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# THEORY AND PRACTICE OF MEAT PROCESSING

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

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

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

The journal "Theory and practice of meat processing" publishes scientific and review articles, reports, communications, critical reviews, short research communications (letters to the editorial office), informative publications covering themes from the raw material base of the meat industry to innovative technologies of meat processing and food production, including the use of food biotechnology, from quality and safety management and risk analysis to information technologies, modeling in the meat industry as well as trophological chain management, marketing, economics, consumer demand analysis.

The primary objectives of the journal "Theory and practice of meat processing" are to present, preserve and distribute results of the new scientific studies in all directions of the research activities in meat science; create a favorable information environment to support the innovative development of the fundamental and applied research in the meat and poultry processing industries; attract attention to promising and relevant directions of the meat science development.

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# DIOXINS AND DIOXIN-LIKE COMPOUNDS IN MEAT AND MEAT PRODUCTS

**Keywords:** *nutrition, additive, bioactive compounds, slaughter characteristics, meat quality*

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***Abstract***
*Dioxin and dioxin-like compounds are persistent organic pollutants that received considerable attention in recent years due to their high potential toxicity, wide distribution and extreme stability. Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) mainly occur in the environment as a result of several human activities including combustion, incineration and many other industrial activities, whereas polychlorinated biphenyls (PCBs) congeners were intentionally manufactured and widely used in various fields. Since dioxin and dioxin-like compounds are found in various environmental compartments (air, water, soil, sludge, sediment, food, feed, blood, animal and human tissues), humans could be exposed to them via inhalation, dermal contact or food ingestion. However, 90% of human exposure to dioxin is through food ingestion particularly foods from animals and foods that are rich in fat. In contrast, only low levels have been found in food items of plant origin. Exposure to dioxin compounds is associated with various adverse health problems. However, their toxicity varies dramatically according to the type of dioxin, species of exposed organism, as well as exposure frequency and duration. Dioxins are mainly determined by instrumental chromatographic methods such as GC-HRMS and GC–MS/MS. Many efforts have been made to remove, reduce and prevent these hazardous substances from the environment. However, the best method for reducing human exposure to dioxins and dioxin-like compounds is controlling and minimizing their production. In this article, structures, sources, exposure, toxicity and analysis methods of dioxin and dioxin-like compounds in meat and other foods were reviewed.*

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## Introduction

Persistent organic pollutants (POPs) are the organic compounds that remain in the environment for a relatively long period of time and resist different types of degradation. POPs were reported to cause adverse effects on the environment, animal and human health. The presence of these compounds in the environment occurs as a result of both natural processes and human activities (unintentional or intentional). POPs are often semi-volatile compounds and show hydrophobic properties so they could be transported long distances via atmosphere and tend to bioaccumulate in food chain (especially fatty tissues). Human exposure to POPs occurs through diet and contact with other environmental compartments [1,2]. POPs are categorized into two main groups including both the polycyclic aromatic hydrocarbons (PAH) and some halogenated hydrocarbons.

Dioxins and dioxin-like compounds, known as a major group of POPs, are a group of polyhalogenated aromatic hydrocarbon compounds that share similar chemical structures and properties. They are highly toxic chemicals that are associated with harmful effects to humans and animals, widespread, found almost everywhere in the environment throughout the world being persistent pollutants that have long half-lives and remain in the environment for long periods of time. Generally, dioxins and dioxin-like compounds are poorly soluble in water and have strong lipophilic properties, so they tend to accumulate in adipose tissues of humans and animals. These compounds are mainly formed as a result of human activities such as chemical production and incomplete combustion processes [3–5].

Dioxins released into the environment as a result of washing soda ($NaCO_3$) production in a German chemical plant were characterized for the first time in 1827, but they were not identified until the 1980s [6]. Another action resulted in releasing huge amounts of dioxins to the environment was the use of the herbicide called "Agent Orange" that contained small amounts of 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD). It was intensively used by the U.S. military during the Vietnam War and resulted in spraying of 150 kg of 2,3,7,8-TCDD over southern Vietnam. Other accidents of dioxin pollution, such as the Times Beach, Love Canal and Seveso disasters were also reported. In 1976, in a town called Seveso located in the north of Italy, an explosion at the chemical factory known as Industrie Chimiche Meda Societa` Anonima (ICMESA) occurred and resulted in releasing chemicals including

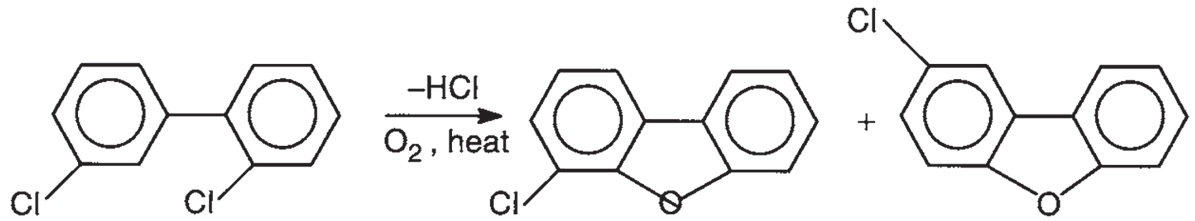


**Figure 1.** Thermochemical generation of the PCDFs from PCBs [13]

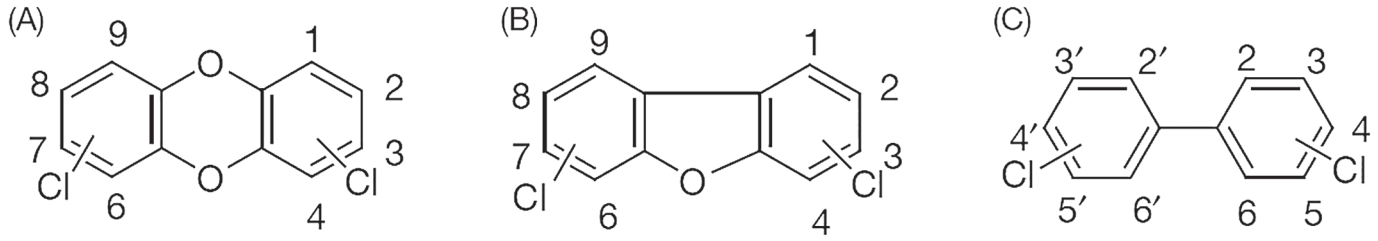


**Figure 2.** The general chemical structures of PCDDs (A), PCDFs (B) and PCBs (C) [8]

2,3,7,8-TCDD and contaminating an area of 2.8 km$^2$ with a huge dose of dioxins [7,8].

The term "dioxin" is usually used to refer to polychlorinated dibenzo-p-dioxins (PCDDs) and sometimes used to refer to the most toxic compound (2,3,7,8-TCDD) produced by human beings [5,8–10]. The dioxin family could be classified into two main categories: polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) [10]. In addition, polychlorinated biphenyls (PCBs) are referred to as dioxin-like compounds since some coplanar congeners exhibit dioxin-like toxicity (binding the aryl hydrocarbon "Ah" receptor) and have similar features with dioxins. Moreover, heating PCBs in the presence of $O_2$ can lead to PCDFs formation (Figure 1); additionally, close relationships between the formation mechanisms for PCBs, PCDDs and PCDFs were also reported [11–13].

The general chemical structures of PCDDs, PCDFs and PCBs are shown in Figure 2. As seen from the figure, PCDFs and PCDDs are polyhalogenated tricyclic aromatic molecules that contain two benzene rings joined by either one or two oxygen molecules, respectively. On the other hand, PCBs consist of two benzene rings directly connected to each other. Each hydrogen atom in the rings could be substituted by a halogen atom such as chlorine [14,15].

Pure dioxins are colorless solids. They are lipophilic, lowly soluble in water, have the low vapor pressure, high melting point. They are extremely stable substances against acids, bases and high temperatures ( < 600 °C) [5,10,16,17]. Depending on the number of chlorine substitutions (regardless of the position of chlorine atoms), 10 homologues of PCBs and 8 homologues of PCDDs and PCDFs could be found [1,5]. According to both position and number of chlorine atoms in the rings, 75, 135 and 209 possible congeners of PCDDs, PCDFs and PCBs could be found (Table 1). In fact, not all of these congeners reported to have dioxin-like toxicity and their toxicity degrees are not equal but in turn depend on the degree and the pattern of chlorine substitution on the benzene rings. Lateral substituted congeners with ≥4 chlorine atoms at 2, 3, 7 and 8 positions are reported to have toxic effects [8,13,14].

**Table 1. Possible number of PCDD, PCDF and PCB congeners [8,19]**

| Number of chlorine atoms | Number of congeners | | |
|---|---|---|---|
| | PCDD/ PBDD | PCDF/ PBDF | PCB |
| Mono | 2 | 4 | 3 |
| Di | 10 | 16 | 12 |
| Tri | 14 | 28 | 24 |
| Tetra | 22 | 38 | 42 |
| Penta | 14 | 28 | 46 |
| Hexa | 10 | 16 | 42 |
| Hepta | 2 | 4 | 24 |
| Octa | 1 | 1 | 12 |
| Nona | 0 | 0 | 3 |
| Deca | 0 | 0 | 1 |
| Total | 75 | 135 | 209 |

In addition to the chlorinated dioxins, brominated structural analogues (polybrominated dibenzo-p-dioxins — PBDDs and polybrominated dibenzofurans — PBDFs) could also be formed as a result of the substitution of chlorine atoms by bromine ones (Figure 3). Similar to chlorinated analogues and according to the number and position of bromine atoms, 75 and 135 congeners of PBDDs and PBDFs, respectively, could be found (Table 1). The brominated analogues have similar physicochemical properties and biotoxicity (or even more toxicity) compared to chlorinated dioxins. Brominated dioxins have higher molecular weights, lower vapor pressures, lower water solubility and higher melting points than chlorinated ones. Incineration of electronic waste is considered the main source for brominated dioxins since they contain polybrominated diphenyl ethers (PBDEs) that are regarded as an important precursor of PBDDs and PBDFs [18,19].

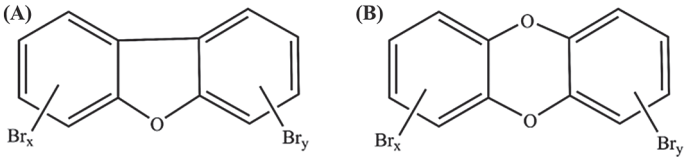


**Figure 3.** Chemical structures of PBDDs (B) and PBDFs (A) [18]

**Sources of dioxins and dioxin-like compounds**

The anthropogenic sources are the main sources of dioxins. Only a tiny part of dioxins is of natural origin. Actually, there is no commercial production of PCDDs and PCDFs (except production for research usage), they are formed as an unintentional by-product of some chemical and industrial processes such as herbicides industry (e. g., 2,4,5-trichlorophenoxy acetic acid), metals recycling and industry, pulp bleaching and synthesis of some common solvents (e. g., perchloroethylene-PCE and trichloroethylene-TCE). Dioxins are mainly released into the environment by incineration and combustion processes. In addition, dioxins could be formed during some natural processes such as forest fires, volcanic eruptions, geological processes and biological processes [6,8,10,13,17,20]. However, natural sources of dioxin release a relatively smaller amount of dioxins compared to that released from human activities [17].

Many classifications of dioxin sources can be found in the literature. While Kulkarni [8] categorized the major sources of dioxins into four main groups including incineration, combustion, industrial, and reservoir processes, the U. S. Environmental Protection Agency grouped these sources into five major classes including combustion, metals smelting, refining, and processing, chemical manufacturing, natural sources and processes and reservoirs [21]. Figure 4 summaries the main sources of dioxins according the U. S. Environmental Protection Agency. Dwyer and Themelis [22] grouped PCDDs and PCDFs emission sources into two categories: controlled industrial and open burning sources. The controlled industrial sources include waste-to-energy, waste incineration, electricity and heat generation, metallurgical processes, cement and asphalt production. Whereas the open burning sources include backyard barrel burning, agricultural burning, construction debris, yard waste and fires (forest, vehicle, landfill, building). U.S. EPA also grouped dioxin sources depending on the time between their formation and releasing to the environment into two categories which include contemporary formation sources (dioxins formed and immediately released to the environment) and reservoir sources (previously formed dioxins and dioxin-like compounds are stored in these sources, then they are re-released to the environment) [21].

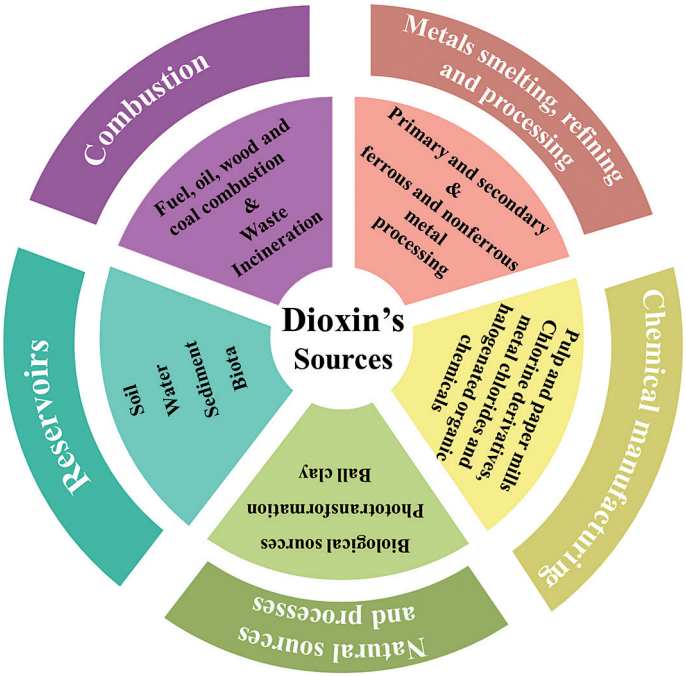


**Figure 4.** Main sources of dioxin substances

Dioxins and furans occur in most of the combustion and incineration processes Kulkarni et al. [23] indicated incineration processes as the main source of PCDDs and PCDFs generation and release to the environment including municipal solid waste, medical waste, hazardous wastes and sewage sludge incineration processes. In 2012, Dwyer and Themelis [22] reported that the electricity and heat generation processes (which include combustion of wood, coal, gasoline, diesel and other fuel oils) as the major dioxin source that is responsible for 66.2% of the total dioxin emission in the USA. Dioxins are also formed during manufacturing bleached pulp and paper by chlorination of phenolic compounds found in wood pulp and during metal smelting, refining and processing [8]. PCDDs and PCDFs can also be unintentionally formed during manufacturing of some insecticides (e. g., DDT), herbicides (e. g., 2,4-D and 2,4,5-T), disinfectants (e. g., hexachlorophene) and chlorinated aliphatic compounds (e. g., PVC) [21,24]. Finally, PCDDs and PCDFs could be also formed from chlorinated phenolic substances by microorganisms under specific environmental conditions or by photolytic radical reactions of highly chlorinated phenols [8,21].

On the other hand, PCBs occur in the environment intentionally a result of their commercial production or unintentionally as a by-product of combustion and industrial processes [1]. PCBs were commercially produced since 1929 by chlorination of biphenyl in many countries over the world. Due to their unique physical and chemical properties, manufactured PCBs (usually containing congener mixtures with different chlorination degrees) have been extensively used in various industrial and commercial applications including electrical transformer and capacitors (as dielectric fluids), heat transfer systems (as a heat-conduction fluid), hydraulic systems (as a hydraulic oil) and pesticides (as an extender). PCBs were also used in flame retardants, carbonless copy paper, oil-based paints, lubricants, plastics, inks and waterproofing compounds [1,21]. PCBs are also formed as by-products in combustion (e. g., fossil fuel and biomass), incineration (e. g., waste), thermal industries (e. g., ferrous and nonferrous metal smelting) and production of some commercial chemicals (e. g., pigments, 2,4-dichlorophenoxyacetic acid) [11,25–27]. PCBs production was then banned in the late 1970s because of their adverse effects on the environment and human health. However, because of their chemical stability, broad usage and their inadvertent production, PCBs are considered important persistent environmental pollutants [1,21].

Kanan and Samara [2] in their review mentioned that major sources of dioxin and dioxin-like compounds varied across countries, i. e., while the fuel combustion for generation of electricity and heat was reported as the principal source of these compounds in the United State [22], burning the household garbage was responsible for the largest dioxin emissions in Canada. In Turkey, the contamination of aquatic organisms was associated with industrial discharges, whereas the highest dioxin emissions resulted from waste incineration in Spain, France and Italy. Higher concentrations of dioxins were reported in the urban areas when compared to those at remote areas in the Middle East. Similarly, samples collected from the high industrial activity areas exhibited high amounts of dioxins [2].

**Exposure to dioxins and dioxin-like compounds**

Dioxin and dioxin-like compounds are usually released to the environment in form of mixtures, which vary widely in their individual congener content and proportions depending on a source. They can also be changed over time, transported over long distances apart from production or releasing areas or redistributed within environmental media [14,21]. After releasing dioxins and dioxin-like compounds from their sources, they are distributed to most environmental media and can move between these media. They have been detected in air, water, sediment and soil. The wide presence of dioxins in the environment is mainly related to physical and chemical properties especially persistency and bioaccumulation [2,21,28]. Dioxins, which are formed by incineration and combustion processes, accidental release and explosions, are responsible for the presence of dioxins in the atmosphere. Whereas they enter soil and water from industrial discharges or deposition of atmospheric dioxins. Dioxins can also enter water as a result of contact with contaminated soil. Due to their hydrophobic properties, they tend to be associated with the organic matrix instead of being dissolved in water; consequently, they deposit in sediment with a very low concentration in the dissolved phase. Dioxins movement from water or soil to the air is less common because of their low vapor pressures [2,10,14]. Booth et al. [29] estimated that 57% and 40% of annual dioxin emission is deposited to soil and ocean water respectively, whereas 3% of it remained in the air. On the other hand, EFSA [16] revealed that dioxin-like PCBs contribute to about 63% of human exposure, whereas PCDD and PCDF groups account for 14% and 23%, respectively. Regarding the individual congeners, PCB-126 had the highest contribution (54.7%) to the total human exposure, followed by 2,3,4,7,8-PeCDF (10.7%), 1,2,3,7,8-PeCDD (7.4%), 2,3,7,8-TCDF (4.9%), PCB-169 (3.7%) and 2,3,7,8-TCDD (3.4%).

Humans are exposed to dioxin and dioxin-like compounds via inhalation, dermal absorption, ingestion of contaminated soil attached to fruit or vegetables and consumption of contaminated food. With the exception of accidental and occupational exposures, inhalation and dermal contact are not considered important pathways of human exposure. However, more than 95% of human exposure to dioxins is attributed to food consumption, particularly food of animal origin [14,16,21]. The total dietary exposure of the European population to 29 congeners of dioxins (17 PCDD/Fs) and dioxin-like (12 PCBs) compounds were estimated to be between 0.4 and 2.6 pg $WHO_{2005}$-TEQ/kg body weight (bw)/day. Furthermore, it was reported that the estimated dietary exposures of toddlers and children were almost two-fold higher than those estimated in teenagers and adults [16]. Due to the persistency and lipophilic features of dioxins and dioxin-like compounds, they are known to concentrate and bioaccumulate in adipose tissues of animals, fish and humans. Additionally, dioxins can be excreted from edible products containing fat such as milk and eggs. Some specific congeners also tend to accumulate in the liver. Generally, foods with higher fat content (especially, animal fats) tend to have higher levels of dioxins [30]. Consequently, food items such as meat, dairy products, fish and eggs (especially seagull eggs, fish liver and offals) are most likely to contain high concentration of dioxins [8,16,17]. Moreover, organisms with higher trophic levels are known to contain high levels of dioxins due to their biomagnification through the food chain [14]. Table 2 shows the levels of total dioxins and dioxin-like compounds in some food groups collected from different regions and during different periods. As seen from the table, dioxin compounds are reported in all foods categories, including fruit and vegetables. However, only foods from animal origin show high levels of dioxins. The authors also reported differences in the dioxin levels in food items within the same group. For example, EFSA [31] indicated that salmon fish had the highest level of dioxins compared to the other fish species and kinds of seafood. Anonymous [30] also reported that fatty fish such as salmon, full-fat cheese, butter and high-fat beef had higher levels of dioxins than other food items within the same category. On the other hand, zucchini was reported to have higher levels of dioxins due to the fact that, unlike other plants, zucchini and pumpkin belonging to the genus *Cucurbita* are able to absorb dioxins from contaminated soils and translocate them to the other parts of the plant, including the fruit [32].

Regarding the contribution ratio of different foods to human exposure to dioxins, it is reported that consuming contaminated fish accounts for 30–75% of total human exposure to PCDD/Fs and PCBs [34]. EFSA [16] indicated that the consumption of fatty fish contributed up to 56% of total human exposure to dioxins, whereas cheese and livestock meat consumption contributed up to 21.8% and 3.8%, respectively, of the total exposure. However, the contribution ratio of food items to human exposure does not only depend on their contamination level, but the consumption frequency among the population is also considered an important factor [16]. The percentage contribution of different sources and food items to human exposures to dioxins and dioxin-like compounds is summarized in Figure 5.

Table 2. Levels of PCDD/Fs and PCBs in some food groups

| Food Group | pg TEQ/g | pg TEQWHO98/g | pg WHO05-TEQ/g |
|---|---|---|---|
| Meat and meat products | 0.005–0.46 | 1.97 | 0.105 (0.003–2.067) |
| Poultry & poultry products | 0.004–0.06 | — | 0.068 (0.007–0.782) |
| Fish, seafood & their products | 0.01–0.33 | 4.42 | 0.284 (0.005–12.365) |
| Hen eggs | 0.01–0.05 | 1.01 | 0.052 (0.011–0.202) |
| Milk | 0.0006–0.01 | 1.49 | 0.030 (0.003–0.149) |
| Dairy products | 0.0001–0.24 | 1.29 | 0.087 (0.002–0.505) |
| Fats & oils | 0.002–0.22 | — | 0.090 (0.010–0.305) |
| Nuts | 0.003–0.006 | — | 0.020 (0.014–0.024) |
| Cereals & cereal products | 0.0001–0.05 | — | 0.018 (0.002–0.050) |
| Fruit | 0.0007–0.01 | — | 0.005 (0.001–0.027) |
| Vegetables | 0.0001–0.05 | — | 0.007 (0.001–0.295) |
| Region | US | Europe | Taiwan |
| Period of time | 1999–2001 | 1999–2008 | 2004–2018 |
| Other | Unspecified | Results based on ww, except for fish based on fat | Based on wet weight (ww) |
| Reference | [30] | [31] | [33] |

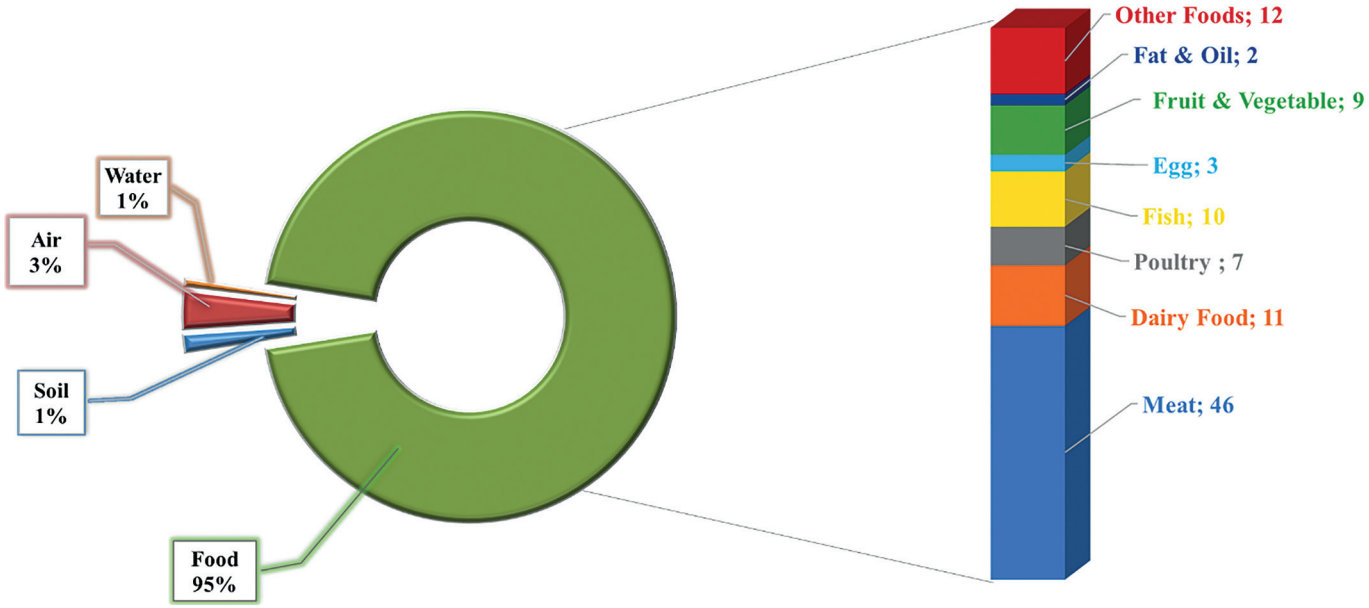


**Figure 5.** Contribution (%) of different sources and food items to human exposures to dioxins and dioxin-like compounds [24,30]

It is worth mentioning that food processing can lead to significant losses of dioxin compounds. Lower levels of PCDD/Fs and PCBs were observed in the processed food compared to the raw ones. Lower chlorinated congeners could be released during cooking when high temperature is used. Dioxin intake could also be minimized during food processing by removing fat from food. Otherwise, using contaminated cooking oil during cooking results in processed products with higher amounts of dioxin compared with raw materials [16]. Planche et al. [35] reported significant losses (18–48%) of PCBs in meat as a result of pan cooking; the losses also increased with increasing the intense of cooking conditions. However, no significant losses in PCDDs and PCDFs amounts were observed. Hori et al. [36] reported that grilling or boiling mackerel slices reduced the levels of PCDD/Fs by 31% or 14%, respectively. Whereas the reduction in beef slices was about 42% when treated by boiling. Domingo [37] in his review indicated that cooking processes that caused reducing or eliminating fat from food led to a decrease in the concentration of some contaminants like as PCDD/Fs and PCBs.

