic and carcinogenic properties. These compounds include heterocyclic aromatic amines (HAA), which are formed in foods as a result of high-temperature treatment. Heterocyclic aromatic amines are a group of compounds with at least one aromatic ring and one amino group in their structure. HAA are formed mainly during high-temperature treatment (for example, frying, grilling, etc.) of animal products due to their relatively high contents of creatine/creatinine, which are the basis for HAAs [1]. According to [2], the HAA formation reaction proceeds by cleavage of water from creatine and its further cyclization to creatinine, which forms the amino-imidazole part of the HAA molecule being its basis. Then pyrazines and pyridines formed from unbound amino acids and hexoses during the Maillard reaction complete the HAA molecule formation. The effect of unbound amino acids on the formation of amino-imidazoarene HAAs was studied by Meurillon, M. et al. [3]. An important participant in the HAA molecule formation is the Strecker aldehyde or the corresponding Schiff base (Figure 1).

A number of studies carried out on laboratory animals and protozoans have found that HAA have carcinogenic and mutagenic effects. Using the Ames test in studies on Salmonella, it was found that HAA have the highest mutagenic and carcinogenic activity compared to other mutagens and carcinogens consumed by humans with food. However, with a normal diet, a person consumes "insufficient" amount of HAA with food for the formation of malignant tumors. In addition, the consumption of HAA with other types of carcinogens and mutagens leads to additional synergistic effects due to their ability to cause gene instability and increase sensitivity to tumor promoters [4]. HAA genotoxicity was proven by the authors in [5]. The carcinogenic potential of HAA may be enhanced in the presence of tumor promoters and agents that enhance cell proliferation. This was published in [6]: according to the results, skin tumors on the back of mice coated with Trp-P-2 HAA began to develop only after the subsequent application of TPA (12-O-tetradecanoylphorbol-13-acetate), a promoter of skin carcinogenesis. HAA may form new mutagenic compounds or enhance existing mutagenic pro-

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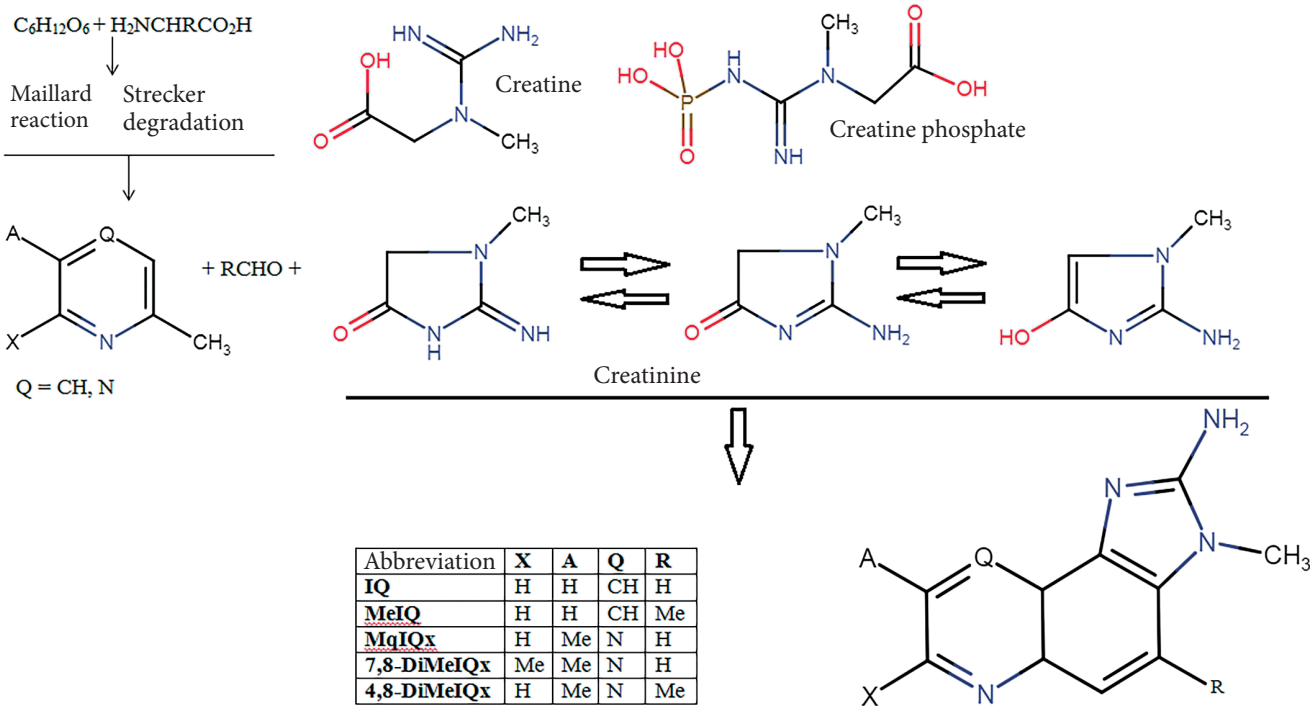

Abbreviation	X	A	Q	R
IQ	H	H	CH	H
MeIQ	H	H	CH	Me
MqIQx	H	Me	N	H
7,8-DiMeIQx	Me	Me	N	H
4,8-DiMeIQx	H	Me	N	Me

Figure 1. HAA formation mechanism

perties. For example, non-mutagenic aniline and norharman form aminophenyl norharman, which, upon further activation with a mixture of S9 enzymes to hydroxyaminophenyl norharman and its final transformation into acetoxy derivatives, leads to the formation of DNA adducts (Figure 2) causing mutations. In experiments on rodents, the formation of aminophenyl norharman from aniline and norharman was established *in vivo*; and it also had carcinogenic properties along with mutagenic ones [7]. The works [8–10] show that consumption of products with high HAA contents may cause colorectal cancer.

The carcinogenic and mutagenic effect of HAA shown in [5–10] requires minimization of the HAA amount in meat products and conducting the corresponding studies. As noted above, the temperature and duration of heat treatment play an important role in the reaction of HAA formation. An increase in temperature and duration proportionally increases the amount of HAA [11], therefore, the most effective way to reduce them is to influence precisely these parameters. But this will have a strong effect on product sensory properties. In this regard, a large number of studies on the ways to reduce the HAA amount are based

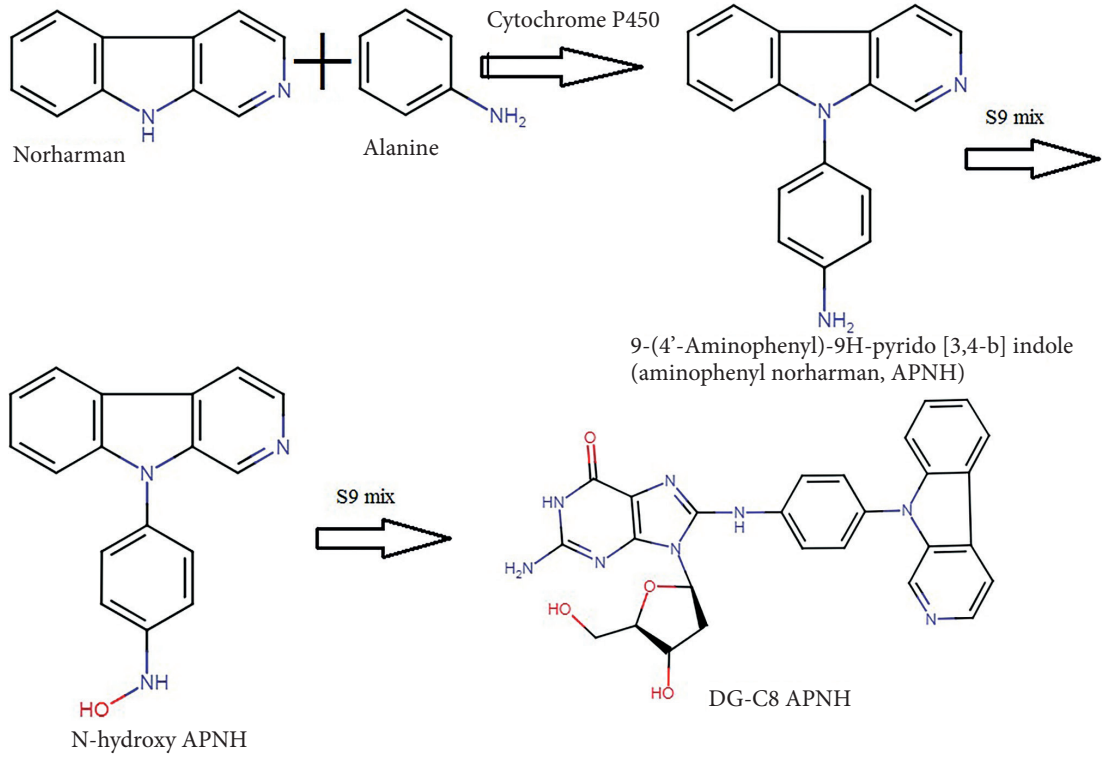

Figure 2. The reaction of aminophenyl norharman formation with the subsequent formation of DNA adducts from it

on changes in product formulations, in particular, introduction of the components that have an inhibitory effect in the HAA formation reaction. Sugars are inhibitors in the HAA formation reaction. During the Maillard reaction, 5-hydroxymethyl-2-furfural is formed from sugars, which simultaneously reduces the amount of HAA formed, as it reacts with creatin/creatinine [12]. Honey, a rich source of glucose and fructose, is the most effective among low molecular weight carbohydrate sources in reducing the amount of HAA formed [13]. Antioxidants have a strong inhibitory effect on the HAA formation reaction [14]. The effect of introducing rosemary and grape seed extracts into a meat product was investigated. The greatest reduction in the MeIQx and PhIP amounts was achieved when 0.8% grape seed extract in the form of a water-in-oil emulsion and 1.5% solution of rosemary extract in sunflower oil were added to the formulation. The effect of adding pomegranate seed extract was also investigated [15]. According to the results, most of the samples showed an increase in HAAs, but it is worth noting that there were many more factors in this work that may affect the amount of HAA, since the product contained many more components in addition to meat and a potential inhibitor. The results of a study on the hawthorn extract inhibitory potential in the reaction of HAA formation were published [16]. These results were rather contradictory, as both an increase and a decrease in HAAs formed were observed in the test samples. In [17], the inhibitory effect of artichoke extract was investigated. According to the results, artichoke extract has a strong inhibitory effect on the HAA formation reaction.

The role of fat in the HAA formation reaction should be noted. Less fat in the raw material results in more HAA formed [18]. This may be due to the fact that fat, being a good heat carrier, leads to faster cooking, which reduces the HAA contents in the finished product. In addition, replacing animal fat with vegetable oils may reduce the amount of HAAs formed by up to 100%. Gunter, F. et al. [19] studied the effect of replacing animal fat with vegetable oils. They made patties from lean pork with the addition of pork fat (60% of the total fat content), sunflower oil, olive oil or pomegranate oil (40% of the total fat content). The results showed that replacing 40% of animal fat in a meat product with vegetable oil led to a significant decrease in the amount of HAAs formed during heat treatment. The decrease in total HAA content in the products ranged from 83% to 100% (the largest decrease was found when adding pomegranate oil) [19]. Like various vegetable oils, the type of animal fat also affects the amount of HAAs. The addition of broadtail fat to the meat product leads to more HAA formation than the addition of beef fat [20].

But prior to studies on reducing the amount of HAAs, it is necessary to develop a methodology for their determination. The development of methodological approaches includes two main stages: sample preparation and setting the conditions for chromatographic analysis. Currently, the majority of HAA quantification studies are based on solid phase extraction (SPE) of the analytes to be determined followed by analysis on an HPLC–MS/MS system. The SPE method of sample preparation was proposed in 1992 and is based on alkaline hydrolysis of the sample followed by extraction of analytes using Oasis MCX SPE cartridges. The HPLC–MS/MS method is preferred because of its high selectivity, which makes it most suitable for the quantitative determination of HAAs. But it is worth noting that HAAs can also be determined by other chromatographic methods, such as detection in the ultraviolet spectrum or the use of an ion trap (Orbitrap) [21,22]. This certainly makes the analysis even more accurate, but the ion trap technology combined with mass spectrometry is relatively new and the equipment required is not so widespread as the HPLC–MS/MS systems. It is important to note that there are other ways to prepare samples. For example, the method of magnetic SPE with iron oxide (II, III) nanoparticles [23]. HAA extraction is possible using QuEChERS cartridges, but they have been tested only on baked foods and may not work with meat matrices [24].

Based on the above, the purpose of this work was to substantiate the conditions for the chromatographic determination of HAA in meat products using HPLC–MS/MS and to compare the slightly modified method of sample preparation with solid-phase extraction (SPE) used in foreign laboratories and the method of sample preparation with liquid extraction by an organic solvent developed by the authors of the work.

Materials and methods

Among more than 30 currently known heterocyclic aromatic amines formed in meat products during heat treatment, 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) were selected, since they account for 80–90% of the total amount of HAAs. Thus, they may be considered markers of HAA presence in meat products. In this connection, when setting up the method for determining HAAs in meat products, we used:

— standard sample of 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (MeIQx) manufactured by Toronto Research Chemicals (Canada) with a basic substance content of at least 99.0%;
— standard sample of 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) manufactured by ChemCruz (USA) with a basic substance content of at least 95.0%.

When selecting the conditions for chromatographic identification, the following reagents were used: acetonitrile for HPLC manufactured by Panreac (France), formic acid by Merck (USA), deionized water obtained on MilliQDirect 8 system (France).

Standard samples were dissolved in methanol with subsequent dilution to achieve concentrations of 1000, 100, 10, and 1 ng/cm^3.

The analysis was performed on Agilent 1200 high performance liquid chromatography system (USA) with Agilent 6410B three-quadrupole mass spectrometer.

To determine HAA, C18 chromatographic column, 4.6 × 50 mm, 1.8 μm (Agilent, USA) was used.

Results and discussion

Chromatographic separation of the analytes to be determined was carried out in the gradient elution mode (two-component mobile phase);

injected sample volume is 0.01 cm^3;

mobile phase flow rate is 0.4 cm^3/min;

column thermostat temperature is 35 °C.

Despite the fact that aromatic compounds are not fragmented, due to the presence of a methyl radical in the substances selected in the work, their fragmentation is possible under certain conditions with the formation of a daughter ion. This makes it possible to use MRM technology in the determination of HAAs. So, the possibility of false positive or false negative identification of the analytes is minimized. The developed conditions for the detection of analytical signals in the MRM mode are presented in Table 1.

Table 1. Ion exposure parameters in MRM mode and electric spray ionization (ESI) conditions with positive (+) and negative (–) ion registration

Analyte	Molecular ion, m/z	Daughter ions, m/z	Fragmentor voltage (Frag), V	Cleavage energy (CE), V
MeIQx	214.6 (+)	199.5	130	30
PhIP	225.6 (+)	210.5	130	30

For the chromatographic separation of substances, the two-component mobile phase was used:

— eluent A is 0.1% formic acid solution in acetonitrile;

— eluent B is 0.1% formic acid solution in deionized water.

Gradient elution conditions are shown in Table 2.

Table 2. Gradient elution conditions

Time, min	A, % об.	B, % об.	Flow rate, μL/min
0	10	90	400
3	40	60	400
4	60	40	400
6	90	10	400
8	90	10	400
8.1	10	90	400
12	10	90	400

The selected conditions and the analyte release time shown in Figure 3 (illustrating the chromatogram of the total ion current superposition (red) on the MRM of the PhIP daughter ions (green, release time ≈5.9 min) and the MeIQx daughter ions (blue, release time ≈4.5 min)) indicate that the MeIQx substance is washed out from the chromatographic column at the A: B eluent ratio of 60:40%, and the PhIP substance is washed out from the chromatographic column at the A: B eluent ratio of 90:10%.

The described chromatography conditions made it possible to detect HAAs in concentrations as low as 1 ng/cm^3, which is the optimal lower limit of detection for HAAs in meat products. Figure 4 shows chromatograms of HAA standard mixture at a level of 1 ng/mL with chromatograms of their daughter ions.

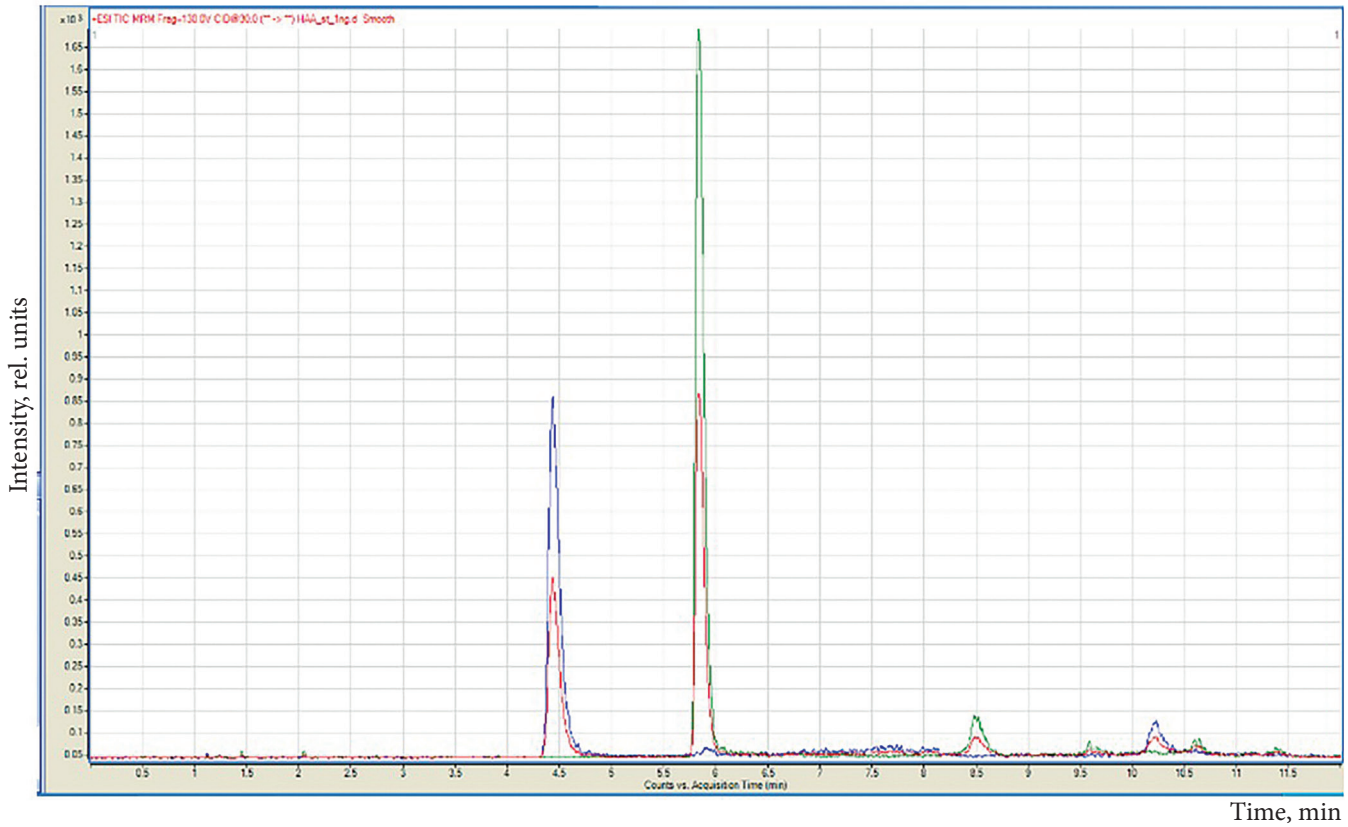

Figure 3. Chromatogram of the total ion current superposition on the PhIP daughter ions and the MeIQx daughter ions

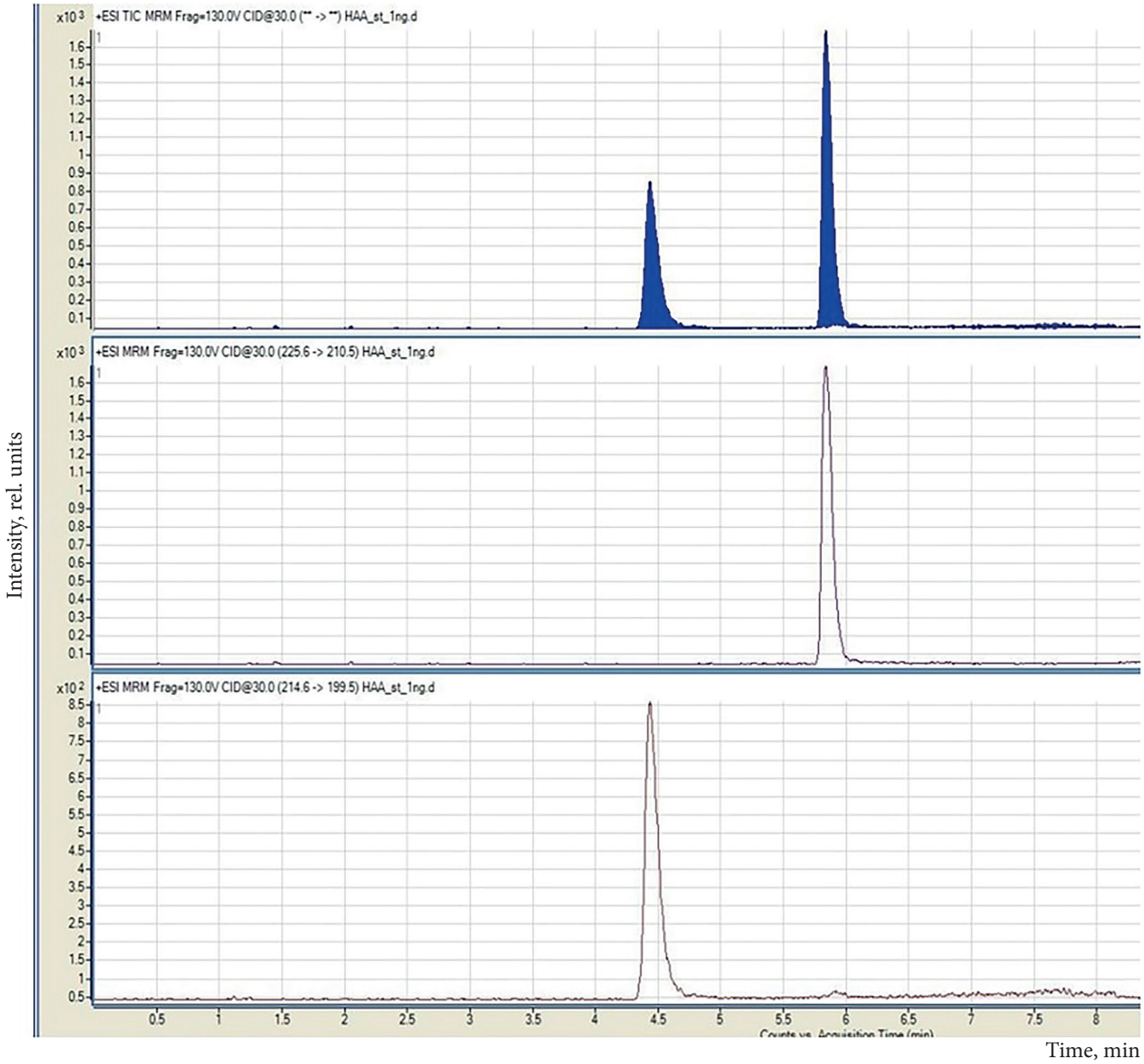

Figure 4. Chromatogram of HAA standard sample solution. Release time of the MeIQx analyte is 4.5 minutes; release time of the PhIP analyte is 6 minutes

Sample preparation

In foreign practice, preparation of meat product samples to determine the HAA content in it is carried out by the alkaline hydrolysis with SPE, for example [25,26]. In our studies, this method was optimized taking into account the available equipment and reagents. The method for determining HAAs include preliminary alkaline hydrolysis of the sample followed by the solid-phase extraction (SPE) and identification using HPLC–MS/MS.

A sample of the analyzed product (3.00 ± 0.01 g) was placed in a 250 cm^3 round-bottom flask with a sleeve. Then 50 cm^3 of 1 M sodium hydroxide solution in ethanol was added. The flask was connected to a reflux condenser, placed in a water bath and heated at a temperature of 80 ± 2 °C for 30 minutes or until the sample was completely dissolved. The flask contents were periodically stirred with a borosilicate glass rod. After that, the flask contents were cooled by adding 30–50 cm^3 of distilled water. Cooling with water is necessary to prevent solidification of the hydrolysate, which may complicate further sample preparation. The hydrolysate was mixed with 6 g of diatomite and transferred to a 500 cm^3 separatory funnel. Hydrolysate transfer to the separatory funnel must be carried out carefully to minimize the amount of diatomite in the funnel, as it may complicate handling the separatory funnel. 10 cm^3 of ethyl acetate was added to the hydrolysate, mixed thoroughly and allowed to settle. After separation, the lower layer was transferred to a second separatory funnel for re-extraction with 10 cm^3 of ethyl acetate. The upper ethyl acetate layer obtained after the first and second extractions was pooled into a 50 cm^3 centrifuge tube. After that, the sample was centrifuged for 10 minutes with an acceleration of 5000 g for complete separation. The supernatant obtained after centrifugation was used for SPE.

For SPE extraction, Oasis MCX 6cc (150 mg) LP Extraction Cartridges (manufactured by Waters, Ireland) were used. The SPE cartridges were pre-activated by passing

6 cm^3 of methanol, and then 6 cm^3 of 0.1 M hydrochloric acid in deionized water. The solution to be analyzed was applied to the cartridge at a rate of 1 cm^3/min, while collecting the ethyl acetate extract. The cartridge was washed by passing 6 cm3 of 0.1 M hydrochloric acid in deionized water and 6 cm^3 of methanol, while discarding the washings. Analytes were eluted with 6 cm^3 of methanol-ammonia mixture in a ratio of 19:1 at a rate of 1 cm^3/min and transferred to a round-bottom flask, while pooling with an ethyl acetate extract. The solution was evaporated to obtain a dry residue on a rotary evaporator at a temperature not exceeding 40 °C. Then, 1 cm^3 of methanol was added to the dry residue, transferred for 5 minutes into an ultrasonic bath until the residue was completely dissolved. The solution was passed through a membrane filter with a pore diameter of 0.45 μm into a 2 cm^3 chromatographic vial for HPLC–MS/MS analysis.

The degree of extraction in this sample preparation technique was determined by the "added/found" method. For this purpose, solutions of standard samples were added to the sample initially free of HAAs to obtain approximate values of 10 ng/g. Sterilized canned beef was used as a HAA-free sample, as the temperature of their heat treatment is closest to the temperatures at which HAAs are formed, but insufficient for their formation.

According to the results, the samples contained 9.09 ng/g MeIQx and 8.94 ng/g PhIP. Thus, the extraction degree was ≈90.9% and ≈89.4% for MeIQx and PhIP, respectively.

Further, the stability of the extraction was determined. For this, solutions of HAA standard samples were added to the sample initially free of HAA. Sterilized canned beef was also used as a HAA-free sample. Nine samples were prepared: 3 samples with the addition of HAA standard solutions to achieve the concentration of 10 ng/g; 3 samples to achieve the concentration of 25 ng/g; and 3 samples to achieve the concentration of 50 ng/g. The extraction degree for all samples was about 90%.

Despite the fact that the described method allows the extraction of HAAs from meat products with a high and stable degree of extraction, it may be not convenient in laboratory practice, because the SPE process significantly increases the analysis time.

To solve this problem, other methods of sample preparation were tested, i. e. salting out with elution with acetonitrile, salting out with elution with methanol, acid hydrolysis, alkaline hydrolysis, followed by liquid extraction with hexane. However, the use of the above approaches led to an extremely low degree of extraction and insufficient purification from extraneous organic compounds. Optimal results were obtained using alkaline hydrolysis of the sample followed by extraction of HAAs from the hydrolysate with an organic solvent, diethyl ether.

A sample of the analyzed product (3.00 ± 0.01 g) was placed in a 250 cm^3 round-bottom flask with a sleeve. Then 50 cm^3 of 1 M sodium hydroxide solution in ethanol was added. The flask was connected to a reflux condenser, placed in a water bath and heated at a temperature of 80 ± 2 °C for 30 minutes or until the sample was completely dissolved. The flask contents were periodically stirred with a borosilicate glass rod. After that, the flask contents were cooled by adding the distilled water. The resulting hydrolysate was transferred into a separatory funnel with a volume of at least 250 cm^3. Then 25 cm^3 of diethyl ether was added to the separatory funnel and allowed to settle for 1–5 minutes for separation of layers. Then the bottom layer was transferred into another separatory funnel with a volume of at least 250 cm^3 for re-extraction. Then 25 cm^3 of diethyl ether was added to it and for separation of layers. After that, the lower layer was poured off, and the ether layer obtained after the first extraction was added to the upper ether layer. The resulting ether was washed 3 times with 25 to 30 cm^3 portions of distilled water for sample purification from alkali. Then the ether layer was passed through a membrane filter with 15 to 20 g of sodium sulfate for dehydration. The resulting ether was evaporated to dry residue at a temperature not exceeding 40 °C. 1 cm^3 of methanol was added to the dry residue and transferred to an ultrasonic bath until the residue was completely dissolved. The solution was passed through a membrane filter with a pore diameter of 0.45 μm into 2 cm^3 chromatographic vial for HPLC–MS/MS analysis.

Similarly to sample preparation with SPE, the degree of extraction was determined by the "added/found" method. The results showed that the extraction degree for the analytes to be determined was ≈78.3% for MeIQx and ≈82.0% for PhIP, which is a satisfactory.

When using this sample preparation method, it is necessary to take into account the possibility of incomplete separation of organic solvents in liquid-liquid extraction. In this case, a mixture of ethanol and water enters into the ether layer leading to the sample contamination. Also, after the evaporation of the ether layer, a mixture of ethanol and water may remain in the flask, which must be further evaporated at higher temperatures.

Tables 3 and 4 show the amounts of reagents for sample preparation by SPE and liquid-liquid extraction, respectively.

Table 3. Calculating the amounts of chemical reagents in the HAA analysis with sample preparation by the SPE

Reagent name	Reagent amounts per 1 analysis
Sodium hydroxide	4 g
Ethanol	100 ml
Diatomite	12 g
Ethyl acetate	40 ml
Methanol	38 ml
Hydrochloric acid	0.44
Ammonia	0.6
SPE cartridge	2

Table 4. Calculating the amounts of chemical reagents in the HAA analysis with sample preparation by the liquid-liquid extraction

Reagent name	Reagent amounts per 1 analysis
Sodium hydroxide	4 g
Ethanol	100 ml
Diethyl ether	100 ml
Sodium sulfate dehydrated	50 g
Methanol	2 ml

The elimination of the SPE process reduces the analysis time and significantly reduces the cost of the sample preparation. The SPE advantage is the reduction in total solvent amount during sample preparation, which has a positive effect on the method safety. In HAA determination, SPE elimination does not reduce the accuracy and reliability of the results.

The use of chromatography with mass spectrometry detection for qualitative HAA analysis is associated with the so-called "matrix effects". To confirm the absence of cross interferences in the mass spectrometry at the collision chamber of the quadrupole analyzer, "matrix effects" were investigated by analyzing the target substances in blank samples and deionized water. The ratio of the blank sample peak area to water was calculated. The studies have shown that the biological matrix suppressed signal for the analytes determined, which decreased the accuracy and reliability of the method. The degree of signal suppression by the matrix was ≈22% for MeIQx and ≈19% for PhIP. To take into account the matrix effect, a matrix calibration was carried out.

To do this, a solution of standard samples was added to previously analyzed samples not containing analytes until concentrations of 6, 10, 15, 30, 60, 100, 150, 300, 600 ng/ml and 5.5, 9.1, 13.75, 27.5, 55, 91, 137.5, 275, 550 ng/ml were reached for MeIQx and PhIP, respectively. These concentrations were selected on the basis of the analyzed literature on the amounts of HAAs formed in meat products.

When preparing solutions for calibration, 200 µL of solutions with established concentrations were added to 800 µL of a HAA-free sample to achieve concentrations of 6, 10, 15, 30, 60, 100, 150, 300, 600 ng/ml and 5.5, 9.1, 13.75, 27.5, 55, 91, 137.5, 275, 550 ng/ml for MeIQx and PhIP, respectively. The chromatographic analysis of the obtained solutions showed a strong decrease in the signal suppression due to the matrix effect, and the results allowed linear matrix calibration. The linear regression correlation coefficient R was not less than 0.99. The linear regression equation of the linear calibration for MeIQx is as follows:

$$y = 6048.379788 * x - 60190.588646 \quad (1)$$

The linear regression equation of the linear calibration for PhIP is as follows:

$$y = 14433.213689 * x + 21370.000524 \quad (2),$$

where y is the analyte concentration, x is the peak area.

Calibration plots for MeIQx and PhIP are shown in Figure 5 and Figure 6, respectively.

The range of values established in the calibration is optimal for HAA studies, since the ranges of the detectable concentrations are 5.5 to 550 ng/ml and 6.0 to 600 ng/ml on average for MeIQx and PhIP, respectively.

Based on the results, the limit of detection and the limit of quantification were determined. The method with the sample preparation technique for liquid extraction was validated and the indicators of accuracy and specificity were established. The method was validated in accordance with EU Commission Directive 2002/657[1]. To perform validation, the results were obtained and processed according to the following parameters: specificity; linearity; correctness (degree of extraction); limit of detection (LOD), limit of quantification (LOQ). To confirm method specificity, 10 samples of meat products not containing MeIQx and

[1] Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.

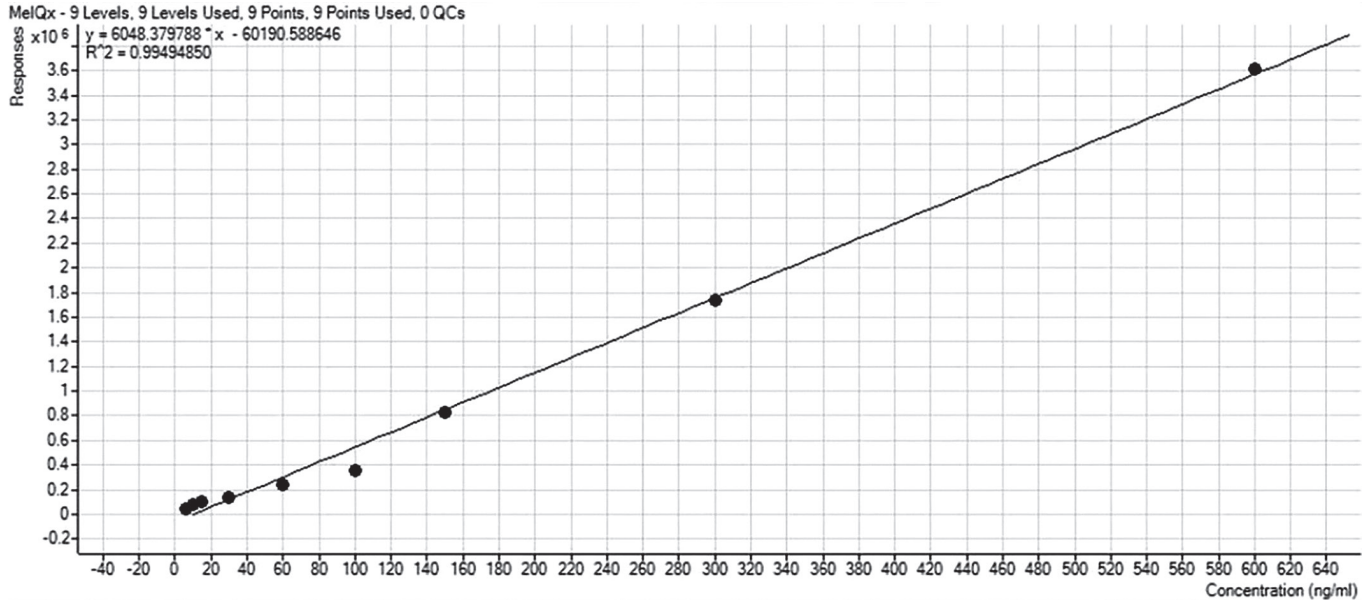

Figure 5. Linear calibration plot for MeIQx

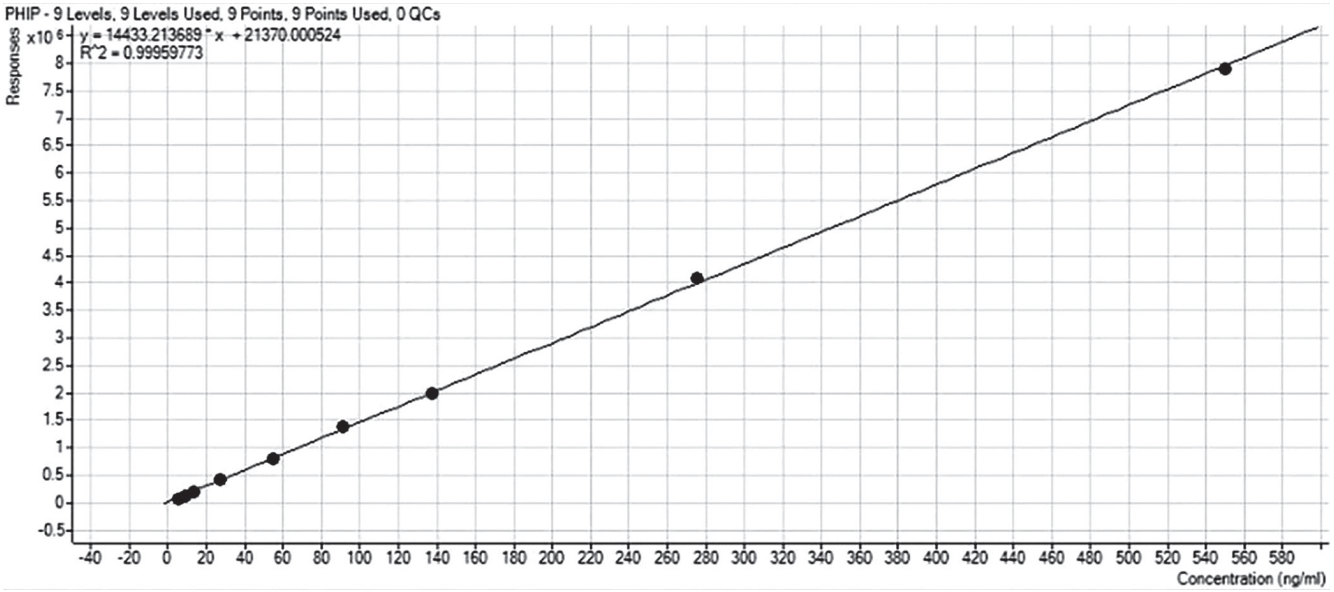

Figure 6. Linear calibration plot for PhIP

PhIP were examined. All 10 samples did not have chromatographic peaks interfering with the determination of MeIQx and PhIP. The technique allows for reliable determination of MeIQx and PhIP in the presence of impurities and systemic peaks of the mobile phase. The linear correlation coefficient of the obtained calibration curves was no less than 0.99 in the concentration range of 5.5 to 550 ng/ml and 6.0 to 600 ng/ml for MeIQx and PhIP, respectively. LOD and LOQ were defined as the signal-to-noise ratio (S/N) for daughter ions of at least 1:3 for LOD and at least 1:10 for LOQ. The measurement results were obtained using 12 samples of a meat product with added MeIQx and PhIP at the concentration of 0.1 ng/ml, 0.5 ng/ml, 1.0 ng/ml, 2.0 ng/ml, 3.0 ng/ml, 4.0 ng/ml, 5.0 ng/ml, 6.0 ng/ml, 7.0 ng/ml, 8.0 ng/ml, 9.0 ng/ml, 10.0 ng/ml. The limit of detection (LOD) for MeIQx and PhIP was 0.5 ng/g. The limit of quantification (LOQ) for MeIQx and PhIP was 3 ng/g.