**Toxicity of dioxins and dioxin-like compounds**

Dioxin and dioxin-like compounds have attracted considerable interest throughout the world due to their potential high toxicity. They had received public attention in 1976 when the highest known exposure to dioxins mainly TCDD happened as a result of releasing a huge amount of toxic chemicals to the environment by an explosion at ICMESA plant in Seveso, Italy [8,38]. Several studies demonstrated the reverse health effects of dioxins on several organs and systems in both humans and animals [16]. It is reported that the toxicity of dioxin compounds strongly depends on the dioxin type i. e., the substitution degree and pattern [2]. As we mentioned previously, there are 75, 135 and 209 possible congeners of PCDDs, PCDFs and PCBs, respectively. But only PCDD/Fs congeners that are halogenated (chlorine or bromine) at 2, 3, 7 and 8 positions; and only coplanar congeners of PCBs that are substituted with ≥4 chlorine (or bromine) atoms are considered toxic. Thus, only 29 congeners of dioxin and dioxin-like compounds (7 PCDDs, 10 PCDFs, and 12 PCBs) exhibit dioxin-like toxicity (compounds are shown in Table 3) [16,21,39]. 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) is considered the most toxic and widely investigated congener. Furthermore, the International Agency for Research on Cancer (IARC) has classified 2,3,7,8-TCDD as carcinogenic to humans (Group 1) [40]. However, oral reference dose (RFD) for TCDD is defined as the dose that is probably to be without considerable risk of adverse health effects over a lifetime. It was estimated by the US EPA in 2012 as 0.7 pg TCDD/kg bw per day. Similarly, the minimal risk level (MRL) for chronic oral exposure to 2,3,7,8-TCDD was determined by the Agency for Toxic Substances and Disease Registry (ATSDR) as 1.0 pg/kg bw/day [16]. The 29 toxic congeners of PCDD/Fs and PCBs not only exhibit the harmful effect at low doses but also possess relatively long half-lives in the human body that vary depending on the type of dioxins. For instance, the half-lives of 2,3,7,8-TCDD, PCB-126 and 2,3,4,7,8-PeCDF are estimated to be 6.5, 1.6 years and 7.0, respectively [16,40,41]. In addition to the dioxin type, the frequency and duration of exposure are also important to determine their toxicities. In order to express the toxicity of different dioxins and dioxin-like compounds, the World Health Organization (WHO) created the toxic equivalency factor (TEF) system. In this system, the toxicity of dioxin and dioxin-like congeners was compared to the toxicity of the most toxic member (2,3,7,8-TCDD). Thus, the TEF value given to TCDD is 1.0 and the other compounds have TEF values relative to TCCD and ranging from 1 to 0.00001 (Table 3). In other words, TEF values express the possible toxicity of certain congeners relative to TCDD (the reference congener). Regarding the toxicity of the dioxin mixture (since they are released to environment in a form of mixture), a total toxic equivalency (TEQ) is used and calculated by multiplying the concentration of each congener by its TEF value, then the products are summed. TEF values have been revised and developed many times since their establishment. In 2005, the World Health Organization (WHO) introduced the final TEF values and proposed separated values for mammals, birds, and fish since different species show different sensitivities to specific dioxin members. The current TEFs are termed $WHO_{2005}$-TEFs. Previously proposed TEF values were termed $WHO_{1998}$-TEQ, I-TEQs and Nordic-TEQs. These TEFs were used to express a dioxin level in many studies particularly those performed before the year 2005. So, it is important to be aware of which TEF values were used when evaluating dioxin levels or exposures. Unlike $WHO_{1998}$-TEQ and $WHO_{2005}$-TEQ, I-TEQs and Nordic-TEQs do not include the TEF values for dioxin-like compounds (PCBs) [8,14,16,21].

**Table 3. Toxic equivalency factors (TEFs) for PCDDs, PCDFs and dioxin-like PCBs [42].**

| | Congener | $WHO_{1998}$ TEF | $WHO_{2005}$ TEF |
|---|---|---|---|
| PCDDs | 2,3,7,8-TCDD | 1 | 1 |
| | 1,2,3,7,8-PeCDD | 1 | 1 |
| | 1,2,3,4,7,8-HxCDD | 0.1 | 0.1 |
| | 1,2,3,6,7,8-HxCDD | 0.1 | 0.1 |
| | 1,2,3,7,8,9-HxCDD | 0.1 | 0.1 |
| | 1,2,3,4,6,7,8-HpCDD | 0.01 | 0.01 |
| | OCDD | 0.0001 | 0.0003 |
| PCDFs | 2,3,7,8-TCDF | 0.1 | 0.1 |
| | 1,2,3,7,8-PeCDF | 0.05 | 0.03 |
| | 2,3,4,7,8-PeCDF | 0.5 | 0.3 |
| | 1,2,3,4,7,8-HxCDF | 0.1 | 0.1 |
| | 1,2,3,6,7,8-HxCDF | 0.1 | 0.1 |
| | 1,2,3,7,8,9-HxCDF | 0.1 | 0.1 |
| | 2,3,4,6,7,8-HxCDF | 0.1 | 0.1 |
| | 1,2,3,4,6,7,8-HpCDF | 0.01 | 0.01 |
| | 1,2,3,6,7,8,9-HpCDF | 0.01 | 0.01 |
| | OCDF | 0.0001 | 0.0003 |
| Non-ortho PCBs | 3,3',4,4'-tetraCB (PCB 77) | 0.0001 | 0.0001 |
| | 3,4,4',5-tetraCB (PCB 81) | 0.0001 | 0.0003 |
| | 3,3',4,4',5-pentaCB (PCB 126) | 0.1 | 0.1 |
| | 3,3',4,4',5,5'-hexaCB (PCB 169) | 0.01 | 0.03 |
| Mono-ortho PCBs | 2,3,3',4,4'-pentaCB (PCB 105) | 0.0001 | 0.00003 |
| | 2,3,4,4',5-pentaCB (PCB 114) | 0.0005 | 0.00003 |
| | 2,3',4,4',5-pentaCB (PCB 118) | 0.0001 | 0.00003 |
| | 2',3,4,4',5-pentaCB (PCB 123) | 0.0001 | 0.00003 |
| | 2,3,3',4,4',5-hexaCB (PCB 156) | 0.0005 | 0.00003 |
| | 2,3,3',4,4',5'-hexaCB (PCB 157) | 0.0005 | 0.00003 |
| | 2,3',4,4',5,5'-hexaCB (PCB 167) | 0.00001 | 0.00003 |
| | 2,3,3',4,4',5,5'-heptaCB (PCB 189) | 0.0001 | 0.00003 |

When dioxins and dioxin-like compounds enter the human body, they are distributed to the liver and fatty tissues as well as blood lipids. Most of these compounds are poorly metabolized and have long half-lives in the human body. They vary depending on type and level of

dioxin, age, body mass index (BMI) and gender. PCCD/Fs and dioxin-like PCBs are associated with several health impacts including chloracne, endocrine disruption, immune system disorder, reproductive disorder and cancer [8,16]. The acute toxicity of dioxins has been associated with the development of chloracne that is considered to be the clearest and most specific sign for the dioxin toxicity. However, this condition is only observed in high exposure cases such as accidental or occupational exposure [10,16]. Other adverse health effects such as irritation in the respiratory and gastrointestinal tracts, headache and fatigue can also be a result of acute or short-term exposure to dioxins. Moreover, many animal studies indicated adverse effects on many organs such as the thymus and adrenal glands, liver and heart. However, the toxicity of these compounds varies dramatically between animal species. For example, the oral $LD_{50}$ values (the dose responsible for killing 50% of the exposed animals) for TCDD are 0.6 μg/kg for guinea pigs, 20 μg/kg for rats and 1175 μg/kg for hamsters [10]. Concerning health risks related to chronic exposure to dioxins, many studies indicated the association between dioxin exposure and the disruption of hepatic function, immune disorder, cardiovascular problems, reproductive disorders and cancers, even though there are no sufficient data to confirm this association. Studies on rats exposed to relatively low levels of TCDD showed reduction in sperm production, delayed puberty, hepatic implications and alteration in bone parameters [10,16]. The toxicity mechanism of dioxins and dioxin-like compounds is complicated and not completely elucidated. However, toxic congeners of PCDD/Fs and PCBs are believed to have a common mechanism of toxicity by disrupting the function of the aryl hydrocarbon (Ah) receptor. They are generally known as Ah-receptor ligands. Most dioxin congeners can bind the Ah-receptor that acts as a transcription factor and controls genetic transcription from DNA to RNA [8,10]. The binding affinity of dioxin compounds to the Ah-receptor varies between different congeners and different species. For example, the binding affinity of the Ah-receptor to TCCD in humans is lower than that in rats and mice [16]. The toxicity of PCDD/Fs and PCBs is initiated by binding to the Ah-receptor. Accordingly, the inappropriate and continuous activation of this receptor subsequently induce the production of several proteins, especially cytochrome P450 1A1, which, in turn, can affect the metabolism of important substances such as steroid hormones, leading to several changes and disorders in biological functions and cellular processes [8,14].

Dioxins are suspected to be associated with immunosuppression by affecting the development of T-cells in the thymus [14]. Many studies reported a correlation between parents' exposure to dioxins and the incidence of allergies and infections in their children during childhood [16]. Additionally, immunosuppression by dioxin exposures is suggested as the reason of mass fatalities of seals and dolphins in the 1980s in Europe [14]. However, EFSA [16] reported insufficiency in the available evidence to confirm the relationship between exposure to PCCD/Fs and dioxin-like PCBs and negative effects on the immune system in adults or children.

PCCD/Fs and dioxin-like PCBs are widely known to adversely affect the function and development of the reproductive system (particularly, in males) based on the results of both animal and human cohort studies [16]. Experimental animal studies reported the abilities of TCDD and some dioxin compounds to reduce the number of estrogen receptors, interrupt the testosterone hormone and affect the development of the prostate. Thus, it is thought that the alteration of sex hormone levels in serum is the possible mechanism to explain the final adverse reproductive symptoms [10,14]. However, the Panel on Contaminants in the Food Chain (CONTAM Panel) in the European Food Safety Authority (EFSA) did not consider the hormonal changes, per se, in adults and children to be a causal negative effect of dioxin exposures [16]. Animal studies on rats, mice and other rodents indicated symptoms such as reduction of sperm synthesis, sperm count and postponement of sexual maturity in males; deformation in the urogenital system, irregularity of the estrous cycle and reduction of the ovulatory rate in females due to exposures to dioxins, particularly, TCDD [14,16]. Semen quality, cryptorchidism and development of puberty are affected adversely by dioxin exposures. The CONTAM Panel in the EFSA indicated a causative relationship between exposure to PCDD/Fs (especially TCDD) during infancy or before sexual maturation and reduced semen quality. This relation depends on the results of both experimental animal studies and human prospective studies including those performed after the Seveso accident [16]. On the other hand, there are limited evidence to support a causative relation between exposure to PCDD/Fs or PCBs and both cryptorchidism (undescended testicle) and postponed puberty. A reduction in the gender ratio (lower probability of male birth) in offspring of males exposed to high levels of 2,3,7,8-TCDD (accidentally or occupationally) has been indicated in many cohort studies [43–45]. This reduction was suggested to be causal by the expert team of ESFA [16]. However, no changes were observed in the gender ratio in offspring of exposed females in the same studies. Additionally, the association with other birth outcomes such as low birth weight, preterm birth could not be proven by the available studies. Concerning the adverse effects on the female reproductive system, no relationship was observed between exposure to dioxins and female pubertal development. The existing studies also did not provide sufficient evidence to associate dioxin exposures with effects such as endometriosis, altered menstrual cycle, altered ovarian function and changes in time of menopause [16].

Again, the existing studies did not show enough evidence to prove the causative adverse effects of dioxin exposures (including TCDD congeners) on the thyroid function or disorders [16]. However, a causative relation was

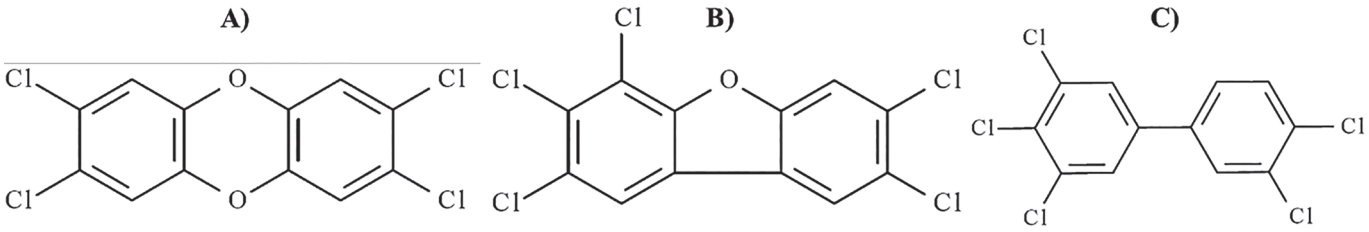


**Figure 6.** The chemical structures of 2,3,7,8-TCDD (A), 2,3,4,7,8-PeCDF (B) and PCB-126 (C) [40].

observed between children born to mothers exposed to high levels of 2,3,7,8-TCDD in Seveso and increased concentrations of TSH in the serum of newborns [46], whereas no adverse effects were reported with low-moderate exposure to dioxin and dioxin-like compounds including 2,3,7,8-TCDD [47,48].

Regarding the cardiovascular risk as a result of dioxin exposures, an increment of the risk has only been indicated with occupational exposure to very high levels of 2,3,7,8-TCDD (serum TCDD > 1,000 pg/g fat) [49], whereas exposure to relatively lower levels of TCDD or other dioxin congeners was either associated with increased cardiovascular risk [50,51] or not [52,53].

Even though epidemiological studies proposed a possible relationship between exposure to PCDDs and PCDFs and hepatic dysfunction, the EFSA expert team concluded that hepatic diseases were not causally associated with exposure to PCDDs and PCDFs due to the insufficient evidence from these studies [16]. EFSA [16] suggested a dose-related and causal association between childhood exposure to PCCD/Fs, particularly TCDD, and enamel defects or hypomineralisation. Similarly, exposure to dioxin and dioxin-like compounds is suggested to be related to changes in bone parameters such as mineral density, size and strength. Finally, positive association was found between occupational, accidental or environmental exposure to toxic PCDD/Fs and PCBs and all cancers combined. They can promote tumors in experimental animals at many sites such as skin, ovary and liver. However, there is no obvious link to any certain cancer site [16,40]. Due to the sufficient evidence obtained from both epidemiological and experimental animal studies as well as the common mechanism of action; 2,3,7,8-TCDD, 2,3,4,7,8-Pentachlorodibenzofuran (2,3,4,7,8-PeCDF) and 3,3′,4,4′,5-Pentachlorobiphenyl (PCB-126) (Figure 6) were classified as carcinogenic to humans (Group 1) by IARC [40].

It is worth mentioning that the WHO determined the tolerable daily intake as 1–4 pg TEQ/kg body weight/day for dioxin and dioxin-like compounds, whereas it was reported as 2 pg TEQ/kg bw/day in the United Kingdom [8,9,16].

## Analysis methods of dioxins and dioxin-like compounds

The analysis of dioxin and dioxin-like compounds is crucially demanded due to the high toxicity and widespread occurrence of these compounds in different environmental and biological matrixes, in addition to the need to monitor their levels in these matrixes and control their releases from sources. Since they are usually found in a very low concentration (at levels of pg/g or fg/g) as congener mixtures and attached/adsorbed to other organic compounds, analytical methods should provide the efficient extraction, purification, separation and accurate determination of toxic congeners at trace levels [9,32,54]. Thus, the analytical methods for dioxin and dioxin-like compounds determination are required to have high sensitivity, selectivity, and specificity, as well as high accuracy and precision with the low limit of detection (LOD) and limit of quantification (LOQ) [9].

To assess compliance with various legislation and regulations, many analytical methods have been developed for dioxin detection and determination. They are mainly determined by instrumental chromatographic methods usually coupled to mass spectroscopic- or bioassay-based methods that are mainly used to determine dioxins in environmental specimens. Chemical and biological methods commonly used for determination of dioxins substances are summarized in Figure 7. Bioassay-based methods have a strong probability to differentiate between the more stable congeners (Ah-receptor ligands) and the other dioxin congeners. Unlike the chromatographic methods, bioassay-based methods generally have lower costs, are fast and, thus, allow handling a relatively larger number of samples. On the other hand, they are considered semiquantitative methods, their results (some methods) are expressed as Bioanalytical Equivalents (BEQs) and results that exceed the cut-off-level need to be re-analyzed using confirmatory methods. Since chromatographic methods are able to identify individual dioxin compounds and provide their exact concentration, they are considered confirmatory methods (gas chromatography/high-resolution mass spectrometry "GC-HRMS" and gas chromatography/tandem mass spectrometry "GC–MS/MS"). However, there is a good correlation between the results obtained by bioassays methods and those obtained by chromatographic ones such as GC-HRMS and GC–MS/MS [16,32,55,56].

Bioassay-based methods used for dioxin determination depend on screening specific responses resulted from organisms or cells when exposed to dioxins, or the capability of some receptors, enzymes, antibodies or any other biological molecules to identify the structural property of dioxin and dioxin-like compounds [55,56]. They could be grouped into *in vivo* and *in vitro* assays. *In vivo* bioassays are based on experimentally exposing the laboratory animals to dioxin compounds and investigating the response

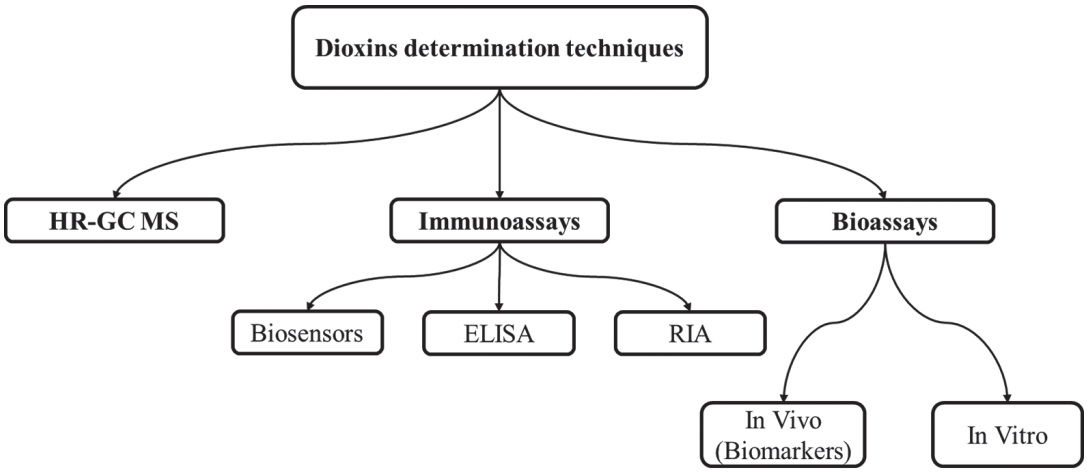


**Figure 7.** Chemical and biological techniques for determination of dioxins

or the resulted abnormality in different organs such as thymus for immune toxicity and liver hepatotoxicity, or assessing some *in vivo* biomarkers of natural exposure to dioxin in humans or wildlife (e. g., cytochrome P450 1A gene- CYP1A and induction of aryl hydrocarbon hydroxylase-AHH). Whereas, the *in vitro* bioassays include methods based on DNA-binding, receptor binding, cell culture and reporter gene assays. In addition, changes in gene expression or enzyme inhibition assays in cultured cells and several immunoassay-based methods, particularly, fluorescence immunoassay, the enzyme-linked immunoassay and radioimmunoassay were also applied to determine dioxin compounds [55,56].

The Chemical Activated LUciferase gene eXpression (CALUX) is the most widely used bioassay for dioxin and dioxin-like compounds detection. It uses genetically modified hepatoma cells that contain the Ah-receptor responsive luciferase reporter gene. This gene reacts to any substance which can stimulate the Ah-receptor (including dioxins), so exposing these cells to dioxins leads to induction of luciferase gene expression and consequently increases the luciferase levels that can be measured by light reaction. Since, the CALUX analysis responds to all chemicals that activate the Ah-receptor, adding a clean-up step over an acid silica column could decrease the interfering compounds and increase the specificity of the analysis for the PCDD/Fs and DL-PCBs. Ethoxyresorufin-O-deethylase (EROD) assay is another bioassay analysis for dioxin and dioxin-like compounds determination but it is less commonly used [16,32,57].

Chemical instrumental methods used for dioxin and dioxin-like compounds determination by chromatographic analysis include gas chromatography (GC) and high-performance liquid chromatography (HPLC) coupled with different types of detectors such as mass spectroscopy, high-resolution mass spectroscopy, fluorescence spectroscopy, electron capture detector or photodiode array [55,56]. Many techniques were reported for separation and detection of dioxin and dioxin-like compounds such as two-dimensional gas chromatographic (GC×GC) separation, GC–MS/MS, GC–HRMS, high-resolution gas chromatography coupled to mass spectrometry (HRGC–MS), high-resolution gas chromatography-high resolution mass spectrometry (HRGC–HRMS), GC coupled to electron capture detector (GC-ECD) and gas chromatography (GC) triple quadrupole mass spectrometry (GC–QQQMS/MS). High-performance liquid chromatography coupled to a photodiode array (HPLC-PDA) was also used for dioxin analysis [9,55,56,58]. However, only methods fulfilling the criteria laid down by the European Commission are considered as confirmatory methods. These methods should provide clear congener-specific identification and quantification of dioxin and dioxin-like compounds in the samples and they usually use GC–HRMS and GC–MS/MS. Moreover, the CONTAM Panel in the EFSA only included the data obtained with GC–HRMS, GC–MS/MS, HRGC–HRMS, GC–HRMS or GC–QQQ-MS/MS in their Comprehensive European Food Consumption Database [16]. Each step in the analysis process is crucial in order to reduce the interferences from other compounds and to avoid analyte losses. Therefore, it is important to pay attention during all stages of the analytical process including sampling, handling of samples, extraction, clean-up, separation, detection, and quantification [9].

First, representative samples should be collected with equipment precleaned by acetone or hexane and/or heat-treated at 450 °C for 20 min. Lipid determination is a critical step in the analysis of dioxin compounds, as the internal standard should be added before the fat extraction in the samples of food and feed of animal origin that contain less than 10% fat, whereas, it could be added prior or later fat extraction in those that contain more than 10% fat [16]. Because of the hydrophobic nature of the dioxin and dioxin-like compounds, extraction methods are based on fat extraction from the samples including liquid-solid extraction (e. g., Soxhlet, accelerated solvent extraction-ASE, microwave-assisted solvent extraction-MAE and supercritical fluid extraction-SFE), solid-phase extraction (SPE), and liquid-liquid extraction (LLE). However, an extraction method is chosen depending on the sample type, amounts and the nature of other interfering substances. Another critical step in the analysis process is extract purification

that provides eliminating interfering substances. Dioxins, as stable compounds, could be cleaned-up from other interfering compounds (e. g., protein and fat) by treating with a strong acid such as sulfuric acid and/or a base. Gel permeation chromatography (GPC) has been also used in dioxin extract purification. However, multistep purification using chromatographic adsorbents (silica, florisil, alumina, and activated carbon) is routinely applied for the isolation of dioxin from other interfering substances [16]. Finally, the $^{13}C\text{-}_{12}$ labelled standards (17 $^{13}C\text{-}_{12}$ labelled PCDD/Fs congeners and 12 $^{13}C\text{-}_{12}$ labelled PCBs) are used as internal standards to determine the losses (recovery) of their corresponding analytes [32].

**Conclusion**

Dioxin and dioxin-like compounds have received considerable attention in recent years, especially after many accidental events that led to releasing huge amounts of these compounds to the environment. They attracted attention and became familiar chemicals between populations due to the high potential toxicity to humans and other organisms, wide distribution over the world and extreme stability. PCDDs and PCDFs mainly occur in the environment as a result of several human activities such as combustion, incineration and many other industrial activities. A very small amount is also reported from some natural processes such as volcanic eruptions and forest fires. Unlike PCDD/Fs, the PCB congeners were intentionally manufactured and widely used in various fields. Huge amounts of PCBs were produced in the period 1929–1970, but their production was suspended in the late 1970s because of their adverse effects on the environment and human health. Once dioxin and dioxin-like compounds are released from their sources, they are spread almost everywhere throughout the world and enter various environmental compartments (air, water, soil, sludge, sediment, food, feed, blood, animal and human tissues) via direct or indirect ways. Humans are exposed to dioxin via inhalation, dermal contact or food ingestion. However, 90% of human exposure to dioxin is through food ingestion particularly foods from animals and foods that are rich in fat. In contrast, only low levels of dioxin and dioxin-like compounds are found in food items of plant origin. These compounds show various adverse health effects started from chloracne, irritation in the respiratory and gastrointestinal tracts, headache to serious problems in the reproductive, immune, thyroid, cardiovascular and hepatic function. They can also promote many types of cancers. However, the toxicity of dioxin and dioxin-like compounds varies dramatically according to species of exposed organisms and the type of dioxin i. e., a degree of chlorine substitution and pattern, moreover, the exposure frequency and duration are also important factors. These health effects were documented based on many experimental animal studies and human cohort or epidemiological studies. However, some of these health effects are not supported by sufficient evidence that confirms the causal association between dioxin exposures and health problems. Regarding dioxin type, only 29 congeners of a total of 419 PCDDs, PCDFs and PCBs are reported to show toxic effects on humans and many other living organisms. Their toxicities are expressed as the TEF value that exhibits the possible toxicity of certain congeners to a reference congener (TCDD- the most toxic congener). TEF values were developed many times and different versions of TEFs were used in different studies. Several methods have been developed to measure dioxins in environmental and biological samples, since they usually occur as a mixture of congeners at very low concentrations and are often attached to other organic compounds. So, analytical methods should provide an efficient extraction, purification, separation and accurate determination of toxic congeners at trace levels. Dioxins are mainly determined by instrumental chromatographic methods or bioassay-based methods. The latter are generally fast, have lower cost, allow to handle a relatively large number of samples but they are considered semiquantitative methods, so their results need to be confirmed by other confirmatory methods such as GC-HRMS and GC–MS/MS. Because of the high toxicity, wide distribution, accumulation ability, poor degradation and stability for a very long period of time, many efforts have been made to remove, reduce and prevent these hazardous substances from the environment. The best method for reducing human exposure to dioxins and dioxin-like compounds is prevention and minimization of production and contamination of foods and animal feeds. Moreover, processing food, sometimes, can lower the concentration of PCDD/Fs and PCBs in food items by discarding the fat during the process or releasing lower chlorinated congeners. Trimming fat from meat, consuming low-fat dairy products and avoiding foods from contaminated areas could also minimize the exposure to dioxin and dioxin-like compounds. Under certain conditions, dioxin and dioxin-like compounds in the environment undergo biodegradation by both aerobic and anaerobic organisms. Highly chlorinated PCDD/Fs and PCBs are dechlorinated via anaerobic organisms, whereas aerobic organisms are responsible for mineralization of the resulted less-chlorinated compounds. Many microorganisms including yeasts, fungi and bacteria are able to degrade dioxins. However, this process strongly depends on the position and degree of chlorine substitution, the species of microorganisms and the status of the medium. Various methods have also been developed to reduce emission of dioxin and dioxin-like compounds in fly ash and flue gases released from incineration and combustion processes including the particulate matter collection, scrubbers or spray absorber, sorbent or flow injection process for flue gases, thermal treatment, non-thermal plasma, UV irradiation for fly ash.

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# SCIENTIFIC CHALLENGES IN MODELING MASTICATION OF MEAT USING ENGINEERING TOOLS

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**Keywords:** *meat characteristics, meat engineering, FMEA, meat quality*

***Abstract***

*This paper gives an overview of scientific challenges that may occur while performing modelling meat (as a product) and simulating mastication by using engineering tools. To evaluate these challenges, Failure Mode and Effect Analysis method has been employed to assess six engineering tools often used in analyzing different perspectives of food oral processing. As a result, a risk priority number comprising of severity of the failure, occurrence probability of a failure and difficulty to detect the failure has been calculated. Results show that finite element method and emotion detection are two tools with highest levels of risks. The first method is a known engineering solution used for analyzing different types of materials, but when it comes to meat as a very complex and anisotropic material, risk of inadequate calculations is high. Emotion detection is not so much dependent on meat as a product consumed but on imperfections of software and risk of recognizing false emotions is high. Findings indicate that more research is needed for a more sophisticated use of these engineering tools. Further studies should include other engineering models that simulate meat breakdown during mastication, the role of saliva and jaw movement with the aim to carry out full modelling of mastication of an average meat consumer.*

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## Introduction

Modelling of food is very complex due to three constraints: knowledge about a food product, reliability of experimental data and uncertainties associated with food properties [1]. Predictive models and simulations enable development of new scientific approaches and optimizations of food products and food processes [2]. Also, modelling of food behavior can provide information related to food characteristics [3]. This is even more pronounced for multiscale modelling of food tissues with different mechanical properties [4]. Therefore, the main purpose for food engineering is to understand a certain engineering phenomenon combining existing theoretical understanding and available measurements [5].

Meat is considered as a postmortem skeletal muscle tissue of different animals used for human consumption [6]. After slaughter, it undergoes numerous changes, both physiological and biochemical [7]. As a material, it is considered as a matrix comprised of three main elements: muscle fibers, intramuscular connective tissue, and intramuscular fat [8]. However, its standardization is difficult as all these elements depend on a variety of factors such as the species/breed of the animal, age of the animal when slaughtered, type of feeding and other different animal husbandry aspects and finally position of the specific sample in the carcass [9].

Consumption of meat and meat products on a global scale demonstrates two tendencies: (i) an overall rise in consumption mainly caused by the growth of the global population, and (ii) an increase in consumption of meat expressed per capita [10]. Several authors have determined reasons for such a trend, just to mention two most important: dietary habits and nutritional needs for food with animal origin (meat) proteins [11], and sensory enjoyment when consuming meat and meat products [12]. Besides these two trends associated with nutrition and hedonisms, mastication also plays its role in overall perception of meat associated with textural perception) [13,14]. To better understand this quality attribute, it is of utmost importance to understand meat as a material and its mechanical characteristics.

Depending on mechanical properties under load, material science recognizes three types of materials, commonly associated with food: (i) isotropic materials directionally independent; (ii) orthotropic materials (interchangeable across the three main orthogonal axes), and (iii) anisotropic with different mechanical properties in all directions [15]. Meat is a very complex system dependent on the interaction between processes and forces of the meat matrix [16]. As such, meat is an anisotropic material, but for different studies/simulations authors consider it as an

orthotropic material [17]. Figure 1 depicts some key words associated with modelling meat.

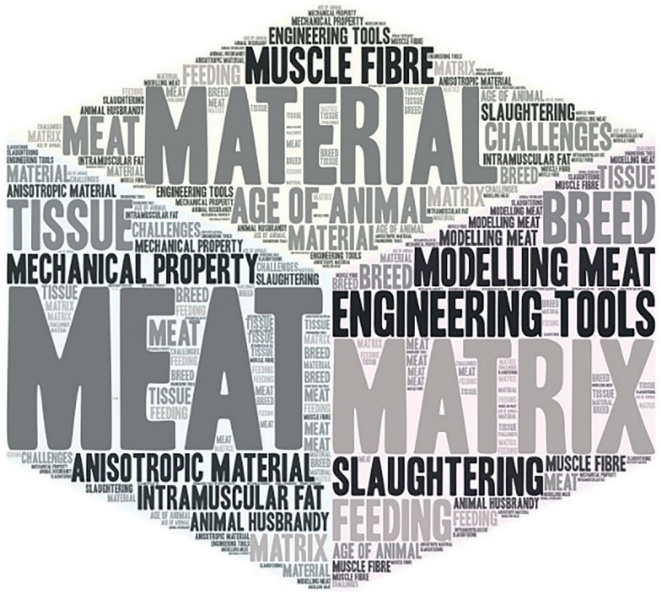


**Figure 1.** Word cloud figure associated with meat modelling

Mastication starts from the first bite and ends with swallowing. Food undergoes several phases, from the first bite when incisors initiate the food breakage and first deformation, followed by fractures, bite acceptance in the oral cavity, initial comminution, transportation and distribution of particles inside oral cavity, further comminution, formation of a swallowable bolus and finally swallowing [18]. The latest research confirms that the mastication behavior is more a rhythmic action that creates a pattern and it is dependent on mechanical properties of food rather than its predominant taste [19].

The main objective of this study was to evaluate challenges when modelling mastication of meat using available engineering tools through the Failure Mode and Effect Analysis and as a result weigh potential risks associated with selected tools. As the mastication process has many activities that can be modelled, only the following steps have been investigated: modeling first bite by using results from the following engineering tools — the Warner-Bratzer (BW) test, compression test and Finite Element Method (FEM); modelling bolus characteristics necessary for swallowing using the computer vision system (CVS) for particle size distribution and analyzing the mastication process itself, through video capturing and emotions detection.

## Objects and methods

Ranking of risks associated with using engineering tool in modeling meat was performed by professionals with expertise in meat science and food quality, also holding engineering and technological skills. To calculate these risks, the Failure Mode and Effects Analysis (FMEA) has been used as an analytic tool [20]. This technique is very useful as it identifies possible failure modes as well as their causes but in parallel investigates effects of the failures [21]. When using FMEA, it is necessary to use previous knowledge related to similar items or problems [22]. Therefore, it is common to develop an inventory of possible failure modes and evaluate associated risks [23]. For the purpose of this study, a list of potential nonconformities has been populated. The FMEA risk also known as the "risk priority number — RPN" was calculated as follows [21]:

$$RPN = S \times O \times D \quad (1)$$

where:

($S$) is the severity of the failure;
($O$) stands for occurrence probability of a failure;
($D$) stands for difficulty to detect the failure.

**Table 1. Severity, Occurrence and Detection rating scale**

| Severity | | |
|---|---|---|
| **Rank** | **Consequence** | **Description** |
| 1 | None | No failure(s) |
| 2 | Minor | Failure(s) associated with results for one characteristic, not critical-to-quality |
| 3 | Low | Failure(s) associated with results within one critical-to-quality meat characteristic |
| 4 | Major | Failure(s) associated with results within more than one critical-to-quality meat characteristics |
| 5 | Severe | Failure(s) associated with results affecting entire quality of meat |
| **Occurrence** | | |
| **Rank** | **Probability** | **Description** |
| 1 | Very unlikely | Minimal probability of occurrence of failure(s) because of *force majeure* |
| 2 | Unlikely | Occurrence of failure(s) only because of misuse of software / instrument |
| 3 | Possible | Occurrence of failure(s) only because of errors in previous calculations/estimations |
| 4 | High probability | Occurrence of failure(s) because of human errors / mistakes |
| 5 | Certain | Occurrence of failure(s) because of lack of knowledge |
| **Detection** | | |
| **Rank** | **Criteria** | **Description** |
| 1 | Very high | Failure(s) associated with results is easily detected |
| 2 | High | Failure(s) associated with results is detected during initial calculations/estimations |
| 3 | Low | Failure(s) associated with results is detected during simulation / validation |
| 4 | Remote | Failure(s) associated with results is detected during verification |
| 5 | Never | No possibility of identifying failure(s) associated with results of modelling |

Table 1 outlines weighting factors of the three factors, adopted and modified from [22,24,25]. Experts who participated in the session confirmed that all important nonconformities that might occur while modelling meat were identified. Consensus for each weighting factor was reached with no opposed and/or conflicting opinions linked with final RPN score.

## Results and discussion

### *FMEA Analysis*

Results of the FMEA analysis are depicted in Table 2 for the following six selected engineering tools: the Warner-Bratzer (BW) test, compression test, Finite Element Method (FEM), computer vision system (CVS), video capturing and emotion detection.

#### *Warner-Bratzer test*

The WB test has been used for many years in order to assess meat tenderness. It measures maximum force calculated as a function of knife movement and compression shear off showing the hardness of meat [26]. As pointed in Table 2, two main issues may occur associated with this test — choice of device parameters and preparation of meat.

When it comes to the choice of test parameters, it has been confirmed that the angle of the cutting edges of the blade may affect results (an increase in shear force), as well as different blade thicknesses and the width between the blade that may influence rupture force values [27]. The RPN value of this issue is 18 mainly as this test is standardized, with the standardized 'Warner-Bratzler' blade used for different texture analysis instruments.

Preparation of a meat sample for testing is of utmost importance. The first criterion is the diameter of the sample as it must be uniformly round. This is easier to obtain from large animals' muscles with a proposed diameter of 1.27 or 2.54 cm [26]. A culinary method used for preparation is the second criterion [28] followed by chilling to 2–5 °C for obtaining consistency of the material [26]. The final criterion is the direction of fibers as this test has to be performed normally on the axis of the muscle fibers [28]. For this test, the RPN value is 36, mainly dependent on human errors associated with preparation of meat samples.

#### *Compression test*

Compression tests are used to assist true stress and strain calculations as proposed by Vallespir et al. [29] and Nieto et al. [30]. In parallel, it enables rupture stress (σR, MPa) and strain (εR) to be extracted from the first peak of the stress-strain curve. Like the WB test, this test also has two main issues that may occur related to the instrument and meat sample.

Opposed to the WB test that may be considered as standardized in terms of the blade characteristics and other instrument parameters, for the compression test a larger number of variables occur such as the test speed, compression percentage, load cell and probe selection. Also, since different mechanical properties associated with texture as a quality characteristic are obtained and/or calculated, the RPN values is 36.

When it comes to meat sample preparation, first, it has to be performed by thin-bladed sharp knives to minimize the damage of the fibers [31]. Second, besides preparation of samples, direction of fibers is in direct correlation with the results [26]. When it comes to 3D modelling, this issue is even more pronounced when it comes to under-

**Table 2. Failure Mode and Effect Analysis of modelling meat using engineering tools**

| No | Tool | Non-conformity | Potential Failure Effect | Severity (S) | Occurrence (O) | Detection (D) | Risk |
|---|---|---|---|---|---|---|---|
| 1 | Warner-Bratzler | Inadequate instrument parameters | Variations in maximum force value | 3 | 2 | 3 | 18 |
| | Warner-Bratzler | Wrong direction of fibers | Inadequate reading of maximum force values | 3 | 4 | 3 | 36 |
| 2 | Compression test | Inadequate instrument parameters | Variations in values of tested parameters | 4 | 3 | 3 | 36 |
| | Compression test | Wrong direction of fibers | Inadequate reading of tested parameters | 4 | 4 | 3 | 48 |
| 3 | Finite element method | Inadequate assumptions | Wrong values and inadequate modelling | 5 | 4 | 4 | 80 |
| 4 | Computer vision system | Inadequate color detection | Wrong reading of color parameters | 3 | 2 | 4 | 24 |
| | | Inadequate particle preparation | Incorrect calculation of particle size distribution | 2 | 4 | 4 | 32 |
| 5 | Video capturing | Video clips of low quality | Difficulty in oral processing characterization | 4 | 4 | 3 | 48 |
| | Video capturing | Inadequate categorization chews / consumption time | Detection of wrong oral processing characteristics | 4 | 2 | 4 | 32 |
| 6 | Emotion detection | Video clips of low quality | Difficulty in detecting emotions | 5 | 4 | 4 | 80 |
| | Emotion detection | Inadequate categorization of emotions | Detection of incorrect emotions | 5 | 2 | 3 | 30 |

standing the direction of compression/expansion related to the fibers [17].

*Finite element method*

One of the most popular engineering tools is FEM and as such it has found its application in food science and food engineering. This tool enables performing different types of analyses and modelling focused on solving complex mechanical problems [32]. In meat science, its common use is mass/heat transfer [33–35], but with limited application in other dimensions of meat science such as simulating the first bite [17]. When modelling meat is performed using FEM, it is typical to define the shape of the piece (usually as cubic pieces leading to 3D simulation) to enable the use of different software. This is the first assumption when modelling meat. In parallel, other assumptions are usually a type of material (orthotropic for 3D simulation), direction of fibers and direction of forces within the material [17].

To simulate the first bite, the following assumptions are needed: the shape and size of the sample, direction of the first bite related to the direction of fibers, and position of the incisors when initiating the first bite [17]. Also, the following rules apply: (i) WB test values divided by two correspond to the first bite force; (ii) compression test values allow assumption of expansion of meat when subject to specific loading direction and values for this test enable calculation of the Poisson's ratio. These inputs are minimal requirements for mesh construction in FEM using four-node tetrahedral elements [36]. The RPN value for this tool is very high as it is directly dependent on all assumptions and pre-calculations serving as inputs in FEM simulations. Any mistake in pre-calculations and assumptions directly causes wrong values and inadequate modelling.

*Computer vision system*

CVS is considered as a novel tool used for instrumental evaluation of the meat color [37]. It has advantages compared to traditional colorimeters as latest studies confirm significant differences between L*, a*, b* color values of different types of meat and meat products measured with CVS opposed to colorimeters traditionally used [16,38]. This equipment also has the potential of being used for particle size distribution analysis, as these high-quality photos enable further computational processing of the number of particles and 2D calculation of the surface area of each particle [28,39].

The use of this tool for color evaluation has a low RPN as this method has been developed and validated [16] and potential failures may be associated with misuse of software for color processing and/or CVS itself. However, for particle size distribution analysis, computational processing of the number of particles and their surface is more dependent on humans (in terms of spreading out the boluses with care in order not to damage the size of the particles, [28]) and consequently RPN has been calculated as 32.

*Video capturing*

In order to perform oral processing studies for the purpose of calculating the number of chews and consumption time, and calculating different attributes such as chewing cycle duration (s/chew), chewing rate (chews/s), eating rate (g/s) and average bite size (g) [19,40,41], it is common to video capture the mastication process involving human subjects. It is important to position the camera so that the complete upper part of the subject's body is visible and recorded [42]. When video clips are replayed, two potential solutions occur. The first one is the use of a software video analysis that has the feature to analyze graphs of time (x-axis) vs. vertical jaw displacement (y-axis) where chews are visible as peaks [40]. The second solution is the use of humans to count chews while replaying clips and cross checking for accuracy [43]. The latest research on food oral processing confirms that mastication characteristics and behavior from a consumer point of view affects consumer satisfaction in parallel with sensorial properties [19].

Video clips of low quality may cause wrong calculation of key parameters (time/number of chews) and consequently all other oral processing attributes. Therefore, the calculated RPN value is 48. However, if validated software is used, the similar issue may arise, but the RPN is 32 as all potential mistakes and errors are minimized.

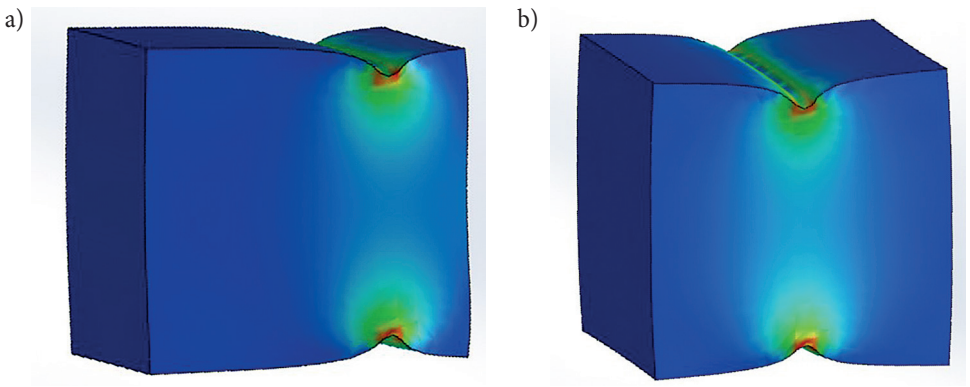


**Figure 2.** Stress distribution during impact of the upper and lower jaw shown for two positions:
a) at the edge of the sample; b) at the exact middle of the sample. Colors indicate gradient areas of the stress in the direction of pressure

*Emotion detection*

It is common to use collected video clips (from oral processing/mastication studies) for analyzing emotions [43]. The first criterion for performing this type of studies is good illumination of the face of the panelists to ensure reliable results [44]. The second one is the categorization of different emotions based on internally developed models using some databases such as DeepFace — face recognition and facial attribute analysis framework [45]. Some publications have detected five types of emotions during mastication — 'neutral', 'angry', 'sad', 'happy', and 'surprise', [43] while others have up to seven — 'angry', 'disgusted', 'happy', 'neutral', 'sad', 'scared', 'surprised' [46]. Finally, before starting these types of studies, it is necessary to avoid all types of biases such as unintended detection of wild facial expression [47] and taking off glasses (if any) as some may mask emotions [46]. Therefore, clear protocol for this type of studies is to have panelists being instructed to look directly into the camera from the first bite to swallowing [43].

Two issues may occur when detecting emotions using video capturing. The first one is in case of low quality of video clips. This issue seriously affects emotion detection and causes incorrect results and as such, the RPN value is 80. The second issue is in case of inadequate categorization of emotions mainly caused by using inadequate software and/or incorrect programing of software. Hence, the calculated RPN value for this failure is 30.

**Conclusion**

The FMEA-based approach for evaluating risks in using engineering tools needed for modelling meat can provide guidance to meat scientists and food engineers to concentrate efforts on the hot spots that are most influential. Our results recognize the finite element method and emotion detection as two tools with the highest level of risks and tools that are still evolving their industrial and scientific application in meat modelling. Results for the first tool are mainly linked with the complexity of meat as a material and difficulties in modelling, in spite of developed software. On the other side, emotion detection is a promising tool but dependent on the human factor and settings for video capturing of emotions associated with meat consumption and hedonism.

Limitation of this paper is the fact that only six engineering tools have been analyzed associated with the first bite, swallowing of bolus and mastication. Further studies should deploy these (and other) tools for modelling food breakdown from the first bite to swallowing, saliva incorporation, understanding jaw movement and finally modelling the chewing trajectory of an average meat consumer. As the final goal, modelling should utilize all meat changes and bolus breakdown and clearly enable validated simulations in relation with the mechanical and physical properties of all types of meat.

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# SHOCKWAVE EFFECTS IN THE TECHNOLOGY OF MEAT RAW MATERIAL PROCESSING

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**Keywords:** *hydrodynamic shock wave, meat, tenderization, tenderness*

***Abstract***

*Meat tenderness is recognized as the most important quality characteristic determining consumer acceptability of fresh meat and meat products. Therefore, the development of effective methods for meat tenderization is a topical direction. The review considers the main aspects of the development of shockwave (SW) technology as an alternative method for meat tenderization. The paper analyzes the means of shockwave formation as well as possible mechanisms responsible for meat tenderization caused by shockwave treatment and related to the energy dissipation and mechanical load on the boundary zones of a biological material under processing. The results of the investigations of a shockwave effect on meat tenderness, microbial inactivation, structure and assimilability of muscle protein and other functional and technological properties of a product are presented. The majority of researchers who studied a SW effect on meat tenderization showed different degrees of the improvement in the Warner-Bratzler shear force and increase in sensory scores of meat tenderness. This review shows the main problems linked with commercialization of the meat treatment process using SW based on electrical discharges under water. This method of SW generation is considered safest but infeasible today due to occurrence of restrictions such as damage of packaging materials after treatment, a need to ensure effective SW propagation in a commercial unit and determine optimal treatment parameters in the process of shockwave tenderization. Furthermore, potential possibilities of using shockwave technologies in the food industry are discussed. In particular, shockwave treatment upon extraction is an effective method for extracting juice/ oil/ bioactive components from various plant materials, which can be used as the pretreatment or independent process.*

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## Introduction

An increase in consumer demand for high quality and minimally processed meat products has led to an extension of investigations and adaptation of several new technologies for the meat industry.

Over the last two decades, the use of high pressure in food systems especially ultra high pressure (>100 MPa) has attracted a significant attention of the scientific community and, as a consequence, their commercial application became a reality [1,2,3,4]. The reason is that the use of high pressure in food systems opens possibilities that cannot be achieved by conventional processing methods and, consequently, has a high potential in the development of new food technologies and optimization of the existing ones. Among these technologies are meat processing with the hydrodynamic shockwave and high pressure processing, which allow improving tenderness of meat raw materials depending on conditions during application of the technology [5].

High pressure can be used in two different forms of process organization: static (i. e., product treatment in a vessel) and dynamic (i. e., product treatment in a fluid flow).

The third method of pressure impact on foods is hydrodynamic pressure processing or shockwave treatment, which represents an instantaneous development of pressure waves up to 1 GPa in fractions of milliseconds. The pressure front can be generated both by detonating explosives and electrical discharge under water. In both cases, a result is the generation of a pressure wave or shockwave. The wave is characterized by the achieved intensity and speed of its propagation in time (that is, a pressure level and build-up time). A shockwave propagates through a liquid medium at a speed that exceeds the speed of sound. As meat is composed of 75% of water, a wave passes through a meat sample and ruptures muscle proteins. This gives what can be called "the rupture effect" and as a consequence favors meat tenderization [6]. Meat tenderness is an important quality parameter, which facilitates the total perception and acceptability of a product for consumers and can influence its cost [2,6,7].

## Retrospective of the development of shockwave technologies and equipment for meat tenderization

The shockwave technology appeared for the first time as an alternative method for meat tenderization at the beginning of the 1970s. In 1970, Godfrey [8] patented a method and an apparatus for tenderizing foods, including meat, with the use of the explosive charge, which generated a shockwave, and called it hydrodynamic pressure processing (HDP). Then, Long [9,10] changed the technology to

overcome its shortcomings and called it the Hydrodyne® process. The Hydrodyne® process was extensively studied by prof. M. Solomon el al. from the Food Technology and Safety Laboratory (Beltsville, MD, USA) [11], who demonstrated significant improvements in meat tenderness. However, the shockwave generation by explosives has certain drawbacks and problems related to the development of the equipment and potential product contamination with explosive residues as well as problems with regard to the safety of operators [6].

At the beginning of 2000, a new concept of the development of the shockwave technology was devised, which enabled the electrical generation of a shockwave by the capacitor discharge system. Also, three additional patents were filed for continuous shockwave food processing with shockwave reflection and shockwave food processing with acoustic converging wave guide. As a result of the integration of these concepts, the commercial system called the TenderClass System (TCS) was developed by Hydrodyne Incorporated. In 2007, Long et al. [12] patented a system, in which a shockwave was transmitted to meat through the diaphragm.

In Russia, a method and device were patented for meat tenderization and destruction of microorganisms in it due to the fact that meat was subjected to an effect of plasma shockwaves or pulses generated by the capacitor discharge between two electrodes [13]. According to this patent, meat is exposed to a shockwave propagating through an incompressible fluid medium. With that, a meat raw material is placed adjacent to the first surface of the drum-shaped diaphragm, which has the acoustic resistance approximately the same as the acoustic impedance of the incompressible fluid medium. The incompressible fluid medium is adjoining to the second surface of the drum-shaped diaphragm, which separates meat from the incompressible fluid medium. When meat is subjected to an impact, its movement is restricted; the shockwave passes through the incompressible fluid medium, then through the drum-shaped diaphragm and after that enters the product. The shockwave generation chamber is used for containing an incompressible fluid, which has the first acoustic impedance. The invention allows improving meat tenderization while destroying microorganisms in meat.

Studies on meat tenderization using a plasma sparking device were carried out based on the patent of Cooper and Solomon [14]. In general, about 20 patents linked to this technology were registered in the whole world [6].

During 2008–2011, an experimental unit for shockwave generation for meat tenderization by electro-hydraulic underwater discharges was developed and realized in the German Institute of Food Technologies (DIL) within the framework of the German research project. A prototype with an average power of 2 kW and a peak power of 40kW with a vessel volume of 50 l was designed. The effective energy conversion from electrical energy to mechanical energy was achieved and its effectiveness in meat tenderization was demonstrated. The developed equipment was successfully used to reduce the time of ageing for beef cuts from 14 to 7 days. Currently, an industrial prototype for continuous shockwave treatment has been under development within the framework of the European-funded project Shockmeat. The aim of the project is to overcome shortcomings of the first prototype developed by DIL and ensure safety in the industrial conditions. Shockmeat is aimed towards continuous treatment of meat rather than a batch system [2,6].

The shockwave technology applied for meat tenderization is a relatively inexpensive and non-invasive method that does not exert a negative effect on the microbiological and chemical stability of a product. However, the commercial application is infeasible up to date as it is necessary to study and overcome such restrictions as a damage of a packaging material after shockwave treatment, ensure effective shockwave propagation in an industrial unit and determine parameters of treatment ensuring tenderization of a particular meat type, that is, to develop an effective process of product tenderization reducing treatment duration, energy consumption and expenditures for obtaining high quality meat products [6].

**Methods for shockwave generation and mechanisms of its effect on meat under treatment**

The shockwave technology or hydrodynamic pressure processing (HDP) is considered a potential method for tenderization due to instantaneous creation of high pressure [2,6,15].

The mechanism of the shockwave impact is related in this case with energy dissipation and mechanical load on the boundary zones of materials having different speeds of sound propagation and acoustic impedance. A wave is characterized by an achieved intensity and the speed of its propagation in time (that is, the level of pressure and built-up time). Packed meat is placed in the working container with water and is exposed to shockwaves. The created shockwave passes through a liquid with the high energy, extremely high speeds and passes through a product placed into the unit. A wave passing through a meat sample ruptures muscle proteins, which allows destruction of the muscle structure leading to an instantaneous tenderization effect and accelerated ageing of meat (duration of processing is reduced from 14 to 7 days). After cooking treated meat, a decrease in the shear force was observed. The total energy expenditure is only several kJ per kilogram of a product, which corresponds to a temperature increase by less than 1 °C [15].

The hydrodynamic shockwave for meat tenderization can be generated either by detonating explosives or by creating electrical discharges under water [2,6,16]. For example, Solomon et al. [11] showed that detonating explosives under water can tenderize meat.

Shockwaves impact meat both directly and after reflection from the floor and walls of the working chamber creating a tenderizing effect. High-pressure shockwaves created by a small amount of explosives significantly increase tenderness of beef, pork, mutton and poultry meat [17,18,19].

The meta-analysis that compares studies on an effect of a shockwave impact on different meat types and muscles shows that explosive shockwaves can reduce the peak shear force after meat treatment by 17.7 N and the electrical shockwave by 7.5 N, respectively [5].

However, safety problems related to the use of explosives significantly limit commercial applicability of this method.

An alternative to shockwave generation by explosives is generation of shockwaves by electrical discharges under water. This method allows avoiding problems linked with the use of explosives, increasing automation of the process, ensuring its continuity, reducing the treatment duration and facilitating modulation of the shockwave intensity by supplying different electrical intensities and/or number of pulses per treatment [6].

Propagation and impact of a shockwave through food depends on the acoustic impedance, which is directly proportional to the product density [20]. The pressure of the wave front can vary from 30 to 100 MPa depending on the distance from the energy source [2], and a shockwave impact can cause a damage of cell walls and destruction of connective tissue depending on a food matrix [21] and wave intensity. Upon an impact on a food product, a shockwave divides into the wave of propagation and the wave of reflection due to changes in the density. Foods differ significantly by the density and matrices, which ensures variable resistance to shockwaves and, consequently, the mechanism of wave action for particular products [20] and a possibility of using the method can be individual.

Initially, it was shown that potentially safer method of the underwater electrical discharge (electrohydrodynamic shockwave) had an ability to tenderize poultry meat [22].

The high effective compact sparkers favor emergence of the electrohydrodynamic shockwaves for meat tenderization [23,24].

A sparker is an electrically driven acoustic source that creates high pressure shockwaves similar to explosives. A sparker operates by pulsing high voltage across an electrode gap, which leads to a plasma discharge that creates a pressure pulse or shockwave. The discharge leaves behind a high-pressure vapor cavity (bubble), which expands and then collapses creating an additional pressure peak. The process is repeated until the bubble energy is fully dissipated. The spark sources also provide an opportunity of electronic pressure control, which is potentially useful for tenderizing different meat cuts and types.

Claus [25] as well as Sagili and Claus et al. [26] showed earlier an improvement in product tenderness when beef and pork cuts were treated using a focused type sparker.

Bowker et al. [23] studied interrelation between working parameters of a sparker and the meat tenderization process with its use. The sparker source system used in this research consisted of the annular head with a pair of concentric cylindrical electrodes separated by an annular insulator. The sparker head was placed into water in a 19 L cylindrical plastic container. Vacuum packed meat samples were put on the bottom of the container on a flat steel plate (with a thickness of 1.3 cm) at a distance of either 3.75 or 7.5 cm from the sparker head located above the beef samples. Both the medial and lateral portions of each steak were treated with the sparker applying either 40 or 80 pulses at each position [23].

The tenderizing effects of beef loin treatment with high-pressure shockwaves from a sparker were assessed by the Warner-Bratzler shear force (WBSF) measured on days 0 and 7. The results of the research show that the distance between the sparker head and a muscle sample and, therefore, the shockwave peak pressure play a crucial part in determination of the tenderization degree. When the sparker head was set at a distance of 7.5 cm above the samples, an improvement in tenderness was on average only 5–10% on day 0 with a maximum improvement of 24%. At this sparker setting, a reduction in overall WBSF by more than 10% compared to the control was observed in 44% of treated steaks on day 0. When the sparker head was placed at a distance of 3.75 cm above the samples, the peak pressure increased from 6.6 to 12.3 MPa. At this distance, the average increase in tenderness was 20–25% on day 0, while the maximum increase in tenderness was 37%. Moreover, at a distance of 3.75 cm, a decrease in WBSF by more than 10% was recorded in all treated steaks; whereby, 70% of the treated samples showed a reduction in WBSF by at least 20% [23]. The WBSF value reduced both in the treated and control samples from day 0 to 7.