Accuracy was assessed by nine repeated measurements of standards at the concentration of 5.0, 10.0, and 20.0 ng/ml. Intralaboratory reproducibility was calculated from the results of the analysis with a solution obtained by another chemist in the laboratory using the same equipment. The relative standard deviation of the peak area did not exceed 5%. The analyte determination accuracy varied in the range of 95 to 98%. Accuracy parameters are shown in Table 5.

The method specificity was confirmed by the absence of analyte peaks in the analysis of 10 pure samples after analyzing the sample containing the highest calibration concentration (550 ng/ml and 600 ng/ml for MeIQx and PhIP, respectively). No HAA peaks were observed during the analysis of blank samples.

Table 5. Accuracy and reliability of the developed method for determining HAAs in meat products

Analyte	Concentration, ng/ml	Relative standard deviation, %	Accuracy, %
MeIQx	5.0	5.40	96.15
	10.0	2.47	97.08
	20.0	1.93	96.54
PhIP	5.0	3.59	97.18
	10.0	2.45	97.98
	20.0	1.60	98.40

Conclusion

The developed technique using the HPLC–MS/MS method allows detecting the heterocyclic aromatic amines in meat products at the concentrations (the limit of quantification of target substances) of as low as 3 ng/g. Sample preparation by polar solvent extraction with diethyl ether may significantly reduce analysis time and cost relative to sample preparation method with SPE. In this connection, the method of sample preparation with liquid extraction may be recommended for routine tests, but for arbitration studies, the method of sample preparation with SPE is more preferable due to high analyte extraction degree. For the technique developed, the limit of detection and the limit of quantification of HAAs, the degree of their extraction for both methods of sample preparation, and indicators of accuracy and specificity were determined. The work performed allows to recommend the developed method for monitoring the accumulation of heterocyclic aromatic amines in meat products.

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LACTOFERRIN: PROPERTIES AND APPLICATION. A REVIEW

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Abstract

The current state, prospects for using and priorities in studying multifunctional protein lactoferrin (LF) in the food industry are discussed. Over the last decades, the studies of iron-binding, antibacterial, antiviral and antiparasitic properties of this representative of transferrins have determined quite a wide sphere of its use. The data on the lactoferrin composition, structure and activity are presented. The authors describe the modern methods for lactoferrin extraction and production from dairy raw materials both in the domestic and foreign productions using chromatographic methods for extraction and membrane methods for production. The practical experience in its application for food production, in particular, for meat and fermented dairy products, child and sports nutrition is discussed. An effect of technological process parameters in food production on the LF activity is highlighted. The study analyses an influence of new processing technologies such as high pressure or pulsed electric field in combination with classic methods for thermal processing and drying on the structure and activity of lactoferrin in food. The reviewed studies show that the use of lactoferrin in the meat industry, especially, in finished meat products, has limitations. The data presented in the review suggest a need for searching ways of lactoferrin introduction into meat systems to obtain functional products. One of the top-priority method for LF incorporation into meat products is LF encapsulation as one of the production stages.

Introduction

Multifunctional protein lactoferrin (LF) is promising for production of different medicines, biologically active additives, cosmetic preparations, food products. It was first identified in 1939 in bovine milk. This happened due to its iron-binding capacity. Lactoferrin was classified as a member of the transferrin family along with serum transferrin and membrane ovotransferrin. Later on, it was recovered from milk of humans, guinea pigs, sheep, goats, pigs, horses, mice and dogs [1]. The primary structure of LF from human milk was discovered in 1984; then, in 1991, the structure of LF from bovine milk was determined. The amino acid sequence identity between human and bovine lactoferrin is about 70%.

It is known that lactoferrin is synthesized by the epithelial cells of mammalian exocrine glands and is a constituent of different secretory liquids. It was found in saliva, lacrimal fluid, nasal discharge, digestive juice, bile, urine, seminal fluid, cervical mucus, colostrum and others [2].

The problem of studying LF properties and ways of using this component in food production has been topical over many years due to its wide functional characteristics such as the iron-binding capacity, antibacterial, antifungal, antiviral, antiparasitic activities, anti-inflammatory action, enzymatic properties [3]. Modern studies are aimed towards extending a line of LF-containing products. Attempts are made to produce not only lactoferrin-containing fermented dairy food products, but also to introduce this component into meat products. The aim of this paper is to summarize information about the recent achievements in LF production for using in food manufacture.

The main lactoferrin source is milk, which is a unique biological liquid. Its protein content varies on average from 2.9% to 3.2%. Milk proteins have complex composition; they are different in terms of physico-chemical properties and functions. Milk proteins are classified into three groups. The first group includes casein and its complex of four fractions; the proportion of its fraction relative to other milk proteins is 80%. The second group includes milk serum proteins accounting for 19%. This group is represented by different globular proteins such as β-lactoglobulin, a-lactoglobulin, immunoglobulin and serum albumin, lactoferrin and other minor proteins. They differ from each other by structure and biological properties. The third group includes fat-globule membrane proteins accounting for only about 1% of all milk proteins.

Lactoferrin

Milk serum proteins, in particular, lactoferrin, which is the most potent antimicrobial agent in milk, have aroused considerable interest in the scientific community. This component is glycoprotein with a molecular weight of about 80 kDa. The lactoferrin composition is presented in Table 1.

The molecular structure of lactoferrin is a polypeptide chain folded into two globular homologous fragments linked with the a-helix. Each of these fragments consists of two domains, to which the iron ion is attached. The molecular structure of lactoferrin has two forms — apolactoferrin and hololactoferrin. Apolactoferrin is the iron-free lactoferrin form, which is more sensitive to the proteinase action. Hololactoferrin is the lactoferrin form containing the iron ion, which is stable and resistant to

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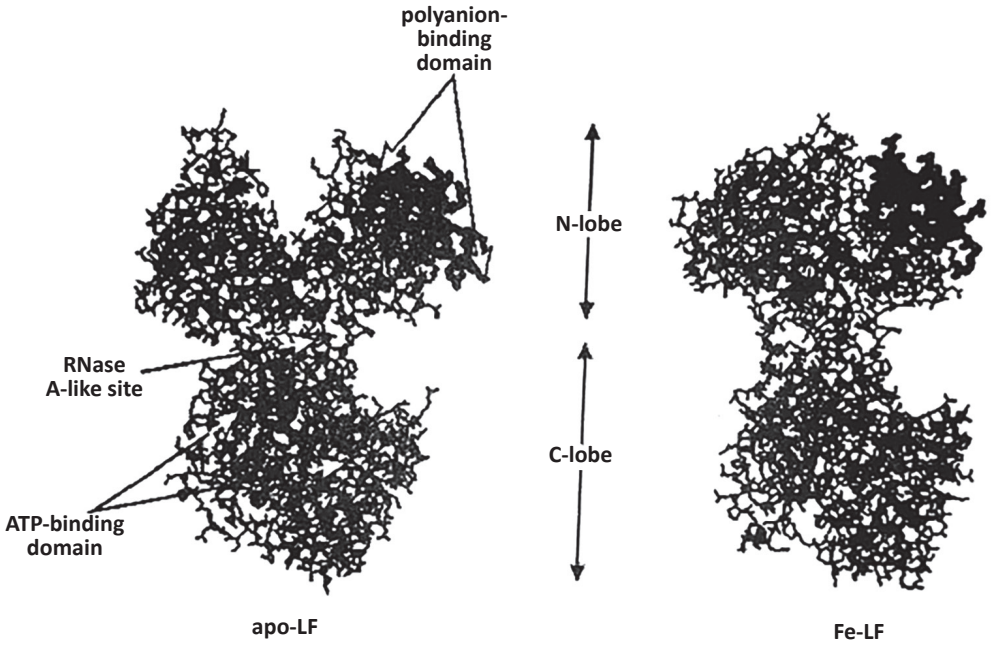

Figure 1. Spatial organization of apo- and Fe- lactoferrin

the action of enzymes [4]. Figure 1 presents the apo- and Fe-lactoferrin forms.

Table 1. Lactoferrin composition

Protein part	Carbohydrate part
— monomeric β-globulin containing more than 690 amino acid residues	— sialic acid residue (5-acetamido-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid) — fucose (6-deoxy-galactose) — hexoses; — N-acetylglucosamine

Functional properties of LF are considered with account for its molecular structure. Glycoprotein is capable of iron binding and its transportation through intestinal mucus, thereby allowing regulation of iron ions in the body. It has the antibacterial and antiviral activities, facilitates tissue regeneration, regulation of cell growth and differentiation.

It was found that lactoferrin has the bacteriostatic activity and showed the special activity towards iron. LF is secreted and synthesized mainly in the iron-free form, which subsequently leads to its binding with free iron ions from the environment. This lactoferrin property significantly delays microbial growth including Gram-negative and Gram-positive bacteria and some yeasts. In physiological concentrations, lactoferrin facilitates membrane damage in Gram- negative bacteria, which results in liposaccharide liberation [4,5].

LF protects mucous membranes from pathogenic microorganisms, has anti-inflammatory and immunomodulatory properties [6].

The antimicrobial properties of lactoferrin have been well studied in *in vitro* and *in vivo* experiments. For example, digestion of lactoferrin from maternal milk in the child stomach stimulates the growth of bifidobacteria, suppresses the growth of other microorganisms and determines the composition of indigenous intestinal microflora that is typical for infancy. This effect was proved when using mixtures for newborns [4,7].

Besides the direct antimicrobial action, lactoferrin can influence the inflammatory process of infectious genesis. It was proved experimentally that lactoferrin protected from gastritis induced by *Helicobacter pylori*, maintained the integrity of intestinal mucosa, reduced lethality during infection with enterotoxigenic *E. coli*. The glycoproteid also takes part in the regulation of inflammation in neurodegenerative diseases, inflammatory bowel disease (IBD), skin allergy, lung diseases and arthritis [4].

Lang J., Yang N. et al. established that LF blocked cells with SARS-CoV [8]. This allowed carrying on investigations of LF clinical application in treating patients with COVID-19 [9].

GOST 33600–2015[1] is used to determine lactoferrin in milk. The essence of the method consists in removal of fat and casein fraction of protein from a sample using a laboratory centrifuge and detection of the lactoferrin content by the method of reverse-phase HPLC with the use of a spectrometric detector at a wavelength of 205 nm.

There are different methods for lactoferrin detection from bovine milk and whey obtained in cheese production. Another source for LF production at the industrial level is curd whey. Whey fractions or colostrum are considered the best LF sources for laboratory studies [10].

Modern methods for lactoferrin extraction and production

Chromatographic methods (ion-exchange, affinity, cation-exchange chromatography)

Most methods of LF extraction that allow obtaining protein in the native state are based on using chromatography with different preliminary stages of raw material preparation [11].

[1] GOST 33600–2015 "Milk and dairy products. Method for determination of the lactoferrin by high performance liquid chromatography". Moscow: Standartinform, 2019. — 11 p.

Lactoferrin is mainly obtained from raw milk. There is a method for LF extraction by cation-exchange chromatography with the following purification and concentration using micro-, ultra-filtration and freeze drying. The choice of a method for LF extraction is based on the molecular properties of glycoproteid [12].

Another method for lactoferrin production from raw bovine milk is linked with chromatography on a column with sorbent SP Sepharose (Sulfopropyl-Sepharose) Big Beads equilibrated with 0.05 M sodium phosphate buffer at pH=6.5. Sorbent SP Sepharose Big Beads is a strong cation exchanger, which has granules with a size of 200 μm. Due to the large size of granules, milk flows through the column without its clogging. The use of this sorbent allows eliminating stages of fat removal and protein desalting, which shortens to a great extent the extraction process time. A degree of lactoferrin extraction by this method is more than 90% with protein purity of 95% [11,13].

The method of chromatographic single-stage industrial extraction and purification of bovine lactoferrin from dairy raw materials enables simultaneous recovery of not less than 90% of contained lactoferrin. This method uses the ion-exchange sorbent based on the hydrophilic macroporous acrylic polymer. This allows obtaining highly purified lactoferrin with preserved biological properties. With that, dairy raw materials used for this method can be subjected to following processing in the food industry.

Membrane technologies for LF production

Bovine lactoferrin was obtained for the first time on the industrial scale in Belgium in 1985. In 1989, the German company MILEI GmbH began production of lactoferrin from whey obtained in cheese and milk production by the technology developed by the Japanese company Morinaga Milk Industry Co., Ltd. engaged in production of food ingredients and functional products. About 600–700 thousand tons of milk per year were processed by the scheme of non-waste production. Lactoferrin yield was about 30 tons per year. The membrane technology was mainly used for lactoferrin extraction from defatted milk and cheese whey; purity of produced lactoferrin was not less than 96% [10].

The method for production of the lactoferrin concentrate by the membrane adsorption method is well known. The method is quite expensive due to the use of import membrane cassettes and there is a possibility of penetrating particles of membrane fibers into dairy raw materials in the process of protein extraction [14].

The method for lactoferrin production enriched with immunoglobulins for prophylactic dietetic nutrition was patented. Colostrum of the first 24 hours of lactation, which is diluted with water to the density of milk, is used as a raw material. The obtained mixture is defatted using a separator; then, the casein fraction is precipitated by rennet; whey is obtained and sent to bactofugation with the following two-stage membrane filtration. As a result of filtration, lactoferrin and immunoglobulin remain between membranes. After that, retentate is dried or filled into containers. The obtained product preserves its native properties [15].

The price on lactoferrin from bovine milk on the world market depends on the level of its purification and quality, and varies from 50 to 500 dollars per 1 kg [16].

At present, more than 20 enterprises producing lactoferrin operate worldwide. The largest production units are in Germany, Japan and USA.

Lactoferrin is produced on the industrial scale by such companies as MILEI (Germany), DMV International (The Netherlands), DOMO Food Ingredients (Belgium), Fonterra (New Zealand), Tatura and Bega Cheese (Australia) and Glanbia Nutritionals (Ireland).

Preparations of imported highly purified lactoferrin are presented on the Russian market. They are intended mainly for the pharmaceutical industry, biochemical and medical investigations.

In Russia, food products with bovine lactoferrin are manufactured by small enterprises. A positive trend in whey processing using innovative technologies has been observed [17]. In infant formula production in the RF, the demand for lactoferrin is 50 tons per year [16].

The use of lactoferrin in the food industry

The use of lactoferrin in the meat industry

In the meat industry, lactoferrin is used to inhibit microbial vital activities. Meat raw material treatment consists in spraying of a LF-based preparation over a carcass or chilled cuts, which allows not only prevention of the microbial growth but also neutralization of the endotoxin activity [18].

In the USA, a dose of the lactoferrin application for meat preservation is not more than 65 mg/kg. The USDA approved the use of lactoferrin as “processing aid” for carcass rinse treatments in 2002 [18]. Large companies began to introduce carcass treatment in production. A commercial form of lactoferrin, which is available as a processing aid for carcass treatments, is Activin provided by aLF Ventures LLC, joint venture between National Beef Packing Co., DMV International and Farmland Industries, located in Kansas City [19].

The antimicrobial action of lactoferrin is expressed in its ability to block attachment of bacteria to the surface of meat tissues. Moreover, interaction of lactoferrin with iron inhibits the microbial growth and prevents iron participation in oxidation reactions.

Spanish scientists studied the bactericidal activity of lactoferrin, its derivatives in combination with high hydrostatic pressure against six strains of three Gram-positive bacterial species *Listeria monocytogenes*, *Staphylococcus aureus* and *Enterococcus faecalis* in chicken breast.

Lactoferrin and its derivatives have a wide spectrum of antibacterial properties. However, these properties can be influenced by different factors such as temperature, water activity, pH, composition of the medium, presence of salts, different protein, lipid or carbohydrate components [20].

For example, high hydrostatic pressure is used to increase microbial safety and extend product shelf life. However, its use in meat and meat products can lead to undesirable changes in color and texture. In addition, pressure influences the lactoferrin structure and activity, its antimicrobial properties and ability to bind and hold iron. Under the action of 400 MPa, the lactoferrin structure does not change significantly, but treatment under 500 MPa and higher strongly affects LF properties, reduces its antimicrobial action [21]. For these reasons, the combined treatment of chicken breasts was used in the study.

During the experiment, samples of chicken breasts were inoculated with bacterial cultures, treated with antimicrobials based on lactoferrin and its derivatives in the concentration of 0.5 and 5 mg/g and subjected to high hydrostatic pressure processing (HHP) at 400 MPa for 10 min at 10 °C. During the study, the following groups of samples were investigated: samples prepared with addition of the antimicrobials and HHP treatment, samples treated only with pressure, and samples without added antimicrobials and without HHP treatment. Microbiological analyses were carried out on days 1, 3 and 9 and included counting of total viable bacteria [20].

The results of the study showed that the use of the antimicrobials based on lactoferrin and its derivatives with high hydrostatic pressure enhanced the bactericidal effect. Under the action of pressure, bacteria membranes are destroyed leading to an increase in sensitivity to the antimicrobials. This method provides food product safety without changes in quality indicators [20,22].

An effect of hydrolysis and microwave treatment on the antibacterial activity of native bovine milk lactoferrin against *Cronobacter sakazakii* in different media was investigated in another study. The results showed that the antibacterial activity of the LF hydrolysates obtained with pepsin, chymosin and microbial rennet was higher than that of intact LF. The combined use of the LF hydrolysates with microwave heating demonstrated an increased inhibitory properties and inhibition of *C. sakazakii* after treatment at 450 W for 5 s (42 °C) and 550 W for 5 s (50 °C). Low intensity microwave treatment maintained product properties and enhanced the antibacterial activity of LF hydrolysates obtained with pepsin, chymosin and microbial rennet against *C. sakazakii* [23].

The antibacterial action of lactoferrin and high hydrostatic pressure (HHP) was also studied on the samples of cured beef carpaccio. The authors used activated lactoferrin (ALF), lactoperoxidase system (LPOS) and high hydrostatic pressure (HHP) treatments at 450 MPa for 5 min against *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Escherichia coli* O_{157}: H_7. Treated cured beef carpaccio was stored at 8 °C and 22 °C for 7 days. It was found that the use of the antimicrobials and HHP treatment gave the synergistic effect and facilitated an increase in the microbiological safety of beef carpaccio [24].

A similar experiment with the use of lactoferrin, lactoperoxidase and high hydrostatic pressure (HHP) treatments (at 450 MPa for 10 min) against microorganisms (*Listeria monocytogenes* and *Salmonella* Enteritidis) was also carried out for dry-cured ham. After treatment, dry-cured ham was stored for 60 days at 8 °C. Microbiological analyses were carried out on 1, 15, 30 and 60 days of storage. In addition, the texture and color of the samples were investigated. The results of the experiment indicated that lactoferrin and lactoperoxidase did not affect *L. monocytogenes* and *S.* Enteritidis when applied individually, while the use of antimicrobials and HHP treatment inactivated *S.* Enteritidis. However, HHP treatment slightly changed color and texture of the samples [25].

Spanish scientists studied the lactoferrin antimicrobial effect in meat products and proved that lactoferrin, reuterin, lactoperoxydase in combination with high hydrostatic pressure (HHP) processing had the antimicrobial effect against *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Escherichia coli* O_{157}: H_7 in cooked ham. Two large pieces of cooked ham (900 g each) obtained from a market in Madrid and aseptically sliced in the laboratory conditions were used in the experiment. Ham slices (20g) were inoculated with pathogenic microorganisms and held at 4 °C for 20 h before further treatment. The samples were divided into eight different groups: control (without using HHP and antimicrobials), samples with reuterin, samples with lactoperoxidase, samples with lactoferrin, samples treated with HHP, samples with reuterin + HHP, samples with lactoperoxidase + HHP and samples with lactoferrrin + HHP. All antimicrobials were added immediately before HHP treatment. Then, the samples were treated with HHP at 450 MPa for 5 min. After treatment, the samples were stored at temperatures of 4 °C and 10 °C for 35 days.

The results of the study showed that individual application of reuterin and lactoperoxydase affected the survival of pathogens; the *S. Enteritidis* and *E. coli* levels decreased. However, *L. monocytogenes* counts increased at a storage temperature of 4 °C. No effect was observed upon individual application of lactoferrin. When reuterin or lactoperoxydase were applied in combination with HHP, the synergistic antimicrobial effect against *L. monocytogenes*, *S.* Enteritidis and *E. coli* O_{157}: H_7 was found in cooked ham stored at 4 °C and 10 °C for 35 days. The pH and a_w values were not changed significantly in all treated samples. Slight changes were found in color and shear strength values in treated cooked ham. Application of HHP individually or in combination with lactoperoxydase or lactoferrin reduced formation of volatile compounds in cooked ham during 35-day storage at 4 °C and 10 °C. As a result, HHP treatment in combination with reuterin or lactoperoxydase was recommended as a hurdle technology against *L. monocytogenes*, *S. Enteritidis* and *E. coli* O_{157}: H_7 in cooked ham [26,27].

Synergetic antimicrobial effect of activated lactoferrin and rosemary extract *in vitro* was studied to improve meat

storage technologies. The antimicrobial action was tested on *Escherichia coli* O_{157}: H_7, *Salmonella* Enteritidis and *Listeria monocytogenes* during meat storage. The researchers determined the minimum inhibitory concentrations (MICs) of activated lactoferrin, which were 1% for *E. coli* O_{157}: H_7, 0.5% for *S.* Enteritidis and 0.1% for *L. monocytogenes*. For example, while 15% rosemary extract inhibited *L. monocytogenes*, 30% rosemary extract partially inhibited the growth of *E. coli* O_{157}: H_7 and *S.* Enteritidis. The use of activated lactoferrin with rosemary extract enhanced the antimicrobial action of reagents, which allowed increasing storage duration of meat products [28].

Scientists from the Istanbul University studied an effect of different concentrations of nisin and lactoferrin (Lf), and their combination on the microbiological quality of Turkish-style meatballs. Meatballs were made by the traditional recipe from ground veal and treated with different concentrations of lactoferrin and/or nisin (0, 100 and 200 μg/g). Samples were analyzed on days 0, 1, 3, 5, 7, 10 and 12 for microbiological parameters (total mesophilic aerobic bacteria, lactic acid bacteria, coliforms, *Escherichia coli*, total staphylococci, *Staphylococcus aureus*, total psychrophilic bacteria, *Pseudomonas* spp., sulfite-reducing anaerobic bacteria, yeasts and molds) and physicochemical characteristics (pH, water activity and moisture). It was found that the lowest level of microbiological spoilage was in the samples treated with the mixture of Lf (200 μg/g) and nisin (100 μg/g), which allowed extending shelf life of meatballs up to 10 days compared to the control samples with shelf life of only 3 days [29].

A search for effective methods for increasing microbiological safety of meat products is continued. The lactoferrin presence is not always regarded as the universal means for prevention of microbial colonization on the meat product surface. In 2006, for example, Korean scientists showed that low-fat, low-salt sausages treated with lactoferrin did not have longer shelf life compared to sausages not treated with it. According to the data of the model study, more than 0.25% of lactoferrin was required for a distinctive antimicrobial activity against the growth of *E. coli* O_{157}: H_7. However, extended shelf life during refrigerated storage was not achieved in the low-fat, low-salt sausages produced with the use of lactoferrin. Moreover, the synergistic effect of lactoferrin with sodium lactate was not found. The study showed that addition of lactoferrin into the low-fat sausages did not have the antimicrobial activity against *E. coli* O_{157}: H_7 due to the denaturation of lactoferrin or the complex process of sausage production, even though the distinctive antimicrobial effect was partly observed in the model study [30].

The use of lactoferrin in other branches of the food industry

The practical use of lactoferrin in the food industry began from production of foods for child nutrition. Addition of lactoferrin into infant formulas facilitated an increase in the resistance of neonates to infections, improvement of intestinal microflora and reduction in the number of respiratory diseases [19]. From the technical viewpoint, lactoferrin in infant formulas prevents lipid oxidation allowing an increase in product shelf life. Production of infant formulas without lactoferrin in their composition is forbidden in some countries.

Bovine lactoferrin is used in production of different fermented products such as yogurt, kefir. These products facilitate stimulation of the bone tissue growth and elimination of rotavirus infection. The technology for functional yogurt preparation with prolonged shelf life was developed in the All-Russian Research Institute of Dairy Industry (VNIMI). Yogurt was produced by the reservoir method using the protosymbiotic starter culture. To increase product shelf life, whey protein lactoferrin having antimicrobial and bifidogenic properties was used. Addition of lactoferrin improved yogurt rheological characteristics and increased shelf life up to 14 days.

Besides dairy products and food additives, purified lactoferrin is added into canned foods, pharmaceutical preparations, sports nutrition, oral care products and cosmetics. Products with lactoferrin prevent the development of infections, strengthen the immune function, have the anti-inflammatory and antioxidant action. For example, a food supplement based on bovine lactoferrin and vitamin C encapsulated into a lipid bubble and having medicinal properties was registered in the European Union [9]. All the above allows classifying it as a novel, safe food product.

Lactoferrin also plays a part in wine production as a preserving agent. It inhibits the growth of yeasts *Dekkera bruxellensis*, which are a factor of wine spoilage, facilitating an increase in wine shelf life [31].

Conclusion

The application of lactoferrin as biologically active protein is expanding. The use of lactoferrin in the food industry should be considered promising for provision of food products with iron-binding component, enhancement of their immunomodulating character and antioxidant action, as well as an increase in product shelf-life. Based on the iron-binding capacity of lactoferrin, Russian scientists propose to use "iron-saturated forms of hololactoferrin from bovine milk as a domestic raw material for production of biologically active food additives and specialized food products".

Therefore, analysis of modern studies on the use of lactoferrin in food production shows that this component is actively used in dairy product manufacture and is of special importance for child nutrition. With that, studies on the use of lactoferrin in the meat industry are limited only to investigation of its bactericidal properties in treatment of meat semi-finished products.

Questions of protein introduction and preservation of its properties in meat products are still insufficiently investigated. In finished meat products, protein is not used as a biologically active additive. This is linked with the fact

that lactoferrin is destroyed in the process of long thermal processing and loses its unique properties. A search for a method for production of a finished meat product containing lactoferrin is topical as meat consumption is growing in the conditions of the growth in income and living standards. An increase in animal proteins in diets of the population set a problem of increasing the biological activity of meat products. The use of lactoferrin in these products can change meat product quality by not only increasing shelf life but also by increasing their healthy properties.

Introduction of lactoferrin into meat systems will give an opportunity to obtain a functional product, which will have antiviral and anti-inflammatory properties and will also allow correction of the immune processes and enhance resistance of the body to overstrain and stress. To obtain such functional product, it is promising to use different lactoferrin modifications including encapsulation, for increasing thermal stability of protein during thermal treatment, which is one of the stages in meat product manufacture.

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TECHNO-FUNCTIONAL, TEXTURAL AND SENSORIAL PROPERTIES OF FRANKFURTERS AS AFFECTED BY THE ADDITION OF BEE POLLEN POWDER

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Keywords: *texture, sensory, Warner-Bratzler, emulsion, FTIR-ATR*

Abstract
The objective of this study was to determine whether the addition of different pollen powder concentrations (0.5; 1.0 and 1.5 g/100 g) had an influence on techno-functional, textural and sensorial traits of frankfurters. Examining the techno-functional characteristics of pollen, a conclusion was reached that the higher the concentration, the higher the emulsification and better techno-functional properties. Also, FTIR-ATR analysis has shown that specific pollen molecules provided good emulsifying properties of sausages. On the other hand, sensory analysis showed that sausages with the addition of 1.0% and 1.5% of pollen powder have a more pronounced floral odor. Warner-Bratzler shear force test has shown that the incorporation of pollen caused a more stable product throughout sixty days of storage than the control sample. It could be explained by the formation of more protein-protein interactions due to the addition of non-meat proteins in the formulation of frankfurters and obtaining a more stable product than the control one. All things considered, it can be concluded that pollen exhibits good techno-functional properties and could be utilized in the formulation of frankfurters with improved and steady techno-functional properties during two months of refrigerated storage.

Introduction

The most common type of emulsified meat products in the world are frankfurters [1]. The world leader in the number of different varieties and the birthplace of frankfurters is Germany. Their popularity is reflected by the convenience in preparation, tastefulness and time-saving. The wide variety in physicochemical properties of emulsions is affected by the diversity of components used during technological process of the production of sausages. Emulsions, also called meat batters, are a complex mix of different systems of dissolved proteins and salts, suspensions, gels made from myofibrillar proteins and emulsions that contain stabilized fat in a gel and fat which is partially present in liquid form [2].

One of the most important quality parameters of the emulsified meat products, such as frankfurters, is emulsion stability. It is highly dependant on the amount of fat and water, as well as myofibrillar proteins, previously extracted from the muscle tissue. Mechanical energy from cutter knives destroys sarcolemma and additives such as nitrite salt and phosphates and activate the protein released. The solubilized protein simultaneously immobilizes the water and emulsifies the added fat, stabilizing them in a tridimensional matrix [2].

Nowadays, there is a growing concern in the meat industry to produce healthier products and to change the perception of them as being unhealthy. Thus, many natural additives such as mushroom decoctions [3,4], mixed extracts of green tea, olive leaves and stinging nettle [5], garlic extract [6], are used for the production of sausages, in order to partially or totally replace the use of commercial antioxidants. Two key processes take a part in this technology: reformulation, by the reduction of ingredients that some consumers perceive as unhealthy (commercial antioxidants, nitrites and sodium chloride) and enriching the product with highly nutritious compounds that consumers perceive as health-beneficial. However, the question arises how these extracts affect the texture, techno-functional (emulsifying) and sensory properties of sausages.

Pollen is a natural product that contains a high amount of nutrients and bioactive compounds, such as polyphenols and flavonoids [7], which give it excellent antioxidant activity. Pollen, as a multi-component natural product, contains significant quantities of proteins, carbohydrates and lipids. These constituents give pollen good emulsifying, foaming and gelling properties [7,8]. Therefore, this complex structure of pollen allows its application in the formulation of numerous food products. Until now, several food products such as yogurt [9], cheese [10], bread [11], etc. have been enriched with pollen. Additionally, it is known that lyophilized pollen polyphenol extracts have been added to sausages [12], as well. However, to the best of our knowledge, it has never been investigated before how pollen powder affects the texture and sensory properties of meat products. Unlike pollen polyphenol extract, which has only antioxidant potential, pollen powder as a multi-component system can have a multifunctional impact in the production of high-fat products such as frankfurters.

Therefore, the objective of this research was to characterize bee pollen powder and frankfurters containing

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it using FTIR-ATR. Also, this study aimed to investigate techno-functional properties of pollen in three different concentrations and to determine the influence of pollen addition on the textural and sensorial traits of the final product.

Objects and methods

Bee-collected pollen (hereafter referred as pollen) was purchased from local beekeepers situated in East Serbia. According to the producers' declaration, the pollen predominantly originated from rapeseed (*Brassica napus*) and ash tree (*Fraxinus spp*.).

Techno-functional properties of pollen

Techno-functional properties such as emulsifying and foaming properties, as well as water/oil absorption capacity (WAC/OAC) were determined according to the methods previously described by [13]. Both, emulsifying and foaming properties were determined for three different pollen concentration (0.5; 1; 1.5 g/100 g aqueous dispersion of pollen sample at pH 7) at which pollen was added to Frankfurter-type sausages.

Frankfurter's formulations

Sausages were manufactured in a commercial meat factory (BigBull Foods, Bačinci, Serbia). Fresh pork meat (*Biceps femoris*), without connective tissue, pork back fat and ice were used in the amounts of 50%, 25% and 25%, respectively. Meat was transferred to a bowl chopper, and salt and polyphosphate were added. After that, meat tissue was comminuted for 3 minutes at low speed, in order to extract myofibrillar proteins until the temperature reached 6 °C, when other ingredients were slowly added. The temperature of the mixture was not allowed to exceed 12 °C [14]. Pollen is added in the amount of 0.5% (T1), 1.0% (T2) and 1.5% (T3). Each treatment was repeated in triplicate, as well as a control sample, only without pollen in its formulation. These mixtures were blended until they reached 12 °C. After preparation of emulsions, sausages were stuffed into polyamide casings (Edicas, Girona, approximate 22 mm diameter) and cooked at 80 °C in a smokehouse (EL–C-Q 1900, Kerres Anlagenststeme GmbH, Backnang, Germany) until the core temperature of 72 °C. The cooked samples were cooled using the shower and placed in vacuum bags (4 frankfurters approximately 50g each) and coded according to appropriate treatment production.

FTIR-ATR analysis

FTIR-ATR spectra of pollen powder and small portions of frankfurters enriched with pollen were recorded by using an ALPHA FTIR spectrophotometer (Bruker) equipped with a Platinum ATR diamond module. The FT-IR-ATR recording parameters were as follows: the spectral resolution was 4cm^{-1}; 32 scans; absorbance mode was in the range of 400 to 4000 cm^{-1}. Date collection and analysis were performed using OPUS7.5 software.

Warner-Bratzler Shear force measurement

Textural properties of frankfurters were conducted using Warner-Bratzler Shear force (WBSF) test [15]. For the purpose of the investigation through this method, Warner-Bratzler 'V' slot blade was attached to the Universal testing machine (TA.XT Stable Micro System Corporation, UK). The load cell capacity was 50 kg, while shearing speed was 1.50 mm sec^{-1}. The parameter obtained in this method was the maximum shear force (N) and represents the highest peak of the curve, which is the maximum resistance of the sample to shearing. At least six specimens were tested from each sample (about 24 per one treatment). All analyses were done in triplicate.

Sensorial traits of frankfurters

Five trained and experienced panel members were selected to participate in the development of sensory profiles for the three different concentrations of pollen in frankfurters. They were chosen based on their ability to provide similar responses on repeated occasions, smell acuity, interest in the project and availability for the duration of the study. In order to ensure that panelists were not influenced in any way, no information with regard to the nature of the samples was provided. Panelists were reminded not to use perfumed cosmetics and to avoid exposure to foods and/or fragrances at least 30 min before evaluation sessions.

A 100-point category scale was used to measure the intensity of each sensory attribute (pork flavour, sweet, floral, woody, salty, juicy) for the different concentrations of pollen in frankfurters. One (1) on the category scale denoted the total absence of sensory attribute (e. g., no floral aroma), fifty (50) denoted a moderate presence and one hundred (100) denoted the most intense condition (e. g., extremely floral frankfurter aroma).