Preliminary experiments showed that the tenderizing effect was reduced after a certain number of spark pulses. It was found during the experiment that the number of sparker pulses necessary for achieving the tenderizing effect significantly depends on the height of sparker head setting relative to the product under treatment, which, possibly, is linked with a decrease in the shockwave pressure upon an increase in the distance. When the sparker head was set closer to the sample surface, the higher degree of tenderization was achieved with the lower number of sparker pulses (5–10 pulses in three places compared to 40–80 pulses in two places). The use of the lower number of pulses is beneficial with regard to maintenance of muscle tissue and packaging integrity.

The results of this research show that high-pressure shockwaves generated by a sparker are an effective technology for post-slaughter processing to tenderize beef.

**Effects of shockwave treatment on meat**

*Effect of shockwaves on microbial inactivation in meat*

There are few studies on an effect of shockwaves on microbial inactivation in meat; however, their results are quite contradictory [2].

For example, it was reported that explosive shockwave treatment allows reduction by up to a 4.5 log10 CFU in ground beef stored aerobically (5 °C) for 14 days, while the results of other studies showed no effect on coliforms and aerobic plate counts in pork loins treated with explosive shockwaves [7].

McDonnell et al. [7] assessed an effect of electrical shockwave treatment on the microbial load during long-term storage in the experiments on beef samples: striploin (longissimus lumborum) and brisket, point end deckle off (pectoralis profundus). Treatment in the unit (Shockwave, DIL German Institute of Food Technologies, Quakenbrueck, Germany) included placing the vacuum packed sample directly in the impact area under the emitting head (a source of a shockwave) located 13 cm from the sample. The treatment regime was as follows: 25 kV with the treatment intensity of 8 pulses with duration of 1 s in the stationary mode with a water temperature of 22 °C throughout the process. The total process time from sample loading to unloading was about 5 min. After treatment, the sample temperature increased from 3.7 ± 0.4 to 5.5 ± 1.0 °C.

The control and experimental (shockwave-treated) samples had six storage points (0, 4, 8, 12, 16 and 20 weeks).

Total viable counts (TVC) were similar in the shockwave treated and untreated control samples; whereby, the mean counts in all sample/treatment combinations at all time points were regarded as microbiologically acceptable when a cut-off of 7 log10 CFU/cm$^2$ was applied. The similar trends for lactic acid bacteria (LAB) and TVC in the storage experiments suggest that the microbial population consisted mainly of LAB, which corresponds to the previous results observed in striploin (longissimus lumborum) stored under similar conditions [7].

*Effect of shockwaves on an increase in meat tenderness and changes in the functional-technological properties*

Shockwaves largely exert the mechanical action on a processed product facilitating tenderization of muscle and connective tissues. The majority of scientists studying an effect of shockwaves on meat tenderization showed different degrees of improvement in Warner-Bratzler shear force and scores of sensory tenderness [2,5,6,28].

Solomon [19] showed that high-pressure shockwaves increased beef tenderness as effective as meat ageing with instantaneous improvement in tenderness by 37–57%. When meat is processed using shockwaves, muscle proteins are destructed [6]. The high-intensity shockwave changes the structure of meat collagen breaking peptide bonds and causing disruption in the myofibril structure [29].

A possibility to use shockwaves in the meat processing technology was studied in the North Caucasus Federal University. The experiments were carried out in chilled pork in a medium of modeled brine contained salt, sugar and nitrite. The treatment conditions were as follows: discharge of 1.81 kJ at treatment intensity 300 pulses [30].

The fluid medium, in which the high-voltage discharge occurs, is a transformer of energy released in the channel. The pulsed release of electrical energy in the latter leads to an increase in pressure in the system under treatment due to low compressibility of the fluid. High pressure forms and spreads intensive excitations in the environment. It is necessary to note that from the hydrodynamic point of view, an electrical discharge in fluid can be regarded as a process of non-stationary expansion of an impenetrable cavity. Due to high pressure near the discharge channel, the formation of the excitation is significantly influenced by non-linear effects that can lead to an increase in the steepness of the compression wave and to the shockwave generation.

The effect of shockwaves on pork samples was assessed by their histological analysis.

The histological investigation of the muscle cross-sections showed that the highest fiber diameter was observed in the experimental pork samples achieving 65–70 μm compared to 35–40 μm in the control sample.

In general, muscle fibers had the polygonal shape with restricted roundness. Part of muscle fibers of the experimental sample with a diameter of more than 60 μm had the round or oval shape and more uniform color. In the experimental samples, arrangement of individual fibers in the primary bundle was quite loose with well pronounced light spaces between muscle fibers. In these samples, a space between muscle fibers in bundles was notably lower than in the control groups.

An effect of shockwaves on muscle tissue was manifested in muscle fiber swelling, weakening of cross-striation, an increase in the development of transverse microfractures or slot-shaped spaces in muscle fibers as well as destruction of myosin and actin myofilaments. The observed changes in muscle tissue correspond to higher sensory indices — tenderness and juiciness of the finished product [25].

Schilling [31] demonstrated a 42% increase in tenderness upon treatment with the explosive generated shockwave. The similar result was obtained in the experiments on chicken broiler breasts exposed to hydrodynamic shockwaves generated in a cylindrical processor with the 40-gram explosive 25 min after deboning (77 min after slaughter) or after 24-hour storage (4 °C), respectively [17].

Analysis of an effect of the electric shockwave process on tenderness of chicken breasts (80 samples, 45 min. after slaughter) and turkey breasts (21 samples, 72 hours after slaughter) revealed a decrease in the Warner-Bratzler shear force by 22% and 12%, respectively, compared to the control after treatment. Cooking losses in turkey breasts were higher than in chicken breasts [20]. Meek et al. [18] also found an increase in tenderness by 19.1–28.1% in chicken breasts with early deboning. The electrical shockwave process can provide processors with a possibility of early deboning and obtaining tender chicken breasts as well as turkey fillets with increased tenderness [17].

To study an effect of hydrodynamic shockwave treatment on beef tenderization and ageing, samples of beef muscles *M. longissimus thoracis* and *M. semitendinosus* were vacuum packed in polyamide/polyethylene packages and subjected to shockwave treatment in a prototype unit produced by the German Institute of Food Technologies (DIL, Quakenbrück, Germany). Muscles were processed using electrical discharges under water in the following mode: 35 kV (corresponding to 11025 J per pulse) and a distance of about 20 cm from the meat sample to the shockwave spark at the frequency of 1 pulse every 3 cm. Subsequently to shockwave treatment, the muscle samples were cut into three pieces with a length of 10 cm and vacuum-packed before aging during up to 21 days at 4 °C. Texture, color, drip losses, cooking losses and the muscle structure (by scanning electron microscopy (SEM)) were analyzed in all meat muscle samples [32].

Shockwave treatment of *M. longissimus thoracis* led to a significant decrease in the Warner-Brazler peak force values at all storage points compared to the control (untreated) samples: 12.4% at day 1, 8.2% at day 11 and 5.8% at day 21, respectively. The results of the scanning electron microscopy revealed some differences between muscles treated with shockwaves and control samples on the 1st day of storage showing slightly larger intermuscular fiber space, which, possibly, led to increased tenderness [29,32]. Shockwave treatment did not significantly influence cooking losses and changes in color parameters (L*, a*, b*) in beef muscles during storage. In general, beef muscle color depended on storage duration. The value of lightness (L*) increased in the samples with storage time and redness (a*) slightly decreased both in *M. longissimus thoracis* and *M. semitendinosus*.

In systematization of studies using meta-analysis of publications, no effects of shockwave on changes in meat color characteristics were found [28].

Schilling et al. [33] determined protein functionality of bovine *Biceps femoris* (BF) muscle proteins after treatment with the hydrodynamic shockwave generated by the explosive method, which created hydrodynamic shockwaves with pressure fronts of 83, 104 and 124 MPa. The explosives were nitromethane and ammonium nitrate in amounts of 105, 200 and 305 g. In general, hydrodynamic shockwaves reduced the shear stress values in beef streaks by 20%; whereby, no differences in solubility of myofibrillar and sarcoplasmic proteins were found between the control and experimental beef samples. The results of gel-electrophoresis showed that proteolysis (protein breakdown) of myosin or actin was not visually observed on the myofibrillar gels, while proteolysis of myoglobin was not visually observed on the sarcoplasmic gels as a result of hydrodynamic shockwave treatment compared to the control. Myoglobin denaturation and, consequently, color changes in shockwave treated beef did not occur [33]. Cheftel J. C. and Culioli J. (1997) [34] reported that pressure from 200 to 350 MPa for 2–5 min after achieving the targeted pressure was required for meat color changes due to myoglobin denaturation.

Frankfurters made from the experimental shockwave treated beef samples and control beef samples did not have differences in cooking losses and color characteristics [33].

Shockwave treatment as a method for increasing tenderness of beef muscles up to 15% compared to the control (untreated) muscles showed a high potential that minimally influenced meat quality characteristics.

Taking into account that shockwave (SW) processing changes the muscle structure [35], it was suggested that these changes have a probable effect on the biological availability of food enzymes for their substrates, which can influence the nutritional value of meat products. To this end, an effect of shockwave processing on the molecular structure of beef muscle protein was studied using a FT-IR microspectroscopy [36].

Steaks were obtained from Simmental beef briskets (21–22 month old) 11 days after slaughter and exposed to hydrodynamic shockwaves (intensity = 11 kJ/pulse, one pulse per step, continuous system) with the following sous-vide cooking at 60 °C for 12 hours. After that, gastric digestion process was simulated for 1 hour at pH 3 and 37 °C in the presence of pepsin.

The infra-red spectra of both myofibers (MF) and connective tissue (CT) obtained using a FT-IR microspectroscope (Thermo Scientific, Nicolet iN10) were analyzed to study changes in the structure. It was found that shockwave (SW) treatment changes the native α-helix structure of connective tissue protein [37]. After sous-vide processing, the intensity of 1655 $cm^{-1}$ band of the myofibers from the SW treated beef samples was significantly lower than that from the control untreated meat sample, which indicates more profound protein denaturation in the treated sample during thermal processing. After an hour of *in vitro* gastric digestion, the intensity of 1655 $cm^{-1}$ band in the center of the cooked meat sample treated with SW was significantly higher than that in the control cooked meat sample suggesting that the acidic gastric condition exerted the higher and faster effect on the untreated control sample, which led to a higher degree of α-helix denaturation of the myofibers in the latter. Therefore, hydrodynamic shockwave treatment changes the protein secondary structure, which can influence functional and nutritional quality of meat and meat products [36].

Shockwave treatment causes changes in the muscle structure such as fragmentation of myofibrils along Z-discs and destruction of collagen fibrils. However, a shockwave effect on the molecular structure of muscle protein is unknown [38].

To optimize the technology of meat tenderization with shockwaves with its following commercialization, the spatial modeling of hydrodynamic shockwave distribution was carried out. Quantitative assessment of distribution and penetration of hydrodynamic shockwaves was performed using laminated pressure sensitive paper (Fujifilm

low pressure 2.5–10 MPa, Bestech, Australia). After shockwave treatment (15–30 kV) by electrical discharge under water in a specialized SW unit (DIL, Quakenbrück, Germany), the pixel intensity on the paper was analyzed using an Epson Perfection V370 Photo Scanner.

Systematization of the experimental data revealed the front of pressure alterations upon shockwave treatment, whereby the green, red and yellow zones on the laminated paper indicated pressures of < 2.5 MPa, 2.5 to 10 MPa and > 10 MPa, respectively. The results demonstrated the even pressure distribution from top (5.32 MPa) to bottom (4.70 MPa) in the treatment chamber with an insignificant increase in pressure towards the shockwave source. The predicted and measured values were comparable, which enabled creating a model that could simulate pressure at various distances from the shockwave source [39].

Therefore, the use of shockwave treatment for meat tenderization is promising. However, up to date, the majority of studies on a shockwave impact on foods, including meat, are at the stage of laboratory experiments and verification, and require further research aimed to an increase in the effectiveness, development of rules and safe methods of using before large-scale commercialization as well as evaluation of consumer acceptability.

One of other possible methods for application of the shockwave technology is processing oysters. Raw oysters are placed into water and exposed to shockwaves. The adductor muscle is relaxed and an oyster is opened. Nowadays, samples of such equipment for treating individual batches have been already applied in practice and it is planned to develop a continuously operating unit [15].

**Using shockwaves in the technology of plant raw material processing**

Over the last decade, studies on using shockwaves for softening fruit and vegetables were carried out to increase the effectiveness of extracting juice/oil from them. The researchers found that treatment of plant materials with shockwaves enables obtaining extraction products with higher quality than those produced with the use of thermally processed fruit due to the minimum comparative effect on nutritional and sensory properties [40]. It was suggested that an increase in the extraction effectiveness upon shockwave treatment is conditioned by increased damage of plant cells before extraction [41]. Underwater shockwaves passing through the plant tissue collide with plant cells and exert high pressure on the cell wall. This creates cracks on the cell wall destructing the cell structure, softening and even liquefying the plant tissue. Therefore, the cell content of plants, including juice, oil and bioactive compounds, penetrates easier through the cell wall destroyed by a shockwave compared to intact cells [42]. Kuraya et al. [42] reported that upon shockwave pretreatment of yuzu, the juice yield increased up to 170% compared to the conventional squeezing methods (yuzu is Japanese citrus fruit with the characteristic pleasant aroma and antioxidant capacity, which is usually consumed as juice).

Yasuda et al. [41] found that carrot subjected to shockwave pretreatment showed a significant increase in juice yield to 44.5% compared to 0.79% in the control raw carrot and 34.4% in the preliminary heat-treated sample. In addition, a significant increase in the carotenoid content was observed in the experimental carrot juice compared to the control (147.6 vs. 103.7 GAE μg/mL). Moreover, the energy input was about 40–50 kJ/kg for the SW treatment compared to heat treatment at 90 °C (which will require, as a minimum, 300 kJ/kg) leading, therefore, to the higher rate of extraction. This shows that energy consumption was significantly lower for SW compared to the methods of heat treatment [41].

It was also established that shockwave treatment increased the rate of extraction compared to various conventional methods including Soxhlet extraction, liquid-liquid extraction, microwave-assisted extraction, and ultrasonic extraction (USE). Molina G. A. et al. [43] reported that the use of shockwaves increased the rate of polyphenol extraction compared to conventional extraction methods with or without solvents. In particular, phenolic compounds and flavonoids from heartwood were extracted over 5 min using shockwave extraction (EP-SW), while the conventional Soxhlet extraction method required 96 hours. In addition, SW extraction did not require the use of organic solvents and led to extraction of significantly higher levels of reducing sugars and lower levels of phenolic acids than Soxhlet extraction. The results of the studies also showed that the time necessary for extraction in the shockwave treatment method was less than that in conventional methods (12.5 min for SW at 0.5 Hz; 20 min for ultrasonic-assisted extraction at 40 kHz; 96 h for Soxhlet extraction) [43].

In general, the use of shockwave treatment in extraction is an effective method to extract juice/oil/bioactive components from different plant materials. This method can be used as a pretreatment or independent process.

**The main problems in commercialization of the shockwave use**

*An effect of shockwave treatment on packaging materials*

Commonly used food packaging materials are prone to damages caused by high-intensity shockwaves generated during processing. Bolumar and Toepfl [2] reported that meat swelling caused by shockwave treatment led to disruption of plastic packages. They came to a conclusion that today there are no packaging materials that are completely resistant to SW and it is important to develop not only such a material but also a technology for their use. At present, the most promising packaging material is probably polypropylene due to the fact that its acoustic resistance is similar to acoustic resistance of water, which reduces shockwave absorption [44].

To eliminate the problem with package damage during shockwave treatment, the researchers studied treatment

of food products without packaging, when a product was in the direct contact with water during the process. Claus et al. (2001) [17] applied shockwave treatment of chicken breasts packed with water to simulate meat treatment in water. The results showed an increase in cooking losses in chicken breasts submerged in water, which can be caused by increased water absorption.

Furthermore, it is necessary to note that in shockwave treatment, water used in reservoirs is a potential source of product contamination, and it is necessary to develop corresponding mandatory requirements to label a product treated in this way.

*Consumer acceptance of the shockwave treatment technology*

To introduce new technologies, it is necessary to overcome natural resistance to changes. A consumer perception of the use of different beef processing technologies including shockwaves as a method for food quality improvement was analyzed within the framework of the European project ProSafeBeef. Researchers came to a conclusion that consumers regard as undesirable multiple impacts on meat that move a product away from its initial state. Beef processing technologies were mainly considered valuable options for consumers' convenience. In general, consumers supported the development of technologies that could provide more wholesome and quality food; if such technologies were "not invasive", the chances of their acceptance by target audience increased. The final conclusion showed a serious skepticism about excessive interventions into food technologies and a strong desire to make food and beef processing as "simple and natural" as possible [6].

It was noted that the fact of using low-grade beef as a raw material for shockwave treatment could cause doubts about product quality among the participants of the study and, therefore, this could have a negative effect on the results. Moreover, the participants said that such processing technology would be suitable only for consumers with limited budgets ("it might be okay for others, but not for me") and several respondents linked it with a probable carcinogenic risk.

Focusing their attention on the shockwave technology, the researchers noted that consumers had doubts about the effects of this technology and consequently their perception was different. On the one hand, the tenderizing effects and non-invasive character of the technology were regarded as quite positive. On the other hand, the absolute lack of knowledge about the technology exerted a significant negative effect on its acceptance by consumers due to unknown risks that it may pose. However, the lack of knowledge can be eliminated by proper consumer education and communication campaigns [6].

**Conclusion**

Studies on the use of shockwave treatment of foods began in the 1990s. However, their development has not led to large scale commercialization and remains to be at the experimental and pilot stages.

Recently, a great success has been achieved in the understanding of the fundamental mechanisms underlying the SW treatment. The use of shockwaves is a non-thermal and non-invasive technology and a promising method for accelerated meat ageing and its tenderization, as well as for increasing the yield and nutritional value of juice/oils extracted from plants.

The main problems in the industrial introduction of the underwater shockwave technology include an absence of the corresponding packaging materials resistant to the destructive effect of shockwaves, necessary capital investments, absence of the normative-legal base regarding the use of shockwave technologies and assessments of consumer opinion. Up to now, the majority of the studies on the shockwave effect on foods are at the stage of laboratory investigations.

The development of the innovative technologies extends the technological tools in the food industry due to introduction of new processing methods into the circle of the verified convenient technologies.

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# INFLUENCE OF PRESLAUGHTER STRESS ON POULTRY MEAT

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**Keywords:** *poultry meat, stress, quality, PSE, DFD, RYR receptor, color, texture*

***Abstract***

*In the present article the authors consider the importance of issue of the poultry meat quality. The increasing demand for poultry meat provides for the rapid growth of industrial stock of poultry, which contributes to appearance of meat with various defects in muscle tissue: PSE meat, that features low pH, pale color, soft and watery texture, and DFD meat — it is more dense and drier, of dark saturated color. Till now, the causes and mechanisms of appearance of those anomalies still haven't been unambiguously formulated, however, a large number of publications prove the influence of the genetic characteristics of modern crosses of broilers and turkeys on disturbances in $Ca^{2+}$ metabolism process in the sarcoplasm of muscle fibers. The uncontrolled release of calcium along with the high temperature of slaughtered poultry carcasses immediately after slaughter provokes an intense decrease in pH and launches denaturation processes in proteins. The numerous studies have shown deterioration in functional and technological properties of meat in stress-sensitive poultry, such as moisture-binding capacity and high acidity, which increases loss of meat juice during its storage and its weight during heat treatment. Recent publications have been devoted to development of a strategy for use of PSE poultry meat and search for efficient processing of PSE poultry meat, since the scientific community does not provide direct evidence on possibility of genetic adjustment of the poultry in order to exclude the occurrence of PSE quality of meat.*

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## Introduction

Poultry meat is one of the important sources of full-range animal protein for nutrition of a most part of the population. Relatively low prices in comparison with the other types of meat, the absence of cultural or religious food prohibitions, the dietary and nutritional properties of poultry meat provide for its high demand. Current forecasts and predictive researches confirm the further expansion of the poultry meat market.

According to foreign studies [1,2], the increasing demand for poultry meat determines the rapid growth of the poultry industrial herds. Large number of the poultry contributes to the appearance of meat with various defects in muscle tissue: PSE quality — pale, soft and exudative texture, and DFD quality — dark, firm and dry meat of dark saturated color [3,4]. As a result of intense breeding selection, broiler chickens are the most efficient in terms of meat productivity and growth rate. However, the meat of broiler chickens raised in intensive industrial conditions, feature quality defects most often. According to Karunanayaka D. S. et al. [5] the rate of detection of PSE broiler meat is about 70%.

PSE poultry meat, in addition to defects in color and texture, is characterized by pretty low water-holding capacity and increased loss of muscle juice during its cooling and processing. The quality features of PSE meat increase the probability of significant economic losses during sale of these raw materials, including sale the separate parts of carcasses, chilled and frozen semi-finished products [6]. According to Alvarado C. [7], the value of PSE meat is limited, and it cannot be considered as a full-fledged culinary raw material and it is not advisable to send it to retail shops. According to the researchers, DFD poultry meat also has limited use, as it is prone to rapid microbiological deterioration due to high pH level > 6.3, even when it initially had a relatively low level of microbial contamination [8,9]. The purpose of the research is to summarize the available data on pre-slaughter and post-slaughter factors contributing to the production of poultry meat with abnormal course of autolysis (PSE, NOR and DFD meat). The research is also aimed to show the effect of abnormal autolysis on pH value and color properties of poultry meat.

## Causes of poultry meat with an abnormal course of autolysis

In this regard the study of the qualitative features of PSE and DFD poultry meat has great scientific and practical interest, as well as the reasons of their appearance.

The causes contributing to occurrence of PSE and DFD poultry meat have not yet been fully determined. As evi-

denced by numerous publications [10,11,12,13,14], there is a number of factors, which influence the quality of broiler meat. Those factors include the influence of genetic characteristics and influence of stress. Stress is provoked by environmental factors and poultry keeping conditions — temperature and humidity, air flow velocity, exposure to thermal radiation. Stress can be also caused by the pre-slaughter factors, like rest and activity mode, mode of watering and starvation, inadequate conditions of keeping, transportation conditions, length of rest and season of the year. According to data obtained by some researchers (Abdullah A. Y. et al., 2010) [15], the influence of slaughter conditions and arrangement of post-slaughter processing shall also be taken into account.

One of the factors that affect the quality of the obtained meat is the linear targeted selection of poultry, which contributes to the development of stress syndrome (PSS — porcine stress syndrome). Some researchers [16] believe that genetically determined growth of muscle mass leads to deterioration of meat quality. Those issues are associated with muscles stiffness, poor muscle fiber connectivity and deterioration in color and water-holding capacity of the muscle tissue. The connective tissue in poultry like that (endomysium), associated with individual muscle fibers, does not keep pace with the rapid growth of muscle fibers and, as a result, becomes less developed and immature, which leads to a deterioration in tissues fibers connectivity [17].

Genetic mutation in RYR1 gene proteins makes the poultry quite perceptible to drugs that reduce the sensitivity of muscles to anesthetics like halothane. Stress syndrome in the pectoral muscles of poultry is caused by a mutation in guarding proteins of the calcium channel (ryanodine receptors, RYRs), which control the release of $Ca^{+2}$ from the sarcoplasmic reticulum. It is known that many biological processes, which are controlled by intracellular calcium signals, depend on intracellular reserves that provide controlled release of calcium into the cytoplasm. The concentration of intracellular calcium in the sarcoplasm of muscle fibers is regulated by the absorption and release of calcium from the sarcoplasmic reticulum due to formation of channel by the ryanodine receptor (RYR) [18].

The increase of calcium ions in the cell sarcoplasm accelerates metabolism. If the carcass is exposed to the elevated temperatures immediately after slaughter, it leads to a violation of the ABB of the carcass [12]. That launches another stress syndrome — the malignant hyperthermia (MH). Calcium imbalance caused by unregulated flow of calcium ions into various parts of the muscle cell can dramatically alter energy metabolism and muscle activity due to point mutations in the $Ca^{2+}$ release channel (RYR) embedded in the sarcoplasmic reticulum. However, the authors believe that the influence of genetic factors is not obvious for commercial lines of turkeys and broiler chickens, and at the moment the genetic factors should be perceived as background, which is capable of manifesting itself depending on exposure to various stress factors.

Later studies [19] showed a relationship between the concentration of calcium ions in the sarcoplasm of the broiler pectoral muscles and manifestations of PSE meat quality. The results of the cationic elements analysis found an excessive amount of $Ca^{2+}$ in the sarcoplasm of pectoral cells of abnormal quality meat during the initial period of autolysis. At the same time, thermogravimetric analysis with inductively coupled plasma optical emission spectrometry showed that the mass fraction of moisture in PSE meat is 4% lower than in the meat of normal quality.

Percival A. L. et al. [20] showed that, unlike pigs, the skeletal muscle tissue of poultry contains two forms of the RYR protein, each of which form has two replicas. All identified variants can be either normal or defective, but due to the greater number of proteins potentially susceptible to mutations, the probability and number of defective combinations significantly increases. When the mutated RYR replicas are present in the poultry meat, the poultry will be susceptible to the influence of stress factors, while the mechanism of ionized calcium circulation in the cells will be disturbed and the intensity of metabolism will be changed.

While researching the differences in RYR activity in poultry of different crosses, the authors [21] showed that in turkeys of commercial line the affinity for ryanodine is three times higher than the studied indicator in the control genetically unimproved group of poultry. The binding of ryanodine by the sarcoplasmic reticulum is recognized by a wide number of authors as a method for determining the stress sensitivity of animals and poultry. Post-mortem hypermetabolism of turkey skeletal muscles defines the development of PSE meat quality. According to the review given by the authors in the research [22], the cause of change in the autolysis dynamics is a disturbance in exchange of calcium ions in the cell due to the RYR1 point mutation at nucleotide 1843.

It follows from the published data [23] that an excess of calcium ions in the sarcoplasm of PSE meat enhances the activity of proteinase (calpain), which manifests itself in the destruction of myofibrils and the disorganization of Z-lines in the muscle fiber sarcomere. The contribution of increased concentration of $Ca^{2+}$ ions to the degradation of the membranes of the main cellular structures of muscle tissue with autolysis deviations was reported by Kuchenmeiser U. et. al. [24].

Myopathy of the pectoral muscles, expressed as the weakened or impeded motor activity of the muscles, also leads to pale soft and exudative breast meat of broiler chickens. Recently it has been found that the PSE defect of meat builds up and manifests itself as white stripes on the section of the pectoral muscles due to presence of lighter muscle fibers or as a result of pectoral muscle hardening (so called “wooden” breast defect), as well as in the form of connective tissue defects manifested in the form of low connectivity of fibers in the structure of the pectoral muscles (so called “spaghetti meat” defect). Those anomalies of

muscle tissue quality significantly reduce the commercial marketability of poultry meat, deteriorate nutritional, functional and technological properties of muscle tissue [25,26].

Van Laack R. L.J.M. et al. [27] showed that protein solubility in pectoral muscles of PSE meat is lower than in normal ones, which indicates increased protein denaturation in exudative meat. The authors believe the low final pH of *pectoralis pallidum* and its high temperature to be the main factors determining the poor water-binding capacity of the meat.

According to some researchers [28], the prevalence of pathological conditions of meat indicates that a further increase in the efficiency of poultry meat production may be limited by the physiological capabilities of broiler chickens, since their internal organs, vascular system and skeleton are close to their functional limit.

**Preslaughter and post-slaughter factors affecting the quality of poultry meat**

According to some authors [29], the delivery of poultry for slaughter and its further processing do contribute to the formation of muscle tissue defects [29]. However, some researchers believe that transportation, which lasts no more than 3 hours, and unloading do not influence significantly on the quality of the pectoral muscles of turkey and chicken [30].