Frankfurters were sliced into 25-mm slices and coded with a random three-digit code. The serving temperature was 40 °C. One frankfurter was served at a time. When served to the panel, a coded sample was placed on a white plastic tray. Care was taken to ensure uniformity of each sample (volume served and serving temperature) and both replication of the different samples. Samples were randomized to exclude any bias due to the position effect. Acquisition of evaluation scores and data analysis was performed by Smart Senso Box software (Smart Sensory Solutions S.r.l., Sassari, Italy). Panel members were provided with water at room temperature, toasted bread and apples, which served as palate cleansers in between evaluation sessions.

Statistical analysis

Statistical analysis software SPSS17.0 (Chicago, Illinois, USA) was used for data analysis. Data from techno-functional properties, FTIR and sensorial traits of frankfurters were tested using One-Way Anova, while textural characteristics were performed using Two-Way Anova with repeated measurements, considering treatments and storage

as fixed effects and replicate as a random effect. Significant differences ($P < 0.05$) between means were determined by Tukey's HSD post-hoc test.

Results and discussion

Techno-functional properties of pollen

Emulsions and foams are usually crucial in the formation of texture and taste of food. Therefore, the potential application of pollen as a functional ingridient in different food products requires the identification of its techno-functional properties (Table 1). The values obtained for the emulsifying stability index (ESI) ranged from 14.04 min to 11.53 min, whereas emulsifying activity index (EAI) varied from 58.59 to 156.60 m^2/g, that depending on the pollen concentration in aqueous dispersion. All pollen aqueous dispersions exhibited the complete absence of the ability to form stable foams. The observed characteristic confirmed the anti-foaming properties of pollen, which is in accordance with a previous report from [13]. The oil and water absorption capacities of analysed pollen were 1.5 and 0.9 g/g, respectively, which is in line with results reported by [13]. It can be concluded that pollen possesses excellent emulsifying properties, oil and water absorption capacities, which qualify it for application in meat products, primarily in heterogeneous colloidal products such as Frankfurter-type sausages where these features are desirable.

Table 1. Techno-functional properties of pollen sample

Techno-functional properties					
Emulsifying properties		Foaming properties		Water/Oil absorption capacity	
ESI (min)	EAI (m^2/g)	FS (%)	FC (%)	WAC (g/g)	OAC (g/g)
14.04 ± 1.66	58.59 ± 10.70	n.d.	n.d.	9.1 ± 0.2	8.5 ± 0.1
11.53 ± 0.33	112.26 ± 8.24	n.d.	n.d.		
12.93 ± 0.26	156.60 ± 21.87	n.d.	n.d.		

* "n.d." — not detected; The results in the table are presented as means ± standard deviations (mean ± SD; n=3); Abbreviations: ESI — emulsion stability index; EAI — emulsion activity index; FS — foam stability; FC — foam capacity; WAC — water absorption capacity; OAC — oil absorption capacity

FTIR-ATR analysis

FTIR-ATR spectra of frankfurters and pollen-enriched frankfurters show the same absorption bands that mainly correspond to proteins, lipids and water (Figures 1a, b, c, d). The broad band at 3272 cm^{-1} corresponding to O-H stretching vibrations which are most likely originated from water, because the ratio of added ice during the production of frankfurters was as much as 25%. Vibrations observed in the FTIR spectrum at 2960 cm^{-1}; 2920–2924 cm^{-1} and 2852 cm^{-1} originated from CH_3 asymmetric, CH_2 asymmetric and CH_2 symmetric stretching and mainly belong to lipids [16,17]. The strong and dominant peaks at 1650cm^{-1} (Amide I) and 1540cm^{-1} (Amide II) originated from C=O stretching, N–H bending and C–N stretching of proteins. Peak at 1736 cm^{-1} was corresponding to C=O carbonyl stretching which can be derived from cholesterol and triacylglycerol esters [17]. Vibration at 1455 cm^{-1} represents C–O–H bending of lipids and proteins, while vibration at 1396 cm^{-1} can be linked with COO^- symmetric stretching of fatty acids. Peak at 1240 cm^{-1} can be connected with PO^{2-} asymmetric stretching indicating the presence of nucleic acids (mainly), phospholipids, phosphorylated proteins in the analyzed samples [16,17]. Vibrations at 1172 cm^{-1} may be derived from CO stretching vibration of C–OH groups of serine, threonine and tyrosine residues [16]. Peak at 1082 cm^{-1} can be correlated with PO^{2-} symmetric stretching; C–O stretching and C–H deformation which was corresponding to the nucleic acids, phospholipids and glycogen [16,17].

Spectrum of pollen powder (Figure 1e), showed dominant peaks belonging to the region of 3000–2800 cm^{-1} (oil's regions); 1700–1500 cm^{-1} (protein region) and 1200–900 cm^{-1} (carbohydrate regions) [7,18,19]. A strong peak at 1025 cm^{-1} indicated the presence of polysaccharides, and less pronounced bands in the range of 700 to 900 cm^{-1} can originate from C-O-C and C–OH vibrations [7,20]. Broad band in the range of 1420–1370 cm^{-1} can originate from C–H deformation vibrations of lipids and cellulose, while region between 1350–1200 cm^{-1} can be connected with N–H deformation and C–H stretching of amide III [19,21]. In the spectra of pollen-enriched frankfurters (Figures 1b, c, d), bands originated from frankfurter were dominant, which in most of cases overlap bands originating from pollen. Thus, through this approach cannot be concluded whether there were strong chemical interactions between constituents of frankfurter and pollen or not.

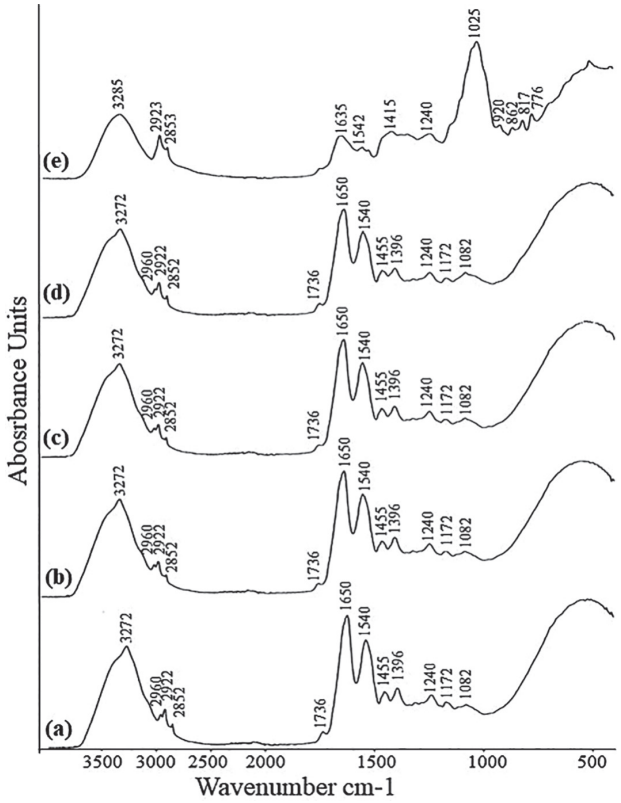

Figure 1. FTIR-ATR spectre of: (a) frankfurters without pollen; (b) frankfurters with 0.5% pollen; (c) frankfurters with 1% pollen; (d) frankfurters with 1.5% pollen; (e) pollen powder

Warner-Bratzler shear force of sausages

One of the main characteristics of meat products that influence consumers' purchasing decision is toughness/tenderness [22]. Three different concentrations of pollen added did not cause a statistically significant difference ($P > 0.05$) between treatments, during 40 days of storage period, regarding Warner-Bratzler Shear force values (N). On the 60th day, significant difference ($P < 0.05$) was observed between control group and treatments with pollen (T1, T2, T3) (Table 2). Usually, the higher the non-meat protein content in sausages, the harder and chewier texture of sausages. In this case, pollen, as a natural ingredient, is rich in protein, but also in lipid content [13]. Hence, these components are crucial in the formation of emulsions, as well as the texture and taste of the final product.

Table 2. Warner-Bratzler Shear force of frankfurters

	Storage time (days)	C	T1	T2	T3
"WBSF" (N)	1	$2.76 \pm 0.18^{a, A}$	$2.86 \pm 0.07^{a, A}$	$2.81 \pm 0.15^{a, A}$	$2.81 \pm 0.16^{a, A}$
	20	$2.73 \pm 0.17^{a, A}$	$2.85 \pm 0.2^{a, A}$	$2.87 \pm 0.17^{a, A}$	$2.89 \pm 0.16^{a, A}$
	40	$2.72 \pm 0.17^{a, A}$	$2.85 \pm 0.2^{a, A}$	$2.87 \pm 0.17^{a, A}$	$2.89 \pm 0.16^{a, A}$
	60	$2.24 \pm 0.15^{a, B}$	$3 \pm 0.21^{b, A}$	$3.04 \pm 0.23^{b, A}$	$3.14 \pm 0.2^{b, A}$

[1] **Abbreviations: C = control group of frankfurters, without pollen; T1 = 0.5% pollen added in frankfurters; T2 = 1.0% pollen added in frankfurters; T3 = 1.5% pollen added in frankfurters; TBARS — Thiobarbituric acid reactive substances value.**

[2] **Values are displayed as arithmetic means ± standard errors of means (mean ± SEM). Values with different lowercase letters (a-b) in the same row differ significantly ($P < 0.05$). Values with different uppercase letters (A-C) in the same column differ significantly ($P < 0.05$).**

Throughout the period of storage, there was no statistically significant difference ($P > 0.05$) within treatments with the addition of pollen, while in the control group there was a significant ($P < 0.05$) decrease. Excellent emulsifying properties, oil and water absorption of pollen contribute to obtaining stable product throughout storage, especially when it comes to such a long period as two months are. Also, it is an indicator of the good stability of frankfurters with pollen added during storage. However, although there were no statistical differences, a slight tendency toward an increase in the shear work appears with the longer storage period in treatments with pollen. A similar trend in the increase of WBSF values in sausages reported [23], as well as [24], who explained that this trend was the consequence of the moisture loss and the mechanism that favors hardening of the sausages, such as the formation of cross-links in proteins. In our study, it could be explained with the additional value of proteins added via pollen and more cross-links formed throughout the storage period.

Sensorial traits of frankfurters

Pork flavor was defined as the amount of pork flavor identity. Major notes for pork flavor identity consist of roasted notes that round and fully aromatic and generally associated with pork fat that has been broiled. Bloody and serumy notes that are associated with blood of cooked meat and closely related to metallic aromatic are also present. Third major pork flavor note is liver-like and associated with cooked organ meat, mostly liver. Finally, pork flavor can be described with green-haylike notes (brown/green dusty aromatic associated with dry grasses, hay, dry parsley and tea leaves) and umami notes (flat, salty, somewhat brothy with the taste of glutamate, salts of amino acids and nucleotides) [25]. Unsurprisingly, frankfurter samples without the addition of pollen were perceived by the panelists as samples with the most intense pork flavor aroma (70.5), while the pork flavor intensity was only mild for T1 samples (58) and moderate for T2 and T3 samples (48.1) (Figure 2).

Floral aroma was described as taste and smell associated with different flowers [26]. We can conclude that with the addition of pollen into frankfurters they have obtained floral notes that have not been present in control samples (12). As much as 0.5% of pollen increased the sensation of floral aroma by two fold (25) in T1 samples and were moderately perceived in both T2 (44) and T3 (45) samples. The fact that floral notes reached a plateau with the addition of 1% of pol-

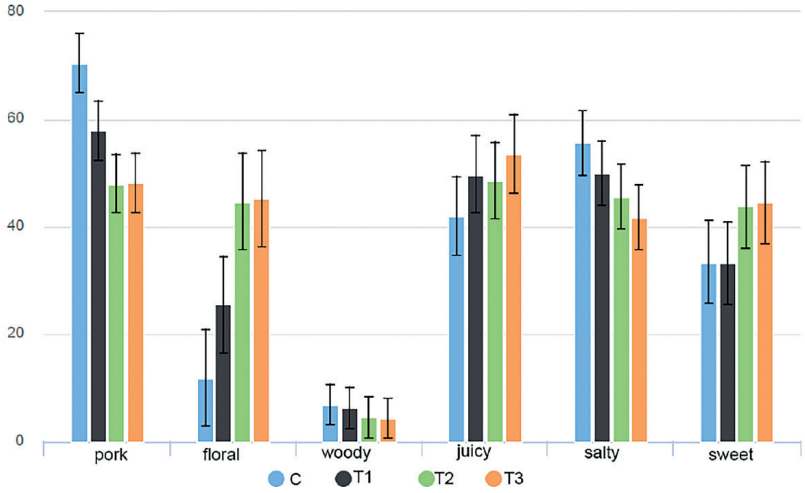

Figure 2. Sensorial traits of frankfurters

[1] Abbreviations: C = control group of frankfurters, without pollen; T1 = 0.5% pollen added in frankfurters; T2 = 1.0% pollen added in frankfurters; T3 = 1.5% pollen added in frankfurters.

len is actually a good thing since the actual "taste" of flowers can be off-putting because, at the end of the day, people don't eat flowers that often. Food that is flavored with floral notes usually push too far. Florals are most pleasing to the palate when they are well balanced and offset by thick rich texture of food like the one present in frankfurters.

Conclusion

The incorporation of pollen resulted in obtaining a stable product during two months of chilled storage, without any adverse effect regarding textural characteristics. Although excellent emulsifying properties of pollen were obtained, a certain limitation in terms of sensory properties occurs, especially due to obtaining specific notes that could provoke certain negative ratings from consumers. FTIR-ATR has shown a multi-component structure of pollen powder which provides good emulsifying properties to sausages and obtaining good consistency. All things considered, each pollen concentration could be used as a potential natural additive that contributes to obtaining a stable emulsion and consistent meat product during the two months of refrigerated storage.

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THE EFFECT OF HERBAL SUPPLEMENTS ON DEVELOPMENT OF INTERNAL ORGANS AND CHEMICAL COMPOSITION OF BROILERS MUSCLES

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Keywords: *phytobiotics, antibiotics, essential oils, safe growth stimulants, feed additive, meat characteristic*

Abstract

The present article presents data on effectiveness of adding a phytobiotic feed additive into the diet of broiler chickens, either additionally or replacing the feed antibiotic in the chicken fodder. It has been established that the introduction of a phytobiotic feed additive into the broilers' diet, both additionally and by replacing the feed antibiotic, provided positive effect on poultry meat quality and gave no negative effect on development of internal organs. By the end of fattening the relative weight of heart, lungs, kidneys, gizzard and intestines in broilers, which consumed the antibiotics-free diet with addition of researched preparation, was higher than in the control group and in the 1st experimental group. At the same time the length of the intestine in researched group significantly exceeded the control group parameters. These changes ranged within the physiological norm, which may indicate the best detoxification capabilities of the chicken body and the activation of enhanced intestinal absorption function. Additional use of the experimental feed additive in formulation of feed for broiler chickens was accompanied by a decrease in total amount of amino acids in broilers' pectoral and leg muscles within acceptable physiological limits. At the same time, a significant decrease, compared with the control parameters, was noted in relation to content of histidine and isoleucine in pectoral muscles, and content of proline in leg muscles. The use of the researched additive as a substitute for a feed antibiotic in composition of mixed feed for broilers decreased the total amount of amino acids in broilers' breast muscle, compared with the control group, and increased level of amino acids in leg muscles. The significant decrease in content of histidine and arginine in the pectoral muscles and proline in the leg muscles was noted. The observed changes varied within acceptable physiological norm. In the experimental groups the energy value of meat was increased.

Introduction

In the 70s-80s of the last century some specific mechanisms of development of microorganisms resistance to antibiotics were shown. This is a great concern of nowadays, and this issue requires an urgent solution. The possibility of using antibiotic agents in animal husbandry has been disputed for infinitely long. Sharpness of such discussions, as well conviction of each of the parties in their right opinion, does not change. However, the focus of scientific forces is gradually shifting: about 10–15 years ago only few people opposed antibiotic growth stimulants, and nowadays there are so many opponents to antibiotics, that they can no longer be ignored.

Antibiotics used for therapeutic purposes and for stimulation of young animals growth accumulate in significant amounts in food products — meat, milk, eggs. The free concentration of antibiotics for a short period of time is excreted from the animal's body with metabolic products — feces, urine, products (milk, eggs), but the antibiotics associated with proteins and other components remains in a body for a long time. Antibiotics, excreted from the body, get to the soil as part of organic fertilizers and after that accumulate in plants [1].

Low efficiency of antibiotics in poultry farming is noted by many experts. For example, employees of the Nizhny Novgorod Research Institute of Epidemiology and Microbiology n. a. Academician I. N. Blokhin note the fact that the difficult ecological situation, imbalance in nutrition contribute to the spread of intestinal infections in poultry farms: *salmonellosis*, *colibacillosis*, *listeriosis* [2]. At the same time deaths of broilers cause significant financial losses and decrease the productivity of the poultry farm. The use of antibiotics in this case is inefficient and environmentally harmful [3].

Antimicrobial preparations operate according to general pharmacological laws; despite their high specificity they are quite effective only under strict adherence to instructions. If the conditions are not met, antibiotic agents show little effect, and in some cases they can even cause harm.

Feed additives provide positive effect on a chicken's body, but are not always necessary for diet. Innovations constantly develop. Farmers keep their eye on the innovations and they are "mentally prepared" to apply them in their farms. It is not easy for everyone to move from a state of crisis and information vacuum to a rapidly developing and turbulent modern market space. However, despite the material difficulties, which are still an integral fact of modern Russian reality, it is clear that the future of livestock and poultry farming lies with new feed additives [4]. Today the Russian poultry industry is 4th largest in the world ranking for meat production and 6th largest in egg production [5].

All over the world there is a trend to increase the share of poultry meat in total volume of meat production [6,7], which is primarily explained by lower production costs

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and, accordingly, lower selling prices, in comparison with beef or pork. To ensure high rates of meat production it is necessary to use the latest advances in breeding, feeding, compliance with growing technology and veterinary protection of animals [8, 9].

Today, in the conditions of modern industrial poultry farming, one of the leading positions is occupied by problem of protection of animals' health with minimal use of antibacterial drugs [10, 11].

In connection with all of the above specified, since July 1, 1999 the EU has prohibited several conventional antibiotics, and in Denmark, Sweden and some other countries all antibiotics used as growth stimulants were prohibited [12].

The World Health Organization in April 2014 published a report, stating that "this serious threat is no longer just a prediction for the future, as it is already manifesting itself right now in every region of the world and can negatively affect everyone in every country, regardless of age. Antibiotic resistance is a peculiar phenomenon when bacteria change so much, that antibiotics no longer have any effect on body of people who need them to fight infection, and this is now one of the most serious threats to human health" [13, 14].

The World Health Organization has concluded that inappropriate use of antibiotics in animal husbandry is a major contributor to the emergence and distribution of antibiotic-resistant microorganisms, and it is necessary to limit the use of antibiotics as growth promoters in animal feed. The International Epizootic Office has added a set of guidelines to the World Veterinary Code with recommendations to run national surveillance and monitoring programs for antimicrobial resistance, thus controlling the amount of antibiotics used in animal husbandry. They also recommend strict compliance with appropriate use of antibiotic drugs in due dosage only. Another recommendation is the implementation of methodologies to help identify factors of associated risk and assess the risk of antibiotic resistance development [15].

In recent years the development of alternative antibiotics has significantly activated. The alternative antibiotics are assigned for maintenance or improvement the health and productive rate of poultry. Alternatively, probiotics, prebiotics, synbiotics, organic acids, enzymes, phytogenics, antimicrobial peptides, hyper immune antibodies to eggs, bacteriophages, clay and metals are offered as additive. Although the beneficial effects of many developed products have been clearly demonstrated, the experts agree that these products are not consistent with each other, and the results of their application vary greatly [16,17].

One of the effective and safe remedy are phytobiotic feed additives with extended sphere of action. The included ingredients should work in collaboration, complementing each other. The result of research proved essential oils, phytoextracts and protected organic acids to be the most efficient. The mechanism of action of complex drugs of this type is very simple. Essential oils weaken the bacterial cell wall. Weak cell wall causes cell lysis. Disruption of ATP synthesis leads to a weakening of the bacterial cell itself. Hydrogen ions are less exported, the cellular environment gets acidified, and bacterial metabolism is disrupted. The bacterium spends its energy for detoxing but not for reproduction. Thus a bacteriostatic effect is achieved. Further, organic acids are included in the feed, providing bactericidal effect. In general, phytoextracts and essential oils with an antibacterial effect prevent development of many intestinal infections, which seriously affects the safety and productivity of the poultry. In addition these compounds provide complex growth-stimulating effect on animal's body and, in addition to the antibacterial effect, they increase the attractiveness of feed (enhance feed palatability), have an anti-stress effect, and increase the secretion of saliva and digestive enzymes. The aim of the research is to assess the meat qualities, development of internal organs and chemical composition of broiler chickens meat when replacing feed antibiotics in their diet with a safe growth stimulant in form of a feed additive, including phytobiotics and protected organic acids.

Objects and methods

The experimental part of the research was run in LLC "Sredneuralskaya Poultry Farm" of Sverdlovsk region. The broiler chickens of the Ross 308 cross in 2019 were exposed to experiment.

The broilers groups were formed in accordance with recommended methodology of Federal Research Center "VNITIP" of RAS[1] (Table 1).

To assess the meat qualities of broilers 3 chick carcasses were anatomically cut at the end of the growing period, each chicken was taken from each experimental group. The development of the internal organs of broiler chickens was assessed during anatomical cutting at the age of 22 and 38 days.

In the breast and leg muscles of broilers, the following parameters were determined: amino acid composition including 17 amino acids (aspartic acid, glutamic acid, serine, histidine, glycine, threonine, alanine, arginine, tyrosine, cystine, valine, methionine, phenylalanine, isoleucine, leucine, lysine and proline); mass fraction of moisture, dry matter, protein, fat; amount of ash. Based on data on chemical composition of muscle tissue, the meat quality index (the ratio of fat and protein) and the energy value of meat were calculated.

The amino acid composition of meat was determined according to the SOP (standard operating procedure) "Determination of the amino acid composition by high performance liquid chromatography (HPLC) with precolumn derivatization with OPA and FMOC agents in food" at the V. M. Gorbatov VNIIMP test center on the device Agilent 1260 Infinity II. Dansyl chloride, phenyliso-thiacyanate, and other reagents were used for derivatization.

[1] Egorov, I.A., Manukyan, V.A., Lenkova, T.N. et al. (2013). Methods of conducting scientific and industrial research on poultry feeding. Sergiev Posad: VNITIP. 2013. — 52. ISBN: 978–5–91582–047–9

Table 1. Scheme of scientific and economic experiment

Group	Number, sex	Feeding
Control	♂ 80 ♀ 80	The conventional diet (CD) is a complete feed ration, with a nutritional value according to the recommendations for the chickens cross. Feed antibiotic was included in the CD: since the 1st to the 21st day — *Albacin*, dosage: 300 g / t of compound feed and since the 22nd to the 30th day *Nosiheptide* — 250 g / t of compound feed
1 experimental	♂ 80 ♀ 80	CD + researched additive in amount of 1 kg / t of compound feed. Period of use: from the 1st day until the end of fattening
2 experimental	♂ 80 ♀ 80	Experimental diet (ED): the feed antibiotic in CD has been replaced by the researched additive in amount of 1 kg / t of compound feed. Period of use: since the 1st day until the end of fattening.

Standard research methods were used to study the physical and chemical parameters of mass fraction of protein[2], fat[3], and the content of minerals (ash)[4].

The development of the internal organs of broiler chickens was assessed during the experiment at the age of 22 and 38 days.

The data obtained were statistically processed on a personal computer, Microsoft Excel editor, using the methods of biometric analysis according to N. A. Plokhinsky. The reliability of difference was established in relation to the control group using the Student's t-test, while determining three reliability thresholds: * $P \le 0.05$; ** $P \le 0.01$; *** $P \le 0.001$.

Results and discussion

The main parameters of poultry meat qualities are: the pre-slaughter live weight, the weight of the eviscerated carcass, the yield of the eviscerated carcass.

At 38 days in order to establish the effect of the studied feed additive on the meat quality of broilers, the average chicks for the group were selected for anatomical cutting. The live weight of selected broiler chickens in the control group was 2,130 g, in 1st experimental group it was 2,131.33 g, in 2nd experimental group it was significantly higher than in the control group by 5.13% ($P \le 0.01$) and reached 2,239.33 g (Table 2).

In terms of the weight of the bloodless carcass, the 2nd experimental group was in the lead with a value of 2,162.0 g, which is significantly more than the control by 5.15% ($P \le 0.01$). This parameter in chickens of the 1st experimental group was lower in comparison to the control group by 0.13%.

One of the most important poultry products is the eviscerated broiler chicken. It was found that replacing the feed antibiotic in the diet of chickens of the 2nd experimental group with the researched phytobiotic additive contributed to increase in weight of eviscerated carcass compared to the control group by 3.3%, thus amounting to 1,513.67 g. In the 1st experimental group this parameter was 1.5% lower than in the control group.

The slaughter yield of eviscerated carcasses in the group of chickens that instead of a feed antibiotic received an additive, including phytoextracts, essential oils and protected organic acids, was slightly lower in comparison to the control group — by 1.2%. This circumstance is associated with a higher mass of internal organs in poultry of this experimental group, in particular: the mass of heart, lungs, kidneys, gizzard, intestines and spleen. These changes remained within physiological norms. The slaughter yield of eviscerated carcasses in broilers of the 1st experimental group was 1.1% lower than the control group parameters.

In composition of a carcass the amount of meat, bones and skin was analyzed, and the meat-and-bone index was calculated.

The total amount of meat in carcass of chickens in the control group was 1,146.7 g, which is 3.9% more than in the chicken of the 1st experimental group. The chickens of the 2nd experimental group featured the highest value of this parameter — 1,163 g, which exceeded the control group by 1.4%. In percentage terms, i. e. by the weight of muscle tissue apart from the carcass weight, the greatest value — 78.3% was reached in the control group, in 1st and 2nd experimental groups this ratio was less than in the control group by 1.9 and 1.4%, respectively.

The bone tissue in the carcass of chickens from the control group was 122.62 g, and relative to the weight of the entire carcass of chickens in this group, the level of bone mass was 8.4%. Chickens of the 1st experimental group had a lower bone mass than in the control group by 5.1%, while the relative value of the chicken carcass weight was 0.3% less in comparison with the control group. The cockerels of the 2nd experimental group had the greatest value in terms of bone mass, higher than similar parameter of the control group by 2.9%, while in terms of the relative bones weight to overall carcass weight this value was less than the control by 0.1%.

The amount of skin in the carcass of chickens of the 1st and 2nd experimental groups exceeded the control value by 9.4 and 29.3%, respectively.

To assess the meat qualities of the carcass, the meat-bone index (the ratio of muscles weight to bones weight) was calculated. In the control group this parameter was 9.4, in the 1st experimental group it was higher than in the control by 0.1 units, and in the 2nd experimental group it was lower by 0.2 units.

[2] GOST 25011–2017 "Meat and meat products. Protein determination methods". Moscow: Standartinform, 2018. — 14 p. (In Russian)

[3] GOST 23042–2015 "Meat and meat products. Methods of fat determination". Moscow: Standartinform, 2019. — 8 p. (In Russian)

[4] GOST 31727–2012 "Meat and meat products. Determination of total ash". Moscow: Standartinform, 2019. — 12 p. (In Russian)

Table 2. Results of anatomical butchering of chickens (M ± m), (n = 3)

Показатель	Groups		
	Control	1st experimental	2nd experimental
Live weight, g	2,130.0 ± 11.02	2,131.33 ± 2.91	2,239.33 ± 5.9 **
Weight of a bloodless carcass, g	2,056.0 ± 12.7	2,053.33 ± 5.21	2,162.0 ± 3.06 **
Eviscerated carcass weight, g	1,465.33 ± 14.62	1,443.33 ± 15.76	1,513.67 ± 9.28
Slaughter yield of eviscerated carcass, %	68.8	67.7	67.6
Total composition of the chicken carcass:			
Muscles, g	1,146.72 ± 15.10	1,102.01 ± 7.65	1,163.22 ± 12.25
% of the carcass weight	78.3	76.4	76.9
Bones, g	122,62 ± 17.93	116,40 ± 11.37	126,12 ± 16.86
% of carcass weight	8.4	8.1	8.3
Skin, g	136,10 ± 6.02	148,95 ± 12.27	175,97 ± 14.41
% of carcass weight	9.3	10.3	11.6
Bone and meat index	9.4	9.5	9.2

The results of anatomical cutting and further deboning of the chicken carcass allowed determining the development of its specific and the most valuable parts of chicken exposed to effect of the researched feed factor (Table 3).

The meat of the broiler chicken breast features special nutritional value. The total weight of the breast in the control group slightly exceeded the weight of breast of the 1st and 2nd experimental groups by 1.7 and 1.39%, respectively. The ratio of breast weight to eviscerated carcass weight was higher by 0.1 and 1.7% in the control group in comparison with this ratio of the 1st and 2nd experimental groups. The bony part of the breast in 1st and 2nd experimental groups amounted to 24.9 and 26.2 g respectively, and exceeded the weight of the control group value by 29 and 35.7%, respectively. Due to the higher content of bones in the chickens breast from the 1st and 2nd experimental groups, the meat-to-bone index (the ratio of meat to bone weight) was lower than the control by 6.5 and 7.4 units, respectively.

The 2nd experimental group (33.3 g) took in first place in breast skin yield, while the control group showed lower breast skin yield by 6.9% (31 g), in the 1st experimental group this parameter was 1.29% higher than in the control group.

According to the results of deboning of leg quarter, it was found that the greatest total weight of the leg quarter was recorded in the chickens of the 2nd experimental group — 146.67 g, which was 8.91% higher than the control value. In the 1st experimental group 1, there was a significant decrease in the weight of the leg quarter relative to the control by 15.8% ($P \leq 0.05$), amounting to 113.33 g. The ratio of the weight of the leg quarter to the weight of the eviscerated carcass was also higher in the 2nd experimental group, exceeding the control group by 0.5%. The weight of muscle tissue in the leg quarter of broiler chickens of the 2nd experimental group exceeded the control value by 3.5%, and in the 1st experimental group, this parameter was significantly decreased in comparison with the control group by 17.1% ($P \leq 0.05$). In relation to the weight of muscle tissue in leg quarter to the mass of the eviscerated carcass, the lowest value was recorded in the 1st experimental group — 6.4 units, which is 1.2% less than in the control group. This

Table 3. Results of the deboning of specific parts of carcass, (M ± m), (n = 3)

Parameter	Groups		
	Control	1st experimental	2nd experimental
Breast			
Total weight, g	557.33 ± 13.49	548.0 ± 14.0	549.67 ± 8.76
% of eviscerated carcass	38.0	37.9	36.3
muscles, g	500.0 ± 16.29	482.0 ± 17.78	484.67 ± 11.62
% of eviscerated carcass	34.10	33.4	32.0
bones, g	19.3 ± 0.66	24.9 ± 2.56	26.2 ± 2.6
meat and bones index	25.9	19.4	18.5
skin, g	31 ± 1.7	31.4 ± 1.4	33.3 ± 2.65
Quarter			
Total weight, g	134.67 ± 3.71	113.33 ± 2.4*	146.67 ± 5.21
% of eviscerated carcass	9.2	7.8	9.7
muscles, g	112.0 ± 3.46	92.82 ± 3.0*	116.0 ± 3.46
% of eviscerated carcass	7.6	6.4	7.7
bones, g	8.44 ± 0.19	8.1 ± 0.2	9.2 ± 0.84
meat and bones index	13.3	11.5	12.6
skin, g	15.5 ± 4.14	11.2 ± 2.3	18.95 ± 0.9
Drumstick			
Total weight, g	102.67 ± 1.76	103.33 ± 2.67	102.0 ± 3.06
% of eviscerated carcass	7.0	7.1	6.7
muscles, g	83.93 ± 3.23	79.54 ± 3.31	81.01 ± 2.71
% of eviscerated carcass	5.7	5.5	5.3
bones, g	12.45 ± 1	11.3 ± 0.6	10.9 ± 0.5
meat and bones index	6.7	7.0	7.4
skin, g	4.9 ± 0.34	0.3 ± 2.1	8.84 ± 3.1
Wing			
Total weight, g	152.00 ± 2.00	152.00 ± 4.16	157.33 ± 1.76
% of eviscerated carcass	10.37	10.53	10.39
muscles, g	55.6 ± 1.2	55.0 ± 2.2	55.9 ± 1.1
% of eviscerated carcass	3.8	3.8	3.7
bones, g	10.3 ± 0.25	9.8 ± 0.26	9.9 ± 0.7
meat and bones index	5.4	5.6	5.6
skin, g	9.65 ± 0.25	16.1 ± 6.7	10.6 ± 0.46
Bones structure			
Total weight, g	268.67 ± 13.33	269.33 ± 12.77	304.67 ± 11.57
% of eviscerated carcass	18.33	18.66	20.13
muscles, g	143.67 ± 1.45	165.33 ± 7.69	172.67 ± 7.69*
% of eviscerated carcass	9.80	11.45	11.41
bones, g	61.7 ± 8.8	52.9 ± 4.1	59.0 ± 2.6
meat and bones index	2.3	3.1	2.9
skin, g	45. 0 ± 6.8	42.3 ± 9.8	65.9 ± 7.35

parameter in the 2nd experimental group exceeded the control group by 0.1%. The control group showed the highest meat-bone index of the leg quarter — 13.3 points, which is 1.8 and 0.7 points higher than in the 1st and the 2nd experimental groups, respectively.

The analysis of the drumstick deboning showed no significant differences between the groups. The percentage of the eviscerated carcass weight for leg muscles was higher in the control group, accounting to 5.7 units. Despite the fact that in the 1st experimental group this parameter was lower than the control value by 0.2%, the total weight of the drumstick exceeded the control value by 0.64%.

The analysis of anatomical cutting of the wing showed that the highest total weight of the wing was achieved in the 2nd experimental group of chickens, amounting to 157.33 g, thus exceeding the control group and the 1st experimental group by 3.5%. The wings of broilers of the 1st experimental group featured the highest skin weight — 16.1 g, which exceeded the same parameter of chicken peers in the control group and in the 2nd experimental group by 66.8 and 51.8%.

The analysis of the anatomical cutting of the chicken frame showed that the highest weight of muscles was recorded in the 2nd experimental group — 172.7 g, which is significantly higher than in the control group by 20.1% ($P \leq 0.05$) and 4.47% higher than in the 1st experimental group. The number of skeleton bones was lower in the 1st and the 2nd experimental groups compared to the control by 14.2 and 4.4%, respectively. In terms of skin weight of the frame, the 2nd experimental group was in the lead, exceeding the control group by 46.4%, and the 1st experimental group by 55.6%. The meat and bone index was higher in the 1st experimental group, amounting to 3.1 points, which is 0.8 points higher than the control value.

The results of analysis of the amino acid composition in the breast and leg muscles of broiler chickens are presented below in Tables 4 and 5.

Table 4. Amino acid composition of the breast muscle of broiler chickens

Amino acids, mg / 100 g sample	Control group	1st experimental group	2nd experimental group
Aspartic acid	1,701.33 ± 118.38	1,648.00 ± 65.57	1,629.67 ± 116.65
Glutamic acid	3,963.33 ± 464.16	3,633.00 ± 181.24	3,576.67 ± 373.37
Serine	1,084.00 ± 42.59	858.00 ± 68.77	906.67 ± 129.14
Histidine	1,063.00 ± 124.50	972.00 ± 47.43*	714.33 ± 46.6*
Glycine	995.00 ± 95.77	942.33 ± 35.75	876.33 ± 96.56
Threonine	1,204.33 ± 62.86	1,070.67 ± 70.43	991.00 ± 94.52
Arginine	2,100.33 ± 132.63	1,731.00 ± 346.20	1,280.00 ± 149.84**
Alanin	1,239.00 ± 116.98	1,151.67 ± 8.67	1,133.00 ± 147.55
Tyrosine	779.33 ± 44.13	634.00 ± 47.88	658.00 ± 57.73
Cystine	227.00 ± 17.62	208.33 ± 47.56	184.67 ± 12.60
Valine	958.67 ± 111.79	962.67 ± 63.05	822.00 ± 32.05
Methionine	551.67 ± 32.20	577.00 ± 48.42	533.67 ± 38.35
Phenylalanine	830.00 ± 74.67	774.33 ± 18.85	721.67 ± 91.48
Isoleucine	1,213.00 ± 29.50	1,103.33 ± 14.19*	1,010.67 ± 90.34
Leucine	1,602.67 ± 153.53	1,560.67 ± 44.30	1,133.00 ± 271.93
Lysine	3,841.67 ± 117.14	3,715.33 ± 119.88	3,318.67 ± 309.71
Proline	274.33 ± 42.06	291.67 ± 27.23	333.00 ± 27.02
Total amount of amino acids	23,628.00 ± 1,562.34	21,834.00 ± 503.95	19,822.33 ± 1,818.29

Table 5. Composition of amino acids in the leg muscles of broiler chickens

Amino acids, mg / 100 g sample	Control group	1st experimental group	2nd experimental group
Aspartic acid	1,335.33±110.60	1,323.33±17.29	1,422.00±79.68
Glutamic acid	3,057.67±386.61	3,027.67±95.84	3,844.67±99.42
Serine	762.33±100.76	727.00±18.01	935.33±48.08
Histidine	767.00±75.41	711.00±40.51	754.00±61.26
Glycine	836.33±72.79	785.33±17.48	896.33±55.81
Threonine	829.67±95.62	719.67±28.83	969.00±22.30
Arginine	1,440.33±102.57	1,313.33±15.62	1,492.33±68.34
Alanin	1,055.33±101.99	989.67±31.83	1,118.33±43.54
Tyrosine	645.00±42.04	591.33±21.17	663.33±25.26
Cystine	135.33±20.99	125.67±8.69	162.67±14.66
Valine	826.33±52.04	739.00±37.75	882.67±50.13
Methionine	495.67±69.66	391.00±46.32	472.33±53.45
Phenylalanine	745.33±34.71	692.00±26.65	670.00±23.59
Isoleucine	823.33±106.95	816.33±19.55	994.33±52.06
Leucine	1371.67±22.00	1,256.67±56.22	1391.00±67.73
Lysine	2,295.67±450.76	1,956.67±263.61	3,110.67±142.69
Proline	383.00±11.79	357.33±34.07	284.33±9.17**
Total amount of amino acids	17,810.00±1,733.02	16,524.33±389.28	20,064.00±626.45

Based on the research it was found that the total amount of amino acids in the pectoral muscles of chickens that in addition to the main diet received a feed additive containing phytobiotics and protected organic acids, and in broilers whose diet included the researched additive instead of the feed antibiotic this value was 7.6% and 16.1% lower than the control value respectively. As for the amount of some individual amino acids in the pectoral muscles of chickens of the 1st and 2nd experimental groups, some amino acids showed decrease in comparison with the control group: aspartic acid — by 3.13% and 4.2%, glutamic acid — by 8.3% and 9.8%, serine — by 20,8% and 16.4%, histidine — by 8.6% ($P \leq 0.05$) and 32.8% ($P \leq 0.05$), glycine — by 5.3% and 11.9%, threonine — by 11.1% and 17.7%, arginine — by 17.6% and 39.0% ($P \leq 0.01$), alanine — by 7.0% and 8.6%, tyrosine — by 18.6% and 15.6%, cystine — by 8.2% and 18.6%, phenylalanine — by 6.7% and 13.1%, isoleucine — by 9.0% ($P \leq 0.05$) and 16.7%, leucine — by 2.6% and 29.3%, lysine — by 3.3% and 13.6%, respectively. With regard to the content of valine and methionine, their increase in pectoral muscle of chickens of the 1st experimental group was recorded by 0.4% and 4.6%, and decrease among the broilers of the 2nd experimental group by 14.3% and 3.3%, respectively. The amount of proline in the breast of chickens of the 1st and 2nd experimental groups exceeded the control group by 6.3% and 21.4%, respectively.

The total amount of amino acids in the leg muscles of chickens, which received the researched substance based on phytobiotics and protected organic acids added to the conventional diet, was 7.2% lower than in the control group. The introduction of the researched feed additive instead of the feed antibiotic increased the amount of amino acids by 12.7%.

The following changes were observed in content of some individual amino acids in the leg muscles of chickens exposed to the experiment. The broilers of the 1st experimental group featured the decrease in all analyzed amino acids in comparison with the level of the control group: aspartic acid — by 0.9%, glutamic acid — by 0.98%, serine — by 4.6%, histidine — by 7.3%, glycine — by 6.1%, threonine — by 13.3%, arginine — by 8.8%, alanine — by 6.2%, tyrosine — by 8.3%, cystine — by 7.1%, valine — by 10.6%, methionine — by 21.1%, phenylalanine — by 7.2%, isoleucine — by 0.85%, leucine — by 8.38%, lysine — by 14.8%, proline — by 6.7%. Among the broilers of the 2nd experimental group, an increase in their level in the leg muscle was noted for most of the amino acids in comparison with the control group: aspartic acid — by 65%, glutamic acid — by 25.7%, serine — by 22.7%, glycine — by 7.2%, threonine — by 16.8%, arginine — by 3.6%, alanine — by 5.97%, tyrosine — by 2.8%, cystine — by 20.2%, valine — by 6.8%, isoleucine — by 20.8%, leucine — by 1.4%, lysine — by 35.5%. The content of histidine, methionine, phenylalanine and proline in the leg muscles of the chickens of the 2nd experimental group was lower than the control group by 1.7%; 4.7%; 10.1% and 25.8% ($P \leq 0.01$).

Analysis of the chemical composition of the pectoral muscles in broiler chickens (Table 6) proved that the moisture content in the meat of the control group was 76.1%, in the 1st and 2nd experimental groups this parameter was lower in comparison with the control group by 2.7% ($P \leq 0.05$) and 1.3%, respectively.

Table 6. The chemical composition of the pectoral muscles of broilers, % (*M ± m*), (*n = 3*)

Parameter	Groups		
	Control	1st experimental group	2nd experimental group
Total moisture	76.1 ± 0.49	73.4 ± 0.21*	74.8 ± 0.3
Dry matter	23.9 ± 0.49	25.6 ± 0.206*	25.2 ± 0.27
Protein	20.12 ± 0.452	19.17 ± 0.483	16.64 ± 1.903
Fat	2.91 ± 0.37	5.76 ± 0.59*	7.92 ± 0.24**
Ash	1.13 ± 0.017	1.02 ± 0.015*	0.91 ± 0.103
Meat quality index (fat / protein)	0.14	0.30	0.47
Energy value, kJ / 100 g	446.0 ± 14.6	537.3 ± 14.0*	576.2 ± 12.3**

Dry matter content in the chickens pectoral muscles within the control group was 23.9%, the chickens of the 1st experimental group featured significantly higher value than the control group — by 1.7% higher ($P \leq 0.05$), and the broilers of the 2nd experimental group showed higher dry matter content by 1, 3% than in the control group.

The protein content in the muscle tissue of breast of chickens in the control group was 20.12%, in the 1st and 2nd experimental groups this parameter was lower than in the control group by 0.95 and 3.48%.

Fat content in the muscle tissue of the broilers of the 2nd experimental group was the highest — 7.92%, exceeding the control group value by 5.01% ($P \leq 0.001$). In the 1st experimental group the fat content was higher than the control level by 2.85% ($P \leq 0.05$) and amounted to 5.76%.

Crude ash content was the highest in the control group — 1.13%, which value is higher than this parameter in the 1st experimental group by 0.11% ($P \leq 0.05$). In the 2nd experimental group the amount of crude ash was minimal — 0.91%, which was 0.22% less than this parameter in the control group.

Due to the lower content of fat in the control group, the energy value of meat was the smallest and amounted to 446 KJ in 100 g. In the 1st experimental group the energy value in 100 g of breast muscle tissue was 537.3 KJ, which is 20.5% more than in the control group ($P \leq 0.05$). The highest energy value was recorded in the pectoral muscles of the chickens of the 2nd experimental group — 576.2 KJ, which exceeded the control value by 29.2% ($P \leq 0.01$).

To characterize the quality of meat and meat products, the fat / protein ratio or meat quality index (MQI) is used. In the control group the MQI was 0.14; in the 1st and 2nd experimental groups this value reached 0.3 and 0.47, respectively.

The data on the chemical composition of the leg muscles are presented below in Table 7.

The moisture content in the broilers leg muscles in the 1st and 2nd experimental groups was lower in comparison with the control group by 1.3 and 3.46%, respectively. On the contrary the amount of dry matter was higher in the 1st and 2nd experimental groups than in the control group by 1.3% and 3.46%, respectively.

Table 7. The chemical composition of the broilers leg muscles, % (*M ± m), (n = 3)*

Parameters	Groups		
	Control	1st experimental group	2nd experimental group
Total moisture	75.5 ± 0.32	74.2 ± 0.93	72.04 ± 0.31**
Dry matter	24.5 ± 0.32	25.8 ± 0.42	27.96 ± 1.31
Protein	10.49 ± 1.02	9.4 ± 0.087	6.47 ± 1.58
Fat	13.58 ± 1.28	15.88 ± 0.59	21.35 ± 2.69
Ash	0.62 ± 0.053	0.53 ± 0.0057	0.41 ± 0.95
Meat quality index (W / W)	1.30	1.69	3.30
Energy value, kJ / 100 g	687.2 ± 18.2	754.6 ± 21.5	911.5 ± 16.9**

Protein content in the leg muscles was the highest in the control group — 10.49%, which exceeded the same parameter in the 1st experimental group by 1.09%, and by 4.02% in the 2nd experimental group. The highest percentage of ash was observed in the leg muscles of the chickens in the control group — 0.62%, this value exceeded the value in the 1st experimental group by 0.09% and by 0.21% in the 2nd experimental group.

The analysis of fat amount in the leg muscles showed the increase of fat in broiler chickens of the 1st and 2nd experimental groups, compared with the control group by 2.3 and 7.77%, respectively.

During estimation of the energy value of the leg muscles, a natural relation of energy value with fat content was traced. The energy values were increased in broilers in the experimental groups. So, in the control group 100 g of the leg muscles contained 687.2 KJ, in the 1st experimental group — 754.6 KJ, in the 2nd experimental group this value reached 911.5 KJ ($P \leq 0.01$).

The meat quality index of the control group was equal to 1.3, in the 1st and 2nd experimental groups it was 1.69 and 3.3 units, respectively.

At the age of 22 days three broiler cockerels of average live weight were selected from each experimental group in order to measure the mass of some internal organs (Table 8). According to the weight of the liver, the 2nd experimental group exceeded the other compared groups, its prevalence over the control and the 1st experimental groups was 8.24% and 17.9%, respectively. The weight of broilers liver from the 1st experimental group was 1.6 g lower than the liver of chickens from the control group.

The relative weight of the kidneys and heart in the compared groups did not differ significantly and varied within 0.72–0.78% and 0.53–0.58%, respectively. The undoubted leaders in intestinal weight were broiler chickens of the 1st experimental group, outdoing the control group by 15.6%, and the 2nd experimental group by 30%. This trend was also peculiar for relative weight of the intestine and its length.

Thus, in terms of the relative intestinal weight, the 1st experimental group exceeded the control value by 1.59%, and the 2nd experimental group by 3.31%. By the length of the intestine, the 2nd experimental group was significantly lower than the control by 10.3% ($P \leq 0.05$) and less than the value of the 1st experimental group by 10.8%.

Table 8. The mass of the internal organs of broiler chickens at the age of 22 days (M ± m), (n = 3)

Parameter, UOM	Groups		
	Control	1st experimental group	2nd experimental group
Live weight, g	765.33 ± 2.91	751.33 ± 1.76*	841.0 ± 1.53***
Liver weight, %	19.52 ± 0.11	17.92 ± 1.13	21.13 ± 1.62
Relative liver weight, %	2.55	2.38	2.51
Kidney weight, g	5.72 ± 0.2	5.9 ± 0.46	6.09 ± 0.22
Relative kidney mass, %	0.75	0.78	0.72
Heart weight, g	4.46 ± 0.26	4.01 ± 0.23	4.81 ± 0.28
Relative heart weight, %	0.58	0.53	0.57
Intestine weight, g	68.62 ± 5.05	79.33 ± 4.1	61.0 ± 2.53
Relative intestinal weight, %	8.97	10.56	7.25
Intestine length, cm	184.0 ± 5.75	185.0 ± 6.21	165.0 ± 1.0*
Weight of the Fabritius bursa, g	1.7 ± 0.46	1.66 ± 0.08	1.88 ± 0.06
Relative weight of Fabritius bursa, %	0.22	0.22	0.22
Spleen weight, g	0.88 ± 0.04	0.71 ± 0.02*	1.05 ± 0.15
Relative weight of the spleen, %	0.11	0.09	0.12
Gallbladder weight, g	0.6 ± 0.13	1.18 ± 0.27	0.94 ± 0.13
The relative weight of the gallbladder, %	0.08	0.16	0.12
Stomach weight with fat without cuticle, g	15.8 ± 0.88	15.26 ± 1.59	17.39 ± 2.42
Relative weight of the stomach with fat without cuticle, %	2.06	2.03	2.07
Weight of the glandular stomach, g	5.78 ± 0.29	4.67 ± 0.3	5.02 ± 0.15
Relative weight of the glandular stomach, %	0.75	0.62	0.6

When assessing the relative weight of the Fabritius bursa, no difference was found between the groups: the values were the same in all groups and were equal to 0.22%.

There was a significant decrease in the spleen weight in the 1st experimental group in comparison with the control group by 19.3% ($P \leq 0.05$). On the contrary, in the 2nd experimental group, this value was 19.3% higher than in the control group. The relative weight of spleen in the control group, the 1st and 2nd experimental groups was 0.11; 0.09 and 0.12%, respectively.

The weight of the gallbladder in the 1st experimental group of 22-days-old broilers was the highest and reached 1.18 g, which is higher than the control group and the 2nd experimental group by 49.15% and 20.3%, respectively. The relative weight of the gallbladder was also higher in the 1st experimental group — 0.16%, this value exceeded value of the control group by 0.08% and the value of the 2nd experimental group by 0.04%.

The relative weight of the stomach with fat without cuticle in the chickens of the experimental groups did not differ significantly and ranged within 2.03 to 2.07%.

The relative weight of the glandular stomach was the highest in the control group — 0.75% and exceeded the value of the 1st and 2nd experimental groups by an average of 0.14%.

The weight of the examined internal organs of broiler chickens at the age of 38 days complied with the physiological norms, while the following differences were noted between the groups (Table 9).

The relative heart weight in the compared groups varied within the range of 0.50–0.54%. The relative lung weight was higher among the broilers of the 2nd experimental group — 0.56%, in the control group this parameter was 0.51%, in the 1st experimental group — 0.48%. The relative weight of the kidneys in the 1st and 2nd experimental groups was increased in relation to the control group by 0.11 and 0.15%.

The relative mass of the muscular stomach was higher in the chickens of the 2nd experimental group, amounting to 2.19%, which is 0.45% higher than in the control. In chickens of the 1st experimental group, this parameter was higher than the control value by 0.15%. The relative weight of the liver in the chickens of the experimental groups was within the range of 2.27–2.4%, the gallbladder weight varied within 0.08–0.11%.

As for the weight of intestine the 2nd experimental group showed significantly higher value than the control group value by 30% ($P \leq 0.05$), while the relative weight of intestine exceeded the control by 1.12%. In the 1st experimental group this parameter also exceeded the control group value in absolute weight and relative weight, respectively, by 8.1 and 0.38%.

Along with an increase in the absolute and relative intestinal weight in chickens of the 2nd experimental group, the increase in intestinal length was also observed. Intestine was significantly longer than in control group by 12.3% ($P \leq 0.05$).

The weight of the spleen was the greatest among the chickens of the 2nd experimental group, accounting to 3.09 g, which exceeded the control value by 0.39 g. In the 1st experimental group this parameter was lower in comparison with the control group by 0.24 g. The relative weight of this organ among the experimental groups of broilers varied within the range 0.115–0.138%.

The weight of the Fabritius bursa in the chickens of the experimental groups varied within the range of 0.045–0.055%.

Table 9. The weight of the internal organs of broiler chickens at the age of 38 days (M ± m), (n = 3)

Organ	Groupы		
	Control	1st experimental group	2nd experimental group
Heart mass, g	11.15 ± 0.27	10.67 ± 0.67	12.03 ± 0.33
Relative heart mass, %	0.52	0.50	0.54
Lung weight, g	11.02 ± 0.3	10.19 ± 1.0	12.54 ± 1.32
Relative lung mass, %	0.51	0.48	0.56
Kidney weight, g	12.15 ± 1.77	14.49 ± 2.12	16.07 ± 0.74
Relative kidney mass, %	0.57	0.68	0.72
Mass of the muscular stomach (with fat), g	37.13 ± 2.17	40.37 ± 2.25	49.12 ± 6.09
The relative mass of the muscular stomach, %	1.74	1.89	2.19
Liver weight, g	51.22 ± 1.12	49.09 ± 2.94	50.93 ± 2.26
Relative liver weight, %	2.4	2.3	2.27
Gallbladder weight, g	2.37 ± 0.15	1.7 ± 0.26	2.18 ± 0.12
The relative weight of the gallbladder, %	0.11	0.08	0.1
Intestine weight, g	100.44 ± 6.02	108.62 ± 7.02	130.67 ± 6.67*
Relative intestinal weight, %	4.71	5.09	5.83
Intestine length, cm	217.0 ± 4.9	208.33 ± 6.35	243.67 ± 7.13*
Spleen weight, g	2.7±0.43	2.46±0.27	3.09±0.78
Relative weight of the spleen, %	0.13	0.115	0.138
Weight of the Fabritius bursa, g	1.17±0.38	0.96±0.08	1.02±0.24
Relative weight of Fabritius bursa, %	0.055	0.045	0.046

Conclusion

The introduction of a phytobiotic feed additive into the diet of broiler chickens, both additionally and a way of replacing the feed antibiotic, provides positive effect on the meat qualities of chickens and does not negatively affect the development of internal organs. It was noted that in broilers who received antibiotics-free compound feed completed with phytoextracts, essential oils and protected organic acids, by the end of feeding those broilers showed higher relative weight of the heart, lungs, kidneys, muscle stomach, intestines than in the control group and in the 1st experimental group. At the same time the length of the intestine significantly exceeded the control group value. These changes varies within the physiological norm, which may contribute to the best detoxification capabilities of the chicken body and enhanced activation of the intestinal absorption function. During the research, the amino acid composition was studied, including 17 amino acids. It has been established that the additional use of a feed additive, including essential oils, hot pepper extract and protected organic acids in formulation of compound feed for broiler chickens, feature decrease in the total amount of amino acids in the breast and leg muscles of broiler chickens within acceptable physiological limits. At the same time, a significant decrease, compared with the control group, was noted for histidine and isoleucine in the pectoral muscle, and proline in the leg muscle.

The introduction of the tested additive to chickens mixed feed as a substitute for a feed antibiotic was characterized by a decrease in the total amount of amino acids in the breast muscle of broilers, compared with the control, and an increase in their level in the leg muscle. A significant decrease in the content of histidine and arginine in the pectoral muscle and proline in the leg muscle was noted. The observed changes varied within the physiological norm.

As for the chemical composition of the pectoral muscles and leg muscles, in the course of the research we noted an increase in mass fraction of moisture in chickens of the 1st and 2nd experimental groups. The mass fraction of fat in the pectoral muscles of broiler chickens and in the leg muscles of the 2nd experimental group exceeded the control values. At the same time, an increase in the energy value of meat was observed in both experimental groups.

The results obtained in research on the amino acid composition of the breast muscles and leg muscles of broiler chickens can be used in industrial practice to optimize the introduction of synthetic amino acids into the diet of broiler chickens simultaneously with application of alternative safe growth stimulants instead of feed antibiotics.

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METHODS FOR NONPARAMETRIC STATISTICS IN SCIENTIFIC RESEARCH. OVERVIEW. PART 1

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Keywords: *nonparametric statistics, Rosenbaum Q-test, Mann-Whitney U-test, sign G-test, Wilcoxon T-test*

Abstract

Daily, researcher faces the need to compare two or more observation groups obtained under different conditions in order to confirm or argue against a scientific hypothesis. At this stage, it is necessary to choose the right method for statistical analysis. If the statistical prerequisites are not met, it is advisable to choose nonparametric analysis. Statistical analysis consists of two stages: estimating model parameters and testing statistical hypotheses. After that, the interpretation of the mathematical processing results in the context of the research object is mandatory. The article provides an overview of two groups of nonparametric tests: 1) to identify differences in indicator distribution; 2) to assess shift reliability in the values of the studied indicator. The first group includes: 1) Rosenbaum Q-test, which is used to assess the differences by the level of any quantified indicator between two unrelated samplings; 2) Mann-Whitney U-test, which is required to test the statistical homogeneity hypothesis of two unrelated samplings, i. e. to assess the differences by the level of any quantified indicator between two samplings. The second group includes sign G-test and Wilcoxon T-test intended to determine the shift reliability of the related samplings, for example, when measuring the indicator in the same group of subjects before and after some exposure. Examples are given; step-by-step application of each test is described. The first part of the article describes simple nonparametric methods. The second part describes nonparametric tests for testing hypotheses of distribution type (Pearson's chi-squared test, Kolmogorov test) and nonparametric tests for testing hypotheses of sampling homogeneity (Pearson's chi-squared test for testing sampling homogeneity, Kolmogorov-Smirnov test).

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Introduction

The starting point for state-of-the-art statistical methods development is 1900. In 1901, the English mathematician, statistician, biologist and philosopher Karl Pearson, together with Francis Galton and Walter Weldon, founded the Biometrika journal (https://academic.oup.com/biomet) to promote the introduction of mathematical methods in biology. In 1925, Karl Pearson created another journal, Annals of Human Genetics (https://onlinelibrary.wiley.com/journal/14691809) dedicated to human genetics.

First third of the twentieth century passed under the sign of parametric statistics, which is defined as a section of statistics "assuming that the sampling belongs to the general population, which may be sufficiently accurately and adequately modeled by a probability distribution with specified set of parameters" [1]. Its main object is sampling from distributions described by one or several parameters. Methods based on the data analysis from parametric distributions described by the Pearson curves were studied. The normal distribution (Gaussian distribution) was the most popular one. Exponential and logarithmic normal distributions, Weibull-Gnedenko distributions, gamma distributions, binomial and hypergeometric distributions, Poisson distributions, etc. were used. Pearson test, Student test, and Fisher test were used to test hypotheses. The method of maximum likelihood and analysis of variance were proposed, and the main ideas for experiment design were stated [1–7].

Simultaneously with parametric statistics, in the works of Charles Edward Spearman and Maurice George Kendall, the first nonparametric methods based on rank correlation coefficients were introduced. Now the above methods are named after these statisticians. But nonparametric statistics became a noticeable part of statistics only since the second third of the twentieth century. Originally, nonparametric methods were designed to test statistical hypotheses (about one-dimensional probability distributions). The most famous nonparametric tests are Kolmogorov-Smirnov tests developed in 1930s, Wilcoxon rank tests and the Mann-Whitney U-test developed in 1940s-1950s, and, of course, rank correlation coefficients (1904–1930) by C. Spearman and M. Kendall [8–24].

Nonparametric tests are tests that do not include distribution parameters in the calculation formula and do not suppose knowledge of the distribution function. These tests use frequencies or ranks. They are used when comparing samplings with nominal and serial indicators, as well as for samplings measured on quantitative scales for which the distribution law is unknown or differs from normal one.

A feature of the nonparametric methods is that the probability distribution is considered completely unknown, and the problems are stated only in terms of dif-

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ferences between classes or within classes of unknown distributions.

If an alternative hypothesis contains a statement about the expected direction of differences, then such a hypothesis is called "directional", otherwise the hypothesis is called "non-directional".

The level of statistical significance or critical values of tests are determined differently when testing directional and non-directional hypotheses.

When testing the directional hypothesis, one-sided test is used, while two-sided test is used for the non-directional hypothesis. The two-sided test is more stringent because it tests for differences in both directions, and therefore the empirical value of test that previously met a significance level of ≤ 0.05 now corresponds to a level of ≤ 0.1.

Many problems in research and practical work are associated with the need to compare two or more groups of observations obtained under different conditions, as well as the need to classify observations (rank them to one of several classes). The solution for such problems is possible only when it is determined what is the difference (or identity) for the groups of observations. When the compared sets of numbers are so different that they do not overlap, the researcher has no doubt that the sets are different. Difficulties begin when the sets overlap.

In the first part of the article, we will discuss two groups of nonparametric tests: 1) to identify differences in indicator distribution; 2) to estimate shift reliability in the values of the studied indicator.

The aim of the research was to determine the application limits of the tests and methods in nonparametric statistics.

Nonparametric tests to identify differences in indicator distribution

This group of tests includes Rosenbaum Q-test [21], Mann-Whitney U-test [20].

1. *Rosenbaum Q-test*

The test is used to assess the differences by the level of any quantified indicator between two unrelated samplings [21].

Figure 1 shows a possible arrangement of two sets (samplings). Two compared sets are ideally arranged if they may be represented as in Figure 1 (a): N_1 is the number of observations in one sampling exceeding the maximum value in the other sampling; N_2 is the number of observations of one sampling, the values of which are less than the minimum value in the other sampling.

In case (a), there is no overlap between the values of both sets. Therefore, there is a statistically significant difference between the two sets (i. e., the difference is significant). In Figure 1b, both sets are at the same level. Therefore, the difference is not significant. If there are equal values in the compared sets, they should be placed exactly opposite each other. In Figure 1c, sets partially overlap, but the first set is higher than the second one.

Let's explain why N_1=0 and N_2=0 in Figure 1b.

N_1 are numbers from sampling 1 that are higher than the maximum value in sampling 2. In Figure 1b, there are no such numbers in sampling 2. N_2 are numbers from sampling 2 that are less than the minimum value in sampling 1. In Figure 1b, there are no such numbers in sampling 1. Thus, N_1 and N_2 in Figure 1b are equal to 0.

The Q-value (Rosenbaum Q-test) is equal to the sum of N_1 and N_2. The higher it is, the differences are more reliable, i. e. $\sum_{i=1}^{2} N_i$ (*Figure* 1*a*) ≥ $\sum_{i=1}^{2} N_i$ (*Figure* 1*c*) ≥ $\sum_{i=1}^{2} N_i$ (*Figure* 1*b*). Experimental value of Rosenbaum Q-test is calculated by the formula:

$$Q_{exp} = N_1 + N_2 \quad (1)$$

The following hypotheses are tested:

H_0: Indicator level in sampling 1 does not exceed indicator level in sampling 2.

H_1: Indicator level in sampling 1 exceeds indicator level in sampling 2.

Application of Rosenbaum Q-test

1. Arrange the values of indicator in both samplings in descending (or ascending) order. Consider sampling 1 the one that is supposed to include higher values, and sampling 2 the one that is supposed to include lower values (Figure 1c).

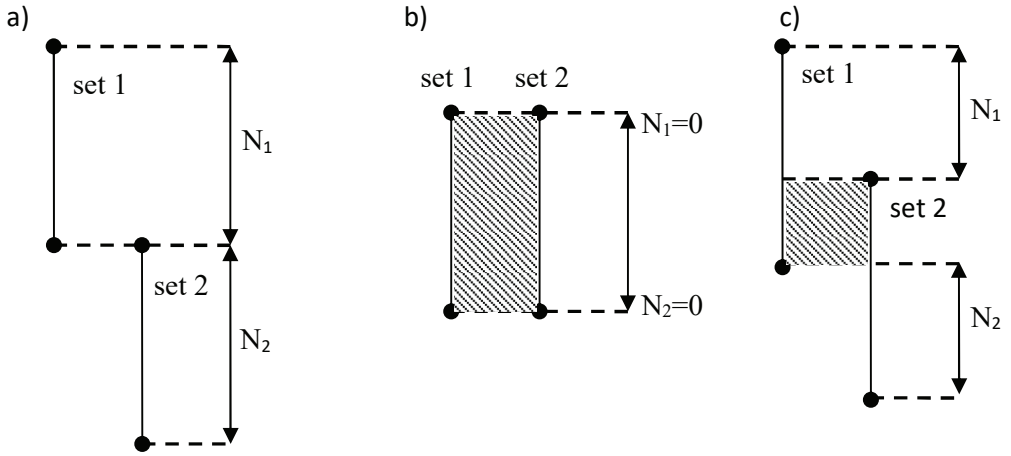


Figure 1. Possible ratios for sets of values in two samplings. N_1 is the area of values in the 1st set, which are higher than the maximum value in the 2nd set; N_2 is the area of values in the 2nd set, which are less than the minimum value in the 1st set; overlapping areas of two sets are marked with hatching

2. Determine the highest (maximum) value in sampling 2.
3. Count the number of values in sampling 1 that are higher than the maximum value in sampling 2 (N_1).
4. Determine the lowest (minimum) value in sampling 1.
5. Count the number of values in sampling 2 that are lower than the minimum value in sampling 1 (N_2).
6. Calculate Q_{exp} by the formula (1).
7. In the table of critical values presented in [7, 21, 25, 26] for given n_1 and n_2 and the significance level α, find the critical value Q_{cr}.
8. If Q_{exp} is equal to Q_{cr} or exceeds it, H_0 is rejected.

Limitations of Rosenbaum Q-test

1. Measurement may be carried out on a scale of order, intervals and ratios.
2. Samplings must be unrelated.
3. Each sampling must contain at least 11 observations. Furthermore, the volumes of samplings should be approximately the same:
   a) if both samplings contain less than 50 observations, then the difference between the volumes of samplings should be not more than 10 observations;
   b) if each sampling contains more than 50 observations, but less than 100 observations, then the difference between the volumes of samplings should be not more than 20 observations;
   c) if each sampling contains more than 100 observations, then it is allowed that one of the samplings is 1.5 to 2 times larger than the other one.
4. If the numbers of observations n_1 and n_2 are ≥ 26, the following values may be used:

$$Q_{cr} = \begin{cases} 8 & \text{at } \alpha \le 0.05 \\ 10 & \text{at } \alpha \le 0.01 \end{cases}$$

5. The ranges of value distribution for the two samplings should not overlap. In this case, the Q-test is not applicable. Therefore, Mann-Whitney U-test should be used.

Example

Given data: Heart rate was measured in two groups of animals. One group (11 animals) includes animals before the experiment, the other group (12 animals) includes animals after the experiment. The results are presented in Table 1. Is it possible to state that one of the groups is superior to the other one in terms of heart rate at the significance level? [25].

Solution. All the Q-test limitations are met, so Rosenbaum Q-test may be used to identify differences in indicator distribution.

Let's arrange the values in both samplings in ascending order, and then state the hypotheses:

H_0: The first group of animals does not exceed the second group of animals in heart rate.

H_1: The first group of animals exceeds the second group of animals in heart rate.

The calculations are presented in Table 2. The first set is the one that is "higher" — set No. N_1, and the second set is the one that is "lower" — set No. N_2. The maximum value in the second sampling is 122 and the minimum value in the first sampling is 92.

Table 1. Given data for the problem

1st group of animals		2nd group of animals	
No.	Heart rate	No.	Heart rate
1	92	1	77
2	98	2	81
3	104	3	81
4	107	4	84
5	107	5	89
6	122	6	95
7	122	7	101
8	122	8	103
9	127	9	107
10	133	10	110
11	137	11	114
		12	122

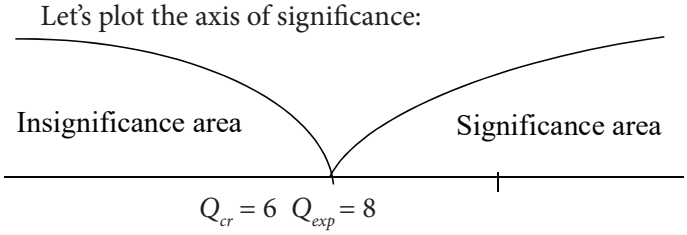
From Table 2, we determine the number of values in the first set (137, 133, 127) that are higher than the maximum value in the second set (N_1 = 3) and the number of values in the second set (89, 84, 81, 81, 77) that are lower than the minimum value in the first set (N_2 = 5).

We calculate Q_{exp} according to the formula (1): $Q_{exp} = N_1 + N_2 = 3 + 5 = 8$. From the table of critical values presented in [7, 21, 25, 26], we can find critical values for the given sampling volume and the significance level. At n_1 = 11, n_2 = 12 and we have Q_{cr} = 6.

Table 2. Calculation results

1st group of animals			2nd group of animals	
No.	Heart rate		No.	Heart rate
1	137	N_1		
2	133			
3	127			
4	122		1	122
5	122			
6	122			
			2	114
			3	110
7	107		4	107
8	107			
9	104			
			5	103
			6	101
10	98			
			7	95
11	92			
			8	89
			9	84
		N_2	10	81
			11	81
			12	77

Let's plot the axis of significance:



Since $Q_{exp} > Q_{cr}$, H_0 is rejected. And is accepted. Thus, the second group of animals is superior to the animals of the first group in heart rate (at $\alpha < 0.05$).

In [27], the authors applied the method of mathematical statistics (Rosenbaum Q-test) when finding the optimal technology for developing professional competencies among the target group of students studying business informatics. In [28], the exercise training effect on the health and condition in two groups of women of different ages was assessed by Student t-test (t) and Rosenbaum Q-test (Q). In [29], to compare and evaluate the data in five groups of 110 students, in a study on how English can be effectively implemented in the training of students of technical specialties for developing professional skills, the statistical method with Rosenbaum Q-test was used.

2. Mann-Whitney U-test

The test is designed to test the statistical homogeneity hypothesis of two unrelated samplings, i. e. to assess the differences by the level of any quantified indicator between two samplings [20]. It allows to differentiate between small samplings when n_1, $n_2 \geq 3$ or $n_1 = 2$, $n_2 \geq 5$ (n_1 and n_2 are volumes of samplings), and is more powerful than the Rosenbaum Q-test. The Rosenbaum Q-test is designed for low-volume samplings. Thus, Rosenbaum Q-test is a particular case of Whitney-Mann test.

Two samplings, $x_1, \ldots, x_n$ (sampling X) and $y_1, \ldots, y_n$ (sampling Y) with volumes of n_1 and n_2 are studied. Let's denote the distribution law of the first sampling by F, and the distribution law of the second sampling by G. The laws of distributions F and G are continuous. From here it follows with probability 1, that among the numbers $x_1, \ldots, x_n$ and $y_1, \ldots, y_n$, there are no overlapped ones.

Let's test the null hypothesis H_0: $F = G$.

All possibilities of $F \neq G$ may be the alternative hypotheses. However, the test is not able to detect all possible deviations from H_0. First of all, this test is intended to test the H_0 hypothesis against the alternative of $F \geq G$ (right-sided alternative, "overflow" of probabilities to the right) or the alternative of $F \leq G$ (left-sided alternative, "shift" of probabilities to the left). Combining both possibilities (two-sided alternative) may also be studied.

The Mann-Whitney U-test is based on a pairwise comparison of the results from the first and the second sampling. For values of $x_i < y_j$, an event means "success", and any event with $x_i > y_j$ means "failure". By changing i from 1 to n_1 and j from 1 to n_2, we have $n_1 \times n_2$ pairwise comparisons of values in samplings X and Y.

Let's denote the number of successes in these pairwise comparisons by U. U-value can take any integer value from 0 to $n_1 \times n_2$. The random U-value is called the Mann-Whitney statistics.

This test is particularly effective when testing an alternative hypothesis of dominance, and its particular case, i. e. the hypothesis of the right shift, when the values in the first sampling are higher than the values in the second sampling.

The test determines if the overlapping values area between the two sets is small enough. The first set, sampling or group is the set of values in which the values, according to preliminary estimates, are higher. And the 2nd set is the one where the values are supposed to be lower. The smaller the area of overlapping values, the more likely the differences are significant. These differences are sometimes referred to as differences in the location of the two samplings. The experimental value U_{exp} reflects how large is the overlapping area between sets. Therefore, the smaller U_{exp}, the more likely the differences are significant. Figure 2 shows three of the many possible relationships between two sets of values.

In the first variant (Figure 2a), the second set is lower than the first one, and the sets almost do not overlap. This means that the values are almost different. The overlapping area is too small to hide the differences between sets. The differences between seem to be significant. In the second variant (Figure 2b), the second set is also lower than the first one, but the area of overlapping values between the two sets is quite large. It may not yet reach a critical value, when the differences have to be recognized as insignificant. Finally, in the third variant (Figure 2c), the second set is lower than the first one, but the overlapping area is so large that the differences between the sets are insignificant.

The following hypotheses are tested:

H_0: indicator level in group 2 is not lower than indicator level in group 1.

H_1: indicator level in group 2 is lower than indicator level in group 1.

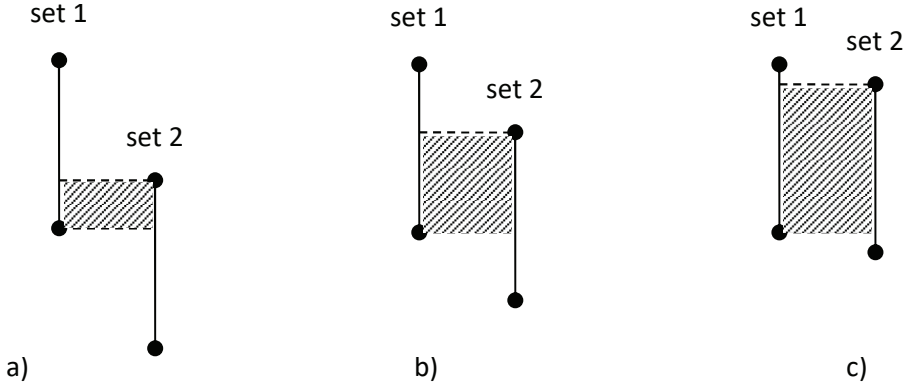


Figure 2. Possible ratios for sets of values in two samplings; overlapping areas are marked with hatching

Application of Mann-Whitney U-test

1. Pool the values for both groups in the order of indicator rising, regardless of what sampling they belong to.
2. Rank the values by assigning a lower rank to a lower value. Total ranks ($n_1 + n_2$).
3. Calculate the sum of the ranks for the values in the first sampling and the sum of the ranks for the values in the second sampling. Determine the larger of the two sums of the ranks.
4. Determine U_{exp} value by the formula:

$$U_{exp} = n_1 \cdot n_2 + \frac{n_x \cdot (n_x + 1)}{2} - T_x \quad (2)$$

where n_1, n_2 are volumes of samplings 1 and 2; T_x is the larger of the two rank sums; n_x is the volume of sampling with the larger sum of the ranks.