Pre-slaughter stresses like heat stress, fighting, packing to cages, transporting, cancellation of feed and delivery to the slaughter point are common in poultry processing facilities. So, it was found in the researches of Debut M. et.al. [31], that stress in broiler chickens is inevitable during the suspension of the poultry, which increases the rate of pH drop and increases the intensity of the color of the breast muscles.

The PSE and DFD defects of poultry meat can develop as a result of short-term and long-term poultry stress [32]. So Owens S. M. et al. [33] point to the significant importance of ambient temperature as a stress factor. Referring to earlier work, the authors conclude that large-size and faster growing poultry are more susceptible to heat stress.

Studies have shown [34] that heat stress (day temperature 38 °C/night temperature 32 °C) causes the low pH value of pectoral muscles, their pale color and higher losses of weight during cooling of broiler chicken carcasses in comparison with muscles obtained from slaughtered poultry not exposed to heat stress.

Studies [35] are devoted to the effect of the cooling rate of poultry carcasses in the post-slaughter period on quality of the obtained meat. It has been established that post-slaughter elevated temperatures of carcass (up to 37–41 °C) in the first 15–20 minutes after slaughter accelerate the breakdown of ATP and contribute to hydrolysis of glycogen to lactic acid, which leads to the formation of pale, soft, exudative pectoral muscles of turkeys and chickens. Slow cooling or insufficiently low cooling temperatures (30–40 °C) may promote the development of PSE meat in normal or rapidly glycolyzing pectoral muscles of the poultry.

According to Molette C. et.al. [36], high post-slaughter temperature (about 40 °C) of turkey muscles activates protein kinase (AMPK), which leads to accelerated glycolysis and results to PSE meat.

**Influence of the nature of autolysis on relation between pH value and the color parameters of the poultry meat**

Deviations in the rate and depth of pH decrease in the autolyzing pectoral muscles are associated with changes in their color. Color is the most important consumer characteristic of meat and it has the highest importance in the market perception of the product. Researchers [37] suggest that light and dark meat differ in density of packing of structural elements of muscle tissue — transverse structural packing of myofibrils and muscle fibers, longitudinal shrinkage of sarcomeres, and different protein composition of sarcoplasm and extracellular space. According to Hughes J. M. et al. [38] color is determined chromatically by myoglobin (measured by its shade and chromaticity) and achromatically — by scattering of light by the tissue structure (measured by luminosity).

Kralik G. et.al. [39] established a relation between color, final pH value and meat juice loss in broiler chicken breasts. Based on the value of the luminosity index (L), breast meat was classified into DFD ($L < 44$), NOR ($L = 44–53$) and PSE ($L > 53$). The PSE breast meat had a higher luminosity value (L), a lower final pH ($pH_f$) and a higher loss of meat juice in comparison with normal meat (NOR). Opposite results were obtained for DFD-quality meat. A negative correlation was established between the L and $pH_f$ values and a positive correlation between the L values and the loss of meat juice.

Similar studies were carried out by Freitas A. S. et al. [1]. Basing on the obtained data, the authors came to the conclusion that in order to classify pectoral muscles of chickens into qualitative groups the threshold values of the luminosity index L and the values of the final RNA should be determined. These values for PSE meat were as follows — $L > 51.0$ and $pH_f < 5.9$; for NOR meat — $5.9 < pH_f < 6.2$ and $45.0 < L < 51.0$; and for DFD meat quality — $L < 45.0$ and $pH_f > 6.2$.

The luminosity index of meat of broiler chickens ($L > 54$) provides over 47% reliable detection of PSE meat with low pH and increased moisture loss during meat storage and heat treatment. According to the authors, sorting meat by its color under industrial conditions does not pose a serious challenge, since fillet sorting is already introduced and being run at enterprises, but according to other criteria [40,41].

The recent publications [42–46] are devoted to the development of a strategy for PSE meat using and to search for efficient processing of PSE poultry meat, since in the

scientific community there is no direct proof on genetic adjustability of the poultry in order to exclude the occurrence of PSE meat.

**Conclusion**

The analysis of the cited references proves that most authors recognize the problem of PSE quality in poultry meat. The study of the mechanisms of occurrence of this anomaly still has not been unambiguously formulated, however, a large number of publications show the influence of the genetic characteristics of modern crosses of broilers and turkeys on disturbances in the mechanism of $Ca^{2+}$ metabolism in the sarcoplasm of muscle fibers. The uncontrolled release of calcium, combined with the high temperature of poultry carcasses immediately after poultry slaughter, provokes an intense decrease in pH and launches denaturation processes. As a result, numerous studies have shown a decrease in the functional and technological properties of meat in stress-sensitive poultry, such as loss of meat juice during its storage and loss of meat weight during heat treatment due to its poor water-binding capacity.

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# COMPARISON OF THE PROTEOMIC PROFILE OF PORK BY-PRODUCTS DURING THEIR STORAGE

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**Keywords:** *by-products, pork, 2-DE, proteomics, by-products proteins, liver, kidneys*

***Abstract***
*In this article, the proteomic profiles of pork by-products (snout, tongue, liver, kidney, spleen) were studied by comparative method on the first day and the fifth day of their storage. Two-dimensional electrophoresis according to O'Farrell was used for the aims of this article, while the results were further processed in ImageMaster software. Proteomic maps of by-products showed clear changes in protein composition after visualization and images analysis. There was a decrease and increase in manifestation intensity of some proteins. The study of the obtained electrophoregrams with the help of references resources allowed identifying various compounds in the by-products. 9 protein fractions with various intensity of manifestation were found on the day 1st and 5th. On the 1st day the following substances were intensively manifested: in the liver — glutathione peroxidase 4 (22.3 kDa), LEAP-2 (8.8 kDa); in the kidneys — quinone oxidoreductase (34.9 kDa); in the spleen — glycoprotein CD59 (13.7 kDa), in the patch — protein flint (49.07 kDa). It is noted that these proteins play their role in stopping certain processes in cells, like oxidation, microbial activity, and accumulation of toxic substances. These processes can worsen the quality of raw materials, and further lead to spoilage of the food product. On the 5th day of storage the highest intensity of manifestation of glyceraldehyde-3-phosphate dehydrogenase (35.8 kDa) in the liver was observed; superoxide dismutase [Cu-Zn] (15.8 kDa) was noted in the kidneys, colony-stimulating factor (16.2 kDa) was observed in the spleen and glutaredoxin –1 (11.8 kDa) in the tongue. In its turn, on the fifth day these chemical processes manifested themselves more intensely, as the fatty acids and glucose broke down. To obtain more accurate results, the proteins were compared by their volume. Among the identified fractions the highest expression was observed in LEAP 2 (8.8 kDa) on the first day, and in glyceraldehyde-3-phosphate dehydrogenase (35.8 kDa) on the fifth day. The least change in the intensity of manifestation was noted for superoxide dismutase [Cu-Zn] (15.8 kDa), which volume increased during storage by 13% for 5 days. The analysis of the obtained electrophoregrams allowed identifying various compounds, tracing the changes in the qualitative composition of protein in by-products during various periods of their storage. The obtained data demonstrate the transformation of protein molecules during storage, which makes it possible to determine the changes and quality of the food products.*

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## Introduction

In the modern world the composition and biological value of meat food is of great importance, as the meat food products are the main source of animal protein and provide a significant impact on human health. Meat products are very closely related to the human diet and its taste needs, which can change over time in direction of some certain types of meat. That is why the variety of choice of types of meat products, as well as the expansion of people's taste preferences, have led to great attention of producers and consumers to by-products as type of raw material.

If we consider the additional source of nutrients, and most importantly protein, then recently there has been an increase in the demand for by-products, which, in their measure, are able to provide people with a daily need for protein, vitamins and minerals, as well as to diversify their diet with various dishes [1]. In terms of nutritional value the pork by-products are not inferior to meat due to their greater variety, and the amount of various trace elements and vitamins, compared to muscle tissue, is many times greater. For example the liver contains a lot of zinc, copper, magnesium and potassium, and pork kidneys contain a large amount of sodium and calcium [2, 3]. The pork tongue is rich in protein, fat, and significant amounts of iron and zinc [4]. As for vitamins, based on studies, it has been noted that their amount is greater than in muscle tissues [5]. For example, the liver contains a large amount of vitamins A and D, as well as many B

vitamins, the tongue is rich in choline and vitamin B12, and pork spleen is rich in A and B3. According to the data on various trace elements and vitamins contained in the by-products, it can be concluded that by-product can serve as an alternative to raw meat, being the source of many trace elements [6].

By-products are sold to consumers, both in original and processed form. The following types of meat products are very popular: various types of sausages (liver sausages, blood sausages, pates), canned food. The most valuable and delicious types of by-products include the tongue, liver, heart and kidneys; in terms of nutritional value they are equivalent to meat [7].

In regards to the nutritional values, to taste and other benefits of by-products, it is necessary to keep in mind such an important factor as the very quality of the product. The quality of raw materials is one of the main priorities in the food industry. In the aggregate it consists of appearance, taste, smell, but the most important thing is the composition of the by-product: the amount of proteins, fats, carbohydrates, minerals and amino acids. All these parameters depend on many factors: method of an animal feeding, feeding conditions, pre-slaughter processing, primary processing and storage conditions.

By-products are quite often used by consumers for cooking of whole variety of dishes. The use of by-products is often caused by their lower cost in comparison with raw meat, and therefore their greater availability. At the same time, there is little information about the changes which occur in the protein profiles of pork by-products over time. During the storage period, they are subject to changes in organoleptic properties, nutritional value and protein composition [8, 9].

To date of great interest are the works that help to determine and analyze the rich proteomic composition of consumed food products of animal and plant origin. Proteomics opens up great opportunities for researchers for the study of this topic. Using of proteomics allows identifying proteins in products, characterizing them, and comparing the proteomic composition of meat raw materials obtained from various animal species [10]. The main proteomics tool that remains relevant to this day is two-dimensional electrophoresis, which is used to study changes which occur to proteins and to identify functional species-specific and tissue-specific compounds [11]. The use of two-dimensional electrophoresis technology makes it possible to define thousands of proteins with high resolution, and characterize the defined protein fractions by mass spectrometric methods. The obvious advantage of this proteomic approach over other methods is explained by its ability to detect alternative protein forms that result from co- and/or post-translational modifications.

Another advantage of proteomic methods is its ability to track changes in the protein composition after slaughter, to assess the influence of storage on quality and food safety of by-products, because they have pretty short shelf life due to increased content of moisture and microorganisms [12,13]. Due to proteomic technologies there is a growing understanding of the various biological processes that define meat quality. This study determines the protein composition and runs comparative analysis of two-dimensional electrophoregrams of various by-products in order to trace qualitative changes during their storage.

**Objects and methods**

The pork by-products served as the objects of this study, in particular: chilled tongue, liver, spleen, kidneys and snout, were taken at the stage of primary processing of slaughtered pigs with a live weight of 120 kg (3 samples of each type were studied). By-product samples for research were taken from 3 animals on the first day and the fifth day of storage at a temperature of 0 to 2 °C.

Immediately before the study, a sample of 100 mg was taken and 2000 μl of a lyse solution (9 M urea, 5% β-mercaptoethanol, 2% Triton X-100, 2% ampholine pH 3–10) was added. The resulting homogenate was clarified by centrifugation at 14,000 rpm for 20 minutes. Further, protein extracts were used in isoelectric focusing.

During study of the samples, the method of two-dimensional electrophoresis (2-DE) according to O`Farrell using isoelectric focusing (IEF) was used [14, 15]. IEF was carried out in tubular gels at 3,650 V/h. After isoelectric focusing, the resulting gels were kept in balancing buffers for 10 min each [16].

Next, the gels were exposed to electrophoresis with sodium dodecyl sulfate; for this, the balanced gels were transferred into a 12.5% polyacrylamide gel. Electrophoresis was run using a buffer containing 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS in amount of 30 mA per gel until the stain front reached the edge of the gel.

The protein zones after their electrophoretic separation in polyacrylamide gel (PAAG) were stained and localized using Coomassie G-250. Each sample was presented in triplicate.

Computer densitometry of two-dimensional wet electrophoregrams was performed using a scanner *Bio-5000 plus* (Serva, Germany), resolution 300 ppi 1D-Gray. The resulting images were analyzed using ImageMaster™ 2D Platinum software based on Melanie 8.0 (GE Healthcare and Genebio, Switzerland). Next, the digitized images were compared by the method of matching. The visualized protein stains were interpreted using the UniProt database [17].

Further, the obtained experimental data were analyzed using Student's t-test and one-way analysis of variance (between gels of different samples) using ImageMaster ™ 2D Platinum software based on Melanie 8.0 (GE Healthcare and Genebio, Switzerland). It was considered that P value $< 0.05$ indicates a significant difference. As part of the work, protein stains were compared by their volume and the Fold index was calculated, the excess of which by more than 2 units is generally considered to be a statistically significant difference. All results are presented as mean ± standard deviation from at least three independent trials.

**Results and discussion**

The study was run on samples of the pork by-products: snout, tongue, liver, spleen and kidney at various periods of storage (the first day and the fifth day) in order to identify significant changes in the protein composition during their storage. A wide range of various protein compounds with molecular masses from 7 kDa and higher was obtained by the method of two-dimensional electrophoresis. As a result of analysis of 2-DE gel images by ImageMaster™ 2D Platinum, about 110 different protein fractions in average were found in each by-product. At the same time, the highest content of proteins was in the liver, kidneys, spleen, and the lowest — in the patch.

Figures 1 and 2 below represent the obtained electrophoregrams of the by-products. When analyzing the proteomes, the researcher can notice differences such as a decrease in intensity of protein manifestation by the fifth day and occurrence of new protein structures that were not found on the first day. Comparative analysis of electrophoregrams showed that the smallest amount of protein stains was revealed in the samples of a snout compared to other by-products, while an increase in the amount of proteins was observed by the fifth day.

When comparing the electrophoregrams of by-products analyzed on the first day and the fifth day of their storage, a difference was revealed in their proteomic profile and in manifestation of individual proteins. Protein fractions were identified, in which the intensity decreased on the fifth day (No. 1 — No. 5 in Figure 3), among which can be noted in liver samples — glutathione peroxidase 4 (22.3 kDa), which protects the cell from oxidative damage and LEAP-2 (8.8 kDa), which has antimicrobial activity [18,19]; kidneys — quinone oxidoreductase (34.9 kDa), which is involved in detoxification of xenobiotics and reduces the load of free radicals in cells [20]; spleen — glycoprotein CD59 (13.7 kDa), which is a powerful inhibitor of action of the complementary membrane attack complex [21]; snout is a kremen protein (49.07 kDa), which can cause cell death, being an addiction receptor [22]. It was noted that the functions of the above described proteins are mainly aimed at protecting cells from various types of damage. It is highly likely that processes started in the by-product cells which adversely affect the composition and quality of meat raw materials, as well as lead to its deterioration.

Among the identified fractions, spots of proteins were noted with high expression by the fifth day (No.6 — No.9 in Figure 4). For liver samples, glyceraldehyde-3-phosphate dehydrogenase (35.8 kDa) was found to be involved in glycolysis [23]; in the kidneys — superoxide dismutase [Cu-Zn] (15.8 kDa), which destroys toxic radicals [24]; in the spleen, a colony-stimulating factor (16.2 kDa) necessary for β-oxidation of fatty acids [25]; in the tongue — glutaredoxin-1 (11.8 kDa), which reduces the content of glycosylated proteins, and is also an antioxidant enzyme [26]. In cells on the 5th day, more intense chemical processes are observed associated with a decrease in the quality of the product, such as glycolysis, the formation of toxic radicals, and β-oxidation of fats.

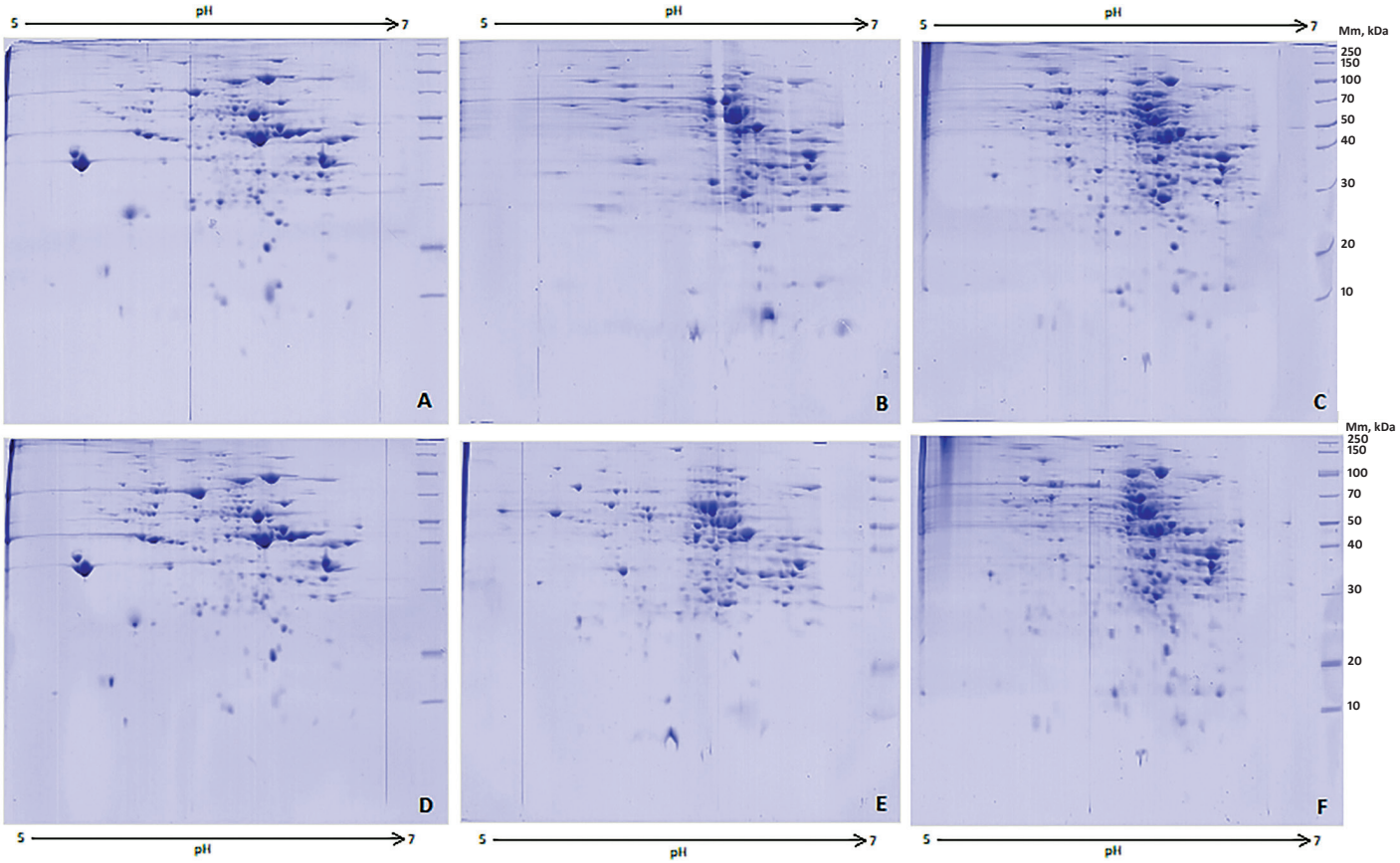


**Figure 1.** Two-dimensional electrophoregrams of the spleen (A — on the first day, D — on the fifth day), liver (B — on the first day, E — on the fifth day) and kidney (C — on the first day, F — on the fifth day)

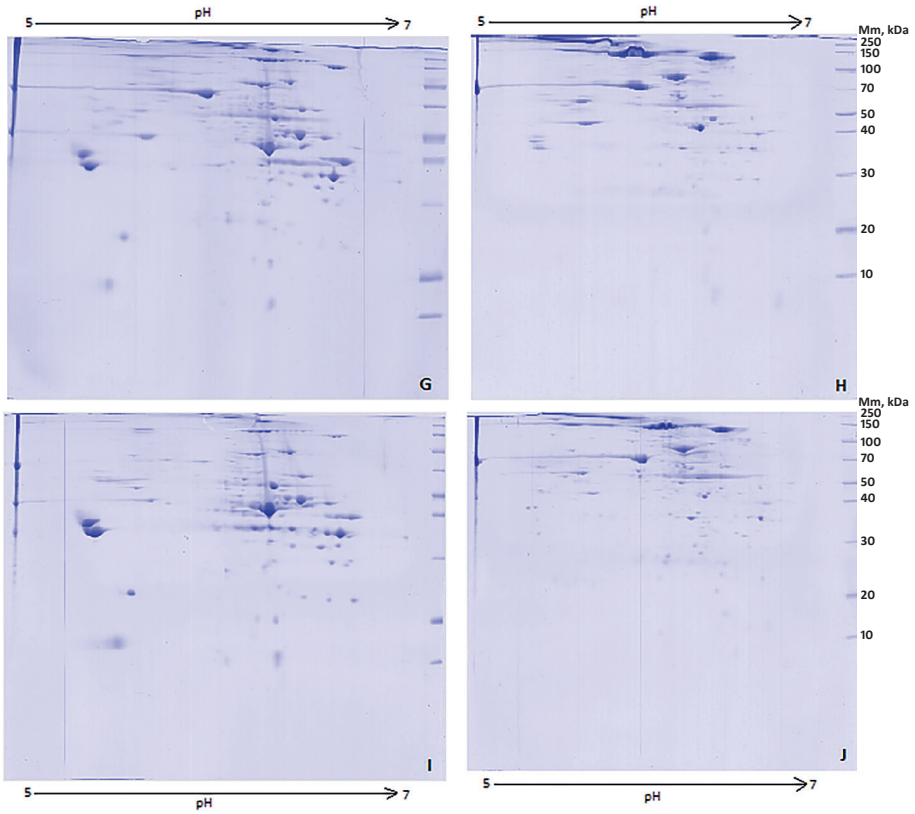


**Figure 2.** Two-dimensional electrophoregrams of the tongue
(G — on the first day, I — on the fifth day) and patch (H — on the first day, J — on the fifth day)

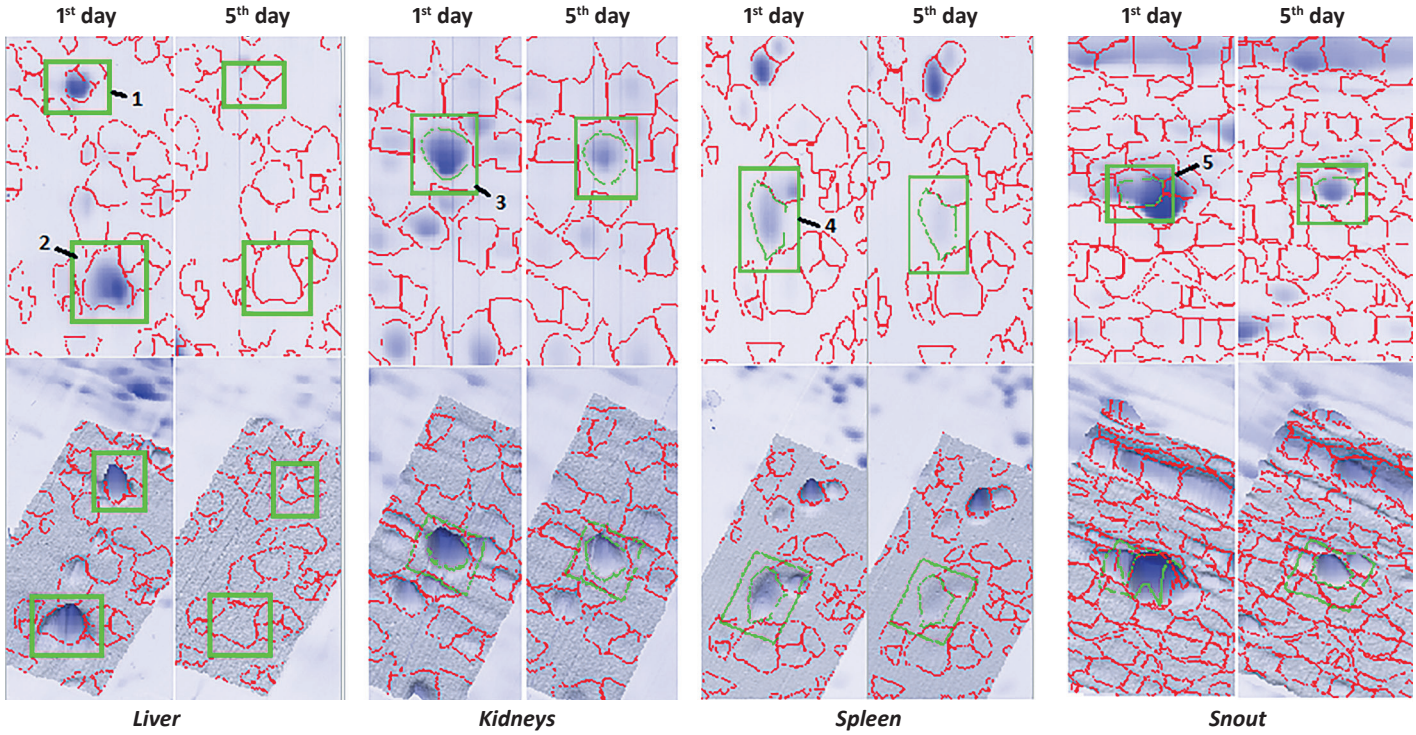


**Figure 3.** Fragments of 2-DE gels of pork by-products on days 1 and 5

As a further part of the research, in order to compare the intensity of protein manifestation in certain days, protein stains were compared by their volume (Figure 5).