5. Determine the critical values U_{cr} for the given n_1, n_2 and the significance level α. If $U_{exp} > U_{cr}$, then H_0 is accepted. If $U_{exp} < U_{cr}$, then H_0 is rejected. The lower the U-value, the higher the reliability of the differences.

Limitations of Mann-Whitney U-test

1. Each sampling must contain at least 3 observations: n_1, $n_2 \geq 3$; it is allowed that one of the sampling includes 2 observations, but then the second one should include at least 5 observations.
2. Each sampling should contain no more than 60 observations: n_1, $n_2 \leq 60$.

Example

Given data. In the control group (without treatment) and in the experimental group (with treatment), the death of animals was established in time intervals (minutes) after the introduction of a toxic substance [7]. The results are shown in Table 3.

Table 3. Given data for the problem

Sampling No.	1	2	3	4	5	6	7	8	9
No. 1 — without treatment	39	37	45	5	27	27	31		
No. 2 — with treatment	45	9	67	42	42	31	34	49	99

Let's state the hypotheses:

H_0: the difference between the indicators in the control group and the experimental group is statistically insignificant.

H_1: the difference between the indicators in the control group and the experimental group is statistically significant.

Solution. In this case, the Rosenbaum Q-test is not applicable, since the volume of both samplings is less than 11. Let's apply the Mann-Whitney U-test.

Let's pool both samplings arranging their values in ascending order, while indicating which sampling they belong to and ranking them. The data are summarized in Table 4.

Rank is a serial number. The lowest value has rank 1.

The value of 5 — rank 1.

The value of 9 — rank 2.

We have the value of 27 twice, so it takes positions (ranks) 3 and 4. We add (3 and 4) and divide by 2 (since 27 occurs twice in sampling). Thus (3 + 4)/2 = 3.5. The rank of 27 is 3.5.

The value of 31 occurs twice in sampling taking positions 5 and 6. Similarly, we add 5 and 6 and divide by 2, so we get rank 5.5, etc.

Table 4. Pooled data from two samplings

Sampling No.	Values	rank	Sampling No.	Values	rank
1	5	1	1	39	9
2	9	2	2	42	10.5
1	27	3.5	2	42	10.5
1	27	3.5	1	45	12.5
1	31	5.5	2	45	12.5
2	31	5.5	2	49	14
2	34	7	2	67	15
1	37	8	2	99	16

Let's check the correctness of the ranking:

$$\sum(R_i) = 1 + 2 + 3.5 + 3.5 + 5.5 + 5.5 + 7 + \ldots + 14 + 15 + 16 = 136$$

Using the formula that calculates the sum of ranks in sampling, we determine the correctness of the ranks. As a result:

$$\sum(R_i) = \frac{N \cdot (N+1)}{2} = \frac{16 \cdot (16+1)}{2} = 136$$

Thus, the ranks are assigned correctly.

Calculating the ranks in the first and the second sampling:

$R_1 = 1 + 3.5 + 3.5 + 5.5 + 8 + 9 + 12.5 = 43$

$R_2 = 2 + 5.5 + 7 + 10.5 + 10.5 + 12.5 + 14 + 15 + 16 = 93$

Checking: 43 + 93 = 136.

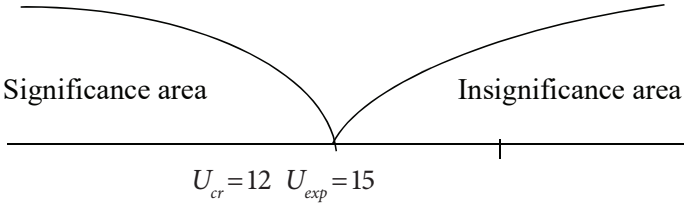
The larger of the two rank sums $T_x = 93$ (corresponds to the second sampling $n_2 = 9$), i. e. $n_x = 9$.

Let's find the experimental value of test by the formula:

$$U_{exp} = n_1 \cdot n_2 + \frac{n_x \cdot (n_x + 1)}{2} - T_x = 7 \cdot 9 + \frac{9 \cdot (9+1)}{2} - 93 = 15$$

We can find the critical value of test according to the table presented in [7, 21, 25, 26]. In this case, we are testing the non-directional alternative hypothesis. With $n_1 = 7$ and $n_2 = 9$, $U_{cr} = 12$ for $\alpha \leq 0.05$.

Let's plot the axis of significance:



Since $U_{exp} > U_{cr}$, H_0 is accepted. Thus, the significant differences between the indicators of the control and the experimental group has not been established.

In [30], the protein damage of individual protease inhibitors of the serpin superfamily in CRSwNP was studied. The damage of protease inhibitors by fibrinolysis in tissues was compared with exosome samples to assess the potential of "liquid biopsy" non-invasive exosomes for CRSwNP. Semi-quantitative analysis of Western blots was performed using the Mann-Whitney U-test. Effectiveness comparison of the standard simulation training in emergency care with a more comprehensive simulation program for emergency care is shown by the authors in [31]. The one-sided t-test and the Mann-Whitney U-test were used to analyze the Objective Structured Clinical Examination (OSCE) scores. The assessment of the effect of ketorolac intravenous injections on pain relief before and after using a tourniquet in orthopedic surgery on the femur and tibia was shown in [32]. This double-blind clinical study enrolled one hundred patients aged 15 to 75, candidates for hip and lower leg surgery. Visual pain tests during recovery were measured and analyzed using descriptive (mean and percentage) and logical statistics (Mann-Whitney U-test). The study [33] assessed the implementation of patient-centered interventions. 34 patients were examined with elective total knee arthroplasty. Endpoints were duration of induction period (primary), duration of hospital stay, pain at rest and pain at physical activity on postoperative day 1 (numeric analogue scale), and surgical release progress (MBF) on postoperative days 1, 3, and 6 (secondary). Group inferiority comparisons were made using the Wilcoxon T-test and Mann-Whitney U-test. Work [34] studied MRI of sodium (^{23}Na) at a field strength of 7 Tesla (T) as a biomarker of tumor stage, isocitrate dehydrogenase (IDH) mutation, and O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation in patients with glioma. The difference in median ^{23}Na concentration throughout the tumor area was compared between gliomas with IDH mutation and wild-type IDH, as well as between glioblastomas with methylated and non-methylated MGMT using the Mann-Whitney U-test.

Nonparametric tests for assessing shift reliability in the values of the studied indicator

This group of tests includes sign G-test and Wilcoxon T-test.

Let a random variable have a distribution $F(x)$, and another random variable has a distribution $S(x)$, and $S(x) = F(x - \Delta)$, where Δ is an abstract parameter characterizing the state of the object under study.

Two samplings, x_1, ..., x_n (sampling from $F(x)$) and y_1, ..., y_n (sampling from $S(x)$), with volumes n are studied. Furthermore, $F(x)$ and $S(x)$ are unknown distribution functions.

It is required to test the null hypothesis H_0: $F(x) = S(x)$, i. e. $\Delta = 0$. Three types of alternative hypotheses may be stated:

Directional hypotheses:

a) H_1^1: $\Delta > 0$;

b) H_1^2: $\Delta < 0$;

non-directional hypothesis:

c) H_1^3: $\Delta \neq 0$.

The following hypotheses are tested:

H_0: indicator level in group 2 is not lower than indicator level in group 1.

H_1: indicator level in group 2 is lower than indicator level in group 1.

Thus, it is known that if the impact changes the indicator distribution, then the impact shifts it in a quite definite way, i. e. by the value of Δ. If the shifts are compared in two unrelated samplings, for example, in the experimental and the control groups of subjects, then the Rosenbaum Q-test and the Mann-Whitney U-test should be used. To determine the shift reliability of related samplings, for example, when measuring indicator in the same group of subjects before and after some exposure, the sign G-test and the Wilcoxon T-test should be used.

1. Sign G-test

Sign G-test is intended to establish a general shift direction of the studied indicator. It allows to determine, in which direction the values of the indicator in sampling generally change during the transition from the first measuring to the second one, i. e. whether the indicators change in the direction of improvement/increase or, conversely, in the direction of deterioration/decrease. Shift is the difference in the values of indicators measured after a certain impact and before it [35, 36].

The sign G-test is applicable both to those shifts that can only be determined qualitatively (for example, a change from a negative attitude to a positive one) and to those shifts that can be quantified.

Shifts that seem to be predominant are usual, and shifts in a rarer direction are unusual. If the indicators do not increase or decrease, then such shifts are called zero shifts and they are excluded from the sign G-test. Furthermore, the number of compared pairs is reduced by the number of such zero shifts.

The sign G-test determines whether there are too many unusual shifts observed to consider shift in usual direction to be predominant? The less unusual shifts, the more likely the prevalence of usual shift is reliable. Figure 3 shows usual shifts as a light area, and unusual shifts as a dark area.

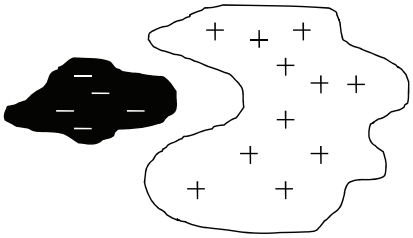


Figure 3. A graphic representation of positive and negative shifts in the form of different areas: light area is positive shifts; dark area is negative shifts [26]

The following hypotheses are tested:

H_0: predominance of usual shift direction is random;

H_1: predominance of usual shift direction is not random.

Limitations of sign G-test

The number of observations in both measurements should be not less than 5 and not more than 300.

Application of sign G-test

1. Check if the limitations are met.
2. Enter the measurement data in the table (Table 5):

Table 5. The filled table

No.	Pre-exposure values	Post-exposure values	«After to before» shift
1			
2			
…			
n			

3. Shift is not quantified, but the difference sign "+" or "–" is added. If the difference is zero, then zero is written in the table.
4. Calculate the number of zero responses n_0 and exclude them from consideration. As a result, the volume of sampling will decrease by the number of zero responses: $n = n - n_0$.
5. Determine the predominant direction of change. Shifts in the predominant direction are "usual".
6. Determine the number of unusual shifts. Consider this number as the experimental value of G-test (G_{exp}).
7. Determine the critical value G_{cr} for a given n and a specified significance level α.
8. Compare G_{exp} and G_{cr}. If $G_{exp} \leq G_{cr}$, H_0 is rejected, i. e. shift in the typical direction may be considered reliable.

Example

Given data: A group of 10 students completed a mathematics test. After checking the examination work, this group was asked to study an electronic manual on the same topic, after which a repeated test was carried out, which was assessed on a five-point scale. Has the knowledge of the students improved after studying the manual? [26].

Solution. The results of double examination work represent measurements in a serial scale. The volume of sampling is higher than 5, so the use of the sign G-test is possible. The results are summarized in Table 6:

Table 6. Given data for the problem

No.	First testing	Second testing	Sign of the difference
1	3	4	+
2	2	3	+
3	2	2	0
4	4	3	–
5	3	2	–
6	3	4	+
7	4	4	+
8	2	3	+
9	2	2	0
10	3	4	+

The number of zero responses $n_0 = 2$. Let's exclude them from consideration, therefore, volume of sampling $n = 10–2 = 8$.

The number of positive shifts is 6, and the number of negative shifts is 2. This means that the predominant direction is the positive one, therefore, shifts in this direction are usual, and shifts in the negative direction are unusual.

Let's state the hypotheses:

H_0: predominance of usual shift direction is random, i. e. students' knowledge did not improve after studying the manual.

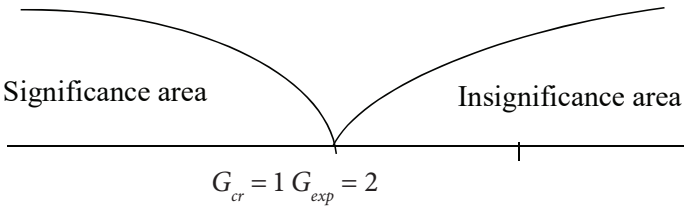
H_1: predominance of usual shift direction is not random, i. e. students' knowledge improved after studying the manual (shift in the positive direction is reliable).

Let's test at the significance level $\alpha \leq 0.05$.

The number of unusual shifts is 2, i. e. (according to item 6).

For $n = 8$ and $\alpha \leq 0.05$ we can find from the table of critical values [7, 21, 25, 26].

Let's plot the axis of significance:



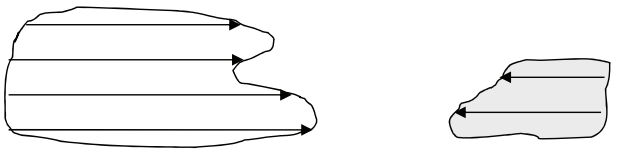
Since $G_{exp} > G_{cr}$, there is no reason to reject H_0, and the predominance of usual shift direction is random, so the students' knowledge did not improve after studying the manual.

2. Wilcoxon T-test

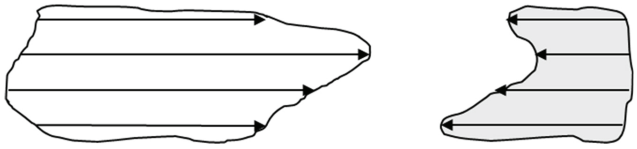
The test is used to compare values measured under two different conditions in the same sampling. It allows to establish not only the direction of changes, but also their intensity. It may be used to determine if the shift of indicators in one direction is more intense than in the other direction. Test is applicable in cases where indicators are measured at least on a serial scale, and the shifts between the second and first measurements can also be ordered. Therefore, they must vary within a considerably wide range [22, 23, 24].

The method compares the shift intensity in one direction with the shift intensity in another direction in absolute value. To do this, you need to rank all absolute shifts, and then summarize the ranks of shifts separately in the positive and negative directions. If shifts occur randomly, then the rank sums of their absolute values will be approximately equal. If the shift intensity in one of the directions outweighs, then the rank sum of absolute shifts in the opposite direction will be significantly lower than it would be with random changes.

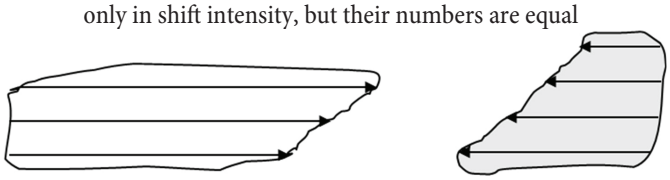
In Figure 4, shifts in opposite directions are represented as different areas.



a) «Light front» predominates over «dark front» in both the number of shifts and their intensity



b) «Light front» predominates over «dark front» only in shift intensity, but their numbers are equal



c) «Light front» is inferior to «dark front» in the number of shifts, but the most intense shifts belong to the "light front"

Figure 4. Types of the "light front" to "dark front" ratios; shifts in two different directions [26]

Initially, it is assumed that usual shift will shift in a more common direction, and unusual shift will shift in a rarer direction.

Let's state the hypotheses:

H_0: predominance of shifts between the initial and final indicators in one of the directions is unreliable (does not differ significantly from zero).

Alternative hypotheses:

Non-directional hypothesis:

H_1^1: predominance of shifts between the initial and final indicators in one of the directions is reliable (significantly differs from zero).

Directional hypothesis:

H_1^2: shift intensity in usual direction exceeds shift intensity in unusual direction (significantly differs from zero).

Application of Wilcoxon T-test

1. Make a list of pairs in any order.
2. Calculate the difference between the individual values in the second and the first measurements (before and after). Determine what will be considered the usual shift and state the corresponding hypotheses.
3. Convert the differences to absolute values and write them down in a separate column.
4. Rank the absolute values of the differences, assigning a lower rank to a lower value. Check the agreement of the received sum of ranks with the calculated one.
5. Note the ranks corresponding to shifts in unusual direction.
6. Calculate the sum of these ranks using the formula:, where is rank of shifts with a rarer sign.
7. Determine the critical values T_{cr} for a given volume of sampling n and the significance level α. If $T_{exp} \leq T_{cr}$, the null hypothesis is rejected, i. e. shift to the "usual" side in terms of intensity reliably predominates.

Limitations of Wilcoxon T-test

The minimum volume of sampling is 5.

Zero shifts are excluded and the number of observations is reduced by the number of these zero shifts. This limitation may not to be used by stating hypotheses that include no change: "Shift towards increasing values exceeds shift towards decreasing values and the tendency to keep them at the same level."

Example

Given data. A pharmaceutical company researches a new drug. A group of 10 volunteers suffering from disease was selected. They had their body temperature measured before and 30 minutes after taking the new drug. The data are presented in Table 7. It is required to conclude about the significance of a decrease in body temperature as a result of the drug administration [7].

Table 7. Given data for the problem

n	Initial indicators (body temperature before drug administration)	Final indicators (body temperature after drug administration)	Difference of indicators (d)	Absolute difference	Rank of the difference
1	39	37.6	–1.4	1.4	7
2	39.5	38.7	–0.8	0.8	5
3	38.6	38.7	0.1	0.1	***1.5****
4	39.1	38.5	–0.6	0.6	4
5	40.1	38.6	–1.5	1.5	8
6	39.3	37.5	–1.8	1.8	9
7	38.9	38.8	–0.1	0.1	1.5
8	39.2	38	–1.2	1.2	6
9	39.8	39.8	0	0	0
10	38.8	39.3	0.5	0.5	***3****

*** Unusual ranks are in bold italics. In this case, the atypical situation is that the temperature should decrease, but it increases.**

Solution. Let's state the hypotheses:

H_0: predominance of shifts between the initial and final indicators in one of the directions is unreliable (does not differ significantly from zero).

H_1: predominance of shifts between the initial and final indicators in one of the directions is reliable (significantly differs from zero).

Let's test the null hypothesis at the significance level $\alpha \leq 0.05$.

The sum of unusual ranks is equal to the experimental value of the Wilcoxon T-test:

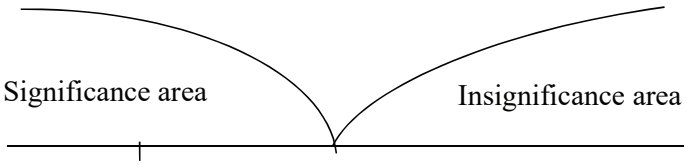
$$T_{exp} = 1.5 + 3 = 4.5.$$

Usual shift of the indicator (body temperature) is its decrease.

From the table of critical values presented in [7, 21, 25, 26], we can find.

Since the direction of the differences was not predicted in advance, we test the hypothesis for the two-sided test. For $n = 9$ (zero shift is excluded) and $\alpha = 0.05$ $T_{cr} = 8$.

Let's plot the axis of significance:



$T_{exp} = 4.5$ $T_{cr} = 8$

Since $T_{cr} > T_{exp}$, the null hypothesis is rejected. It can be stated that the reliability between the initial and final indicators in one of the directions is established.

Example

Given data:

In a group of students, training was conducted on the development of creative thinking. Tests were carried out before and after the training (stimulating effect should increase creativity, i. e. increase the score). The data are presented in Table 8. Is the hypothesis from the training providers confirmed that the training contributes to the development of creative thinking at the significance level $\alpha \leq 0.05$?

Table 8. Given data for the problem and calculation results

n	Before training	After training	Difference of indicators (d)	Absolute difference	Rank of the difference
1	19	17	–2	2	*4.5*
2	20	26	6	6	11
3	18	20	2	2	4.5
4	15	18	3	3	6.5
5	29	30	1	1	2
6	21	25	4	4	8.5
7	21	28	7	7	12
8	18	19	1	1	2
9	21	20	–1	1	*2*
10	23	27	4	4	8.5
11	14	19	5	5	10
12	10	13	3	3	6.5

Solution. The first step in calculating the T-test is to subtract each individual "before" value from the "after" value. The calculation results in the form of the difference of indicators (d) are presented in Table 8. Two of the obtained differences are negative, and 10 of the differences are positive. Therefore, the usual direction is positive.

Let's state the hypotheses:

H_0: shift intensity in usual direction does not exceed shift intensity in unusual direction.

H_1: shift intensity in usual direction exceeds shift intensity in unusual direction.

In the next step, all shifts, regardless of their sign, must be ranked.

We check the correctness of the ranking.

$$\sum(R_i) = 2+2+2+4.5+6.5+6.5+8.5+8.5+10+11+12 = 78$$

We check by the formula:

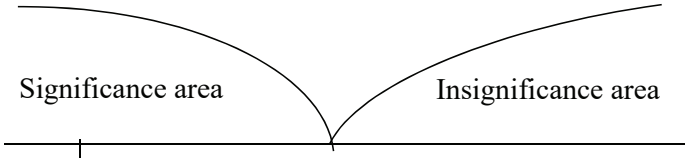
$$\sum(R_i) = \frac{N\cdot(N+1)}{2} = \frac{12\cdot(12+1)}{2} = 78$$

Consequently, ranks are assigned correctly.

Note those shifts that are unusual, i. e. negative ones (in Table 8 they are marked in bold italics). The sum of the ranks of these unusual shifts is the experimental value of the T-test: $T_{exp} = 2+4.5 = 6.5$.

In this problem, the alternative hypothesis is directional because the direction of the differences is predicted in advance, so one-sided test should be used to test hypotheses. For $n = 12$ and $\alpha \leq 0.05$, from the table of critical values presented in [7, 21, 25, 26], we can find $T_{cr} = 17$.

Let's plot the axis of significance:



$T_{exp} = 6.5$ $T_{cr} = 17$

Since $T_{cr} \geq T_{exp}$, the null hypothesis is rejected. It can be stated that shift intensity in usual direction exceeds shift intensity in unusual direction, therefore, the initial assumption about the effectiveness of the stimulating effect is confirmed.

Let the following test scores to be obtained for another group of subjects (Table 9). 6 differences are negative, and 4 differences are positive, i. e. it is impossible to state a hypothesis corresponding to the initial assumption. It is possible to state a hypothesis assuming the insignificance of the indicator shift in the direction of decreasing:

H_0: shift intensity towards decreasing creativity does not exceed shift intensity towards its increase.

H_1: shift intensity towards decreasing creativity exceeds shift intensity towards its increase.

Table 9. Given data for the problem and calculation results

n	Before training	After training	Difference of indicators (d)	Absolute difference	Rank of the difference
1	19	17	–2	2	3.5
2	23	20	–3	3	5.5
3	18	20	2	2	*3.5*
4	15	18	3	3	*5.5*
5	25	17	–8	8	10
6	21	15	–6	6	9
7	21	20	–1	1	1.5
8	18	19	1	1	*1.5*
9	24	20	–4	4	7
10	23	28	5	5	*8*

Let's rank the absolute differences and check the correctness of the ranking.

$$\sum(R_i) = 1.5+1.5+3.5+3.5+5.5+5.5+7+8+9+10 = 55$$

By the formula we obtain:

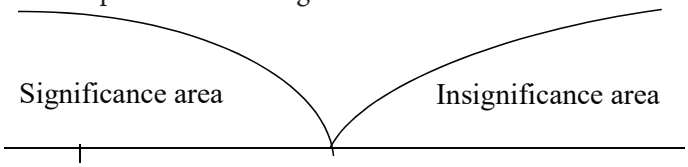
$$\sum(R_i) = \frac{N\cdot(N+1)}{2} = \frac{10\cdot(12+1)}{2} = 55$$

Consequently, ranks are assigned correctly.

Note those shifts that are unusual, i. e. positive ones (in Table 9 they are marked in italics). Let's calculate the experimental value of T-test: $T_{exp} = 3.5+5.5+1.5+8 = 18.5$.

Since the direction of the differences is predicted in advance, a one-sided test is used to test the hypotheses. For $n = 10$ and $\alpha \leq 0.05$ $T_{cr} = 10$. [7,21,25,26].

Let's plot the axis of significance



$T_{cr} = 10$ $T_{exp} = 18.5$

Since $T_{cr} \geq T_{exp}$, the null hypothesis is accepted. It can be stated that the intensity of the negative shift does not exceed the intensity of the positive shift.

The Wilcoxon T-test [37–49] is the most applicable for statistical processing in scientific researches. In [37], a study was conducted to validate the ecfX-targeted qPCR protocol designed to detect all viable *P. aeruginosa* bacteria and identify forms in sputum samples from patients with cystic fibrosis. The study involved 115 *P. aeruginosa* strains of various origins and 10 other strains in 88 cystic fibrosis sputum samples, 41 of which were positive and 47 were negative. The statistical significance of the results was assessed using the of Wilcoxon T-test and Student t-test. The work [38] is aimed at improving diabetes care. The study is to assess the improvement of group care, which directly affects the quality of care for patients diagnosed with diabetes. Changes in laboratory values of A1c hemoglobin and LDL cholesterol were analyzed using the paired t-test and verified using the Wilcoxon T-test. The aim of work [39] was to conduct a study to provide additional information on the health benefits of gluten-free foods and to test the hypothesis that both self-selected and recommended diets are less nutritious when gluten-free diets are followed. Diet differences were assessed using paired t-tests and ranks of the Wilcoxon T-test. Assessment of the amino acid profile in trauma patients and assessment of the nutritional mode effect and the addition of exogenous ALA-GLN dipeptide on plasma amino acid concentration were conducted in [40]. Differences between plasma amino acid concentrations on day 1 and day 6 were assessed using Student t-test, Mann-Whitney U-test, and Wilcoxon T-test. In [41], a study of weight recovery after gastric bypass by increasing the consumption of high-calorie liquid and soft foods was carried out. Each patient with dysphagia was compared with 4 control patients based on the age, body mass index and time after surgery. Statistical analysis was performed using the Wilcoxon T-test. A study on the treatment of equine laminitis with and without acupuncture is reviewed in [42]. The study involved 14 horses with chronic laminitis. Lameness was statistically analyzed using the paired t-test and the sign ranked Wilcoxon T-test at $p < 0.05$. An analysis of the effect of yerba mate daily consumption on serum lipids and body composition in overweight women is shown in [43]. 119 overweight 25 to 50 years old women were randomized into three groups: mate and regime (MD), mate without regime (M), water and regime (AD). For 12 weeks, the M and MD groups were supplemented with mate, while the AD and MD groups followed a hypocaloric regime. Anthropometric measurements and blood tests (total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides) were performed at the beginning and in the end of the study. Statistical analysis was performed using Student t-test and Wilcoxon T-test. Determination of the effect of a new phytosterol emulsion for nutritional supplements (1.5 g/day of phytosterol equivalent) on the concentration of low-density lipoprotein cholesterol was studied in [44]. Thirty-two healthy adults were randomized to receive placebo or treatment with a washout period followed by placebo or treatment. Each phase lasted one month. Secondary endpoints related to cardiovascular health were assessed. Study management, including screening, recruitment, monitoring, compliance and data collection, was carried out remotely (off-site clinical trial) using a new virtual instrument. The effect of phytosterol supplementation was assessed using Student t-test and Wilcoxon T-test. Changes in radiomic characteristics based on cone computed tomography (CBCT) during treatment and quantification of the potential relationship between CBCT-based delta radiomics characteristics and overall survival in locally advanced lung cancer patients have been reviewed [45]. 23 patients were identified and 658 radiomic characteristics were calculated for each of the 11 CBCT images in each patient. The choice of characteristics was made on the basis of repeatability, contour uncertainty resistance, and lack of redundancy. Evaluation of the predictive ability for survival was carried out using the t-test and Wilcoxon T-test. The work [46] investigated the effect of smoking on periodontal disease. Comparison of 441 daily smokers with 441 non-smokers was performed using the of Wilcoxon T-test and t-test. The study [47] evaluated the reproducibility of 9 instruments for the analysis of differential expression in scRNA-seq data. Statistical analysis was performed using the t-test and Wilcoxon T-test. The study [48] was conducted from September 2018 to August 2019 and included 101 athletes with disabilities (13 Paralympic disciplines) from Brasilia, Federal District. Food intake was assessed based on two or four inconsistent nutritional reviews over a 24-hour period, in which para-athletes reported all food, drinks, and supplements consumed in the previous 24 hours. Diet analysis was performed using the method of the National Cancer Institute. Comparisons between fellows and athletes not receiving a fellowship were made using the Student t-test for parametric variables and Wilcoxon T-test for nonparametric variables. Research [49] was focused on the recruitment and retention of women in Type 2 Diabetes Women Health Program, and to assess the program's initial effectiveness in improving well-being. A 12-week wellness event enrolled 70 middle-aged women with type 2 diabetes. Analyzes were performed using chi-squared tests, McNemar test, paired t-test, and Wilcoxon T-test.

Conclusion

The described nonparametric statistical tests are much less laborious than parametric ones, and for distributions far from normal (Gaussian), they are more efficient as well. It is completely insufficient for a current researcher to use one of the methods for statistical assessment of the differences between two groups of observations. In each case, a suitable test must be selected. This allows not only to increase the efficiency of statistical processing and reduce its labor intensity, but also to get a clear idea of the comparison reliability of experimental results. The second part will review nonparametric tests for testing hypotheses of distribution type (Pearson's chi-squared test, Kolmogorov test) and nonparametric tests for testing hypotheses of sampling homogeneity (Pearson's chi-squared test for testing sampling homogeneity, Kolmogorov-Smirnov test).

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CHICKEN EGG WHITE — CHARACTERISTICS OF ITS PROPERTIES AND THE PROSPECTS FOR FUNCTIONAL FOODS DEVELOPMENT

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Abstract
The overview presents the literature data and the results of our own research on prospects of using the chicken eggs as the basis of functional foods. The composition of chicken eggs and their components, characteristics of egg white proteins properties are presented thereto. The biologically active compounds included into egg composition are analyzed. The data on the biological value of egg white are given. The characteristic of egg white foaming ability is presented. It has been shown that the ability of proteins to form stable intermolecular structures, especially with partially denaturated proteins, allows them forming viscoelastic superficial films that ensure foam stability. The high foaming ability of chicken egg protein macromolecules is directly related to their interphase properties, i. e. the ability to form interphase layers at the "liquid — gas" interface. The foaming properties of the various egg proteins are not equal, and therefore they contribute to foaming properties at various extents. The model of egg white proteins gelation is considered and the factors influencing the gelation process are described. It has been shown that very important changes in proteins properties are caused by denaturation. The proteins lose their ability to hydrate; the protective aqueous shell around the globules disappears, the proteins stick together, grow larger and lose solubility. This process is called coagulation. The influence of denaturation and aggregation on variations of protein properties is described below. Data on protein fortification with functional ingredients (calcium, iodine, plant polyphenols) and creation of functional egg and meat foods are presented here.

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Introduction

The priority task of state policy in our country in sphere of nutrition is development of health-improving foods production in order to preserve and enhance the health of population, to prevent diseases caused by insufficient and unbalanced nutrition. The practical implementation of this task assumes, in particular, development, production and supply of wide range of health-improving foods to the domestic consumer market, including functional foods, in accordance to appropriate regulatory and methodological provisions harmonized with international requirements.

The concept of healthy nutrition assumes an increase of efficiency of nutrition factor to the preservation and improvement of public health within the state. This factor provides necessity for development of biotechnologies, targeted to foods modifying by including ingredients of high biological and nutritional value. This leads to creation of an expanded range of functional foods (FF).

The promising source of food raw materials with high biological FF value are chicken eggs. Chicken eggs contain all food and biologically active substances necessary for healthy development [1]. Chicken egg is a natural functional food; it is a unique source of numerous substances of high biological and nutritional value due to significant content of protein balanced in amino acid profile. It contains a complete lipid complex, wide range of macro- and microelements, and vitamins. The average chemical composition is constant, fluctuations in the content of eggs, laid by one species of poultry, depend on the diet of layers (which is especially peculiar for lipid fraction) (Table 1) [2].

Table 1. Chemical composition of a chicken egg

Components	Mass fraction, %				
	Moisture	Protein	Fat	Carbohydrates	Inorganic compounds
Melange	75.33–76.07	11.34–12.31	10.2–10.3	1.0	0.8
White	88.65–87.06	9.45–11.02	traces	0.9	0.6
Yolk	49.91–50.29	15.51–15.57	31.9–32.3	1.0	1.1
Shell	1.61–1.63	3.30–3.41	traces	—	95.1

Eggs contain a lot of biologically active compounds, including those which possess antimicrobial, immunomodulatory, antioxidant, anticarcinogenic, hypotensive and other properties [3–5].

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Information on this extensive issue can be partially obtained from a number of scientific reviews publications [3–8], nevertheless it is necessary to briefly characterize the chicken egg proteins, since they constitute the most important component in the composition of the functional egg foods being developed now.

Egg composition

Egg white contains simple proteins (ovalbumen, ovoconalbumen, ovoglobulin) and complex proteins — glycoproteins or mucoproteins (ovomucoid and ovomucin) (Table 2).

The major egg white proteins are ovalbumen (54%), ovotransferrin (12%), ovomucoid (11%), lysozyme (3.5%) and ovomucin (3.5%). Minor proteins include avidin (0.05%), cystatin (0.05%), ovomacroglobulin (0.5%), ovoflavoprotein (0.8%), ovoglycoprotein (1.0%), and ovoinhibitor (1.5%) [3,5,6].

Biological value of the egg white

In order to give quantitative characteristic to the quality of food protein, the parameter "biological value" (BV) in nutritional science is used. BV is the degree of food nitrogen retention in a growing body (which degree depends on the amino acid composition and other structural features of protein), and efficiency of food nitrogen utilization to maintain nitrogen balance in humans. The amino acid composition, content and ratio of essential amino acids determine the BV of a protein — the parameter that reflects the degree of protein utilization. The degree of digestion, absorption and utilization of protein is significantly influenced by food manufacture technology and methods of food processing at the food processing plants. Therefore, when evaluating BV, its true digestibility is taken into account. The parameter "The protein digestibility-corrected amino acid score" (or PDCAAS for short) [9], which is equal to the amino acid rate multiplied by true digestibility index, has been introduced. Its highest reference value is 1.0. Comparative data on true digestibility and PDCAAS for chicken egg white and proteins obtained from various food sources are presented below in the Table 3.

Table 3. Corrected amino acid index of proteins digestibility

Food	Protein content %	True digestibility	Amino acid score	PDCAAS
Casein	94.7	99	1.19	1.00
Chicken egg white	87	100	1.19	1.00
Beef protein	95.2	98	0.94	0.92
Soy protein isolate	92.2	98	0.94	0.92

Gel-forming properties of chicken egg

Currently the share of eggs and their separate components in formulations of a wide range of foods is increasing steadily, which is explained by their properties: emulsifying ability and thermal gelation (coagulation) [7].

Foaming capacity of egg white proteins

Poultry egg white is an aqueous protein solution with 84–89% of water. It also contains minor amounts of carbohydrates, vitamins and minerals. Proteins, which make up more than 90% of the egg white dry matter, are heat-sensitive and prone to surface denaturation, which explains their unique technological properties. As it's known, foams are the dispersed systems consisting of many gas bubbles separated by thin (only single colloidal particle thick) films of liquid. Adding of surfactants, which reduce surface tension, contribute to foaming. However, stable foaming requires the presence of high-molecular compounds in the system, for example, proteins which form two-sided strong adsorption layers on the surface of thin films of liquid. Egg white proteins are amphiphilic and feature relatively high surface hydrophobic properties; therefore, they diffuse towards the air-water phase interfaces, where they are adsorbed effectively. This flexibility of molecules allows proteins to rearrange their conformational structure at the interface, which leads to significant decrease in the superficial tension at these interfaces. The ability of proteins to form stable intermolecular structures, especially partially denatured proteins, allows them forming viscoelastic superficial films that ensure foam stability. The high foaming capacity of chicken egg protein macromolecules is directly

Table 2. Physical and chemical properties of egg white proteins

Protein	% in protein	pH	Molecu-lar weight (kDa)	Internal viscosity, 100 cm^3/g	$T_{denaturation}$ (°C)	–SH	S–S	Notes
Ovalbumen	54	4.5–4.6	45000	0.043	75–84	4	1	Phosphoglyco-protein; 4 SH-groups
Ovotransferrin	12–13	6.1–6.6	76000–80000	0.084	61–65 (76.5; Al^{3+})	—	15	Glycoprotein, binds to complexes with iron and other metals
Ovomucoid	11	3.9–4.3	28000	0.055	77	—	9	Glycoprotein, trypsin inhibitor
Ovomucin	3.5	4.5–5.0	110000	2.10	—	—	—	Glycoprotein, fibrous, viscous
Lysozyme	3.4–3.5	10.7	14300–14600	0.027	69–77	—	4	Spherical protein; 4 — SS — links; features lytic activity
Ovoinhibitor	1.5	5.1–5.2	44000–49000	—	—	—	—	Inhibits trypsin and chymotrypsin
Ovoglyco-protein	1.0	3.9	24000–24400	—	—	—	—	Glycoprotein
Ovoflavoprotein	0.8	4.0–4.1	32000–35000	—	—	—	2	Binds riboflavin
Ovomacro-globulin	0.5	4.5–4.7	760000–900000	0.065	—	—		Glycoprotein
Avidin	0.5	9.5–10	55000–68300	—	—	—	1	Binds biotin

related to their interfacial properties, i. e. the ability to form interfacial layers at the liquid-gas interface. The foaming properties of various proteins of chicken egg white are not equal, and therefore they contribute to the formation of its foaming properties in various extents (Table 4).

Table 4. Interfacial characteristics of the major proteins of egg white [8]

Protein	Superficial tension (mN/m)	Foaming coefficient (cm^3/g per minute)
Ovalbumen	51.8	0.59
Ovotransferrin	42.4	0.34
Lysozyme	42	0.12
Ovomucoid	39	0
Ovomucin	No data	0
Mixture of these proteins in the same proportion as in egg white	46.7	3.08

Egg white is considered to be reference model for foaming properties, which other animal and plant proteins are compared to [10–15]. Despite numerous comparative studies of the properties of some individual egg white proteins and attempts to rank them according to these properties [10,16,17], it is almost impossible because of complexity of egg white proteins composition and potentially possible synergistic interactions of individual proteins. All those complexities make it almost impossible to identify the special role of each individual protein in the foaming process [18]. It is very difficult to predict the foaming properties of any mixture of proteins, since individual proteins may compete among themselves for the surface, or they may substitute each other at the interface [19]. However, the foaming capacities of individual proteins isolated from egg white are always less than the foaming capacity of egg white as a whole. Egg white foams are the main component of such foods as meringues, nougat and light biscuits. Another peculiar feature of egg white-based foams is the dependence of their characteristics on quality of the original egg white [20]. Since the foaming properties of dense egg white and liquid egg white differ from each other [21], all factors affecting the ratio of these fractions in the egg white (i. e. shelf life of the egg, age and breed of laying hen) also affect the foaming capacity of the egg white [22–26]. Another extremely important parameter is the accuracy of separation of egg white and yolk, since even very small amounts of yolk (0.022%) in the egg white significantly reduce its foaming capacity [27]. Heat treatment also reduces the foaming capacity of the egg white. Industrial pasteurization can reduce by 10% both the foaming capacity of the egg white and the foam stability as well [20,28, 29]. The dripping of liquid phase from the foam can be increased threefold after its pasteurization. Heating the protein during drying also worsens the foaming properties of the egg white, and the foaming may be decreased by 10 to 60% due to pH of the egg white during drying, and may decrease the foam stability by 20%.

Gelation properties of egg white

The process of egg proteins gelation is described in details by the model of thermal gelation of globular structure proteins [5]. In this model gelation is considered as a two-stage process, sequentially including denaturation and aggregation. The scheme is as follows: native protein → denatured protein (long chains) → aggregated protein (associated mesh).

Denaturation results in very important changes in proteins properties. First of all, they lose their ability to hydrate, the aqueous protective shell around the globules disappears, they join together, grow larger and lose their solubility. This process is called coagulation.

When eggs are heated, the proteins that make up their composition, changes first. Due to the thermal denaturation of protein substances at 50–55 °C, local opacities are formed in the transparent mass, and gradually spread out. At 65 °C the entire mass of protein get thicker, and at 75 °C it turns into a solid opaque mass of a very delicate consistency. At 80 °C the gel is obtained which is able to keep its shape, and along with further heating (above 85 °C) it becomes more and more dense. The degree of compaction of the protein gel depends on heating duration. Chicken egg white has 2 main endothermic points at 60–65 °C and 80–85 °C, which corresponds to the temperatures of ovotransferrin and ovalbumen denaturation [30,31].

Differences in opacity of samples with different salt content are associated with differences in protein clots sizes and rate of protein aggregation. Salt NaCl causes changes in conformation of egg proteins proportionally to increase in the ionic strength of the solution. Increasing the solubility of protein molecules increases their susceptibility to thermal denaturation. The high content of NaCl leads to coagulation of protein, thus playing a decisive role in the process of its aggregation.

At the first stage of gelation the "folded" macromolecules of globular proteins "unfold", thus forming the denatured proteins, and release their hydrophobic "internal" structure. At the second stage of gelation (aggregation), macromolecules of denatured proteins interact with each other to form even higher molecular weight aggregates, which in its turn also interact with each other, which ultimately leads to the formation of three-dimensional gel structure [32]. Comparison of the denaturation rate with the aggregation rate helps determine the characteristics of the obtained gel. The wide range of factors affect protein aggregation, which is important because changing the aggregation rate in relation to the denaturation rate affects the characteristics of the obtained gel. Some of these factors are listed below.

Electrostatic charge (pH) can alter the charge distribution among the side chains of amino acids and, accordingly, either decrease or increase protein-protein interactions. The main factor in heat-induced aggregation of ovalbumen (pH 4.5–4.6) is the degree of electrostatic repulsion between the protein molecules of exposed denaturation. The aggregation rate of a heated 1.8% ovalbumen solution

is higher at pH=5.5 and 8.5 than at pH=7.0. At a pH of 5.5 the opacity builds up more rapidly.

The concentration of protein in solution also affects aggregation rate. The high concentration of protein causes the molecules to come closer together to form aggregates at lower temperatures.

The pH value required for achieving the same opacity that indicates protein aggregation, increases as the protein concentration increases, and the pH shifts to alkaline direction from the isoelectric point from 6 to 11, and decreases when the pH shifts to acidic direction from 4.5 to 3.0. There is an assumption [5] that the denaturation rate causes a low rate of aggregation, because the forces of attraction between denatured protein chains are low. The resulting gel will have a fine mesh of protein chains, will be more transparent, and will be characterized by less syneresis than the gel obtained at the high aggregation rate. A larger mesh of protein chains will result to the opaque gel with large internodes capable of retaining a solvent that is easily pressed out from the matrix. Conditions that favor denaturation, such as high or low pH, reduce aggregation of globular proteins. In this case, due to the high charge of the molecules, denaturation based on protein-solvent interactions prevails rather than aggregation caused by protein-protein interactions. A gel mesh with a certain degree of structural regularity can be obtained if the aggregation pace is slower than the denaturation pace — this slower rate "gives time" for denatured molecules to self-orientate before aggregation, thus reducing opacity and increasing elasticity compared to conditions under which aggregation rate is not retarded. If aggregation occurs simultaneously with denaturation, then opaque and less elastic gels are formed.

The efficiency and rate of both stages — i. e. the deployment of molecules and their aggregation — depend on the concentration of the protein, the ionic strength of the solution, the pH value, and other parameters that can affect the number and nature of intermolecular and intramolecular interactions that affect the rheological properties of the resulting gel. For example, in the gels obtained by thermal processing of egg contents, these interactions were predominantly hydrophobic and electrostatic, but in these systems the higher-energy interactions were also found (for example, interactions caused by disulfide bridges); thiol and amino groups are highly reactive, especially in alkaline media. It has been found that sugars protect proteins from heat, but create coarser aggregates, and worsen the rheological properties of the resulting gel [32]. If standard conditions of industrial pasteurization of the protein do not affect its gelation properties, then drying the protein can be detrimental to the gelation. This adverse effect can be prevented by adjusting the pH of the egg white before drying it. At the same time the research [30] recommends pH 8.5, while in the article [33] the best gel stability was obtained at pH 6.5.

Egg white gels

In the process of developing of egg foods technologies, the gelation capacity of eggs has great importance.

Gelation is an ordered aggregation of proteins, which may or may not be denatured, to form a three-dimensional mesh, which mesh can form a well-ordered matrix. The gelation properties of egg white are determined by the included proteins. In general, gelation is associated with an imbalance between mutually attractive (van der Waals) and mutually repulsive (electrostatic, steric) interactions that determine the spatial structure of the protein in solution. The protein structure can be modified in various ways. Examples of the modification are given in the monograph by V. N. Izmailova and P. A. Rebinder "Structure formation in protein systems" and by other authors [34]. Both during acidification and alkalization the native globular proteins can irreversibly "rearrange" into proteins of the fibrillar type. The formation of thixotropic ovalbumen gels due to addition of acetic acid may result from formation of hydrogen bonds between acetic acid and protein. Ovalbumen gets denaturated in presence of urea and alkali, when this protein forms the gel. Alkaline ovalbumen gels melt the faster, the higher is pH of the gel. Ovalbumen gels can also be prepared at high pressures which cause denaturation changes in protein macromolecules.

The results of heat treatment research are of particular interest, as the heat treatment is widely used in the food industry for formation of gels from egg white and yolk. In aqueous solutions of ovalbumen at room temperature "the induction period of structure formation got shorter with increasing protein concentration". At the same time, the strength of the gel structure increased due to an increase in the number of intermolecular contacts per volume unit, accompanied by conformational changes in protein macromolecules. The process of denaturation of ovalbumen, exposed to acid or alkali, was accelerated by heating. While discussing the nature of the interactions that determine the strength of the resulting ovalbumen gels, the authors come to the conclusion that it is determined by hydrogen bonds and hydrophobic interactions. In this case electrostatic interactions are excluded due to the formation of the gel structure in strongly acidic and strongly alkaline media. Thus, the structure of ovalbumen gels is similar to thixotropic coagulation structural meshes, where the particles of the dispersed phase or macromolecules aggregates are bound together by van der Waals forces which occur between hydrocarbon hydrophobic amino acid residues of ovalbumen macromolecules during protein denaturation.

The type and force of interaction between denatured protein molecules depend on their structure and, in particular, on the "extent of unfolding" of protein molecules at the end of the denaturation stage. These interactions also depend on the general physical and chemical conditions in the system, which can both restrict and contribute to gelation, i. e. respectively increase or decrease the level of aggregation and, conversely, decrease or increase the degree of denaturation, after which these interactions begin [35]. These mechanisms were intensively researched on the samples of thermally induced gelation of egg white and ovalbumen, while the pecu-

liar attention was paid to the influence of the ionic strength of a solution on the structure and characteristics of the resulting gels [32, 36–39]. When heated at high ionic strength, ions shield the charges of protein molecules, which process promotes hydrophobic interactions [40]. In these conditions aggregates of partially denatured proteins are formed at random. These aggregates create cloudy opaque gels with low rates of rigidity [hardness], low elasticity and poor water-holding capacity (WHC). On the other hand, at low ionic strength the higher electrostatic repulsive forces slow down the aggregation process [32] and promote protein denaturation. Finally, the further process of aggregation includes the interaction of hydrophobic sections of molecules with the formation of linear polymer aggregates. In media with the higher ionic strength these aggregates can interact to form gels with good processing properties.

The thermal gelation capacity has been used to develop technologies and new types of functional egg foods, as well as meat foods with a high share of eggs included in their composition.

Another important parameter to monitor the quality of protein gelation is the pH value. Close to their isoelectric point (pH ~ 5) the proteins are more prone to the formation of randomly arranged aggregates, similar to those obtained at high ionic force. On the contrary the gelation properties of protein are expressed in the best way within the alkaline pH range [41,42]. On the other hand at low pH (2.0) the gelation temperature decreases and the rheological properties of the gel worsen, which is explained by decrease in protein solubility [32]. The composition of the albumen fraction in chicken egg whites also affects their aggregation. The denaturation temperature of conalbumen, globulin, ovalbumen and lysozyme is 57.3 °C; 72.0 °C; 71.5 °C and 81.5 °C, respectively, ovomucin and ovomucoid do not coagulate at these temperatures. The lysozyme-based gel is the strongest gel, the globulin-based gel is less strong. Conalbumen gels feature the greatest drainage rate. In binary mixtures of the albumen fraction of chicken egg whites, the mixtures get aggregated close to the denaturation temperature of the least thermostable protein. The lysozyme-globulin gel is the strongest, while the ovomucoid-ovalbumen gel features the least strength [43].

Egg white coagulation

Accidental aggregation of already denatured protein molecules, when polymer-polymer interactions dominate over polymer-solvent interactions, leads to protein coagulation.

Coagulation is the process of particles adhesion that leads to formation of large aggregates. As a result of coagulation the system loses its sedimentation stability. There are two stages of coagulation. The first of these is the latent coagulation. At this stage the particles grow larger, but still keep their sedimentation stability. The formation of disulfide bonds and their impact on hydrophobic amino acid residues are involved in the first stage of coagulation. Proteins with a higher percentage of hydrophobic amino acids are classified as the proteins of coagulating type, while proteins with a lower percentage of hydrophobic amino acids are the proteins of gelation type. Further heating causes the egg albumen to polymerize and form a mesh. Many globular proteins with different sulphydryl groups can form thermally induced gels.

The second stage is the evident coagulation. At this stage the particles lose their sedimentation stability. If the density of the particles is higher than the density of the dispersion medium, those particles fall out. Coagulation can be caused by various factors: heat, high pressure, presence of salts, alkalis, acids, alcohols and denaturing agents, e. g. urea. In cooking conditions, the egg white coagulates at a temperature of 57–60 °C, while the yolk or whole egg coagulates at slightly higher temperature (65–70 °C). Adding of certain organic acids or cooking salt can raise the upper threshold of the protein thermal coagulation, which mechanism is used for eggs pasteurization [30,31,43,44].

Changes in the functional parameters of egg white in relation to the heating temperature

During coagulation and aggregation of proteins the disulfide bonds are redistributed, the content of Sh-groups changes, which leads to pH change. Thus, pH is a parameter that characterizes the changes in protein denaturation process — coagulation and aggregation.

The research conducted by Stefanova I. L. et al. [45] shows (Figure 1) that while heating the egg white with citric acid and cooking salt, the pH of the protein changes as follows:

- it decreases till the temperature of 65 °C;
- then it gradually grows up to the original value till the temperature of (75–80) °C;
- at the temperature of 82 °C pH increases sharply and grows up from 7.2–7.4 units to 8.6 units at the temperature of 92 °C.

These data prove that protein coagulation occurs at a temperature of (82 ± 2) °C. At the temperatures above 82 °C the egg white clot keeps thickening at temperatures up to 88 °C.

When the egg white is heated to the temperature of 80 °C, the coagulated protein forms a suspension that slightly compacts, but does not separate from the whey while centrifuging at 3,500 rpm. With a further increase in temperature a clot is formed, the amount of which increase along with the increase of temperature up to 86–88 °C. Then the mass of the clot does not change; only further compaction of the clot occurs.

Composition and yield of coagulated protein depending on the level of egg white heating

The level of heating directly affects the yield and protein composition (Figure 2). The separation of the clot begins at 82 °C. At the temperatures up to 84 °C, the yield of coagulated egg white increases by more than 15%. At 88 °C the yield is the highest. A further increase of temperature leads to a slight decrease in yield of protein.

The temperature of denaturation transitions depends on adding of acids, alkalis and salts to protein solutions [45].

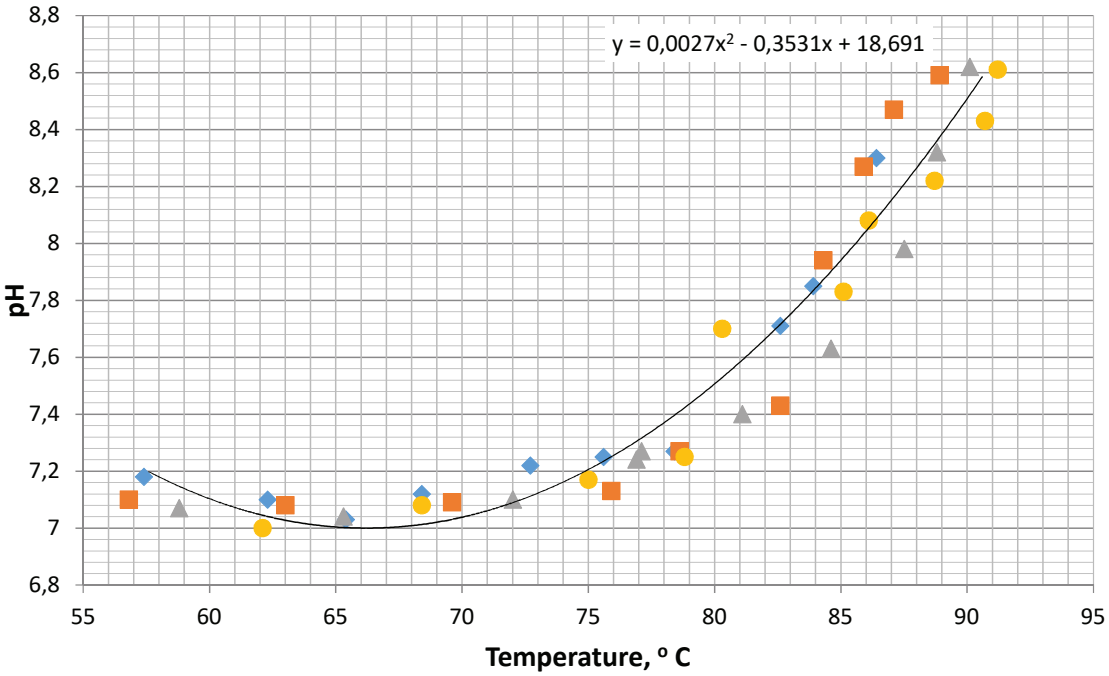

Figure 1. Changes of egg white clot pH during heating

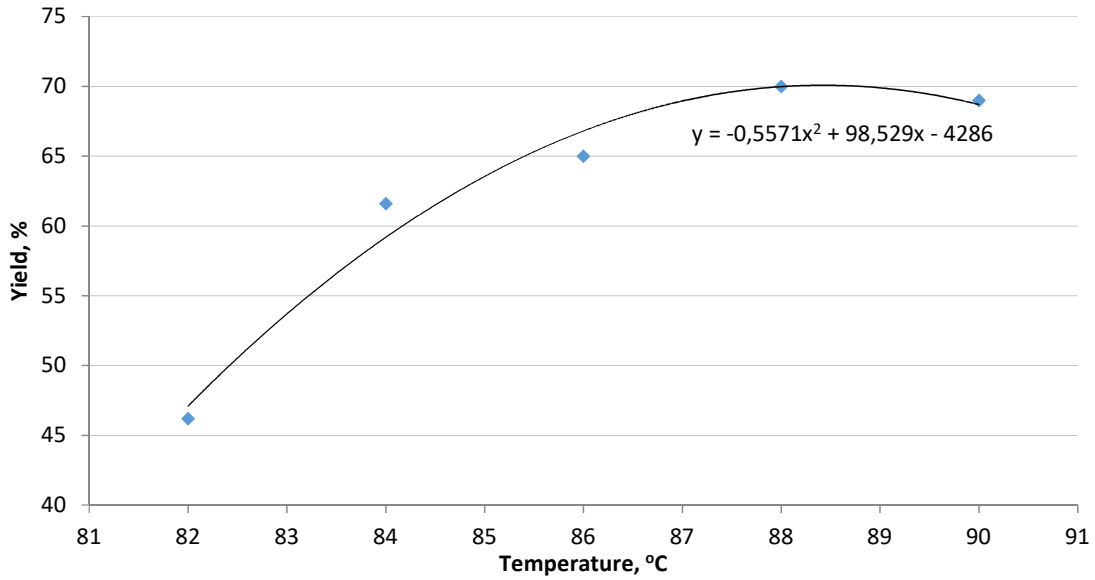

Figure 2. Dependence of coagulated protein yield on temperature of the protein mixture heating

It is shown in the research [45] that while raising the amount of added citric acid from 0.10 to 0.13% simultaneously with heating up to a temperature of 86 °C, the yield of protein increases from 61 to 66% and decreases with a further buildup of acid concentration (Figure 3). The dependence of protein yield on amount of the introduced acid when the mixture is heated to a temperature of 88 °C has the same pattern, but the yield is higher. The highest yield is observed at 86 °C and 88 °C, and it accounts for 66.2% and 68.5%, respectively. Thus, the optimal citric acid concentration is 0.13%.

When the temperature of the final heating raises up to 88 °C, the yield of coagulated protein increases while the amount of drained whey decreases. The composition of the coagulated protein changes (Table 5). It was found when coagulation temperature increases and, accordingly, the yield, the content of protein and dry matter in the coagulated egg white (clot) increases, while 92.65% of the protein remains in the coagulated protein, and only 7.35% of the protein remains in the whey. The dry matter in the coagulated egg white accounts for 85.3% of dry matter in the original egg white.

Table 5. Content of protein and dry matter in coagulated egg white

No. of test	Temperature, °C	Yield, %	Mass fraction of protein, %	Mass fraction of dry matter, %
1	82	46.4	14.8 ± 0.5	17.89 ± 0.35
2	84	61.6	13.8 ± 0.3	16.73 ± 0.33
3	86	65.0	14.1 ± 0.5	17.25 ± 0.28
4	88	70.0	14.1 ± 0.4	17.30 ± 0.28
5	90	69.0	14.2 ± 0.4	17.41 ± 0.30

Note: mass fraction of protein in the original egg white — 10.6 ± 0.3%, mass fraction of dry matter — 12.86 ± 0.26%

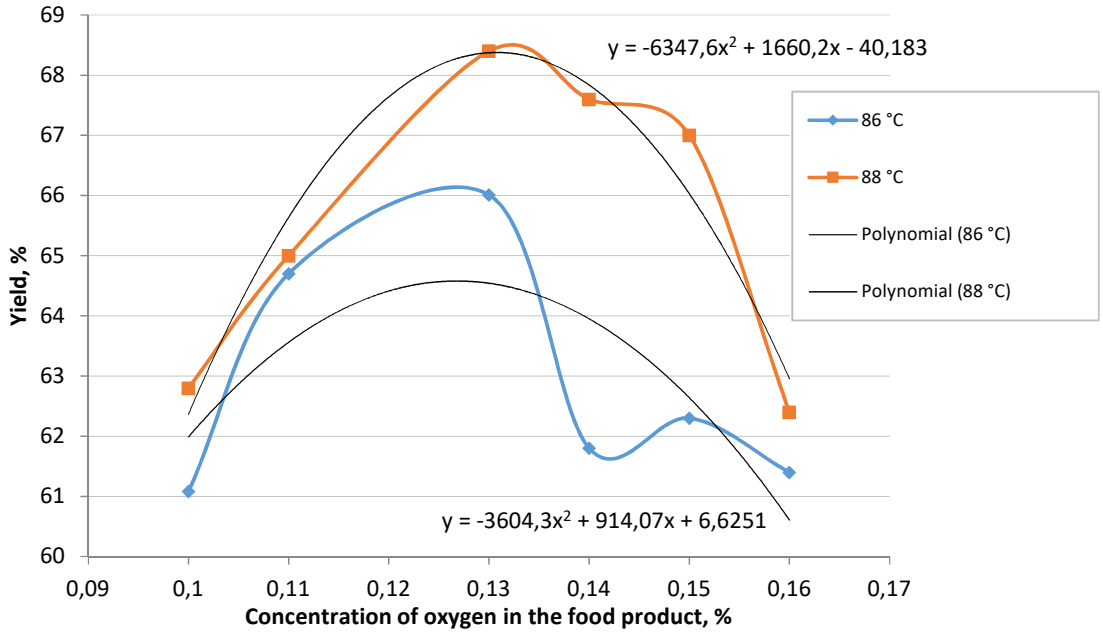

Figure 3. Dependence of coagulated protein yield on amount of added acid at different levels of heating

Table 6. Mineral composition of coagulated protein

Product	µg/kg					µg/100 g
	Ca	Mg	K	Na	Fe	P
Coagulated clot	26.4 ± 1.8	56.9 ± 9.1	1047.3 ± 123	54413 ± 598	4.7 ± 0.3	16.37 ± 2.3

Mineral composition of the coagulated protein is represented by the elements like sodium, potassium, calcium, iron, magnesium, phosphorus, chlorine, etc. and is shown below in the Table 6.

In conclusion, we also provide data on change in the total chemical composition of eggs, depending on the mode of heat treatment (Table 7).

Table 7. The content of protein and fat in the egg, depending on the heat treatment mode

Egg (50 g)	Protein (g)	Calories	Fat (g)
Raw egg	6.3	72	4.7
Hardboiled egg	6.3	78	5.3
Scrambled eggs	5.0	75	5.5
Poached egg	6.2	69	4.7
Omelet	5.3	77	5.8
Coagulated egg white	7.5	30	—
Coagulated melange	7.4	87	6.4

Coagulation of egg white can significantly affect its allergenic potency. Egg allergy is based on the allergy for the egg white. Sensitization to the protein components of chicken eggs is often accompanied with an allergy for the yolk and eggs of some other poultry species. Among young children the allergy for egg white is the second most common food allergy after allergy for cow's milk protein [44]. The overall prevalence of allergy for chicken egg white among the children in European countries is about 2.5% [46]. Reducing the allergenic potency of food proteins is an important problem for food technology, which faces the challenge of manufacturing of specialized hypoallergenic foods. Although food proteins are denatured in result of heat treatment, no decrease in their allergenic potency in the general case can be guaranteed, since the allergenic sectors of the protein in some cases are short fragments of the polypeptide sequence which are resistant to denaturation. Nevertheless, the literature contains data on decrease in the allergenic potencies and properties of food proteins during intense heating and cooking [47].

It may be possibly explained that the denatured food antigens are more actively attacked by digestive proteases in comparison with intact protein and, therefore, the absorption of their antigenic structures is reduced. Heat treatment destroys the conformational epitopes of egg whites, which cause the immune system of an allergic predisposed person to form IgE antibodies. A brief description of the potential allergenic properties of a range of chicken egg whites is presented in the research [48]. The authors note the complexity of the antigenic composition of the raw chicken egg white, and also state that 13 egg proteins have allergenic properties and ovalbumen (OVA) and ovomucoid (OVM) are allergenic to the greatest extent. As noted, OVA is phosphoprotein with MW of 44 kDa. Protein is relatively poorly attacked by proteases and can be absorbed in the digestive tract in an undigested form. OVA is one of the most important food allergens. The protein is thermolabile (it easily denatures when heated to form an insoluble gel), but its allergenic potency decreases slightly. The antigenic determinants of OVA, which are recognized by antibodies of the IgE class, appear to be consequent [49]. OVM, due to its high resistance to proteases of the human gastrointestinal tract, quite easily penetrates the intestinal barrier and causes allergic sensitization. The antigenic structures of OVM are thermolabile, and the allergenic potency of this protein decreases sharply when heated. This is apparently

facilitated by the discovery that the allergenic epitopes of OVM are conformational, in contrast to OVA which are not. The promising use of thermally induced coagulation of egg white and / or chicken egg melange to reduce the allergenic potency of the resulting food is evidenced by the data that quite often people who are allergic to chicken egg whites are able to tolerate them when egg whites are heat-treated [50].

It was shown in the research [51] that thermal coagulation of egg white acidified with citric acid provides a 15-fold decrease in the original antigenicity in comparison with native egg white (Figure 4). The content of intact ovalbumen, the antigenicity of which is taken as 100%, is 2.2% and 33% respectively in the coagulated and original lyophilized egg white. The obtained results prove a decrease in the potential allergenic properties of coagulated egg white and are an important additional argument for the prospects of using the egg whites in the composition of mass market foods and in specialized foods also [52,53].

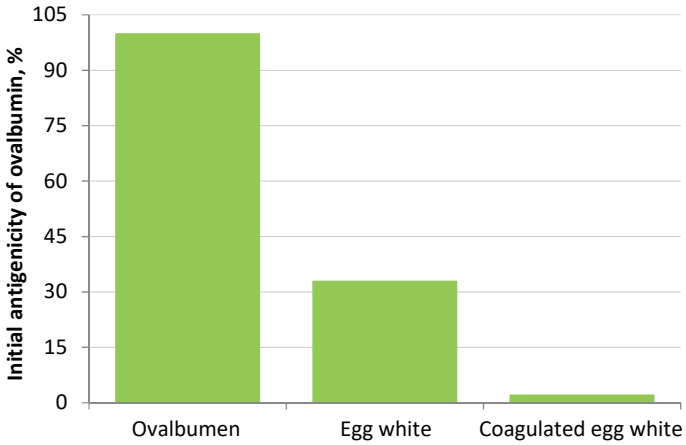

Figure 4. Antigenicity of coagulated egg white

A wide range of coagulated egg foods has been developed on the basis of coagulated egg whites [53].

The coagulation process provides for fortification of chicken eggs with minor compounds. Stefanova I. L. et al. in their research [54] proved that fortification of protein in the coagulation process with iodine and calcium provides 25–30% of the daily demand for calcium and iodine.

The content of calcium in the coagulated protein, depending on the level of its heating during egg white coagulation (i. e. adding of a mineral fortifier in amount of 1% of the egg white mass during fortification of egg white with calcium and iodine) decreases together with rising of coagulation temperature from 84 °C to 90 °C, and calcium content amounts to 551.98; 518.95; 470.86 and 439.00 mg/100 g protein, respectively (Figure 5). When the temperature rises from 84 °C to 90 °C, calcium is lost at level 25.2, 28.4, 35.0, 39.4%. Basically, calcium losses occur due to its excretion with whey.

The content of calcium in the fortified melange (Figure 5) at the introduction of calcium at dose of 725 mg/100 g of melange is 425.4; 392.4; 399.4 and 396.2 mg/100 g within the range of temperatures of 86 °C; 88 °C; 90 °C; 92 °C, respectively, and it practically does not change within the temperature range 88–92 °C. Calcium losses are 44.9–45.9%. Melange binds 54.1–55.1% of the added calcium.

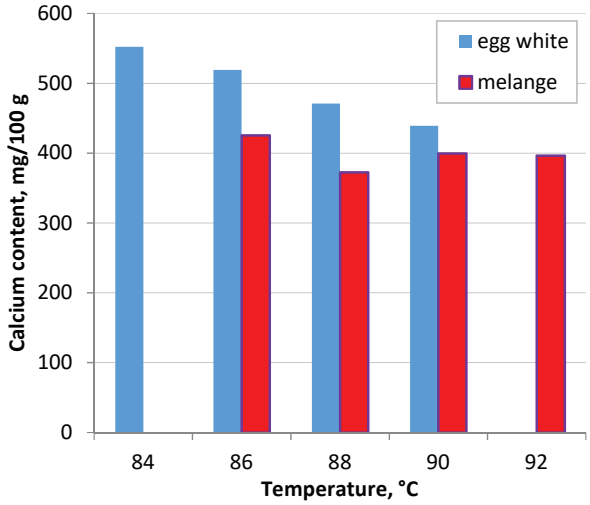

Figure 5. Change of calcium content in coagulated egg white and melange depending on the level of heating

Egg white binds more calcium: 60.6–65.0% versus 54.1–55.1% bound by melange, despite more considerable whey drainage (23.2% for egg white at 88 °C versus 9.2% for melange at 90 °C).

The content of iodine in the coagulated protein, depending on the temperature of final coagulation (86 °C; 88 °C; 90 °C; 92 °C), showed that when fortifying the protein with seaweed powder containing 456 μg of iodine per 100g of egg white, the mass fraction of iodine is respectively 298; 253; 311 and 281 μg/100g of coagulated egg white, iodine losses are 34.6; 44.5; 31.8 and 38.4%, respectively. The loss of iodine during egg whites fortification during the coagulation process is lower than when iodine is added at the moment of the food formulation. When the melange is fortified with seaweed powder in amount of 0.2%, the mass fraction of iodine in the coagulated melange is 0.203; 0.243; 0.258; 0.273 μg/100g respectively, depending on the heating temperature (86 °C; 88 °C; 90 °C; 92 °C). The share of iodine bound with melange is lower in comparison with egg white fortification with iodine, which is apparently related to the lower protein content in melange. (Figure 6).

The high frequency of metabolic syndromes, type 2 diabetes mellitus and concomitant clinical complications determine the relevance of development and creation of a wide range of new functional foods for their use in the diet of people with disorders of carbohydrate and / or fat metabolism. The results of clinical and experimental studies, so far accumulated by world nutritional science, prove the hypolipidemic and hypocholesterolaemic effects of a wide range of polyphenolic compounds.

The authors of the research [55] implemented a significant comprehensive work for development of functional specialized foods based on the egg whites fortified with cranberry polyphenolic compounds. The sorption of anthocyanins by the chicken eggs whites in the process of hydrolysis occurs to the greatest extent when the egg white is

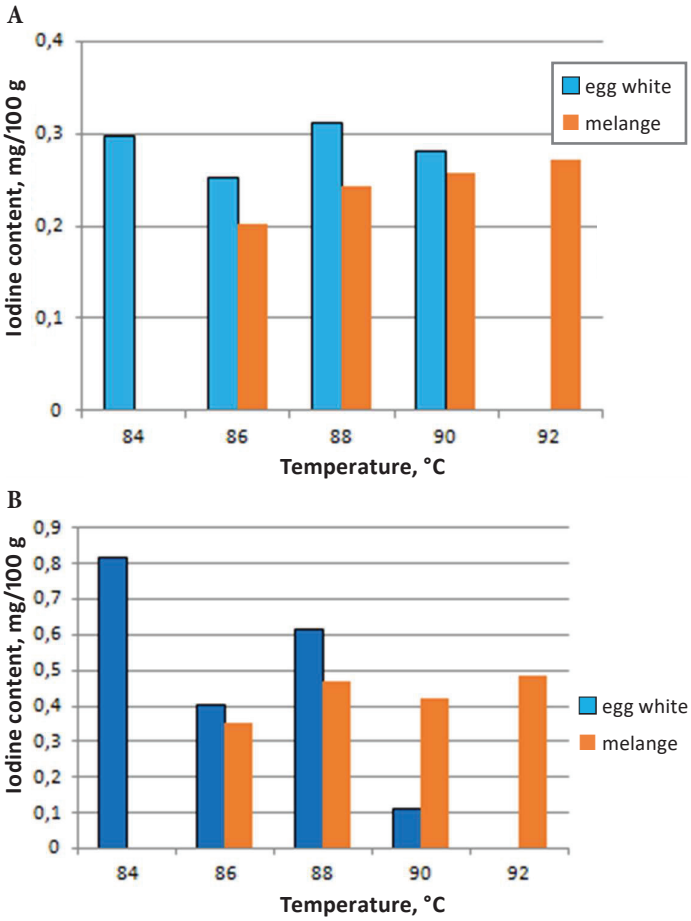

Figure 6. Change in the content of iodine in coagulated egg white and melange, depending on the heating level, with addition of 0.2% seaweed powder (A) and 0.5% seaweed powder (B)

heated to 82 ± 1°C. In this case the yield of the functional food ingredient (FFI) is 70.6–74.0% (Table 8). The anthocyanin profile is represented mainly by cyanidin-3-galactoside, peonidin-3-glalactoside (Table 9).

It is possible to substitute poultry meat with coagulated egg products (egg white, yolk and melange) in amount of 15–25 in the production of semi-finished foods%, which led to creation of whole range of foods with high biological value and low fat content [56–58].

Table 8. Anthocyanin content in FFI depending on the coagulation temperature

Temperature of coagulation, °C	100% juice	80	82	84	86
The amount of anthocyanins, (mg/100 g B*)	17.56	3.14	3.79	2.97	1.96

* Content in the solid food

Table 9. Anthocyanin profile in FFI depending on the coagulation temperature

Anthocyanin	Content, % of anthocyanin amount				
	100% juice	B80 °C	B82 °C	B84 °C	B86 °C
Cyanidin-3-galactoside	28.2	21.1	21.8	21.8	19.1
Cyanidin-3-glucoside	3.0	1.9	2.0	1.9	3.1
Cyanidin-3-arabinoside	19.0	18.7	19.6	19.1	17.1
Peonidin-3-galactoside	31.4	35.2	34.1	34.6	36.3
Peonidin-3-glucoside	5.5	6.8	6.5	6.7	7.4
Peonidin-3-arabinoside	12.9	16.3	16.0	15.9	17.0
Malvidin-3-arabinoside	traces	traces	traces	traces	traces

Conclusions

Eggs are not only the unique mono food necessary in human nutrition. Eggs contain egg whites of a high biological value and biologically significant macro- and micronutrients. Egg whites are also a multifunctional raw material component for development and creation of new modern foods for various aims and tasks. The study of the functional and technological properties of a whole egg and its components, the influence of various parameters of technological processes on the physical and chemical properties of an egg, and ability to preserve the biologically significant key characteristics allows creating a wide range of healthy foods.

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AN EFFECT OF THE RECIPE COMPOSITION ON MINCED MEAT PROPERTIES

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Keywords: *by-products, broiler chickens, chicken muscles, milk fat, ginger, oxidative stability, water holding capacity*

Abstract
The paper describes an experiment on the development of minced meat recipes for poultry-based semi-prepared products. The design principle included a search for optimal compatibility of recipe ingredients to develop a balanced meat system. The model recipes included meat from different animal and poultry species, by-products and dairy products. White and red turkey meat and chicken breast fillet provided recipes with complete animal proteins. Fat meat raw materials were partly replaced with milk fat. A semi-prepared product filled into an intestinal casing was named "sausages for grilling". A technology of sausages for grilling was developed with the indication of the main control technological parameters for the production process and thermal treatment up to product readiness. The incoming control of raw material quality was carried out: dairy cream was assessed by acidity and pasteurization effectiveness; cheese by sensory indicators; meat raw materials by the pH value. The functional-technological properties of raw minced meat for sausages for grilling were compared before and after cooking by the pH level, moisture mass fraction, water binding capacity. The rheological properties of minced meat (adhesion, viscosity, shearing structural-mechanical properties) were studied. The expedience of introducing meat by-products and dairy ingredients into recipes of sausages for grilling to stabilize protein and fat in the meat system was substantiated. It was proved that replacement of the main raw materials in recipes with pork heart, ginger, cheese and chicken liver allows obtaining sausages for grilling with preservation of high quality indicators. Sausage sensory properties after grilling were analyzed.