In proteins No. 3 and No. 4, the saturation of staining evenly decreased by 43% and 50% from days 1 to 5 respectively, and in protein No. 7 it increased by 12%. Presumably, the decrease in the intensity of protein No. 3 was caused by the neutralization of free radicals and increase in toxic substances, and therefore the increase of protein No. 7 volume. The largest difference in optical density is observed in fraction No. 2 — on the first day it was 10 times denser than on the fifth day. Further, proteins No. 6 and No. 9 can also be noted, in which the volume increased by 5 times in comparison with the first day. Protein No. 6 is probably glyceraldehyde-3-phosphate dehydrogenase (35.8 kDa), required for glycolysis. It can be suggested that on the 5th day of the

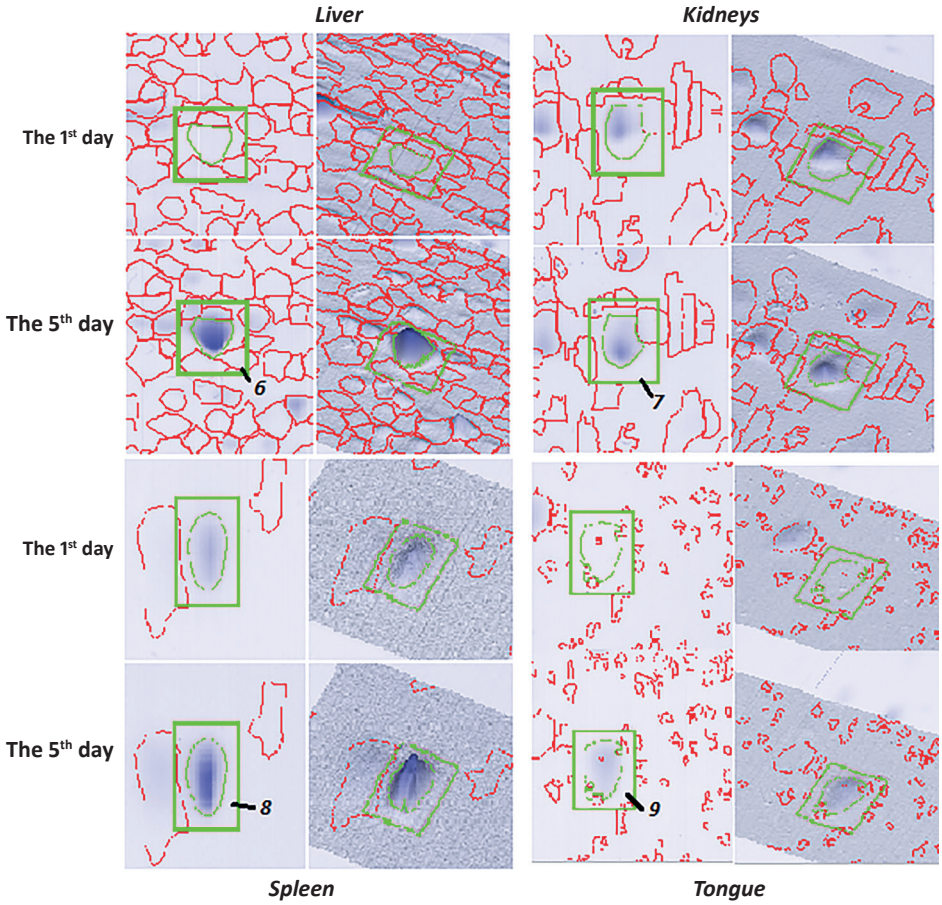


**Figure 4.** Fragments of 2-DE gels of pig by-products on days 1 and 5

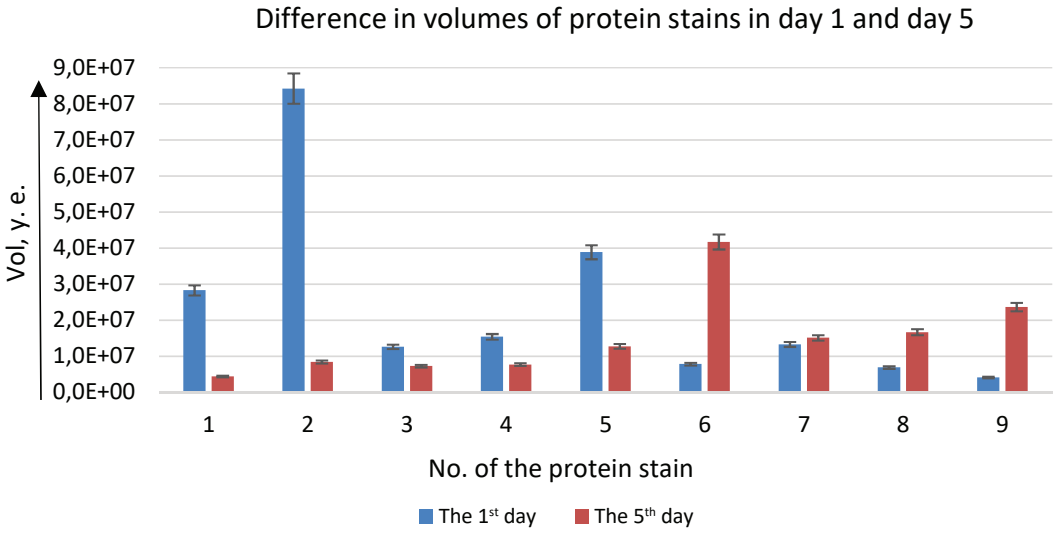


**Figure 5.** Integrated optical density of protein fractions

Note: Spot intensity was normalized by total spot intensity and the average of three analytical replicate gels.

by-product storage, an intensive process of glucose oxidation occurs, therefore, a large amount of lactic acid appears in the liver cells. Also, high staining intensity is observed in proteins No. 1 and No. 5 on the first day, which is respectively 6 and 3 times higher than the staining volume compared to the fifth day, and for protein No. 8 high intensity is noted by the fifth day, namely, it is higher by 59% more than in the first day.

The above listed changes in volume of protein fractions can reflect various processes which run in the cell over several days of storage. The decreased mechanisms of defense and increasing of chemical processes and reactions lead to deterioration of quality of the analyzed product. For example, protein No. 1, which is 85% more intense on the first day, is responsible for reduction of hydroperoxide groups (–OH) of fatty acids in membrane phospholipids. These changes may indicate the beginning of cell membranes destruction, which may lead to other changes in cells that affect the spoilage of the meat by-products.

**Conclusion**

This study allowed determining and comparing the protein composition of by-products at various periods of their storage, as well as it allowed considering the intensity of processes over time of storage. There were clear changes in the electrophoregrams from the first day to the fifth day. The most interesting were the proteins in the kidneys: quinone oxidoreductase (34.9 kDa) and superoxide dismutase [Cu-Zn] (15.8 kDa), where intensity of one protein decreased while the intensity of the other protein increased.

In the liver two protein stains were found, which intensity greatly decreased by the fifth day. Glutathione peroxidase 4 with a molecular weight of 22.3 kDa plays a key role in glycolysis. Presumably, the concentration of glucose in the cells dropped sharply over the course of 5 days in a row, thereby reducing the concentration of this protein, as was shown on fragments of 2-DE gels and on the graph of integrated optical density. The 10-fold decrease in intensity of the LEAP-2 protein (8.8 kDa) by the fifth day, which protein is responsible for antimicrobial activity, contributed to acceleration of microbiological deterioration of the liver samples.

By the fifth day decrease was noted in spleen protein glycoprotein CD59 (13.7 kDa) concentration. This protein is responsible for preservation of cell membranes. This decrease indicates cells destruction. In addition, on the fifth day the colony-stimulating factor (16.2 kDa) grew noticeably, which indicates an increase of fats β-oxidation intensity due to increase of amount of lipid peroxidation products, which include aldehydes, ketones, etc. Formation of above listed compounds leads to deterioration of organoleptic characteristics and nutritional value of by-products.

On the fifth day the amount of some proteins increased actively, namely: in the samples of snout and tongue. In the sample of tongue glutaredoxin-1 was intensely expressed, which protein is an antioxidant enzyme. This fact suggests the formation of a large number of oxidants.

The study showed that on the first day the processes start in the by-products, that negatively affect the quality of raw materials; and by the fifth day the processes begin to develop more intensively. By-products have been proved to be a rich source of protein components with a very short shelf life.

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# METHODS FOR NONPARAMETRIC STATISTICS IN SCIENTIFIC RESEARCH. OVERVIEW. PART 2

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**Keywords:** *nonparametric statistics, null and alternative hypotheses, type I error, type II error, goodness-of-fit tests, tests for homogeneity*

***Abstract***

*The use of nonparametric methods in scientific research provides a number of advantages. The most important of these advantages are versatility and a wide range of such methods. There are no strong assumptions associated with nonparametric tests, which means that there is little chance of assumptions being violated, i. e. the result is reliable and valid. Nonparametric tests are widely used because they may be applied to experiments for which it is not possible to obtain quantitative indicators (descriptive studies) and to small samples. The second part of the article describes nonparametric goodness-of-fit tests, i. e. Pearson's test, Kolmogorov test, as well as tests for homogeneity, i. e. chi-squared test and Kolmogorov-Smirnov test. Chi-squared test is based on a comparison between the empirical (experimental) frequencies of the indicator under study and the theoretical frequencies of the normal distribution. Kolmogorov-Smirnov test is based on the same principle as Pearson's chi-squared test, but involves comparing the accumulated frequencies of the experimental and theoretical distributions. Pearson's chi-squared test and Kolmogorov test may also be used to compare two empirical distributions for the significance of differences between them. Kolmogorov test based on the accumulation of empirical frequencies is more sensitive to differences and captures those subtle nuances that are not available in Pearson's chi-squared test. Typical errors in the application of these tests are analyzed. Examples are given, and step-by-step application of each test is described. With nonparametric methods, researcher receives a working tool for statistical analysis of the results.*

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## Introduction

German philosopher, psychologist and teacher Johann Friedrich Herbart at the beginning of the 19th century wrote: "Any theory trying to be consistent with experience, first of all, must be continued until it accepts quantitative determinations that arise in experience or lie in its foundation. If not, it hangs in the air, exposed to every wind of doubt and being unable to contact with other, already strengthened opinions".

Thus, the researcher, having received data during the experiment, must process them correctly using mathematical methods in order to draw a correct and reasonable conclusion.

As a rule, researchers use methods of parametric statistics, which is not always correct. Many parametric methods have direct analogues in nonparametric statistics. For example, Student test and analysis of variance determine the significance of differences in mean values for two or more groups; and Mann-Whitney U-test determines the significance of differences in the average rank for two groups; Pearson's correlation coefficient allows determining the linear relationship between two numerical indicators; and Spearman rank correlation coefficient allows determining linear relationship between the ranks of two indicators. In some cases, there is no direct analogy with nonparametric method.

Nonparametric methods of mathematical statistics do not require knowledge of the functional form for the theoretical distribution. The name "nonparametric methods" itself emphasizes their difference from classical (parametric) methods, in which it is assumed that the unknown theoretical distribution belongs to some family that depends on a finite number of parameters (for example, the family of normal distributions), and which allow estimating unknown values of these parameters based on the results of observations and testing certain hypotheses regarding their values [1].

Common characteristics for most nonparametric methods [2,3] are: 1) fewer assumptions about the type of distribution; 2) the sample size is less strict; 3) the measurement may be nominal or ordinal; 4) independence of randomly selected observations, except for paired ones; 5) the focus is on the ranking order or data frequency; 6) hypotheses are expressed regarding the ranks, medians or data frequency.

Based on the practice of statistical data analysis, there are three main spheres of nonparametric statistics [4]:

- sphere at the junction of parametric and nonparametric methods;
- rank statistical methods;
- nonparametric estimates for functions, primarily distribution density, regression dependence, as well as statistics used in classification theory.

In the first part of the article [5], a review of simple nonparametric methods is given. Two groups of nonparametric tests are considered: 1) to identify differences in the indicator distribution (Rosenbaum Q-test, Mann-Whitney U-test); 2) estimates of the significance for shift in the values of the studied indicator (sign G-test, Wilcoxon T-test).

In the second part of the article, nonparametric tests for testing hypotheses of distribution type (Pearson's chi-squared test, Kolmogorov test) and nonparametric tests for testing hypotheses of homogeneity (Pearson's chi-squared test for homogeneity, Kolmogorov-Smirnov test) are considered.

The purpose of the article is to give a working tool for solving specific research and applied problems using methods of nonparametric statistics.

**Materials and methods**

The materials of the study are recent publications in the statistical analysis of which methods of nonparametric statistics are used, i. e. goodness-of-fit tests (Kolmogorov test, Kolmogorov-Smirnov test, Pearson's chi-squared test).

***Goodness-of-fit tests***

It is known that one of the most important tasks for mathematical statistics is the establishment of a theoretical law of distribution for a random variable characterizing the studied indicator, based on empirical distribution. The solution of this problem allows: 1) choosing the right method of statistical data processing; 2) determining the type of model that describes the relationship between the analyzed indicators.

Goodness-of-fit tests are used to check the agreement between the experimental data and the theoretical model. So, goodness-of-fit test is a test for testing a hypothesis about an assumed distribution law [6].

The researcher states two hypotheses: null hypothesis ($H_0$) and alternative hypothesis ($H_1$). Next, the hypotheses are tested using various tests.

$H_0$: The resulting empirical indicator distribution does not differ from the theoretical distribution (normal, uniform, exponential, etc.).

$H_1$: The resulting empirical distribution of the indicator differs from the theoretical distribution.

To test the null hypothesis $H_0$, some random variable $U$ is chosen, which characterizes the disagreement between the theoretical and empirical distributions, the distribution law for which is known, for sufficiently large n, and almost does not depend on the distribution law for the random variable $X$.

When knowing the distribution law of the random variable $U$, a critical value $U_\alpha$ can be found, at which the null hypothesis $H_0$ is true, as well as the probability that the random variable U assumes a value greater than $U_\alpha$, i. e. the function $P(U > U_\alpha) = \alpha$ is small, where α is the test significance level.

If the value observed in the experiment $U_i = U > U_\alpha$, i. e. it falls into the critical region, this means that such large $U$ values are practically impossible and contradict the hypothesis $H_0$. In this case, the hypothesis $H_0$ is rejected.

If $U_i = U \leq U_\alpha$, then the difference between the empirical and theoretical distributions is insignificant, and the hypothesis $H_0$ may be considered as not contradicting the experimental data.

In this case, the researcher can make two types of errors when testing hypotheses: type I error and type II error [6].

*Type I error*. If we reject the null hypothesis $H_0$ (i. e., we consider the null hypothesis $H_0$ is false), while in fact the null hypothesis $H_0$ is true, then the researcher makes an error consisting in the incorrect rejection of the null hypothesis.

*Type II error*. If we accept the null hypothesis $H_0$ (i. e., we do not agree with the alternative hypothesis $H_1$), while in fact the null hypothesis $H_0$ is false, then the researcher makes an error consisting in incorrect acceptance of the null hypothesis.

It is worth noting that the probability of making a type I error is established quite easily, because it is equal to $\alpha$, while for type II errors, it must be specially calculated.

***Pearson's goodness-of-fit test or Pearson's chi-squared test***

Pearson's goodness-of-fit test (or Pearson's chi-squared test) is the most commonly used to test the hypothesis that a certain sample belongs to a theoretical distribution law [7,8].

Given data for the problem: let there be a sample of values for a random variable $X$ with size $n$: $x_1$, $x_2$, ..., $x_k$ and a set of corresponding frequencies $m_1$, $m_2$, ..., $m_k$ ($k$ is the number of partition intervals). As a measure of difference between the empirical and theoretical distributions, the value $\chi^2$ is taken, which is equal to the sum of the squared deviations of the relative frequencies $\frac{m_i}{n}$ from the probabilities $p_i$ calculated from the assumed distribution and taken with a certain coefficient $c_i$:

$$\chi^2 = \sum_{i=1}^{k} c_i \left(\frac{m_i}{n} - p_i\right)^2 \quad (1)$$

The coefficient $c_i$ is chosen in such a way that for the same deviations $\left(\frac{m_i}{n} - p_i\right)^2$, the deviations at which $p_i$ is small have more weight, and the deviations at which $p_i$ is large have less weight. Therefore, $\frac{n}{p_i}$ ratio is taken as $c_i$. We obtain the measure of difference of the following form:

$$\chi^2 = \sum_{i=1}^{k} \frac{n}{p_i}\left(\frac{m_i}{n} - p_i\right)^2 = \sum_{i=1}^{k} \frac{{m_i}^2}{np_i} - n =$$

$$= \sum_{i=1}^{k} \frac{\left(m_i - m_i^{theor}\right)^2}{m_i^{theor}} = \sum_{i=1}^{k} \frac{m_i^2}{m_i^{theor}} - n,$$

so that, with $n \to \infty$, the sample distribution of $\chi^2$ tends to the limit distribution of $\chi^2$ with the number of degrees of freedom $v = k - r - 1$, where $r$ is the number of parameters of the hypothetical probability distribution estimated from the sample data. Numbers $m_i$ and $m_i^{theor}$ are called empirical and theoretical frequencies, respectively.

*Application of Pearson's chi-squared test*

1. The measure of difference between empirical and theoretical frequencies is determined by the formula (2) and the experimental value of the test is calculated.

2. For the chosen significance level $\alpha$, using the table of $\chi^2$ distributions, the critical value $\chi^2_{cr}$ is found with the number of degrees of freedom $v = k - r - 1$.

3. If the experimental value $\chi^2_{exp}$ is greater than the critical value, i.e. $\chi^2_{exp} \geq \chi^2_{cr}$, then the null hypothesis $H_0$ is rejected; and if $\chi^2_{exp} < \chi^2_{cr}$, the null hypothesis $H_0$ does not contradict the experimental data.

*Limitations of Pearson's chi-squared test*

1. Sample size must be large enough: $n \geq 30$.

2. The theoretical frequency for each cell should not be less than 5.

3. The selected ranks should cover the entire range of the indicator's variability. Classification into ranks should be the same in all compared distributions.

4. Ranks should be non-overlapping.

*Testing the hypothesis about the normal distribution of the general population*

1. Based on a sample of size $n$, arrange the interval statistical array by classification of the given data into $k$ ranges $[a_i; a_{i+1})$ with the corresponding frequencies $m_i$. Rearrange interval statistical array into statistical array by replacing each range $[a_i; a_{i+1})$ with its mean value: $x_i = \frac{a_i + a_{i+1}}{2}$. Now we have Table 1.

**Table 1. Interval statistical array**

| Ranges for observed values of a random variable $X$ | $[a_1; a_2)$ | $[a_2; a_3)$ | ... | $[a_i; a_{i+1})$ | $[a_k; a_{k+1})$ |
|---|---|---|---|---|---|
| Frequencies $m_i$ | $m_1$ | $m_2$ | ... | $m_i$ | $m_k$ |
| Mean value $x_i$ | $x_1$ | $x_2$ | ... | $x_i$ | $x_k$ |

2. Using Table 1, calculate mathematical expectation estimate $\overline{x}$ and sample standard deviation $\sigma_v$.

3. Calculate $z_i = \frac{a_i - \overline{x}}{\sigma_в}$, $i = \overline{2,3,\ldots k}$, where $a_i$ is the left end of the $i^{th}$ range. Set value $z_1$ equal to minus ∞, and value $z_{k+1}$ equal to plus ∞.

4. Assuming a normal distribution of the general population, determine the theoretical frequencies $m_1^{theor}$, $m_2^{theor}$, …, $m_k^{theor}$ by the formula:

$$m_i^{theor} = n \cdot p_i,$$

where $p_i = \Phi(z_{i+1}) - \Phi(z_i)$ is the probability of a random variable $X$ to fall within the range $[a_i; a_{i+1})$; $\Phi(x)$ is the cumulative Laplace distribution function.

5. Calculate $\chi^2_{exp}$ by the formula:

$$\chi^2_{exp} = \sum_{i=1}^{k} \frac{\left(m_i - m_i^{theor}\right)^2}{m_i^{theor}} \quad (3)$$

or

$$\chi^2_{exp} = \sum_{i=1}^{k} \frac{m_i^2}{m_i^{theor}} - n \quad (4)$$

6. Using the table, calculate $\chi^2_{cr}$ [9,10,11,12], considering the given level of significance $\alpha$ and the number of degrees of freedom $v = k - 3$.

7. Compare $\chi^2_{exp}$ and $\chi^2_{cr}$.

If $\chi^2_{exp} < \chi^2_{cr}$, there is no reason to reject the hypothesis about the normal distribution of the general population.

If $\chi^2_{exp} \geq \chi^2_{cr}$, the hypothesis about the normal distribution of the general population should be rejected.

*Testing the hypothesis about the distribution of a random variable according to a uniform law*

1. Group the sample data by arranging them as a sequence $k$ of the ranges $[a_i; a_{i+1})$ and their corresponding frequencies $m_i$, $i = \overline{1,\ldots,k}$, $a_1 = a$, $a_{k+1} = b$.

2. From a given variational array, calculate the probabilities $p_i$ of $X$ to fall within the range by the formula:

$$p_i = P(a_i < X < a_{i+1}) = \frac{a_{i+1} - a_i}{b - a} \quad (5)$$

3. Calculate theoretical frequencies by the formula:

$$m_i^{theor} = n \cdot p_i,$$

where $n$ is sample size.

4. Calculate $\chi^2_{exp}$ by the formula (4).

5. For given significance level $\alpha$ and the number of degrees of freedom $v = k - 1$, calculate $\chi^2_{cr}$ using the table [9,10,11,12].

6. Compare $\chi^2_{exp}$ and $\chi^2_{cr}$.

If $\chi^2_{exp} < \chi^2_{cr}$, there is no reason to reject the hypothesis of uniform distribution of $X$ within the range [a; b].

If $\chi^2_{exp} \geq \chi^2_{cr}$, then the hypothesis of uniform distribution should be rejected.

***Example 1***. 48 cows were examined for deviations of the annual milk yield from the average. Grouped data are given in Table 2.

**Table 2. Given data for the problem**

| Annual milk yield, kg | 0÷1000 | 1000÷2000 | 2000÷3000 | 3000÷4000 | 4000÷5000 |
|---|---|---|---|---|---|
| Number of cows, animals | 2 | 8 | 23 | 13 | 2 |

Evaluate the hypothesis about the normal distribution of the general population at a significance level $\alpha \le 0.05$ with Pearson's chi-squared test.

***Solution***. Let's rearrange interval statistical array into statistical array by replacing each range $[a_i; a_{i+1})$ with its mean value $x_i = \frac{a_i + a_{i+1}}{2}$. Now we have Table 3.

**Table 3. Statistical array**

| $x_i$ | 500 | 1500 | 2500 | 3500 | 4500 |
|---|---|---|---|---|---|
| $m_i$ | 2 | 8 | 23 | 13 | 2 |

Using Table 3, let's calculate mathematical expectation estimate $\overline{x}$ and sample standard deviation $\sigma_v$.

Mathematical expectation:

$$\overline{x} = \frac{1}{48}\sum_{i=1}^{5} x_i m_i = \frac{1}{48} \cdot (500 \cdot 2 + 1500 \cdot 8 + 2500 \cdot 23 + 3500 \cdot 13 + 4500 \cdot 2) \approx 2604;$$

Sample variance

$$D_v = \frac{1}{48}\sum_{i=1}^{5} x_i^2 m_i - \overline{x}^2 = \frac{1}{48} \cdot (500^2 \cdot 2 + 1500^2 \cdot 8 + 2500^2 \cdot 23 + 3500^2 \cdot 13 + 4500^2 \cdot 2) - 2604^2 \approx 759982.64;$$

Sample standard deviation

$$\sigma_v = \sqrt{D_v} = \sqrt{759982.64} \approx 871.77.$$

Let's calculate $p_i = \Phi(z_{i+1}) - \Phi(z_i)$, the probability of a random variable $X$ to fall within the range $[a_i; a_{i+1})$; $\Phi(x)$ is the cumulative Laplace distribution function, $z_i = \frac{a_i - \overline{x}}{\sigma_e}$.

$$p_1 = \Phi\left(\frac{1000 - 2604}{871.77}\right) - \Phi(-\infty) = -0.4671 + 0.5 = 0.0329;$$

$$p_2 = \Phi\left(\frac{2000 - 2604}{871.77}\right) - \Phi\left(\frac{1000 - 2604}{871.77}\right) = -0.2549 + 0.4671 = 0.2122;$$

$$p_3 = \Phi\left(\frac{3000 - 2604}{871.77}\right) - \Phi\left(\frac{2000 - 2604}{871.77}\right) = 0.1736 + 0.2549 = 0.4285;$$

$$p_4 = \Phi\left(\frac{4000 - 2604}{871.77}\right) - \Phi\left(\frac{3000 - 2604}{871.77}\right) = 0.4452 - 0.1736 = 0.2716;$$

$$p_5 = \Phi(+\infty) - \Phi\left(\frac{4000 - 2604}{871.77}\right) = 0.5 - 0.4452 = 0.0548.$$

Let's calculate $m_i^{theor}$ by the formula $m_i^{theor} = n \cdot p_i$ and complete Table 4.

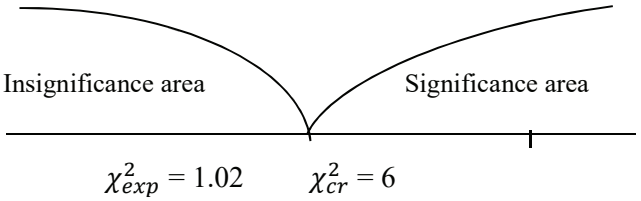
**Table 4. Calculation results**

| № | $x_i$ | $m_i$ | $m_i^2$ | $p_i$ | $m_i^{theor}$ | $\frac{m_i^2}{m_i^{theor}}$ |
|---|---|---|---|---|---|---|
| 1 | 500 | 2 | 4 | 0.0329 | 1.5792 | 2.532928 |
| 2 | 1500 | 8 | 64 | 0.2122 | 10.1856 | 6.28338 |
| 3 | 2500 | 23 | 529 | 0.4285 | 20.568 | 25.71956 |
| 4 | 3500 | 13 | 169 | 0.2716 | 13.0368 | 12.9633 |
| 5 | 4500 | 2 | 4 | 0.0548 | 2.6304 | 1.520681 |
| **Σ** | | 48 | | 1 | 48 | 49.01986 |

$\chi^2_{exp} = 49.01986 - 48 = 1.01986 \approx 1.02.$

Using the table [9, 10, 11, 12], for $\alpha \le 0.05$ and $v = k - r = 5 - 3 = 2$ let's determine $\chi^2_{cr} = 6$

Let's plot the axis of significance:




Since 1.02 < 6 ($\chi^2_{exp} < \chi^2_{cr}$), hypothesis about the normal distribution of the general population should be accepted.

***Example 2***. In some areas, the distribution of cows by live weight was recorded. Grouped data are given in Table 5.

**Table 5. Given data for the problem**

| Live weight, kg | 400÷420 | 420÷440 | 440÷460 | 460÷480 | 480÷500 |
|---|---|---|---|---|---|
| Livestock, animals | 12 | 39 | 88 | 82 | 86 |

Evaluate the hypothesis about the normal distribution of the general population at a significance level $\alpha \le 0.05$ with Pearson's chi-squared test.

***Solution***. Let's rearrange interval statistical array into statistical array by replacing each range $[a_i; a_{i+1})$ with its mean value $x_i = \frac{a_i + a_{i+1}}{2}$. Now we have Table 6.

**Table 6. Statistical array**

| $x_i$ | 410 | 430 | 450 | 470 | 490 |
|---|---|---|---|---|---|
| $m_i$ | 12 | 39 | 88 | 82 | 86 |

Using Table 6, let's calculate mathematical expectation estimate $\overline{x}$ and sample standard deviation $\sigma_v$.

Mathematical expectation:

$$\overline{x} = \frac{1}{307}\sum_{i=1}^{5} x_i m_i = \frac{1}{307} \cdot (410 \cdot 12 + 430 \cdot 39 + 450 \cdot 88 + 470 \cdot 82 + 490 \cdot 86) = 462.443;$$

Sample variance

$$D_v = \frac{1}{307}\sum_{i=1}^{5} x_i^2 m_i - \overline{x}^2 = \frac{1}{307} \cdot (410^2 \cdot 12 + 430^2 \cdot 39 + 450^2 \cdot 88 + 470^2 \cdot 82 + 490^2 \cdot 86) - 462.443^2 \approx 513.5757;$$

Sample standard deviation

$$\sigma_v = \sqrt{D_v} = \sqrt{513.5757} \approx 22.66.$$

Let's calculate $p_i = \Phi(z_{i+1}) - \Phi(z_i)$, the probability of a random variable $X$ to fall within the range $[a_i; a_{i+1})$; $\Phi(x)$ is the cumulative Laplace distribution function, $z_i = \frac{a_i - \overline{x}}{\sigma_e}$.

$$p_1 = \Phi\left(\frac{420 - 462.443}{22.66}\right) - \Phi(-\infty) = -0.4693 + 0.5 = 0.0307;$$

$$p_2 = \Phi\left(\frac{440 - 462.443}{22.66}\right) - \Phi\left(\frac{420 - 462.443}{22.66}\right) = -0.3389 + 0.4693 = 0.1304;$$

$$p_3 = \Phi\left(\frac{460 - 462.443}{22.66}\right) - \Phi\left(\frac{440 - 462.443}{22.66}\right) = -0.0389 + 0.3389 = 0.2991;$$

$$p_4 = \Phi\left(\frac{480 - 462.443}{22.66}\right) - \Phi\left(\frac{460 - 462.443}{22.66}\right) = 0.2794 + 0.0389 = 0.3192;$$

$$p_5 = \Phi(+\infty) - \Phi\left(\frac{480 - 462.443}{22.66}\right) = 0.5 - 0.2794 = 0.2206.$$

Let's calculate $m_i^{theor}$ by the formula $m_i^{theor} = n \cdot p_i$ and complete Table 7.