Introduction

Historically, meat has been an important part of the typical human diet being an important source of macro- and micronutrients, including protein, fat, iron, selenium, folic acid, zinc and vitamins A and B_{12}. According to the FAO forecasts, global meat production should additionally grow by 200 million tons by 2050 to correspond to the current forecasts for food demand [1].

Meat product manufacturers include simultaneously different types of animal and poultry meat into most minced meat recipes. Beef, pork, chicken and turkey meat, mutton and horse meat can have different weight proportions in the matrices of mixed minced meat, which gives an opportunity to produce meat systems balanced by the chemical composition and having different nutritional values [2]. A wide assortment of minced semi-finished products can be seen in retail display cases. These products are more often produced from pork and, consequently, contain significant amounts of intermuscular fat and speck, which is not recommended for people adhering to the healthy life style, as well as for people having problems with the digestive tract and cardiovascular system including atherosclerosis. A solution to this problem consists in the development of poultry-based semi-finished products with the moderate caloricity and high protein content [3].

Over the last years, changes in the food composition have been actively studied. The available base and different methods for recipe changes allow affecting the food profile and create high-quality products that are acceptable, prepared in a simple and economical way, correspond to the food legislation and are suitable for using in the industrial scale [4].

The perception of foods as wholesome is an important factor influencing food behavior of people that are prone to consciously restrict their nutrition to control appetite or weight [5]. Over the last years, researchers and the meat industry focused upon the development of low-fat meat products. However, meat products with low fat content (10% or lower) are tougher, less juicy and flavory, darker, expensive and less acceptable than their analogs with higher fat content [6]. One of the options for producing minced meat for semi-finished products balanced by the chemical composition is introduction of turkey meat having functional properties [7] and turkey by-products [8] into recipes. Turkey meat has lower fat content and higher protein content than pork. Therefore, sausage meat that contains turkey meat can be the healthier solution compared to meat from other animal and poultry species [9]. The protein and fat content in turkey meat is 25% and 4%, respectively. Broiler chicken meat protein contains 92% of amino acids necessary for humans. The content of lipids in broiler chicken meat is about 11% and many of them are unsaturated. These peculiarities of the fatty acid composition of poultry fats determine the low

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temperature of their melting (lower than 40 °C), which ensures their good emulsifying capacity and assimilability by the human body [10]. Fat plays an important role in the development of the desired texture of meat products. The mass fraction of animal fats in a minced meat recipe can be regulated by the use of fats from dairy products [11]. Dairy cream imparts good technological properties to meat systems [12]. The protein composition of dairy cream, stability of fat globules and their resistance to agglomeration are influenced by thermal treatment. Pasteurized dairy cream has more stable fat globules and, therefore, is preferable for using in meat systems [13]. Addition of dairy cream with the increased fat content into recipes enables improving structural-mechanical properties, creating necessary consistency and harmonious taste of meat products [14].

Cheese, a dairy product produced by different methods, is often used in meat product technology providing multiple characteristics such as the unique taste, texture and aroma [15]. Semi-hard cheeses introduced into meat systems are distinguished by low moisture content, firm consistency and flavory, strong taste. These cheese characteristics are taken into account when developing multicomponent recipes [16].

To improve physico-chemical properties and sensory characteristics, plant raw materials [17] and dietary fibers [18] are introduced into minced meat. The use of the dry ginger powder in meat product recipes allows extending shelf-life. The expediency of using ginger for increasing safety and controlling microbial growth in chicken meat was proved. Fermented ginger paste causes reduction of pH in chicken meat from 5.66 to 4.73 and moisture content from 76% to 72%. The paste from fermented ginger can control the growth of spoilage microorganisms and demonstrates the high potential for the large-scale industrial application in manufacturing products from chicken meat [19]. It was shown that ginger can be considered a potential replacement of nitrite both for color formation and for inhibition of the growth of *Escherichia coli*, *Staphylococcus* and *Salmonella* in meat products. Addition of ginger into recipes decreases an amount of nitrite in meat products without meat safety impairment. Compared to nitrite, ginger has additional functional advantages: the nutritional value and absence of toxicity when working with it [20].

Spices and herbs with the bactericidal action are introduced into minced meat. The use of rosemary, garlic, onion, pepper (black, red, Sichuan, chili), sage, lemon and others in meat products is well known. Spices and herbs have the antioxidant activity. For example, they reduce oxidative stress or inhibit oxidation of meat components during processing. Many researchers revealed interaction between a decrease in formation of potential carcinogenic substances and antioxidant properties of spices and herbs [21,22].

The aim of the study was to develop recipes and technologies for mixed minced meat from different types of meat raw materials and dairy products. Addition into minced meat recipes of pork, by-products, dairy ingredients instead of chicken skin confirmed the hypothesis about preservation of functional properties in minced meat and achievement of high sensory characteristics in the finished product. The obtained information can be used to determine product strengths compared to analogs. Comparison of functional-technological properties of experimental minced meat and finished products allows evaluating and revealing ways for improving sensory properties that influence consumer preferences.

Objects and methods

The recipe development, production and quality assessment of samples of sausages for grilling were carried out in the Department of Meat and Dairy Product Technology according to the scheme presented in Figure 1.

The composition of the minced meat recipes under development is presented in Table 1. The following main raw materials were used in the recipes: broiler chicken meat and chicken skin, turkey meat, pork heart, chicken liver, pork belly and speck, semi-hard cheese, dairy cream with 20% fat. The auxiliary materials included fresh garlic, edible salt, sugar, ground white pepper, cardamom, ground ginger, ground paprika. The recipes of the model minced meat are presented in Table 1.

At the stage of mixing, 20% of drinkable water and 1 kg of salt per 100 kg of the main raw materials were added for preliminarily salting. Water addition was not envisaged by the recipe of the control sample according the technical specifications for the product. Part of garlic and sugar was replaced with ground cardamom, ginger and paprika in the recipes of the samples. Spices were not used in the control sample recipe; garlic was added in an amount of 2% of the main raw material weight.

The basis of the meat constituent in the control sample recipe was white meat of broiler chicken breast fillets (50%) and red meat of turkey thigh (38%). In sample No. 1, broiler chicken breast fillets were replaced with pork heart in an amount of 10%, the volume of meat from turkey thigh was reduced by 8% and chicken skin was completely removed from the recipe replacing it with pork belly (17%). Chicken skin was replaced with ingredients containing easily assimilable animal fats in all experimental samples. Skin was replaced with pork speck in sample No.2, with cheese in sample No.3 and partly with pork belly and chicken liver in sample No.4. To impart juiciness and tenderness to the finished product, dairy cream was added into the model sausage meat in samples No.1, No.2, No. 3 in an amount of 3% instead of broiler chicken breast fillets. Sausages were stuffed into natural casings (pig small intestines).

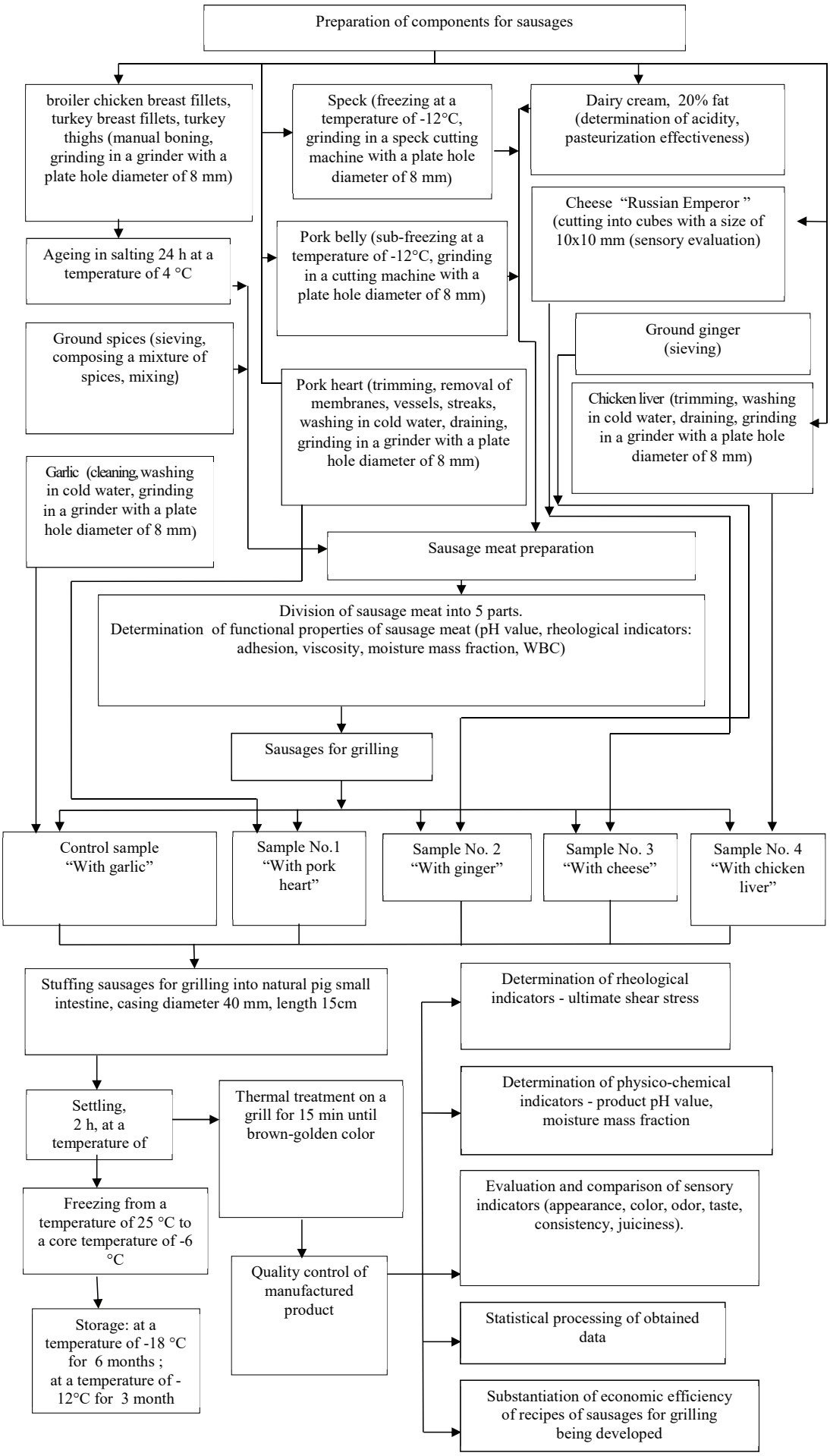

Figure 1. Scheme of the experimental investigations

Table 1. Recipes of minced meat for sausages for grilling

Unsalted raw materials, kg	Control sample "With garlic"	Sample No.1 "With pork heart"	Sample No. 2 "With ginger"	Sample No. 3 "With cheese"	Sample No. 4 "With chicken liver"
Main raw materials, kg					
Broiler chicken breast fillets	50	40	40	45	40
Turkey breast fillets (white meat)	—	—	40	—	25
Turkey thighs (red meat)	38	30	—	35	—
Chicken skin	12	—	—	—	—
Pork heart	—	10	—	—	—
Chicken liver	—	—	—	—	15
Pork speck	—	—	17	—	—
Pork belly	—	17	—	—	20
Semi-hard cheese	—	—	—	17	—
Drinkable dairy cream, 20% fat		3	3	3	—
TOTAL	100	100	100	100	100
Spices and herbs per 100 kg of unsalted raw materials, kg					
Fresh garlic	2	0,4	0,6	0,2	0,4
Edible salt	1	1	1	1	1
Granulated sugar	0,25	—	—	—	—
Ground white pepper	0,3	0,3	0,1	0,3	0,3
Cardamom	—	0,3	—	—	0,3
Ground ginger	—	—	0,5	—	—
Ground paprika	—	—	—	0,3	—
Water, %	—	20	20	20	20
Yield, %	103.55	102	102.2	101.8	102

To determine physico-chemical, structural-mechanical and other indicators of the initial raw materials, ingredients, minced meat and finished products, standard and modified analytic methods and equipment were used. Moisture mass fraction in minced meat and finished sausages was determined by the thermal gravimetric method using a drying apparatus APS-1 (Russia). With that, moisture mass fraction was calculated by the difference in the mass of the initial and dried samples. To determine pH of meat raw materials and minced meat, pH-meter of the model 2696 (Russia) was used. A range of pH measurement was 0 to 14, precision 0.02 pH units. Minced meat viscosity was measured on the Brookfield viscometer RVDVE with RV 7 spindle (USA). A range of viscosity values was 100 to 13,000,000 mPa*s, rotational speed was 0.3 to 100 rpm. Viscosity was measured at 2 rpm. Shearing structural-mechanical properties of the finished product were measured on the laboratory conical plastometer CP-3 (Russia). Detection limit for ultimate shear stress was up to 1 MPa. The adhesion level was determined on the unit for stickiness measurement by S. Tishkevich. Water binding capacity (WBC) of minced meat was determined by the pressing method and then by calculation. Sensory evaluation of finished products after grilling was carried out by the descriptive (profile) method. Cream acidity was determined by the titrimetric method[1], pasteurization effectiveness by the peroxidase reaction[2]. Cheese quality was assessed by appearance of a cheese wheel, consistency, taste and odor, color, pattern on the cut surface of the cheese[3].

Statistical processing of the experimental results was carried out according to [24]. With that, the arithmetic mean and standard deviations were calculated. Then, for these samples, the standard error of the arithmetic mean deviations and limits of its confidence interval were determined with account for the Student's t-coefficient (n, p) at the significance level of 95% ($p = 0.05$) and the number of measurements. Then, the significance of differences between mean values in the experimental and control samples were assessed by the p-value in the variant of the two-sample unpaired t-test with uneven dispersions. Differences were considered significant when inequality $P \geq 0.05$ was true.

Results and discussion

Dairy cream (20% fat) was introduced into the model minced meat recipes in an amount of 3% of the main raw material mass. The incoming control of dairy cream acidity showed the level of 18 °T, which indicated their freshness and wholesomeness. When determining pasteurization effectiveness, a change in the color of the tube content was not noticed; therefore, peroxidase was broken down and dairy cream was pasteurized correctly at the high temperature.

The recipe of sample No.3 contained semi-hard cheese in an amount of 17% of the main raw material mass. When assessing appearance of a cheese wheel, the uniform and strong condition of the crust and paraffin layer was observed. Consistency was moderately elastic, homogeneous throughout the wheel. Taste and odor were cheesy, slightly acidic. The pattern on the cut surface consisted in eyes of the irregular and angular shape that were uniformly located throughout the cheese mass. The color was in a range from white to light yellow. Cheese did not pass its shelf life date and the product was made according to the requirements of the standard.

To classify meat raw materials according to quality groups, hydrogen ion concentrations in thigh and breast muscles were measured immediately after boning poultry carcasses.

Changes in the pH values demonstrate meat system stability and indicate the development of irreversible protein aggregates and the level of meat raw material ageing [25]. It was established that after slaughter the pH level dropped in

[1] GOST 3624–92 "Milk and milk products. Titrimetric methods of acidity determination". Moscow: IPK Standards Publishing House, 2004. — 8 p. (In Russian)

[2] GOST 3623–2015 "Milk and milk products. Methods for determination of pasteurization". Moscow: Standartinform, 2016. — 12 p. (In Russian)

[3] GOST 33630–2015 "Cheese and processed cheese. Methods for control of organoleptic properties". Moscow: Standartinform, 2016. — 58 p. (In Russian)

broiler meat (drumsticks) from the initial value of 6.18 to the ultimate value of 5.96 at 24 hours after slaughter [26]. For turkey meat, pH was 5.93 to 6.0, and in turkey fillet 6.20 (normal) and 6.04 (pale) [27]. In the present study, the pH value in turkey meat was 5.94, in broiler chickens 5.96, which corresponds to the data of foreign scientists mentioned above. The indicators allow making a conclusion about classification of meat as the NOR quality group and the expediency of using poultry meat in the technology of sausages for grilling.

At the stage of minced meat preparation, the pH level was measured again, minced meat properties were analyzed (Figure 2).

Addition of dairy ingredients, by-products, ginger, salt and spices into the experimental minced meat samples did not influence changes in the pH level in raw minced meat. The significant difference between the samples by the level of hydrogen ion concentration was not revealed.

The rheological properties of meat systems are determined by the solubility of muscle protein, a size of minced meat particles, viscosity, capacity to bind water, fat and other minced meat constituents [28]. It was found in the rheological investigation of minced meat (adhesive properties, Figure 3) that the highest adhesion level (399.25 ± 10.34) was in the control sample. Addition of water into minced meat led to a significant decrease in adhesion compared to the control. Addition of cheese into the recipe of sample No.3 facilitated maintenance of minced meat stickiness due to the content of milk fat in the cheese composition. With that, this indicator exceeded stickiness in samples No.1 and No.2 by 43.5 Pa and 34.25 Pa ($P < 0.01$), respectively. The lowest adhesion level was in sample No.1. This means that addition of pork heart into minced meat insignificantly reduces minced meat stickiness, and crumbly texture of the product is possible after thermal treatment.

In terms of rheological properties, structural rigidity and viscosity of raw minced meat play an important role in assurance of the right texture and consistency of the finished meat product. High viscosity of chicken-based minced meat ensures safe consistency of the finished product and can delay the rate of flow for food bolus during swallowing [29]. Absence of water in the recipe of the control sample also determined the high level of minced meat viscosity (532.40 ± 37.16 Pa·s) and strong minced

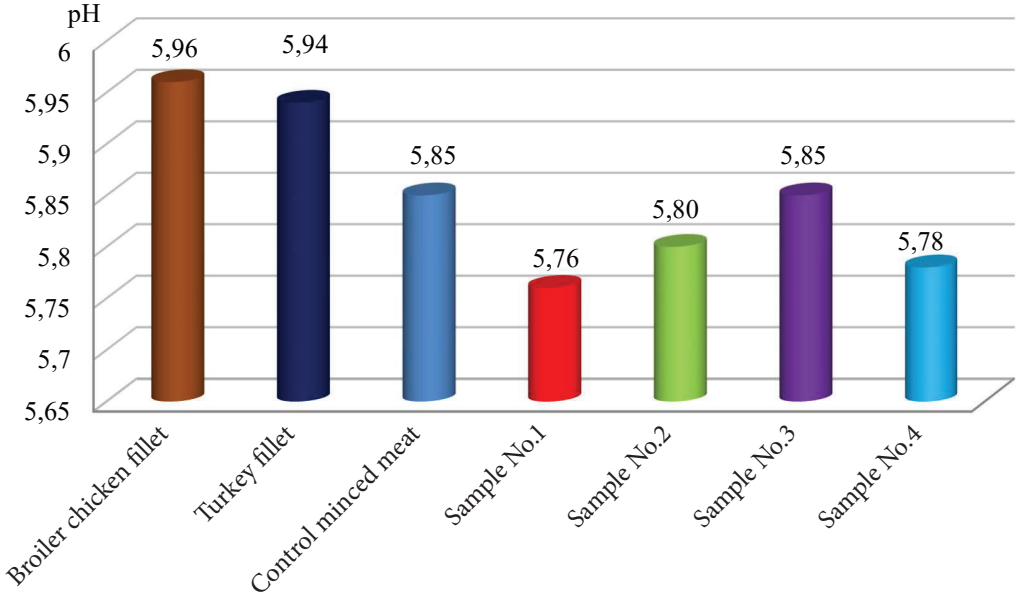

Figure 2. pH level in meat and minced meat

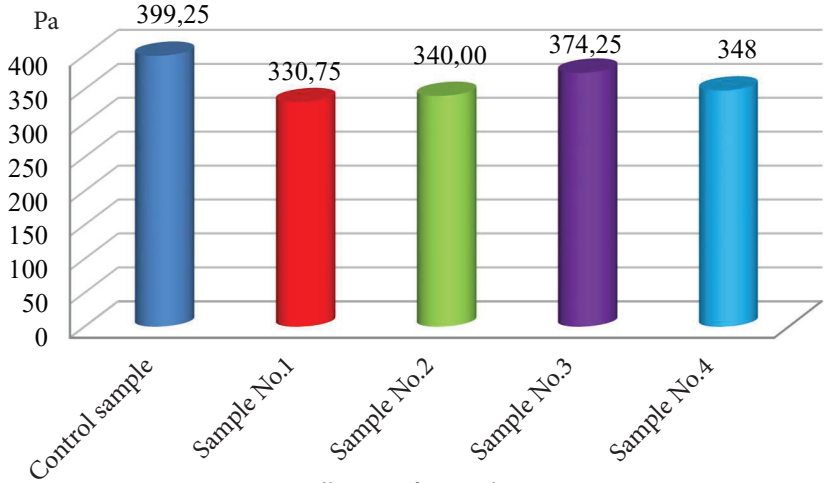

Figure 3. Adhesion of minced meat, Pa

meat structure (P < 0.001). The lowest viscosity level was in sample No.1 (182.60 ± 5.65 Pa·s). This is significantly lower than in samples No.2 and No.4 by 62.8 and 60.8 Pa·s, respectively (P< 0.001), and in sample No.3 by 44.4 Pa·s (P < 0.01). Addition of pork heart into the minced meat recipe imparted a loose structure to it (Figure 4).

The experimental samples of minced meat significantly exceeded the control sample by the level of water binding capacity (WBC) (P < 0.001) (Figure 5). The authors [30] established that the use of ginger with the chicken breast as a model system facilitates water holding capacity, protein solubility, myofibril fragmentation, shear force reduction. A degree of myofibril fragmentation caused by the action of proteases in ginger is a technologically viable alternative to improve tenderness and yield of chicken breast. The data of our research confirm that addition of ginger in minced meat sample No.2 facilitated an increase in minced meat hydration by 15.12% compared to sample No.1, which recipe contained pork heart (P < 0.01). Addition of cheese (sample No.3) and chicken liver (sample No.4) into the recipes led to a significant decrease in WBC compared to minced meat sample No.2, which composition included ginger (P < 0.05).

Calculations of the moisture mass fraction in minced meat confirmed that the experimental samples exceeded the control by the moisture content (P< 0.001) (Figure 6). The moisture mass fraction in the experimental samples was determined by the product recipe. Sample 2 contained more free moisture than sample No.1 by 5.06% (P< 0.01). The moisture mass fraction was 3.12% and 3.96% higher (P < 0.01) in samples No.3 and No. 4 compared to sample No.1. Therefore, addition of ginger into minced meat recipes facilitated binding higher amounts of moisture of the hydrophilic groups compared to addition of cheese and chicken liver.

The pH value, moisture content and rheological characteristics were measured in the finished product after grilling for 15 min. and following cooling (Figures 7, 8, 9).

Finished experimental samples had higher pH value than minced meat before thermal treatment. Significant differences between experiments were not found.

After grilling, the control sample retained the maximum values of ultimate shear stress (Figure 8).

Ultimate shear stress in the control sample was 300.73 ± 25.63, which was significantly higher by 160.6 and 162.39 Pa than in samples No.1 and No.4 (P < 0.05) and by 134.23 Pa (P < 0.01) than in sample No.3. Sausages for grilling made from the control sample had stronger and more resilient consistency compared to the control samples.

Results of the moisture mass fraction measurement in the finished product are presented in Figure 9.

The highest value of the moisture mass fraction (67.34%) was established in sample No.3 (P < 0.01). A significant difference between samples No.2 and No.3 by this indicator was not established. Moisture losses upon bringing to culinary preparedness were in a range from 35% in the control sample to 28% in the experimental samples.

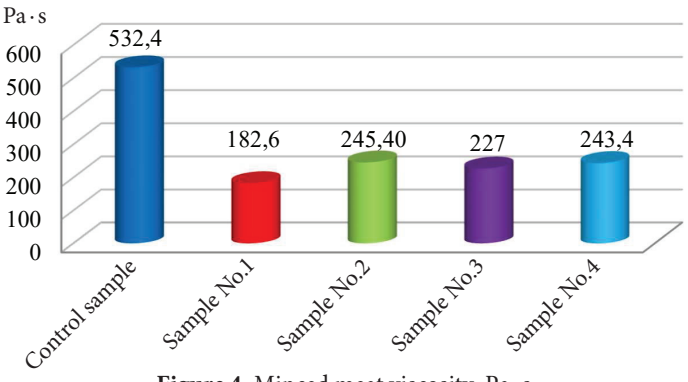

Figure 4. Minced meat viscosity, Pa·s

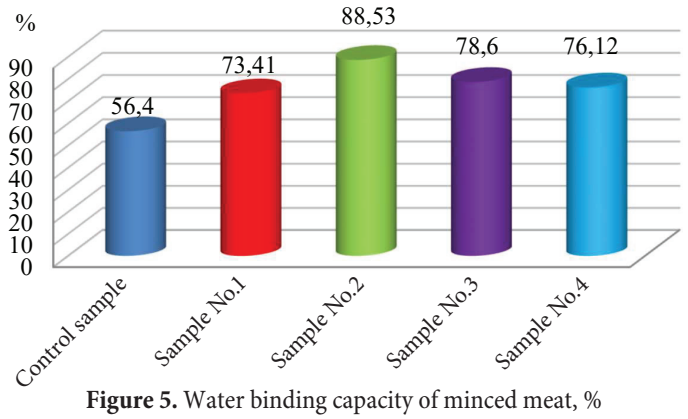

Figure 5. Water binding capacity of minced meat, %

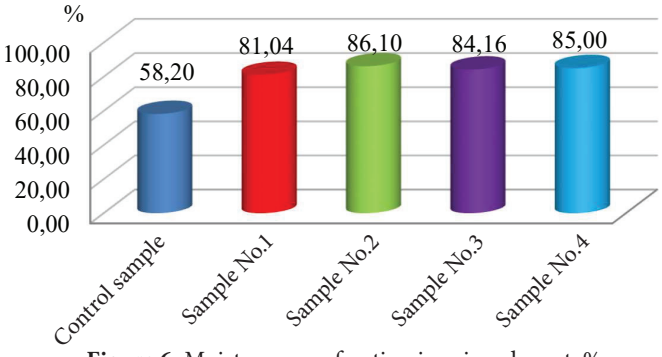

Figure 6. Moisture mass fraction in minced meat, %

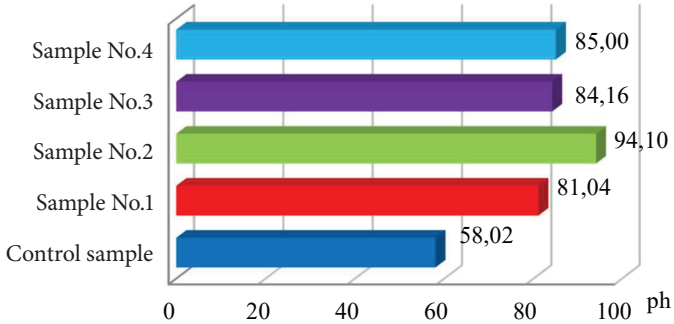

Figure 7. pH value in the finished product

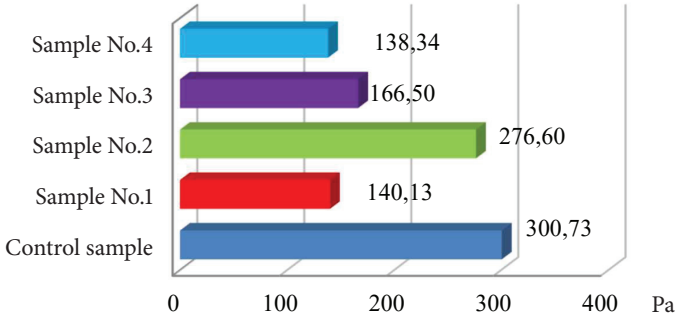

Figure 8. Ultimate shear stress in the finished products, Pa

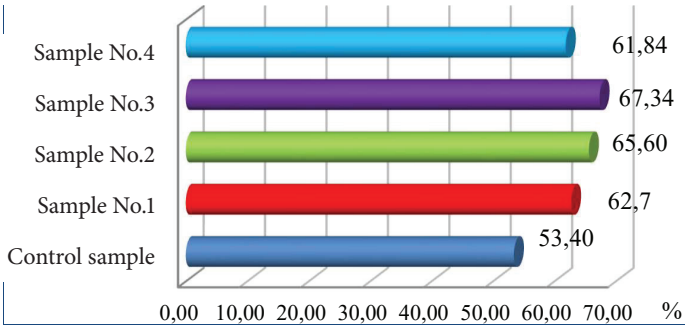

Figure 9. Moisture mass fraction in the finished product, %

The technology for production of sausages for grilling includes the following main stages:

1. cutting chicken broiler and turkey carcasses according to the anatomical organization into leg quarters, breasts, thighs, drumsticks, carcasses, wings;
2. boning turkey thighs, turkey and broiler chicken breasts to obtain fillets
3. pork heart trimming of membranes and rough vessels, chicken liver trimming of membranes, washing under cold water;
4. sub-freezing of speck and pork belly at a temperature of –12 °C;
5. cutting cheese into cubes with a size of 10x10 mm;
6. grinding meat raw materials in a grinder with a plate hole diameter of 8 mm;
7. mixing meat raw materials in a sausage meat mixer with water in a volume of 20% and edible salt in an amount of 1 kg salt per 100 kg raw materials;
8. holding sausage meat for ageing at a temperature of 4 °C for 24 hours to create conditions in the finished products for formation of taste, juiciness, stickiness, increased resistance to the microbial action and water holding at thermal treatment;
9. making model sausage meat in a sausage meat mixer with the following addition and mixing of all required recipe components. At first, lean raw materials and spices were added; then, pork belly, dairy cream, chicken skin and speck preliminarily sub-frozen at a temperature of –12 °C;
10. stuffing sausages for grilling into natural pig small intestine with a diameter of 40 mm using a vacuum syringe and making sausages with a length of 15 cm;
11. hanging on the racks and holding at a temperature of 4 °C for 6 hours for secondary structure formation in sausages, which corresponds to settling;
12. controlling semi-finished product quality; freezing to a temperature of –8 °C;
13. packaging sausages into trays in the packaging machine under vacuum.

To assess finished product quality, the frozen semi-finished product was grilled for 15 min. The product core temperature was recorded at a level of 72 °C using a digital thermometer to guarantee that sausages from poultry meat were brought to culinary readiness. Wider possibilities of using the semi-finished product both frozen and thawed to a room temperature are recommended for a consumer. To bring a semi-finished product to culinary readiness, several methods are suggested: frying on a mangal, frying pan, grill, as well as baking both sides in a baking oven or microwave oven with a grill mode for 15 min. to the gold-brownish color. Grilled sausages are preferably eaten hot. Sausages are served with a side dish, for example, backed potato, rice, buckwheat, fresh vegetables and greens; hot sauces (such as garlic, mustard, tomato sauces) are also suitable.

Methods for profile analysis allow quantitative assessment and graphical description of the whole spectrum of sensory properties of the meat product.

The profile method gives an opportunity to distinguish competitive products from each other by the individual peculiarities of taste, aroma and consistency. When evaluating an appearance of the sausages for grilling, the tasting panel paid attention to uniformity of grilling, tightness of the casing adherence to the surface of the presented samples, integrity of the casings. It was noted that this indicator corresponded to the norms in the control and experimental samples and did not have significant differences (Figure 10).

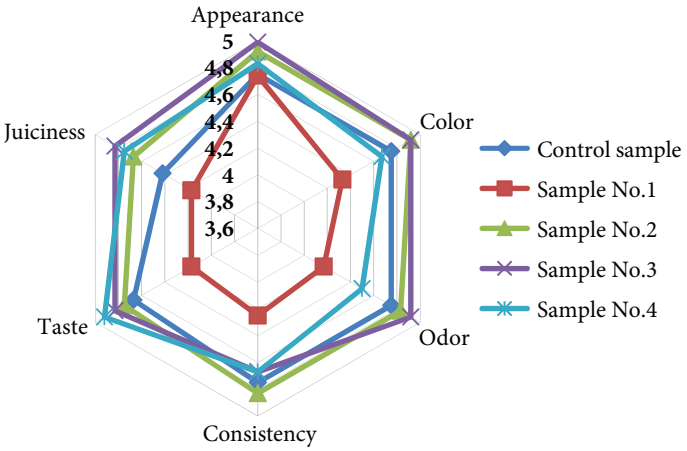

Figure 10. Values of the sensory indicators of the finished products, points

When evaluating color on the cut surface, the tasters noted a difference be-tween samples No. 1, 2 and 3. Samples No. 2 and No.3 exceeded sample No.1 by 11.8% ($P < 0.05$). According to the tasters' opinion, dark inclusions of the pork heart in sample No.1 caused the negative visual perception.

Evaluating product aroma, the taste panel noted that samples No.3 and No.4 exceeded sample No.1 by 0.75 and 0.33 points, respectively ($P < 0.01$) or by 15% and 6.6%. In sample No.3, odor was conditioned by introduction into the product recipe of semi-hard cheese, which was specially intended for grilling and imparted pronounced cream-milk odor that was pleasant for the olfactory organs. In sample No.4, odor typical for liver was noticed, which was also very pleasant, delicate and hardly noticeable.

Properties of meat product texture influence human perception of finished product consistency. Textures,

which are difficult to process in the mouth, are less preferable for people. Changes in meat product texture from juicer, softer and more uniform to harder and lumpy significantly reduce the consumption rate and energy intake, affect food behavior and, consequently, health [31].

Evaluating product consistency, the tasting panel observed the differences in samples No.1 and No.2. Sample No.2 exceeded sample No.1 (P < 0.05) by 0.58 points or 11.6%. Due to inclusion of heart, rough and crumbly consistency in sample No. 1 was conditioned by the by-product structure, which did not become tender even after thermal treatment. Firm and resilient consistency was noticed in sample No.2 as ginger can bind the minced meat structural components between themselves into the uniform monolithic structure. The significant differences were not found between all other experiments and the control; the samples corresponded to the requirements of the standard and had firm and homogeneous consistency.

In terms of taste, the experts preferred samples No. 2,3,4. Samples No.2 and No.3 exceeded sample No.1 by 0.58 and 0.66 or 11.6% and 13.2%. In sample No.1, the specific flavor of pork heart was significantly different from astringent flavor of ginger and pungent flavor of cheese P< 0.05). Sample No.4 had more pronounced flavor of chicken liver compared to sample No.1 with inclusions of pork heart with a difference of 0.75 points or 15% (P< 0.01).

The experts noticed the highest juiciness in samples No.3 and No.4 compared to sample No.1 by 0.66 (13.2%) (P< 0.01) and 0.58 points or 11.6% (P< 0.05), respectively. Minced meat with cheese and chicken liver have the higher water holding capacity and can satisfy the consumer requirements in terms of finished product juiciness.

Statistical processing of all points showed that tasters preferred sample No.3, which exceeded the control sample and sample No.1 in terms of sensory characteristics by 0.28 (P<0.05) and 0.35 points (P<0.01). The second place was given to sample No. 4, which exceeded the control sample and sample No.1 by 0.27 and 0.34 points (P<0.05). The third place was occupied by sample No.2, which exceeded sample No.1 by 0.33 points (P<0.05). Experimental samples of sausages for grilling with cheese, chicken liver and ginger turned to be more attractive by taste characteristics and all other sensory indicators than sausages with pork heart.

Conclusion

When evaluating color on the cut surface, the tasters noted a difference be-tween samples No. 1, 2 and 3. Samples No. 2 and No.3 exceeded sample No.1 by 11.8% (P< 0.05). According to the tasters' opinion, dark inclusions of the pork heart in sample No.1 caused the negative visual perception.

Evaluating product aroma, the taste panel noted that samples No.3 and No.4 exceeded sample No.1 by 0.75 and 0.33 points, respectively (P< 0.01) or by 15% and 6.6%. In sample No.3, odor was conditioned by introduction into the product recipe of semi-hard cheese, which was specially intended for grilling and imparted pronounced cream-milk odor that was pleasant for the olfactory organs. In sample No.4, odor typical for liver was noticed, which was also very pleasant, delicate and hardly noticeable.

Properties of meat product texture influence human perception of finished product consistency. Textures, which are difficult to process in the mouth, are less preferable for people. Changes in meat product texture from juicer, softer and more uniform to harder and lumpy significantly reduce the consumption rate and energy intake, affect food behavior and, consequently, health [31].

Evaluating product consistency, the tasting panel observed the differences in samples No.1 and No.2. Sample No.2 exceeded sample No.1 (P< 0.05) by 0.58 points or 11.6%. Due to inclusion of heart, rough and crumbly consistency in sample No. 1 was conditioned by the by-product structure, which did not become tender even after thermal treatment. Firm and resilient consistency was noticed in sample No.2 as ginger can bind the minced meat structural components between themselves into the uniform monolithic structure. The significant differences were not found between all other experiments and the control; the samples corresponded to the requirements of the standard and had firm and homogeneous consistency.

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FACTORS INFLUENCING MICROBIAL TRANSMISSION IN A MEAT PROCESSING PLANT

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Abstract
The review paper examines the main risk factors for microbial contamination of meat at different stages of its processing. Particular emphasis has been placed on primary animal processing being the most hazardous in terms of microbial contamination of meat. Carcass cross-contamination most frequently occurs during skinning and evisceration since hides and the digestive tract are the primary sources of microbial pathogens. It is necessary to observe stringent sanitary and hygienic rules when performing these operations. Continuous cold chain management along all following stages of meat processing and control of the sanitary status of cold chambers during meat storage are of extreme importance. An increase in the microbial counts due to the high number of manual operations was observed during meat cutting, boning, and trimming. Subsequent stages of meat processing, including mincing, curing, the addition of spices, also promote significant microbial growth. Strict control regarding detection of dangerous pathogens, especially L. monocytogenes, is needed at this stage. In general, to minimize problems linked with meat and meat product safety, it is necessary to take timely measures on sanitary treatment of meat processing facilities, including the prevention of biofilm formation.

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Introduction

The primary task of the food industry is to ensure the microbiological safety of manufactured products. Manufacturers have to prevent possible risks linked with microorganisms causing foodborne infections [1]. Dangerous microorganisms include bacteria (*Salmonella* spp., *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, Clostridium botulinum, Clostridium perfringens, Cronobacter sakazakii, Bacillus cereus, Shigella* spp., *Staphylococcus aureus, Vibrio* spp., *Yersinia enterocolitica,* and others), viruses (Hepatitis A virus and Norovirus) and parasites (*Cyclospora cayetanensis*, *Toxoplasma gondii*, and *Trichinella spiralis*) [2].

Many factors affect microbial composition in food processing plants. Among them, a type of food processing plant, sanitary and hygienic conditions of the working environment, and microflora of raw materials are the main ones. The methodology of sampling and microbial identification influences the knowledge about microflora in a food processing plant. High-throughput sequencing opens wide possibilities for detecting microorganisms in food products being highly efficient and quick compared to the traditional methods [3]. There may be hundreds of different bacteria in a single food processing plant, but, as a rule, only a few bacterial species dominate. Six bacterial groups show high prevalence, such as *Pseudomonas, Acinetobacter, Enterobacteriaceae*, spore-forming bacteria, *Staphylococcus* spp. and lactic acid bacteria (LAB). Non-dominant species can account for more than 10% of the total bacterial counts and show high species diversity, including *Aeromonas* spp., *Brochothrix* spp., *Microbacterium* spp., *Micrococcus* spp., *Neisseriaceae, Psychrobacter* spp., *Ralstonia* spp., *Rhodococcus* spp., *Shewanella* spp., *Sphingomonas* spp., *Stenotrophomonas* spp. and *Vibrio* spp. [4].

Meat is one of the most perishable foods since it contains substances necessary for rapid microbial growth. Causative agents of foodborne infections such as *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes, Clostridium perfringens, Escherichia coli, Yersinia enterocolitica, Staphylococcus aureus* can be present and grow in meat creating risks for consumers' health [5].

The rate of the microbiological changes in meat depends on the initial number of microorganisms, storage conditions (duration, temperature, relative humidity), and physicochemical properties of meat, such as pH and water activity [6]. Various technological operations (curing, heat treatment, cooling, and others) affect microbial communities' qualitative and quantitative composition in meat and production facilities. Production facilities are characterized by a specific temperature, humidity, sanitary and hygienic activities. Consequently, microbial communities composed of different species composition are formed in various production facilities. During processing, meat can be the primary source of microbial contamination or, *vice-versa*, contaminated by the personnel, technological equipment, utensils, containers, floors, walls, ventilation ducts, air and other sources of the production environment [7].

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Slaughter and primary carcass processing

There is a high probability of microbial contamination of meat already at the stage of animal slaughter and primary carcass processing. The preparation of animals for slaughter is an essential factor in the microbiological status of a processing plant [8]. The cleanliness of animals at slaughter is monitored in many countries. In the Russian Federation, this requirement is specified in TR CU034/2013[1].

The high risk of microbial contamination of carcasses occurs upon contact with the surface of the equipment or constructions. The existing conditions in production facilities (mainly temperature and humidity) promote an increase in microbial counts. Various microorganisms (typical for production facilities) can attach to the surfaces and form biofilms and, consequently, retain viability after cleaning and disinfection [9]. It is known that bacteria of the genus *Pseudomonas* can develop mono-species biofilms and multi-species biofilms with pathogenic microorganisms, for example, *L. monocytogenes*. This mutual biofilm formation ensures high resistance of *L. monocytogenes* during cleaning and disinfection of different surfaces (walls, equipment, and others) [10].

Species diversity of microorganisms varies depending on the type of the slaughter line. Bakhtiary et al. [11] conducted a comparative study of the microbiological carcass status on slaughter lines for cattle and small ruminants. *Salmonella*, *Enterococcus faecalis*, *Escherichia coli,* and *Pseudomonas fluorescens* were revealed in all samples by the PCR method. The highest microbial diversity was found on the carcasses of small ruminants. Significant cross-contamination can be explained by their slaughter and carcass processing peculiarities, especially during skinning, evisceration, and scrapping. Carcass contamination is the highest when all these processes are performed manually [11].

The use of manual operations in skinning can negatively affect microbiological carcass parameters. A significant number of microorganisms are present on the surface of tools and employees' hands. The number of microorganisms can reach 2×10^7 CFU/cm^2 on the hands of workers performing skinning and from 6×10^3 to 6×10^8 CFU/cm^2 on knife surfaces (depending on the sanitary state of a food processing plant). In several cases, bacterial pathogens such as *Salmonella* were revealed on the surfaces of tools [8].

Microbial contamination of hides varies from 10^4 to 10^9 CFU/cm^2 depending on the sampling site [12]. The most contaminated hide areas are the distal leg (metacarpus) and brisket due to the extensive contact with fecally contaminated floors during the pre-slaughter phase [13]. Thereby, there is a high risk of microbial carcass contamination at the moment of hide cutting at these areas during manual pre-skinning. Several published studies showed that the highest carcass contamination was observed in the brisket area on the line of skin-opening cuts compared to bovine rump, flank, and neck areas [14,15]. Coldwater washing of carcasses did not lead to significant changes in microbial counts at the sites contaminated after skinning [14].

Mechanical skinning positively influences a decrease in microbial carcass contamination [11]. Contamination with microorganisms occurs mainly due to their transmission to a carcass with dust and dirt at the moment of hide removal. It was found that the average total viable counts of bacteria (TVC) and *Enterobacteriaceae* counts (EC) on bovine hides were 5.0×10^6 and 2.0×10^4 CFU/cm^2, respectively [13]. *E. coli*, *Proteus, B. cereus, B. megaterium, Penicillium* spp., *Aspergillus* spp., and *Mucor* spp. prevailed among the detected microorganisms [16].

Pig slaughtering and porcine carcass processing are production processes with the highest risk of contamination with pathogenic microorganisms. The environment in production facilities quickly becomes contaminated during pig slaughter [17] — the risk of cross-contamination increases. Dangerous pathogens including *Salmonella, Campylobacter, Listeria, Enterococcus, Staphylococcus, Yersinia, E. coli* can be transferred to the carcass surface. *Salmonella*, *Campylobacter*, *Yersinia,* and *E. coli* can enter the production environment and meat from the animal gastrointestinal tract or skin [17]. *Yersinia* spp. can also be found on the porcine tongue and tonsils [18].

In modern production practice, swine processing is carried out without skinning. To reduce microbial contamination of surfaces, porcine skin is washed with warm water leading to an improvement in the sanitary condition of animals and reduces microbiological risks in production [17].

Bleeding of pigs is accompanied by damage of carcass integrity resulting in carcass contamination with microorganisms, including pathogens. There is a high risk of *Salmonella* contamination at this stage [17].

Scalding of pigs is performed at a temperature of 62–70 °C for several minutes. This stage can improve microbiological indices of meat carcasses, particularly inhibiting Salmonella, Campylobacter, and *E. coli* [19]. Nevertheless, the scalding process is accompanied by the accumulation of microorganisms in the water tanks. Specifically, there is a probability of detecting *Salmonella*, which survival increases with a decrease in a water temperature lower than 62 °C [20]. Thus, continuous water temperature monitoring enables minimizing the risk of porcine carcass contamination [21].

Proper evisceration is extremely important to ensure the sanitary and microbiological safety of meat. Microorganisms are constantly present in the gastrointestinal tract, internal organs, and their's lymphatic nodes. During evisceration, workers can accidentally cut the gastrointestinal tract, and meat and the production environment may be contaminated with the content of the digestive system. The studies show that the most contaminated sites when processing the porcine internal organs are the table for receiving and washing the stomach and the table for receiving and separating intestines [22].

[1] TR CU034/2013 Technical Regulations of the Customs Union "On the safety of meat and meat products" Retrieved from http://docs.cntd.ru/document/499050564. Accessed January 15, 2021. (In Russian)

Even healthy animals can be potential carriers of pathogens and opportunistic pathogens. The permeability of the intestinal walls changes as a result of stress during animal slaughter. Microflora of the gastrointestinal tract may penetrate other organs and tissues; therefore, its removal should be done as quickly as possible after slaughtering [19]. When collecting and processing intestinal raw materials, it is necessary to comply with strict sanitary and hygienic requirements, including those for washing and disinfection of production facilities [23].

The investigations indicate that different parts of porcine carcasses (ham, back, jowl and belly) after slaughter and their processing do not significantly differ in the microbiome composition. However, important differences in the carcass microflora were found when comparing carcasses from different slaughterhouses [24].

The effect of seasonality on the microbiological carcass status is ambiguous. On the one hand, it is believed that the highest risk of bovine carcass contamination with gut bacteria occurs in the summer and autumn [26]. Other studies showed that seasonality did not significantly influence microbiological indices of bovine, porcine, and ovine carcasses [27].

Various microorganisms are present on the wall and floor surfaces in production facilities, on the surfaces of equipment and utensils used in slaughterhouses. Among them, the most frequently detected are *Enterobacteriaceae*, *Proteus* spp., *Enterococcus* spp., *Staphylococcus* spp., and pathogens *Salmonella, Campylobacter*, *Yersinia enterocolitica* [28].

During the working shift, the hygienic condition of the floor and walls in a slaughterhouse significantly deteriorates. Analysis of swab samples shows that the total microbial counts exceeded the sanitary norm (1×10^3 CFU/cm^2) by five times after 3 hours of slaughterhouse work, while by the end of the working shift (after 9 hours), they were $(1.6\pm0.23)\times10^6$ and $(8.2\pm1.1)\times10^5$ CFU/cm^2 on the floor and walls, respectively [22].

Cold chambers for chilled meat storage

Cold processing is an integral part of meat production. Hot meat chilling and creating the continuous cold chain at all following stages of the technological process are necessary to achieve stable quality and safety of meat products [29].

After slaughter and processing, hot carcasses should be immediately chilled. Exposure to low temperatures facilitates a decrease in spoilage microorganisms' growth rate and reduces the risks of pathogen growth [29].

Microflora of meat entering cold chambers for storage is diverse in composition and is usually represented by mesophiles, thermophiles, and psychrophiles [6].

During meat chilling, mainly psychrophilic and psychrotrophic microorganisms, such as gram-negative *Pseudomonas* and *Vibrionaceae*, gram-positive *Lactobacillus, Microbacterium, Arthrobacter*, microscopic fungi, yeasts, and coccal forms of microorganisms grow [6, 30].

The higher the initial meat contamination, the higher was the probability of fast multiplication of psychrophilic and psychrotrophic microorganisms during chilling [31, 32]. The presence of moisture on the meat surface can be favorable for microbial growth [33].

Pathogenic microorganisms can retain viability at low temperatures. In particular, *E. coli, Campylobacter* spp., *Clostridium perfringens, L. monocytogenes, Yersinia enterocolitica* were found in meat [34,35].

Intermittent spray-chilling of bovine carcasses using 1% acetic acid or 1% lactic acid slowed down microbial growth [36].

The study of the microbial species composition on the surfaces in the cooling chambers showed the presence of bacteria *B.subtilis, B.mesentericus, Pseudomonas* spp., *Sarcina flava,* yeasts *Rhodotorula,* mycelial fungi *Penicillium, Alternaria, Mucor, Aspergillus, Chrysosporium, Tamnidium, Cladosporium.* The highest numbers of microorganisms that survived after disinfection in the cooling chambers were observed on the surfaces of shelves, tables, and boxes for by-product storage [31].

The high level of the sanitary status of cooling chambers and adherence to storage conditions (temperature, relative humidity, airflow rate) facilitates reducing microbial meat contamination by one order of magnitude and ensures long-term storage of meat raw materials [31].

Meat cutting, boning, and trimming

High hygienic requirements are imposed on meat intended for processing as it is one of the contamination sources for production facilities and final products [37]. Microbial counts and species composition in cutting, boning, and trimming facilities are dependable on the air temperature and humidity. EAEU legislation establishes the requirement for the air temperature of not higher than 12 °C (TR CU034/2013[2]). However, this temperature is too high to effectively limit meat's microbial growth as its microflora is often represented by psychrophilic pathogenic microorganisms such as *Pseudomonas* spp., *L. monocytogenes, Yersinia enterocolitica* [38].

During carcass cutting, meat boning, and trimming, microbial counts increase due to many manual operations. Total microbial counts in meat can increase by 100 times and more compared to the initial values. During meat processing, cross-contamination can occur through cutting knives and contaminated working surfaces [39]. Sources of microbial contamination also include workers' hands and clothes, tools, utensils, the air in the production facilities, and so on [40,41]. As a result of carcass cutting and during the separation of bone-in and boneless cuts, microorganisms are transferred from the carcass and cut surfaces to the inner parts of muscle tissue. The area of meat contact with working surfaces and air increases, which, correspon-

[2] TR CU034/2013 Technical Regulations of the Customs Union "On the safety of meat and meat products" Retrieved from http://docs.cntd.ru/document/499050564. Accessed January 15, 2021. (In Russian)

dently, leads to deterioration of microbiological meat indices [42].

Microbiological examination of the wall, floor, and ceiling surfaces and the air of the production facilities in meat processing plants revealed that their sanitary and hygienic condition worsened as they were contaminated with microorganisms. For example, total microbial counts varied from 10^3 to 10^5 CFU/cm^2. Pathogens and opportunistic pathogens were present in the air, on the wall, floor, and ceiling surfaces, and the surfaces of the technological equipment and ventilation ducts (Lukin, A.A., Golubtsova, Yu.V[3].).

Many authors emphasize the necessity to give particular attention to meat safety assurance and control of dangerous cold-tolerant microorganisms, including *Salmonella, E. coli, Staphylococcus, Pseudomonas, Listeria* [43,44,45].

Meat product manufacture

Meat product manufacture begins from the stage of preliminary meat processing (mincing, curing) and ends at the stage of its packaging. Fine meat grinding and minced meat preparation promote significant microbial growth. Microorganisms are distributed throughout minced meat, which is a favorable environment for their growth [46].

Microorganisms enter sausage meat from different sources: with meat, non-meat ingredients, especially non-treated spices, from air, workers' hands, and utensils [46,47].

When using spices and other recipe ingredients, particular attention is given to the incoming control of their compliance with the microbiological parameters established by regulations, for example, TR CU029/2012[4] for the EAEU states.

Several studies have shown that spices contain high levels of aerobic microorganisms and pathogenic, spore-forming bacteria, yeasts, and molds [48,49]. In particular, increased numbers of *B. cereus* were found in the samples of ground black pepper. Spices are often contaminated with molds *Aspergillus* and *Penicillium* spp. [49]. There are also reports about many cases when *Salmonella* spp. and *Escherichia coli* were detected in spices and herbs such as basil, coriander, black pepper, and peppermint [50]. Dried vegetables and spices are also sources of lactic acid bacteria (LAB), some of which can cause food spoilage. The study results showed that lactic acid bacteria were found in ingredients in 65% of cases. Their content in dried onions and garlic powder was at the highest level. Among revealed LABs, *Leuconostoc citreum, Leuconostoc mesenteroides,* and *W. confusa* were associated with food spoilage [51].

The hygienic properties of sausage casings also affect minced meat microflora. *B. halophilum*, various micrococci, *Sarcina*, aerobic bacilli, actinomycetes, molds, and other halophilic and salt-tolerant microorganisms have been found in natural casings preserved by the wet or dry salting methods [52]. Artificial casings are usually the most hygienic [53].

After minced meat stuffing, any additional external contamination of sausages can occur only upon cutting and packaging the finished product. The primary sources of microbiological contamination are workers' hands and equipment [54].

Risk assessment of finished product contamination with pathogens and spoilage microorganisms during production is essential in monitoring meat processing plants [55].

Among various microbial species, *L. monocytogenes* is the most dangerous one. The available data on *L. monocytogenes* in meat products indicate the different frequency and level of its detection [56, 57]. Analyses of frozen and chilled meat from various manufacturers, semi-finished meat products (lumpy and chopped), raw smoked and dry-cured sausages at different stages of aging, swabs from technological equipment and utensils showed high levels of *Listeria* detection [56]. Particular attention should be given to the cleaning and disinfection of equipment and surfaces in meat processing plants to prevent cross-contamination with *L. monocytogenes* [57].

The correlation between the frequency of *Listeria* spp. and *L. monocytogenes* detection in swabs taken in the production facilities was established, suggesting the possibility to use the presence of *Listeria* spp. as an indicator of the pathogen, reducing, thereby, time for analysis [58].

Sanitary and hygienic conditions of the air in industrial premises

Many studies are devoted to the microbiological assessment of the air in slaughterhouses [59,60]. Air contamination showed a decreasing trend being the lowest in the chiller compared to the early stages of the slaughter process [59].

Some microorganisms such as *E. coli, Salmonella,* and *L. monocytogenes* can survive and circulate in the air of production facilities contaminating the meat. The transmission of microorganisms through the air is influenced by:

- employees;
- moving parts (mechanisms);
- raw meat,
- insufficient separation between each zone;
- open drain ducts;
- plant structures,
- poor sanitary and hygienic conditions of production [60,61].

Employees have a significant impact on microbial air pollution. It was found that a slowly gesturing person can generate about 500,000 particles min^{-1}, and a rapidly ges-

[3] Lukin, A.A., Golubtsova, Yu.V. (2018, 14–16 May). *Research of the sanitary and hygienic well-being of raw materials, the surface of walls, floors, ceilings, air-gas space at a meat processing industry enterprise.* Innovations in food biotechnology. Proceedings of the International Symposium. Kemerovo, Russia (In Russian)

[4] TR CU029/2012 Technical Regulations of the Customs Union "Safety Requirements for Food Additives, Flavorings and Technological Aids" Retrieved from https://docs.cntd.ru/document/902359401. Accessed May 7, 2021. (In Russian)

turing person can produce up to 5,000,000 particles min^{-1}. During an 8-hour working shift, employees potentially can contaminate 8,000 l of air [61].

Sanitary treatment of industrial premises

In meat industry plants, sanitization is conducted to remove residues of raw materials, contaminants, including microorganisms, from working surfaces, including technological equipment and utensils, and other objects (floor, walls) [62].

Disinfection of wall and floor surfaces is critical in all production facilities, including those where animals are slaughtered. Examination of surfaces in the stunning and bleeding area was carried out before and after using the 1% disinfection solution containing surfactants, organic acids, and inorganic buffers [63]. The obtained results showed a possibility to reduce total bacterial counts, coliforms, and molds. It is believed that an increase in the hygienic level in slaughterhouse facilities will help meat industry professionals to establish proper sanitary procedures to prevent or reduce microbiological contamination of meat and meat products [63].

When choosing disinfectants, it is necessary to assess their effectiveness, quality, antimicrobial properties, toxicity, hazard class, corrosive activity, usability, ease of use, and economic feasibility [64].

The following factors affect the effectiveness of disinfection:

- physical and chemical properties of a disinfectant — the ability to inactivate bacteria, concentration, water-solubility, temperature, pH, etc .;
- biological resistance of microorganisms to various disinfectants;
- features of processed objects — the quality of materials, design features, the degree of pollution with organic substances;
- level of microbial contamination of fomites;
- disinfection treatment methodology — large-drop or aerosol irritation, wiping or immersion in a solution;
- exposure time [65].

Non-compliance with an established sanitization schedule, usage of ineffective disinfectants, or long-term application of the same disinfectant can lead to a decreased effectiveness of disinfection [66]. Microflora circulating in the food production environment may become resistant to disinfectants. Antimicrobial resistance is explained by the biofilm-forming ability of microorganisms [67]. The initial stage of biofilm formation is the attachment of microorganisms to the surface of production equipment and utensils covered with organic pollutants. This process is intensified until attachment becomes irreversible and strong [68].

Microflora protected by a biofilm becomes more resistant to disinfectants. Biofilm removal is a rather tricky task [69].

Timely preventive measures taken against molds are urgent for any food industry, including meat processing plants. Molds freely circulating in production facilities for fermented meat products can cause incredibly huge losses [70].

The choice of the most effective antifungal disinfectants and determination of adequate concentrations are of critical importance [71].

The following preparations at the indicated concentrations were used as liquid disinfectants: benzalkonium chloride (5%), biguanide (5%), peracetic acid (3%); quaternary ammonium (5%); sodium hypochlorite (0.2%).

The study [71] showed that the antifungal activity of peracetic acid was highest compared to the other tested chemical disinfectants. It was noted that some fungal strains, for example, *A. westerdijkiae* and *P. polonicum*, showed increased resistance to all disinfectants at the above concentrations.

Benzalkonium chloride and quaternary ammonium salts showed similar antifungal activity against the studied fungal strains. Sodium hypochlorite and biguanide at concentrations mentioned above had the lowest antifungal activity against molds in the production of dry-cured meat products [71].

It should be stressed out that biofilm formation by filamentous fungi must be considered in the selection of production environment and technological equipment sanitization programs. Several studies demonstrated that *Aspergillus* (*A. nigri* and *A. flavi*), *Penicillium, Cladosporium,* and *Alternaria* molds could form biofilms in the aquatic environment and on different abiotic surfaces [72].

Biofilm formation by *Candida* spp. remains to be the most discussed issue. The presence of the extracellular matrix (biofilm) that protects fungal cells against disinfectants may become an additional problem in the meat industry [73]. The simplest solution to this problem is to prevent biofilm formation through scheduled sanitization and strict adherence to preventive measures that minimize the probability of biofilm formation [74].

Conclusion

Nowadays, a wide range of research is devoted to hygiene and sanitation issues in food production facilities. Finished product safety is a result of adherence to many requirements at different production stages. Meat and meat product manufacture is associated with the highest risks. In the past few years, many studies have shown that prudent sanitary and hygienic measures are necessary at all stages of meat product manufacture. The formation of microflora in the production facilities begins from the stage of animal slaughter. Multiple factors take part in the development of the microbial community at each production stage. The recent studies demonstrated that sanitization of workshops must be planned considering the possible biofilm growth on the surfaces of objects in the production environment.

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ASSESSMENT OF GENOTOXIC AND MUTAGENIC EFFECTS OF FOOD PRODUCTS WITH BIOASSAY METHODS

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Keywords: *food safety, meat products, genotoxicity, mutagens, express bioassay, allium test*

Abstract

The current state of studies on application and safety of food additives in various technologies for food production is examined. Considerable attention should be given to studies dedicated to analysis of food safety criteria due to a possibility of appearance of adverse consequences for human health and the trend towards increasing life quality. Special emphasis is placed on such parameters as genotoxicity and mutagenicity. It is shown that the most rapid and convenient tool for complex monitoring of product toxicity can be the bioassay procedure. Based on the review of the literature on bioassays for edible meat and meat products, canned foods, carbonated soft drinks, beer, milk and milk containing products as well as seasonings, the authors show that above mentioned products had the cyto- and genotoxic potential when tested on animal and human cell cultures, microorganisms and plants. With that, it was found that a list of relevant publications is quite small despite a significant growth in scientific research dedicated to food toxicity assessment using bioassays. A review on the conducted research on assessment of genotoxic and mutagenic effects of foods by bioassay methods will make it possible to extend the understanding of the processes and mechanisms of this toxicity and form more rational concept of consumption.

Introduction

Increasing globalization and acceleration of the global food market development impose new requirements for finished products [1]. According to this trend, a spectrum of food product types and their components is expanding, new production technologies and food additives, which extend shelf life and improve consumer characteristics of foods, are appearing. At the same time, according to the "Strategy for improving the quality of food products in the Russian Federation until 2030"[1], adequate nutrition of the population remain to be the key requirement for increasing life expectancy and quality. Therefore, the problem of controlling the risk for food safety has been actively discussed in the scientific community [2].

For example, several studies were published that assessed the correlation between red and processed meat consumption and the development of colorectal cancer [3,4]. Substances in meat with the proved carcinogenic potential (polycyclic and heterocyclic aromatic hydrocarbons) and metabolites increasing the proliferative activity of the intestinal epithelial cells and triggering the process of lipid peroxidation can initiate processes of the malignant tumor development.

The main part

As for now, the methodological aspects of the solution to the problem of food safety assessment are still debatable. One of the realized approaches is aimed to studying certain product components and assessing their individual toxicity or combined effects of every component on each other. This approach is also directed to studying products of component breakdown in the body [5–7]. For example, it was shown that food colorants widely used in production of soft drinks and confectionary products can facilitate the development of allergic reactions, as well as pathological changes in the gastrointestinal tract [8,9]. Also, nano-food additives that are more and more frequently used in the food industry are not classified as fully safe for consumption. Furthermore, when testing zinc nanoparticles and ascorbic acid *in vivo* and *in vitro*, it was found that cytotoxicity in the mixture was significantly higher than upon their individual incorporation [10]. The American scientists showed in their review on the toxicity of the biologically active compounds that 47 known chemical compounds (alkaloids, hormones) in 55 different plant species from 46 families demonstrated harmful side effects [11]. The negative side effects such as hepatic toxicity, the development of pathologies of the cardiovascular, central nervous and digestive systems in the animal and human bodies were revealed after consumption of products with the indicated components.

On the other hand, studies dedicated to assessment of the toxicity effects of complex food matrices using the bioassay methods on different objects such as human and animal cell cultures [12], plants [13] and microorganisms [14] have appeared recently. This approach can provide the complex analysis in food biosafety assessment, first of all, with account for interaction of all indicated components. The undeniable advantage of these methods is also a possibility to obtain the indicated experimental data without using labo-

[1] The strategy for improving the quality of food products in the Russian Federation until 2030 (approved by the Decree of the Government of the Russian Federation No. 1364-r of 29.06.2016).

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ratory animals, which keeping and handling require adherence to modern bioethical standards and significant material expenditures. In addition, a significant period of time is required to obtain results of animal experiments.

Today, analyses of the genotoxic and mutagenic potential of food matrices have become topical in assessment of risks for safe food consumption [15]. Moreover, the recommendations of the European Food Safety Authority (EFSA) indicate the necessity of cyto- and genotoxicological studies in assessment of the finished products when considering the risk of safety of food product consumption for human health [16].

Therefore, the aim of the review was to analyze approaches to assessment of genotoxic and mutagenic effects of foods using bioassays.

Genotoxicity is a property of a substance (agent) manifested in its ability to damage a DNA molecule. This can happen as a result of the direct impact or indirectly, for example, by acting on enzymes taking part in DNA replication. It is necessary to pay close attention to these indicators mainly due to the fact that mutations in cells can be linked with an increased risk of cancer development [17]. In case of chronic exposure, compounds with mutagenic properties can lead not only to appearance of chromosomal aberrations, but also to an increased rate of their accumulation [18]. Based on the above, it can be concluded that the determination of the genotoxic and mutagenic potential of food products can be of utmost importance when assessing risks of safe food consumption [15].

Meat and products of its processing are important sources of protein in the human diet. Taking into consideration this fact, assessment of mutagenicity and genotoxicity of meat and cooked meat dishes is an important component in monitoring food safety of the daily diet. The research was carried out to assess studies that determined mutagenicity of different types of prepared meat using the Ames test (Table 1). Augustsson et al. demonstrated the pronounced mutagenic effect of extracts of six different fried meat dishes on *Salmonella typhimurium* TA98. Apparently, the revealed mutagenic effect can be linked with the development of heterocyclic amines during frying [19]. In another study, effects of frying temperature (100, 150, 200, 250 and 300 °C) and duration (2, 4, 6, 8, 10, 12 min at 250 °C) of cooking patties from ground lamb on mutagenicity was investigated also using the Ames test [20]. The maximum number of revertant colonies per 10 g sample was found in case of frying meat for 10 min (nine times higher than in uncooked meat) and in case of frying at maximum temperature 300 °C (eight times higher than in uncooked meat). It is also necessary to emphasize the presence of the dose-dependent mutagenic effect when considering an influence of both factors.

Gocke et al. analyzed mutagenicity of the extract from fried pork sausages on several cell cultures and microorganisms [21]. They showed that the Ames test was more sensitive to mutagens formed during meat frying compared to other used tests. For example, only slight mutagenic activity was noticed in analysis of the sister chromatid exchange in the cell line V79. Also, the mutagenic action of the extracts was not recorded in analysis of gene mutations (thioguanine resistance) in V79 cells and in analysis of sex-linked recessive mutations in *Drosophila*.

Vikse and Joner studied the mutagenic activity in 16 extracts of different meat types compared to the control beef sample using *Salmonella typhimurium* TA98 [22]. It was found that all extracts had less pronounced mutagenic effect than the control sample. Mutagenicity of extracts varied from 36% in seal meat to 81% in goat meat compared to the control sample. Additionally, the content of water, protein, fat and carbohydrate was measured in the meat samples. The significant correlation coefficients between these indicators and mutagenicity were not revealed.

Furthermore, data on toxicity of commonly consumed canned fish and vegetables were obtained using the yeast cell culture (*S. cerevisiae*) and the standard Ames test [14]. The results of these investigations showed that four selected ready-to-eat products (canned fish, canned spinach, canned tomato and canned fruit cocktail) showed the cyto- and genotoxic effects on these test objects. Canned tuna fish had the highest cyto- and genotoxic potential; namely, the number of spontaneous mutations in the experimental samples was two times higher than the control values. Moreover, a significant dose-dependent mutagenic effect was observed for all concentrations of this product (2, 5, 10, 50, 100 ml of the sample in a well). The authors linked the pronounced mutagenic effect largely with contamination of tuna meat with a high amount of heavy metals (mercury, lead, cadmium). Among canned plant products, canned spinach turned to be most toxic. The researchers suggested that these products contain food additives (mainly, coloring agents) with the pronounced toxic effect.

Biotesting of beer and the reference aqueous solution (7% of ethanol and sugar added according to the quantity indicated on product labels) was carried out on tumor and normal animal and human cells [12]. It is worth noting that in the experimental conditions survival of both tumor and normal cells decreased upon exposure to these low-alcohol beverages. After 72 hours of treatment, the number of normal cells reduced up to 67% and the number of tumor cells up to 3–4% compared to the control, while the exposure to the equivalent doses of the reference solution did not show the cytotoxic effect. Based on these results, the authors concluded that ethanol is not the determining component affecting manifestation of toxicity.

The other test object being used in studies on genotoxicity of foods is onion (*Allium cepa L.*). The onion apical meristem is convenient for cytological investigations as its chromosomes have the large size and are well seen under a microscope [23]. Furthermore, advantages of this method also include its low cost. Therefore, the field of Allium-test application for different foods has been extending recently (Table 1).

Furthermore, data on analysis of ultra-pasteurized milk and milk containing products in the conditions of the

Table 1. Toxicity parameters detected during bioassay of food products

Food product	Bioassay	Exposure time	Bioassay results		
			Mito(cyto)toxicity	Mutagenicity	Genotoxicity
Extracts of 6 meat dishes [19]	Ames test (with *S. typhimurium* TA98))	48 h.	**	+	**
Extracts of lamb patties [20]	Ames test (with *S. typhimurium*)	48 h.	**	+	**
Extracts of pan-fried sausages [21]	Ames test (with *S. typhimurium*)	48 h.	**	+	**
	SCE assay in vitro with V79 Chinese hamster cells	31 h.	**	+	**
	Drosophila sex-linked recessive lethal test	–	**	+	**
Extracts of fried meat from 16 different animal species [22]	Ames test (with *S. typhimurium*)	48 h.	**	+	**
Beer and solutions for comparison, [23]	Human and animal cell culture	48–72 h.	+		
Canned food: tuna, tomato, spinach, fruit cocktail, [14]	Ames test (with *S. typhimurium*)	24–72 h.	+		+
	S. cerevisiae				
Fruit juices, [13]	Allium-test	24–48 h.	+	+	+
Fruit juices, [24]	Allium-test	24–48 h.	+	+	
Seasonings, [25]	Allium-test	24–96 h.	- (*)	+	+
Milk, [26]	Allium-test	24–48 h.	+	+	+
Milk, [27]	Allium-test	24–48 h.	+		+
Coca-cola, [28]	Allium-test	4–8 h.	+		+
Coca-cola, [29]	Allium-test	6–8 h.	+		+
Coca-cola and Pepsi, [30]	Allium-test	2–48 h.	+	+	
Apple juice and model apple juice, [31]	Allium-test	24–72 h.	+ (*)	+	+

Note: * presence of root mass inhibition, ** parameters were not defined.

Allium-test were published [26,27]. It was found that all analyzed samples had the significant anti-proliferative effect. It was also established that the mitotic index in the cells of the onion bulb reduced by 2.5 to 7 times compared to the control value depending on a producer upon the daily exposition of bulbs to the samples of ultra-pasteurized milk. When treating onion bulbs with samples of dry milk diluted with water, the frequency of cells in mitosis also reduced to several tenths of a percent. Moreover, an increased number of micronuclei compared to the control was observed in the studies. According to the authors' opinion, these disorders can indicate the presence of the potential toxicity of this product, which can provoke tumor development, as a positive correlation between the increased frequency of micronuclei and cancer development was demonstrated [32]. The authors also explained such negative changes by the presence in the products of fruit processing and milk-containing products produced in Brazil, as well as by the presence of food additives (anti-caking and alkylating agents, colorants, preserving agents and sweeteners). It is necessary to note, that some of these additives are forbidden for using in production of juice and dairy products in Russia according to the requirements of the Technical Regulations of the Customs Union[2, 3].

Analysis of the toxic effect of domestic apple juice using the Allium-test, showed that juice diluted in a ratio of 1:5 reduced the growth of root biomass by two times and the mitotic index by 18 times, while the MDA level in the root tissue increased by 11% compared to the control [31]. At the same time, fructose and the model juice solution prepared from the main components of its dry solids were less toxic both regarding an effect on cell mitosis and in the process of root development. Thus, the leading role of the minor juice components in inhibition of cell proliferation and the following growth and development of roots was demonstrated.

Mitotoxicity, genotoxicity and mutagenicity were recorded in analysis of the other frequently used soft drink, Coca-Cola, in the experimental conditions using the onion apical meristem [28–30]. The mitotic index decreased in a dose-dependent manner with the extension of treatment duration and the level of aberrant cells increased when roots were treated with soft drinks. For example, in the experiment with a short-term impact, the 8-hour exposure to the drink reduced the mitotic index from 8.5% to 1.3%. The mitotic index reduced almost by half (7.6% vs. 4.4% in the control) in the root cells exposed to the drink over two days. Among the whole spectrum of the revealed chromosomal aberrations in cells upon treatment with drinks, an increased frequency of stickiness of chromosomes in mitosis was recorded. The authors suggested that an increase in the frequency of stickiness of chromosomes during cell division took place due to depolymerization of the DNA molecule and partial dissolution of nucleoproteins in the metaphase. This process can be irreversible and lead to cell

[2] TR TU023/2011 Technical Regulations of the Customs Union "Technical regulations for juice products from fruits and vegetables" (as amended on December 15, 2015, Decision of the Council of the Eurasian economic Commission of December 09, 2011, № 882. .Moscow, 2015. (In Russian)

[3] TR TU033/2013 Technical Regulations of the Customs Union "On the safety of milk and dairy products" (as amended on July 10, 2020, Decision of the Council of the Eurasian economic Commission of October 09, 2013, № 67. Moscow, 2020. (In Russian)

death. With that, significant differences were not found when comparing the response of the bio-tester on drinks of two different trademarks. It is worth noting that in the above mentioned examples, the roots were exposed to the undiluted drink or drink diluted with water in a ratio of 1:2 for 2–48 hours. We assume that testing over such a short period of time can give little information as the average duration of the cell cycle of the onion apical meristem is 18 hours. It is also known that the limits of Allium-test application in terms of the hydrogen indicator begin from 3 units [33], while cola pH is within a range of 2.5–3.5 units [34]. Therefore, bio-testing of the undiluted drink can be associated with inaccurate experimental data as well as with problems in interpretation of indicators.

Conclusion

At present, the problem of food safety assessment, including their toxicological load, is topical. When analyzing studies devoted to this question, it was found that a list of studies on food toxicity assessment by bioassay methods is still quite small despite the growing interest to such investigations. Onion (*Allium cepa*) should be mentioned among frequently used test objects. Several studies showed cyto- and genotoxicity of products in the concentrations being analyzed with this biotester. Extension and addition of studies on food toxicity using a wide spectrum of test objects can be useful for understanding mechanisms of this toxicity and formation of the risk-oriented concept of consumption.

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Completely prepared the manuscript and is responsible for plagiarism.

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