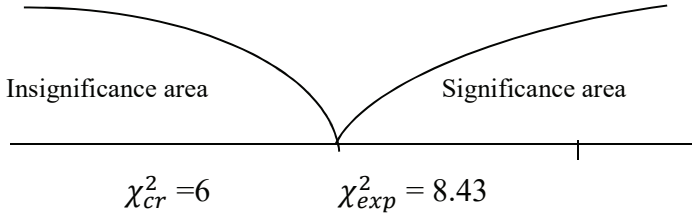
**Table 7. Calculation results**

| № | $x_i$ | $m_i$ | $m_i^2$ | $p_i$ | $m_i^{theor}$ | $\frac{m_i^2}{m_i^{theor}}$ |
|---|---|---|---|---|---|---|
| 1 | 410 | 12 | 144 | 0.0307 | 9.4249 | 15.2786767 |
| 2 | 430 | 39 | 1521 | 0.1304 | 40.0328 | 37.99384505 |
| 3 | 450 | 88 | 7744 | 0.2991 | 91.8237 | 84.33552558 |
| 4 | 470 | 82 | 6724 | 0.3192 | 97.9944 | 68.61616582 |
| 5 | 490 | 86 | 7396 | 0.2206 | 67.7242 | 109.2076392 |
| **Σ** | | 307 | | 1 | 307 | 315.432 |

$\chi^2_{exp} = 315.432 - 307 = 8.43185 \approx 8.43$.

Using Table [9, 10, 11, 12], for $\alpha \leq 0.05$ and $v = k - r = 5 - 3 = 2$ let's determine $\chi^2_{cr} = 6$

Let's plot the axis of significance:




Since 8.43 > 6 (when accepting the null hypothesis, it should be $\chi^2_{exp} < \chi^2_{cr}$), hypothesis about the normal distribution of the general population should be rejected.

Using the example of research in various fields, we will show the application of Pearson's goodness-of-fit test. The article [15] shows the applicability of target hazard quotient (THQ) estimates for communicating the danger of seafood due to metal contamination. The food recall data set was collected by the Laboratory of government chemists (LGC, UK) between January and November 2007. For example, seafood products originating in only 3 countries were recalled more than 10 times due to metal contamination (Spain, 50 times; France, 11 times; Indonesia, 11 times). Products containing swordfish and sharks have been recalled more than 10 times, mostly due to mercury contamination. Based on the food alert/recall system, the application of THQ risk assessment in cases of seafood contamination with metals is questionable, as THQ implies frequent (or even daily) lifetime exposure. Infrequent recalls due to metal contamination and lack of trend make it highly unlikely that a person would be exposed to repeated significant levels of metal ions in seafood. Pearson's goodness-of-fit chi-squared test, nonparametric correlation (Kendall's tau) and Kruskal-Wallis test were used to confirm the hypothesis and perform statistical processing. The work [16] shows a study of perception, belief and behavior in relation to nutritional and complementary practices in inflammatory bowel disease (IBD). 80 patients with IBD completed a closed-ended 16-item questionnaire that was divided into three subsections: 1) baseline/demographic characteristics; 2) disease characteristics; 3) dietary and complementary beliefs and behaviors. One-sample chi-squared goodness-of-fit tests were used for each question, and two-sided Pearson's chi-squared tests of independence were used for testing differences in response to each question between baseline/demographic variables.

The processing time of 1.0 cm, 1.5 cm and 2.0 cm potato cubes with 0.4%, 0.8% and 1.2% aqueous solutions of sodium carboxymethyl cellulose at flow rates of 453 ml/s, 534 ml/s and 599 ml/s was measured for the performance of vertical scraped surface heat exchanger (VSHE) rotating at 60, 110 and 160 rpm, and the particle flow distribution characteristics for each set of conditions were studied in [17]. Statistical data processing using Pearson's chi-squared test showed that most distributions for the residence time of individual particles in the vertical flow in VSHE may be described by the gamma model, while for the horizontal VSHE, many of the individual distributions correspond to the normal model in addition to the gamma model. VSHE orientation turned out to be an important

factor influencing the forces acting on particles during the flow in the VSHE. Interactions of particles with each other, as well as a combination of process parameters, caused a "tail" of some particles, which led to a shift in the distribution to the right. The purpose of the article [18] was to assess the purchasing behavior of consumers and the decision-making process when buying bread and to suggest ways to improve bread positioning in the market. 1601 correctly completed questionnaires were used for the analysis. Results were presented as response rates and statistical tests. The analysis included the evaluation of statistical hypotheses about independence (significance level $\alpha = 0.01$) using goodness-of-fit chi-squared test and Pearson's randomness coefficient. Then the significance level was compared with the p value. For the p value > α, the null hypothesis was not rejected. The most important factors in choosing bread are freshness, appearance and price. Importance of price increases with the age of the respondents and decreases with the income of the surveyed consumers. The importance of a brand, as well as referrals from family and friends, increases slightly as consumer income increases. When making a purchase decision, most respondents do not make a difference between yeast and rye-yeast bread baking technologies. However, it cannot be stated that the preference for rye-yeast bread increases with the age of the respondents to the detriment of yeast bread, or vice versa.

In [19], gender differences were determined in the self-assessment of social functioning in patients with comorbidity of affective disorders and chronic coronary artery disease. The study included 248 cardiac patients (194 men (78.2%) and 54 women (21.8%)) with chronic coronary artery disease and affective disorders. The mean age of patients with chronic disease in men was (57.2 +/– 6.5) years, and in women it was (59.3 +/– 7.1), $p = 0.04$. Qualitative and quantitative indicators were examined using the Mann-Whitney test, Wilcoxon test and T-test; chi-squared test (Pearson's goodness-of-fit test) was used to estimate frequencies. The purpose of the study in [20] was to reveal the parents' ideas about the main trends and structural features of children's Internet addiction. The study was based on the results of a mass survey. The survey was conducted in 2019 on a multi-stage sample (by gender, age, type of location), consisting of the adult population at the Tyumen region. The authors carried out a detailed socio-statistical analysis of Internet risks for children based on self-assessments of all respondents (with identification of socio-demographic groups), risk assessments for children according to parents. The structure of "Parents" subsample by gender and type of location was proportional to the structure of the main sample. According to the authors, "Children" subsample included respondents' children of minority age. The risk of Internet addiction was included in the structure of 12 Internet risks and examined on the basis of 4 components (behavioral, cognitive, social and affective components). The analysis used Cronbach's alpha consistency ratings, index method, Spearman rank correlation coefficients, Pearson's goodness-of-fit test, F-test for equality of several means, case classification and triangulation method. The study [21] examined the relationship between mean micturition volume and urinary incontinence episodes per 24 hours after adjusting for fixed frequencies in children with overactive bladder. Patients were aged 5 to 12 years with >= 4 episodes of daytime urinary incontinence during the 7-day period prior to study entry. Mean number of episodes of urinary incontinence per 24 hours at the end of the study was the dependent variable. Explanatory variables included treatment, mean number of episodes of urinary incontinence per 24 hours at baseline, and change in mean micturition volume from baseline to the end of the study. Statistical significance and degree of conformity were analyzed using Pearson's chi-squared test. The aim of the study [22] was to evaluate the effectiveness of a pediatric mortality index of 3 in predicting mortality at the intensive care unit. This was an observational study conducted in the intensive care unit from January 2016 to October 2018. All patients aged 1 month to 15 years who were hospitalized to the intensive care unit were included. The authors analyzed the relationship between the pediatric mortality index of 3 and mortality. Indicators of the pediatric mortality index of 3 were assessed by calibration and discrimination. Calibration assessed the pediatric mortality index of 3 at various mortality risks using the standardized mortality rate (SMR) and Pearson's goodness-of-fit test (chi-squared test). The study [23] evaluated the impact of health-related quality of life on the use of health services using four different scoring data models. Health-related quality of life was measured using a brief six-dimensional instrument and a functional assessment of colon cancer therapy, while health service use was measured by the number of monthly clinical consultations and the number of monthly hospitalizations. Goodness-of-fit statistics (Pearson's chi-squared test, Akaike information criterion and Bayesian tests) were used to determine the best model. In [24], a cross-sectional diagnostic study was described. 83 medical records of patients with suspected heart failure admitted to the emergency and internal medicine department of the Ramiro Priale Priale National Hospital were examined. Pearson's chi-squared test was used to analyze categorical variables and ANOVA was used for continuous variables. P-values < 0.05 were considered significant.

**Kolmogorov test**

*Kolmogorov goodness-of-fit test* is designed to test the hypothesis that the sample belongs to some distribution law, i.e. to check that the empirical distribution corresponds to the expected model.

In this test, the maximum value of the absolute difference between the empirical distribution function $F_n(x)$ and the corresponding theoretical distribution function $d = max|F_n(x) - F(x)|$ is a measure of difference between theoretical and empirical distributions. This random variable is denoted as $\lambda = D\sqrt{n}$ and is called *Kolmogorov goodness-of-fit λ-test*.

*Application of Kolmogorov test*

1. Arrange the results of observations in ascending order: $x_1 \le x_2 \le \cdots \le x_n$ or represent them as an interval variational array.

2. Calculate the empirical relative frequencies for each rank by the formula:

$$f_j = \frac{m_j}{n} \quad (6)$$

3. Determine the values of the empirical distribution function $F_n(x)$ by calculating the accumulated empirical relative frequencies by the formula:

$$\sum f_j = \sum f_{j-1} + f_j \quad (7)$$

where $\sum f_i$ is the relative frequency accumulated in the previous ranks; $j$ is the order number of the rank;

The obtained values $\sum f_j$ is empirical distribution function.

4. Determine the corresponding values of the assumed theoretical distribution function by counting the accumulated theoretical relative frequencies for each rank by the formula:

$$\sum f_j^{theor} = \sum f_j^{theor} + f_j^{theor} \quad (8)$$

where $\sum f_j^{theor}$ is the theoretical relative frequency accumulated in the previous ranks.

5. Calculate the absolute differences between the empirical and theoretical accumulated relative frequencies for each rank. Designate them as $d$.

6. Determine the largest absolute difference $d_{max}$.

7. Using the table of Kolmogorov test critical values [9, 10, 11, 12], for a given significance level $\alpha$ and a number of observations n, determine the critical value $d_{cr}$.

If $n > 100$, then $d_{cr}$ is calculated by the formula:

$$d_{cr} = \begin{cases} \frac{1.36}{\sqrt{n}} & for\ \alpha \le 0.05 \\ \frac{1.63}{\sqrt{n}} & for\ \alpha \le 0.01 \end{cases} \quad (9)$$

If $d_{max} \ge d_{cr}$, then the null hypothesis is rejected: differences between distributions are significant.

If $d_{max} < d_{cr}$, then it is considered that there is no reason for rejecting the null hypothesis, i.e. the difference between the empirical and theoretical distribution function is not significant.

*Limitations of test*

Ranks should be arranged in ascending order.

***Example.*** When weighing the fattened young cattle (103 animals) delivered to the meat processing plant, the following primary (raw) array was obtained according to live weight (kg):

413 454 419 412 427 435 404 430 421 399 414 386
428 441 397 417 418 423 420 416 407 427 428 417
398 424 419 401 424 411 426 380 419 406 410 409
416 410 403 426 407 400 423 425 394 432 409 418
419 388 423 434 402 431 405 436 405 424 405 412
413 444 392 411 428 394 433 395 433 420 430 398
437 422 394 416 424 434 407 443 406 422 414 429
417 406 419 429 406 388 421 415 417 394 431 411
422 410 432 409 439 421 414

Determine whether the data obtained are normally distributed or not at a significance level $\alpha \le 0.05$.

***Solution.*** Let's rearrange the primary array into the variational array (Table 8).

**Table 8. Variational array by the live weight of young cattle when delivered to a meat processing plant**

| W | 380-389 | 390-399 | 400-409 | 410-419 | 420-429 | 430-439 | 440-449 | 450-459 | Sum |
|---|---|---|---|---|---|---|---|---|---|
| f | 4 | 10 | 16 | 30 | 26 | 13 | 3 | 1 | n=103 |

Let's determine empirical relative frequencies for each rank by the formula:

$$f_j = \frac{m_j}{n},$$

where $m_j$ is the frequency of a given number of points, $n$ is the total number of points appearances.

$$f_1 = \frac{m_1}{n} = \frac{4}{103} = 0.039$$
$$f_2 = \frac{m_2}{n} = \frac{10}{103} = 0.097$$
$$f_3 = \frac{m_3}{n} = \frac{16}{103} = 0.155$$
$$f_4 = \frac{m_4}{n} = \frac{30}{103} = 0.291$$
$$f_5 = \frac{m_5}{n} = \frac{26}{103} = 0.252$$
$$f_6 = \frac{m_6}{n} = \frac{13}{103} = 0.126$$
$$f_7 = \frac{m_7}{n} = \frac{3}{103} = 0.029$$
$$f_8 = \frac{m_8}{n} = \frac{1}{103} = 0.0097$$

Let's determine accumulated empirical relative frequencies by the formula:

$$\sum f_j = \sum f_{j-1} + f_j$$

where $\sum f_i$ is the relative frequency accumulated in the previous ranks; $j$ is the order number of the rank.

$$\sum f_1 = f_1 = 0.039$$

$$\sum f_{1+2} = \sum f_1 + f_2 = 0.039 + 0.097 = 0.136$$

$$\sum f_{1+2+3} = \sum f_{1+2} + f_3 = 0.136 + 0.155 = 0.291$$

$$\sum f_{1+2+3+4} = \sum f_{1+2+3} + f_4 = 0.291 + 0.291 = 0.582$$

$$\sum f_{1+2+3+4+5} = \sum f_{1+2+3+4} + f_5 = 0.582 + 0.252 = 0.834$$

$$\sum f_{1+2+3+4+5+6} = \sum f_{1+2+3+4+5} + f_6 = 0.834 + 0.126 = 0.960$$

$$\sum f_{1+2+3+4+5+6+7} = \sum f_{1+2+3+4+5+6} + f_7 = 0.960 + 0.029 = 0.989$$

$$\sum f_{1+2+3+4+5+6+7+8} = \sum f_{1+2+3+4+5+6+7} + f_8 = 0.989 + 0.0097 = 0.9987 \approx 1$$

Let's determine theoretical relative frequencies for each rank. For the 1st rank, the theoretical relative frequency is calculated by the formula:

$$f_1^{theor} = \frac{1}{k},$$

where $k$ is the number of ranks ($k = 8$).

$$f_1^{theor} = \frac{1}{k} = \frac{1}{8} = 0.125.$$

This theoretical relative frequency applies to all ranks.

Let's determine accumulated theoretical relative frequencies.

$$\sum f_1^{theor} = f_1^{theor} = 0.125;$$

$$\sum f_{1+2}^{theor} = \sum f_1^{theor} + f_2^{theor} = 0.125 + 0.125 = 0.250$$

$$\sum f_{1+2+3}^{theor} = \sum f_{1+2}^{theor} + f_3^{theor} = 0.250 + 0.125 = 0.375$$

$$\sum f_{1+2+3+4}^{theor} = \sum f_{1+2+3}^{theor} + f_4^{theor} = 0.375 + 0.125 = 0.500$$

$$\sum f_{1+2+3+4+5}^{theor} = \sum f_{1+2+3+4}^{theor} + f_5^{theor} = 0.500 + 0.125 = 0.625$$

$$\sum f_{1+2+3+4+5+6}^{theor} = \sum f_{1+2+3+4+5}^{theor} + f_6^{theor} = 0.625 + 0.125 = 0.750$$

$$\sum f_{1+2+3+4+5+6+7}^{theor} = \sum f_{1+2+3+4+5+6}^{theor} + f_7^{theor} = 0.750 + 0.125 = 0.875$$

$$\sum f_{1+2+3+4+5+6+7+8}^{theor} = \sum f_{1+2+3+4+5+6+7}^{theor} + f_8^{theor} = 0.875 + 0.125 = 1$$

Calculate the absolute differences between the accumulated empirical and theoretical frequencies:

$$d_1 = \left|\sum f_1 - \sum f_1^{theor}\right| = |0.039 - 0.125| = 0.086;$$

$$d_2 = \left|\sum f_2 - \sum f_2^{theor}\right| = |0.136 - 0.250| = 0.114;$$

$$d_3 = \left|\sum f_3 - \sum f_3^{theor}\right| = |0.291 - 0.375| = 0.084;$$

$$d_4 = \left|\sum f_4 - \sum f_4^{theor}\right| = |0.582 - 0.500| = 0.082;$$

$$d_5 = \left|\sum f_5 - \sum f_5^{theor}\right| = |0.834 - 0.625| = 0.209;$$

$$d_6 = \left|\sum f_6 - \sum f_6^{theor}\right| = |0.960 - 0.750| = 0.210;$$

$$d_7 = \left|\sum f_7 - \sum f_7^{theor}\right| = |0.989 - 0.875| = 0.114;$$

$$d_8 = \left|\sum f_8 - \sum f_8^{theor}\right| = |1 - 1| = 0.$$

The results are shown in Table 9.

**Table 9. Calculation results**

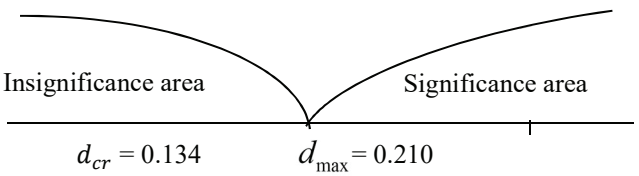
| Number of points | Empirical frequency | Empirical relative frequency | Accumulated empirical relative frequency | Accumulated theoretical relative frequency | Difference |
|---|---|---|---|---|---|
| 1 | 4 | 0.039 | 0.039 | 0.125 | 0.086 |
| 2 | 10 | 0.097 | 0.136 | 0.250 | 0.114 |
| 3 | 16 | 0.155 | 0.291 | 0.375 | 0.084 |
| 4 | 30 | 0.291 | 0.582 | 0.500 | 0.082 |
| 5 | 26 | 0.252 | 0.834 | 0.625 | 0.209 |
| 6 | 13 | 0.126 | 0.960 | 0.750 | 0.210 |
| 7 | 3 | 0.029 | 0.989 | 0.875 | 0.114 |
| 8 | 1 | 0.0097 | 1 | 1 | 0 |
| Sums | 103 | 1 | | | |

Let's determine the largest absolute difference $d_{max}$ (yellow color cell).

Since in this problem $n > 100$, then the critical value $d_{cr}$ is calculated by the formula (9) for a significance level $\alpha \leq 0.05$:

$$d_{cr} = \frac{1.36}{\sqrt{n}} = \frac{1.36}{\sqrt{103}} = 0.134$$

Let's plot the axis of significance:



Since $d_{max} \geq d_{cr}$, then the null hypothesis is rejected, i.e. the empirical distribution for the live weight of cattle delivered to a meat processing plant differs from the normal (uniform) distribution.

Let's give examples for the use of Kolmogorov test in scientific research. The article [25] analyzed the growth of rice, wheat and common food grains in India for the period from 1950 to 2019. The distribution was assessed using Kolmogorov test. It was found that the availability of rice (70.05 kg/year), wheat (70.73 kg/year) and total grains (182.96 kg/year) will decrease in 2021 compared to this year. The article [26] analyzed a questionnaire survey of 227 respondents regarding purchasing preferences for organic food in Slovakia. To achieve the goal and provide a deeper analysis of the results, 3 assumptions and 5 hypotheses were made. According to the survey results, 65% of respondents buy organic products, of which 39% buy organic products at least once a week. Up to 98% of respondents have already heard about the concept of organic food and know what it means. 37% of respondents buy mostly organic fruits and vegetables; 18% of respondents buy mostly organic meat and meat products, and 13% of respondents prefer organic dairy products. The most preferred place to buy organic products are specialized stores (36%); buying organic products directly from the manufacturer is the most popular way for 29% of respondents; hypermarkets and supermarkets are a favorite place to buy organic products for 19% of respondents; and 12% of respondents buy organic products mainly in farmers' markets. Only 4% of respondents prefer another way to buy organic products. The quality of organic products and the absence of pesticides are the most important criteria for purchasing organic products (36%). The results of the study were evaluated using the goodness-of-fit chi-squared test and Kolmogorov test, and the following conclusion was made: there is a difference in the preferences of the respondents. In Slovakia, there is a relationship between consumer preferences for organic food and traditional food, and there is a strong preference to buy organic food. The aim of the study [27] was to present a correct model for probability distribution based on data obtained from surveys on the temperature of food storage in household refrigerators at home. The temperature in household refrigerators was determined as a risk factor for foodborne disease outbreaks for microbial risk assessment. Temperature was measured by visiting 139 homes directly with a data logger from May to September 2009. The overall average temperature for all refrigerators participating in the survey was 3.53 ± 2.96 °C, with 23.6% of refrigerators having temperatures above 5 °C. Probability distributions were generated from the measured temperature data. Statistical ranking was determined by Kolmogorov goodness-of-fit test or Anderson-Darling test to determine the appropriate probability distribution model. This result showed that the LogLogistic distribution (-10.407, 13.616, 8.6107) was the most appropriate for the microbial risk assessment model.

The aim of the work [28] was to study the strong Markov property for stochastic differential equations controlled by G-Brownian motion (G-SDE). First, the authors extended the conditional G-expectancy of deterministic time to optional points of time. The strong Markov property for the G-SDE was obtained using Kolmogorov tightness criterion. The article [29] considers the process of the defect appearance in the body of a workpiece obtained by casting. The medium with many randomly distributed discontinuities was schematically a regular structure formed by a set of elements in the form of a regular tetrahedron with spherical depressions at the vertices. The proposed technique makes it possible to create a model of a continuous homogeneous medium that is equivalent in its deformation properties to the original discontinuous material. Using this approach, a power approximation of the extension curve for a model medium was obtained. The rupture of the material was fixed using Kolmogorov plastic deformation test. This test was used in the evaluation of the limit state of the valve chamber under operating conditions.

**Nonparametric tests for homogeneity**

*Hypotheses of homogeneity* are hypotheses assuming that the samples under study are taken from the same general population.

Let there be two independent samples with sizes $n_1$ and $n_2$ obtained from populations with unknown theoretical distribution functions $F_1(x)$ and $F_2(x)$. Hypotheses are stated:

$H_0$: Empirical distribution 1 does not differ from empirical distribution 2, i.e. $F_1(x) = F_2(x)$.

$H_1$: Empirical distribution 1 differs from empirical distribution 2, i.e. $F_1(x) \neq F_2(x)$.

**Pearson's chi-squared test for homogeneity**

Pearson's chi-squared test may be used to evaluate the homogeneity of two or more independent samples, i.e. to test the hypothesis that there are no differences between two and more empirical distributions of the same indicator. Source data should be presented in the form of Table 10:

**Table 10. Source data template (cross-tab table or contingency table)**

| Empirical frequencies | | Indicator ranking | | | | | Sum |
|---|---|---|---|---|---|---|---|
| | | 1 | … | *j* | … | *k* | |
| Ranks of the indicator | 1 | | | | | | |
| | … | | | | | | |
| | *i* | | | | | | |
| | … | | | | | | |
| | *c* | | | | | | |
| Sum | | | | | | | |

Such tables are called cross-tab tables or contingency tables.

The algorithm for calculating Pearson's chi-squared test is the same as for Pearson's goodness-of-fit test (see above), but for each cell of the i[th] row and j[th] column, its own theoretical frequency is determined by the formula:

$$m_{ij}^{theor} = \frac{\sum_i m_{ij} \cdot \sum_j m_{ij}}{N}, \quad (10)$$

where $N$ is the sum of frequencies of the entire contingency table; $\sum_i m_{ij}$ is the sum of frequencies in all cells of the i[th] row; $\sum_j m_{ij}$ is the sum of frequencies in all cells of the j[th] column.

Pearson's chi-squared test is calculated by the formula:

$$\chi^2_{exp} = \sum_{i=1}^{c} \sum_{j=1}^{k} \frac{\left(m_{ij} - m_{ij}^{theor}\right)^2}{m_{ij}^{theor}}. \quad (11)$$

The number of degrees of freedom is calculated by the formula:

$$df = (k-1) \cdot (c-1) \quad (12)$$

where $c$ is the number of ranks for the indicator (number of compared distributions).

If the number of degrees of freedom is equal to 1, i.e., if the indicator only takes two values, the adjusting for continuity is needed. The adjusting for continuity is applied under the following conditions:

1) when the empirical distribution is compared to the uniform distribution, and the number of indicator rankings $k = 2$, and the number of degrees of freedom $v = k - 1 = 1$.

2) when two empirical distributions are compared, and $k$=2, i.e. number of rows and number of columns is both equal to 2 and $v = (k-1) \cdot (c-1) = 1$.

In these cases, it is necessary to reduce the absolute difference $\left|m_{ij} - m_{ij}^{theor}\right|$ by 0.5 prior to squaring. $\chi^2_{exp}$ is calculated by the formula:

$$\chi^2_{exp} = \sum_{i=1}^{c} \sum_{j=1}^{k} \frac{\left(\left|m_{ij} - m_{ij}^{theor}\right| - 0.5\right)^2}{m_{ij}^{theor}} \quad (13)$$

***Example.*** During the survey, high school students were asked which of the three possible areas of education (mathematics, natural sciences or human sciences) they would prefer in the future. Among the respondents were both young males and young females [30]. The data are summarized in Table 11.

**Table 11. Given data for the problem**

| Empirical frequencies | | Indicator ranking | | |
|---|---|---|---|---|
| | | Mathematics | Natural sciences | Human sciences |
| Ranks of the indicator | Young males 1 | 18 | 10 | 3 |
| | Young females 2 | 10 | 9 | 15 |

Such table is called a cross-tab table with size of 2 × 3.

Is it possible to state that at a significance level $\alpha \leq 0.05$ the preference for one or another area of education is somehow related to the gender factor?

***Solution.*** Let's state the hypotheses:

$H_0$: distribution of preferences for the area of education in young males and young females is not significantly different from the random distribution.

$H_1$: distribution of preferences for the area of education in young males and young females is significantly different from the random distribution.

In Table 12 sums of frequencies are calculated by rows and columns.

**Table 12. Intermediate cross-tab 2 × 3 calculations**

| Empirical frequencies | | Indicator ranking | | | Sum |
|---|---|---|---|---|---|
| | | Mathematics | Natural sciences | Human sciences | |
| Ranks of the indicator | Young males 1 | 18 | 10 | 3 | 31 |
| | Young females 2 | 10 | 9 | 15 | 34 |
| Sum | | 28 | 19 | 18 | 65 |

For each of the cells, a special theoretical frequency related only to this cell should be calculated by the formula:

$$m_{ij}^{theor} = \frac{\sum_i m_{ij} \cdot \sum_j m_{ij}}{N}.$$

There are 65 frequencies in total, of which 28 frequencies correspond to mathematics, 19 frequencies correspond to natural sciences, and 18 frequencies correspond to human sciences. The proportion of each education area is 28/65, 19/65, 18/65, respectively. In all rows, mathematics should be 28/65 of all the answers, natural sciences should be 19/65, and human sciences should be 18/65. Knowing the sums of frequencies for each row, you can calculate the theoretical frequencies for each cell.

$$m_{11}^{theor} = \frac{31 \cdot 28}{65} = 13.35;$$

$$m_{12}^{theor} = \frac{31 \cdot 19}{65} = 9.06;$$

$$m_{13}^{theor} = \frac{31 \cdot 18}{65} = 8.58;$$

$$m_{21}^{theor} = \frac{34 \cdot 28}{65} = 14.65;$$

$$m_{22}^{theor} = \frac{34 \cdot 19}{65} = 9.94;$$

$$m_{23}^{theor} = \frac{34 \cdot 18}{65} = 9.42.$$

Let's complete Table 13.

**Table 13. Calculation results**

| Rank — indicator ranking | $m_{ij}$ | $m_{ij}^{theor}$ | $m_{ij} - m_{ij}^{theor}$ | $\left(m_{ij} - m_{ij}^{theor}\right)^2$ | $\frac{\left(m_{ij} - m_{ij}^{theor}\right)^2}{m_{ij}^{theor}}$ |
|---|---|---|---|---|---|
| Young males — mathematics | 18 | 13.35 | 4.65 | 21.59 | 1.62 |
| Young males — natural sciences | 10 | 9.06 | 0.94 | 0.88 | 0.10 |
| Young males — human sciences | 3 | 8.58 | -5.58 | 31.19 | 3.63 |
| Young females — mathematics | 10 | 14.65 | -4.65 | 21.59 | 1.47 |
| Young females — natural sciences | 9 | 9.94 | -0.94 | 0.88 | 0.09 |
| Young females — human sciences | 15 | 9.42 | 5.58 | 31.19 | 3.31 |

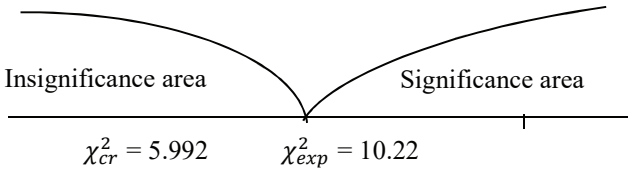
$\chi^2_{exp} = 1.62 + 0.10 + 3.63 + 1.47 + 0.09 + 3.31 = 10.22.$

The number of degrees of freedom is calculated by the formula:

$$v = (k-1)\cdot(c-1) = (3-1)\cdot(2-1) = 2$$

Using the table of critical values [9, 10, 11, 12], $\chi^2$ distributions for $v = 2$ and $\alpha \leq 0.05$ $\chi^2_{cr} = 5.992$.

Let's plot the axis of significance:




Since $\chi^2_{exp} > \chi^2_{cr}$, the null hypothesis should be rejected and the alternative hypothesis should be accepted, i.e. the dependence of preference in choosing a further education on the gender of the respondent was proved.

In the studies [31-35], chi-squared test was used. The study [31] examined the association of interleukin-6 (IL-6) (IL-6-174G/C), transforming growth factor-beta 1 (TGF-beta1-29C/T) and calmodulin 1 gene. 16C/T-polymorphism (CALM1-16C/T) was clinically determined in Pakistani patients with osteoarthritis and corresponding control group. The study included 295 subjects, including 100 patients with osteoarthritis, 105 patients with predisposition to osteoarthritis and 90 patients from the control group. The study design was based on biochemical analysis of osteoarthritis using hyaluronic acid serum enzyme-linked immunosorbent assay and genetic analysis based on PCR with an amplification-resistant mutation system. Allele probabilities were statistically estimated using Pearson's chi-squared test. The authors [32] studied the role and interaction of proteins involved in the control and stimulation of neurotransmission in predisposition to migraine. The study was performed on 183 migraineurs (148 women and 35 men) and 265 non-migraine controls (202 women and 63 men). Labeling of single nucleotide polymorphisms of neurexin was carried out to assess the association between neurexin and predisposition to migraine. Chi-squared test was used to compare allele frequencies in test cases and controls, and odds ratios were estimated with 95% confidence intervals. The authors [33] present a retrospective crossover observational study of the epidemiological profile of all dengue cases confirmed and reported to the Minister of Health in Pernambuco between 2015 and 2017. The data include all municipalities of Pernambuco with the exception of Fernando de Noronha. People infected with dengue were classified according to the type of dengue fever (without and with the symptoms or severe dengue), age, sex, ethnicity, and intermediate geographic region of residence (Recife, Caruaru, Serra Talhada, or Petrolina). The distribution of cases by years was estimated using chi-squared test. The aim of the study [34] was to evaluate eating behavior, health-related and nutrition-related problems among students with symptoms of orthorexia nervosa. The participants were 1120 college students from seven universities in Poland studying health-related (n=547) and other specialties (n=573). Students were examined with ORTO-15 test, the health problems scale and the food intake frequencies questionnaire. Then, based on principal component analysis, eight nutrition patterns were derived ("sweets and snacks", "legumes and nuts", "fruits and vegetables", "refined breads and animal fats", "dairy products and eggs", "fish", "meat", "fruit and vegetable juices"). Pearson's correlation, Pearson's chi-squared test, Student t-test and one-sided ANOVA were used for further analysis. In the work [35], the authors studied the potential roles and mechanisms of si-STOML2 (stomatin-like protein 2) in the migration and invasion of human hepatoma LM3 cells. Stomatin-like protein 2 expression levels in tissues and cells were separately analyzed by quantitative real-time PCR (qRT-PCR) and Western blotting. Cell viability, migration and invasion were assessed using the cell count-8 kit, wound healing and transwell assay kit, respectively. mRNA and various protein factors levels were separately measured by qRT-PCR and Western blotting. The correlation analysis between the expression of stomatin-like protein 2 and the clinical/pathological features of liver cancer patients was assessed using the chi-squared test.

**Kolmogorov-Smirnov test**

Kolmogorov-Smirnov test statistics is the following:

$$\lambda' = \sqrt{\frac{n_1 n_2}{n_1+n_2}} \cdot max|F_1(x) - F_2(x)|, \qquad (14)$$

where $F_1(x)$ and $F_2(x)$ are empirical distribution functions from two samples with sizes $n_1$ and $n_2$. Let's assume that the functions $F_1(x)$ and $F_2(x)$ are continuous.

*Application of Kolmogorov-Smirnov test*

1. Arrange the results of observations in ascending order: $x_1 \leq x_2 \leq \cdots \leq x_n$ or represent them as an interval variational array.

2. Calculate the empirical relative frequencies for each rank for distribution 1 by the formula:

$$f_{1j} = \frac{m_{1j}}{n_1}$$

where $m_{1j}$ is the empirical frequency in the given rank; $n_1$ is the number of observations in the sample.

3. Calculate the empirical relative frequencies for each rank for distribution 2 by the formula:

$$f_{2j} = \frac{m_{2j}}{n_2},$$

where $m_{2j}$ is the empirical frequency in the given rank; $n_2$ is the number of observations in the sample.

4. Calculate the accumulated empirical relative frequencies for distribution 1 by the formula:

$$\sum f_{1j} = \sum f_{1j-1} + f_{1j}$$

where $\sum f_{1j-1}$ is the relative frequency accumulated in the previous ranks; $j$ is the order number of the rank; $f_{1j}$ is the relative frequency of the given rank.

5. Calculate the accumulated empirical relative frequencies for distribution 2 by the same formula.

$$\sum f_{2j} = \sum f_{2j-1} + f_{2j}$$

where $\sum f_{2j-1}$ is the relative frequency accumulated in the previous ranks; $f_{2j}$ is the relative frequency of the given rank.

6. Calculate the absolute differences between the accumulated relative frequencies for each rank. Designate them as $d$. Determine the largest absolute difference $d_{max}$.

7. Calculate $\lambda'_{exp}$ by the formula:

$$\lambda'_{exp} = d\sqrt{\frac{n_1 \cdot n_2}{n_1 + n_2}}_{max} \quad (15)$$

where $n_1$ is the number of observations in the first sample; $n_2$ is the number of observations in the second sample.

8. Using the table of critical values [9, 10, 11, 12], for a given significance level $\alpha$, determine $\lambda_{cr}$. If $\lambda'_{exp} \geq \lambda_{cr}$, then the differences between the distributions are significant. If $\lambda'_{exp} < \lambda_{cr}$, then the differences between the distributions are not significant.

*Limitations of Kolmogorov-Smirnov test*

1. When comparing two empirical distributions, it is necessary that $n_1, n_2 \geq 50$.

2. Ranks must be arranged in ascending or descending order by some indicator. We cannot accumulate frequencies by the ranks that differ only qualitatively and do not represent a scale of order.

***Example.*** To evaluate the effectiveness of a drug, one group of subjects was given a test drug tested on animals, and the other group of subjects was given a placebo (a physiologically inert substance, the positive therapeutic effect of which is associated with the patient's subconscious psychological expectation). Table 14 represents data on the number of occurrences of influenza symptoms over a two-year period in the group taking prophylactic drug at the beginning of the period and in the group taking placebo [12].

**Table 14. Given data for the problem**

| Number of diseases | Number of patients taking the drug | Number of patients taking placebo |
|---|---|---|
| | $m_{1j}$ | $m_{2j}$ |
| 0 | 32 | 26 |
| 1 | 26 | 30 |
| 2 | 15 | 11 |
| 3 | 6 | 14 |
| 4 and more | 6 | 19 |
| Sum | 85 | 100 |

Can we state that at a significance level $\alpha \leq 0.05$ the effect of the drug is sufficiently greater than of placebo?

***Solution***. Let's state the hypotheses:

$H_0$: Empirical distribution 1 differs from empirical distribution 2, i.e. the effect of the drug significantly exceeds the effect of the placebo.

$H_1$: Empirical distribution 1 does not differ from empirical distribution 2, i.e. the effect of the drug does not significantly exceed the effect of the placebo.

Let's determine empirical relative frequencies for each rank for sample 1 (first test) by the formula:

$$f_{1j} = \frac{m_{1j}}{n_1}$$

$$f_{11} = \frac{m_{11}}{n_1} = \frac{32}{85} = 0.3765;$$

$$f_{12} = \frac{m_{12}}{n_1} = \frac{26}{85} = 0.3059$$

etc.

The results of the calculations are represented in Table 15.

**Table 15. Calculation results**

| Number of diseases | Empirical frequencies | | Empirical relative frequencies | | Accumulated empirical relative frequencies | | Difference $\lvert\sum f_{1j} - \sum f_{2j}\rvert$ |
|---|---|---|---|---|---|---|---|
| | $m_{1j}$ | $m_{2j}$ | $f_{1j}$ | $f_{2j}$ | $\sum f_{1j}$ | $\sum f_{2j}$ | |
| 0 | 32 | 26 | 0.3765 | 0.2600 | 0.3765 | 0.2600 | 0.1165 |
| 1 | 26 | 30 | 0.3059 | 0.3000 | 0.6824 | 0.5600 | 0.1224 |
| 2 | 15 | 11 | 0.1765 | 0.1100 | 0.8588 | 0.6700 | 0.1888 |
| 3 | 6 | 14 | 0.0706 | 0.1400 | 0.9294 | 0.8100 | 0.1194 |
| 4 and more | 6 | 19 | 0.0706 | 0.1900 | 1.0000 | 1.000 | 0 |
| Sum | 85 | 100 | 1 | 1 | | | |

Let's determine empirical relative frequencies for each rank for sample 2 (second test) by the formula:

$$f_{2j} = \frac{m_{2j}}{n_2}$$

$$f_{21} = \frac{m_{21}}{n_2} = \frac{26}{100} = 0.2600;$$

$$f_{21} = \frac{m_{22}}{n_2} = \frac{30}{100} = 0.3000$$

etc.

Let's calculate the accumulated empirical relative frequencies for sample 1 by the formula:

$$\sum f_{1j} = \sum f_{1j-1} + f_{1j}.$$

$$\sum f_{11} = f_{11} = 0.3765$$

$$\sum f_{12} = \sum f_{11} + f_{12} = 0.3765 + 0.3059 = 0.6824$$

etc.

Let's calculate the accumulated empirical relative frequencies for sample 2 by the same formula:

$$\sum f_{2j} = \sum f_{2j-1} + f_{2j}.$$

$$\sum f_{21} = f_{21} = 0.2600$$

$$\sum f_{22} = \sum f_{21} + f_{22} = 0.2600 + 0.3000 = 0.5600$$

etc.

Let's determine the absolute differences between the accumulated empirical relative frequencies by the formula:

$$d_j = \left|\sum f_{1j} - \sum f_{2j}\right|.$$

$$d_1 = \left|\sum f_{11} - \sum f_{21}\right| = |0.3765 - 0.2600| = 0.1165;$$

$$d_2 = \left|\sum f_{12} - \sum f_{22}\right| = |0.6824 - 0.5600| = 0.1224;$$

etc.

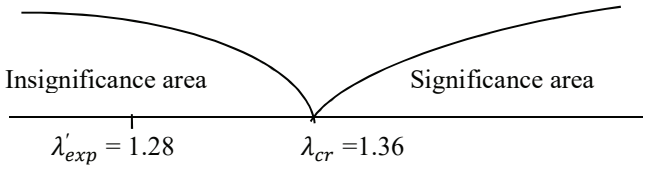
From Table 15, let's determine the largest absolute difference $d_{max}$. This is $d_{max} = 0.1888$ (highlighted in yellow).

Let's calculate $\lambda'_{exp}$:

$$\lambda'_{exp} = d_{max}\sqrt{\frac{n_1 \cdot n_2}{n_1 + n_2}} = 0.1888 \cdot \sqrt{\frac{85 \cdot 100}{85 + 100}} \approx 1.28$$

Using the table of critical values [9, 10, 11, 12], for a given significance level $\alpha \leq 0.05$, let's determine $\lambda_{cr} = 1.36$.

Let's plot the axis of significance:




Since $\lambda'_{exp} < \lambda_{cr}$, then the null hypothesis is not rejected, i. e. the effect of the drug significantly exceeds the effect of the placebo.

In the studies [36–44], Kolmogorov-Smirnov test was used. The study [36] aimed to determine the relationship between the management of household solid waste (HSW) and non-household solid waste (NHSW) (X variable) in Huancavelica County and municipal government (Y variable) in 2016. The population and sample were 12,249 and 140 people, respectively. The collected data were analyzed using Kolmogorov-Smirnov test. The paper [37] represents the results of physicochemical and rheological studies of wet foams obtained from hen egg albumin with the addition of xanthan gum and/or arabic gum using the batch method. Physicochemical analysis included determination of foam density, gas phase volume fraction, overrun, stability and distribution of gas bubbles suspended in liquid. The study of hydrocolloids effect on the distribution of gas bubbles was based on standard descriptive parameter estimation and the use of the nonparametric Kolmogorov-Smirnov test. The study [38] evaluated the expression of basic fibroblast growth factor and the number of osteoblasts during orthodontic tooth movement after administration of *Bifidobacterium bifidum* probiotic in male Wistar rats. Orthodontic tooth movement was carried out using a nickel titanium coil spring with a force of 10 g applied between the first incisor and the maxillary first molar of a Wistar rat. Samples were then removed on days 3, 7 and 14. Maxillary tissue was isolated for immunohistochemical examination and hematoxylin-eosin staining. All data were analyzed using an independent t-test ($p<0.05$), which was implemented based on Kolmogorov-Smirnov test and Levene test ($p>0.05$). In the study [39], it was proposed to use a queuing network to simulate the diffusion of molecules in accordance with Fick's law. The proposed model was tested using Kolmogorov-Smirnov test to compare the results obtained from the simulation with the theoretical standard deviations obtained based on Einstein-Smoluchowski test. The article [40] develops two different approaches to simulative diagnostic procedures for models of Markov chains based on bands. The first approach uses a formal test based on Kolmogorov-Smirnov or Cramer-von Mises statistics.

The article [41] shows a study to determine the effect of consumption of roasted soyabeans and textured soy protein on the clinical and metabolic status of older women with borderline metabolic syndrome parameters. A randomized single-blinded controlled clinical trial included 75 women aged over 60 years with a diagnosis of metabolic syndrome based on ATP III. Participants were randomly assigned to three groups of 25 people who consumed for 12 weeks: 1) soyabeans; 2) textured soy protein; and 3) control diet. Fasting blood samples were taken at the beginning and end of the study to compare metabolic responses. Kolmogorov-Smirnov test, ANOVA, ANCOVA, paired t-test, and repeated measurements analysis of the generalized linear model were used to evaluate the study results. As a result of the study, it was found that nutrition and physical activity of the participants in the two groups did not differ significantly. After 12 weeks of intervention, the soyabean-treated participants showed significant reductions in total cholesterol ($p<0.001$), low-density lipoproteins, and very-low-density lipoproteins. Thus, short-term consumption of roasted soyabeans and textured soy protein improves lipid profile, markers of glucose intolerance and oxidative stress. Although roasted soyabeans were more effective than textured soy protein. Moderate daily intake of roasted soyabeans as a snack or textured soy protein as a food supplement for individuals with borderline metabolic syndrome parameters may be a safe and useful way to avoid disease progression. The work [42] was aimed at analyzing the consumption of sugar (sucrose) by the low-income population of Brazil. A cross-sectional descriptive study was conducted to evaluate typical customers of a popular restaurant (PR) in Brazil (Brazilian food aid program for low-income people). In the final sample, 1232 adult PR clients were interviewed. Exclusion criteria were pregnant women, diabetics, or people on any special sucrose-restricted diet. People were enrolled at

lunchtime while they waited in line to pick up food. The invitation to participate were made to the first person in the queue, then to the 15th person, and so on until the sampling was complete. A three-day, 24-hour review was used to estimate sugar intake. Sociodemographic and anthropometric data were collected so that client profiles could be compiled. To characterize the sample, a statistical analysis of descriptive data (frequency, mean value, median, percentage and standard deviation) was carried out. Statistical normality tests (Kolmogorov-Smirnov test) were performed for all analyzes to test the assumptions of the statistical tests. The average total energy value (TEV) for the estimated three-day period was 1980.23 ± 726.75 kcal. A statistically significant difference was found between income groups ($p < 0.01$). The northern and northeastern regions have the lowest median income in Brazil, statistically different from the south ($p < 0.01$) and southeast ($p < 0.01$) regions. The northern region showed the lowest sugar consumption from industrial products, in contrast to the northeast ($p = 0.007$), southeast ($p = 0.010$) and south ($p = 0.043$) regions. The north region also has the lowest consumption of home-cooked foods among other regions ($p < 0.001$). Total sugar (sucrose) intake did not differ with body mass index ($p = 0.321$). There was no significant difference in sugar (sucrose) intake over the three days ($p = 0.078$). The addition of sugar (sucrose) contributed to 36.7% of all sugar (sucrose) intake, and sweetened beverages contributed to 22.53% of all sugar (sucrose) intake. Home-cooked products accounted for 20.06% of sugar (sucrose) consumption and industrial products accounted for 22.53% of sugar (sucrose) consumption. Thus, consumption of free sugar (sucrose) is still the largest contributor to total sugar (sucrose) intake, followed by sweetened beverages, especially on weekends. The average percentage of sugar (sucrose) intake exceeds the World Health Organization's recommendation of consuming less than 5% of total energy from sugars. Because this population group has a high percentage of overweight and obesity, sugar (sucrose) consumption may increase health outcomes by increasing public health costs.

The article [43] presents a study assessing the consumption of meat and products obtained from hunting by the consumer population. To do this, a survey was conducted on the frequency of eating meat from the four most representative species in Spain, two large species: wild boar (Sus scrofa) and red deer (Cervus elaphus), as well as two small species: rabbit (Oryctolagus cuuniculus) and red partridge (Alectis rufa), as well as processed meat products (salami sausages) made from the meat of these animals. The survey was conducted on 337 habitual consumers of these products. The overall average per capita meat consumption in this population group is 6.87 kg of meat per year or 8.57 kg of meat per year if processed meat products are also considered. The consumption of rabbit, red partridge, red deer, and wild boar was 1.85, 0.82, 2.28, and 1.92 kg per person per year, respectively. Using probabilistic methods, distributions of meat consumption frequencies were estimated for each of the studied hunted species. The distribution of consumption frequencies was statistically proven by the chi-squared test and Kolmogorov-Smirnov test.

The aim of the study [44] was to describe the nutritional value of food and non-alcoholic beverages advertised in a lineup for children compared to a general lineup on two national private free-to-air television channels in Colombia. The methods chosen were: a cross-sectional descriptive study. The recording was made in July 2012 for four days randomly selected from 6:00 am to 12:30 pm. Nutrient content has been classified according to the Food Standards Agency nutrition profile criteria for nutrients indicating risk, the Pan-American Health Organization for trans fats, and Colombian Resolution 333 dated 2011, which classifies foods as a source of protective nutrients. Descriptive statistics was used, i. e. Kolmogorov-Smirnov test to establish normality and Pearson's chi-squared test to compare variables. The p value of $< 0.05$ was taken into account. As a result, the following data were obtained: 1560 advertising clips were shown in 52 hours of recording, of which 23.3% (364) clips advertised food and drinks, of which 56.3% were shown in a lineup for children. In terms of nutritional value, in the lineup for children, a high percentage of foods and non-alcoholic beverages classified as "rich" in sugar, sodium, saturated fats (69.0%, 56.0%, 57.1%) was noted, compared with the general lineup. In contrast, the percentage of foods and non-alcoholic beverages classified as "rich" in total fat content was higher in the general lineup (70.4% vs. 29.6%, respectively). Thus, in the lineup for children, a large impact of food and non-alcoholic beverage advertising was observed characterized by a high content of high-risk nutrients and a low content of foods.

Thus, the possibilities of nonparametric statistics are shown in the analysis of seemingly incomparable results.

**Conclusion**

The second part discusses nonparametric tests for testing hypotheses of distribution type and nonparametric tests for testing hypotheses of sampling homogeneity. Pearson's chi-squared test, Kolmogorov-Smirnov test, Kolmogorov test were reviewed. Using examples, the use of tests was discussed, and their capabilities and limitations were evaluated. Based on the literature review, brief descriptions of studies in which methods of nonparametric statistics have been successfully applied are given. These tests may be used when comparing descriptive characteristics, which allows statistical processing of the results, for example, tasting evaluation of the product or morphological analysis of the section. Nonparametric methods also allow to compare groups with different unequal number of parameters.

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# CERTAIN FEATURES OF USING MODIFIED COLLAGEN-CONTAINING RAW MATERIALS WITH PROLONGED SHELF LIFE IN FOOD TECHNOLOGY

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**Keywords:** *collagen, modified collagen, cattle rumen, freeze-drying, proteinoids, microstructure, rheological properties*

***Abstract***

*In the current circumstances, trends in nutrition of a person striving to lead a healthy life-style require intake of meat products with the reduced energy value, minimal amounts of fat, increased protein mass fraction, presence of substances improving homeostasis of the body. The synergism of the modern nutrition science and meat industry enables creating food products that satisfy consumers' demand. Today, in the Russian Federation, a theoretical and practical base of the technology development has been collected to the full extent in the field of rational processing of secondary raw materials in the food industry, optimal use of animal secondary raw materials, study of the protein ingredients of animal and plant origin and their deep scientifically substantiated processing, improvement of technological processes and equipment, and correspondently, product range extension. The paper broadens the information about the modified collagen-containing raw materials (cattle rumen), examines physico-chemical characteristics of the collagen-containing raw material and its changes in the process of freeze-drying with a special attention paid to the study of changes in the histological structure. The presence of the relatively uniform fibrillar structure was determined, which facilitated discovering the functional potential of proteinoids that form the fibrillar matrix in the composition of products from different groups. Analysis of IR-spectra revealed several significant absorption bands linked with the state of peptide bonds. The character of bands is linked with the complex of valence and deformation vibrations of the N- and C- types. It is believed that IR-spectra reflect conformations in the protein secondary structure, which suggests preserving properties of the tropocollagen particle or collagen molecule. Freeze-dried modified collagen-containing cattle rumen was tested by the example of jellies. The obtained databank broadens information about physico-chemical properties of modified collagen-containing raw materials (cattle rumen).*

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## 1. Introduction

Nowadays, the questions of population nutrition are a huge physiological and hygienic problem. Materials from studies show that over the last years actual nutrition of certain population groups in the country has been characterized by a decrease in consumption of meat, dairy and fish products, as well as fresh vegetables and fruit. A reduced energy intake with food (91%), especially from animal proteins, should be considered an unfavorable fact. The content of vitamins in diets of certain population groups is 55–60% of the recommended level [1,2].

Decree of the RF Government No.1364-p of June 29, 2016 approved the "Strategy for Improving the Quality of Food Products in the Russian Federation until 2030"[1], according to which it is necessary to develop and introduce innovative resource saving technologies within the framework of processing agricultural resources that would enable extending a range and volume of production of specialized, functional and enriched foods.

Within the same context, the national projects "Public Health" and "Demography" were adopted by Decree of the RF President No. 204 of May 7, 2018 "On national goals and strategic objectives of the development of the Russian Federation for the period up to 2024"[2], which determine the priority of supporting the life quality of the population and development of the healthy society with the aim of achieving life expectancy of 80 years in the short-term period (up to 2030).

At present, a deficiency of dietary fibers, peptides, amino acids and other physiologically active ingredients is observed in the nutrition structure. A solution to this problem to a large extent can be based on the use of the best available techniques on the basis of resource and health saving [1,2,3].

Achievements of biotechnology and its increasing use in the food industry enable not only extending product

[1] Decree of the RF Government No.1364-p of June 29, 2016 "Strategy for Improving the Quality of Food Products in the Russian Federation until 2030"/ http://static.government.ru/media/files/9JUDtBOpqmoAatAhvT2wJ8UPT5Wq8qIo.pdf

[2] Decree of the RF President No. 204 of May 7, 2018 "On national goals and strategic objectives of the development of the Russian Federation for the period up to 2024" https://www.economy.gov.ru/material/file/ffccd6ed40dbd803eedd11bc8c9f7571/Plan_po_dostizheniyu_nacionalnyh_celey_razvitiya_do_2024g.pdf

assortment, but also ensuring their correspondence with the concepts of the theory of adequate nutrition. In the existing economic situation, enterprises manufacturing food products from animal raw materials pay more and more attention to the use of non-traditional sources of raw materials — secondary resources, in particular, those with the high content of connective tissue. It is necessary to note that national and foreign professionals of the industry carry out multifaceted work to create protein products as fat replacers and components that are able to show properties of dietary fibers for their use in meat products, which correlate with the scope of the present work. In particular, according to their functional and physiological properties, collagen and products of its destruction can be assigned to fibrillar, anisotropic, three dimensional food systems, which are a template basis for sorption of certain components.

It was established in the complex investigations that the impact of dietary fibers within the framework of functional food products on the processes of the symbiotic and own human digestion in the gastrointestinal tract leads to an improvement in clinical metabolic (normalization of the functional activity of intestinal microbiota) and anthropometric parameters (reduction of the body weight and waist circumference), which predetermines a possibility to use dietary fibers within programs on treatment and prevention of obesity [4,5].

For example, the studies were carried out to determine sorption properties of collagen fermentolysate relative to heavy metals by the example of $Cd^{2+}$and $Pb^{2+}$ ions. The results allow stating that in terms of the ability to bind $Pb^{2+}$ ions, the product of biomodification of the connective tissue protein is comparable with cellulose, for which sorption of ions of this element was recorded in a range of 0.10–0.23 mg/g [6]. Therefore, the hydrolyzed forms of collagen are able to bind heavy metal ions in the digestive tract with formation of insoluble complexes that are not absorbed and are excreted from the body. This mechanism can be used to prevent poisoning with heavy metal salts.

The process of the combined sorption of several protein components and bioactive substances is also of interest. Systematic study of sequential and combined sorption of several binary protein mixtures and some bioactive substances (for example, ion-exchange components of plant origin — ascorbic acid, iodine) show that the process of binding is complicated by the phenomena of synergism. It has been established that the synergetic phenomena in sorption processes are facilitated by strong binding of protein with certain components of different nature, which is possible to determine by the number of fixed ionogenic groups of a sorbent on a protein molecule. A decrease in the local concentration of ionogenic groups of bioactive components of plant origin favors transition to the synergetic mechanism of competitive sorption. As a result of such sorption of bioactive substances on the matrix collagen base, it is possible to increase preservation of components that are easily destructible at heat treatment such as ascorbic acid and iodine in the organic form up to 70% [7].

It is worth noting that the mechanism of such sorption has not been determined. However, it is known that all proteins are characterized by the pronounced ability to non-specific binding to the SH-groups, guanidine group of arginine and other constituents of amino acids. It is possible that biomodification of connective tissue facilitates disruption of peptide chains of collagen; as a result, the above mentioned functional groups become more available for interaction with metals and biologically active substances [8].

Therefore, modified (chemically, physically or by biomodification) connective tissue is a sorbent with the high activity for heavy metals and biologically active substances and can be used in the future as a functional additive in production of foods, in particular meat products [9,10,11].

An acute need for increasing the human adaptive potential stemmed from the increasingly aggressive impact of both ecological and socio-economic factors causes a necessity among professionals in the industry to create a new generation of food products which should not only provide the body with substances that are essential for the growth, development and vital activity but also stimulate its protective functions.

The aim of the research is the development of the technological solutions in production of jellies based on determination of certain peculiarities in the raw materials occurring in the process of alkali-salt modification and freeze-drying.

## 2. Objects and methods

Objects of the research were samples of cattle rumen (15×15 mm) before and after modification. Food-grade gelatin from beef skin Bloom 220 by Gelnex (USA) was used for comparative analysis of IR-spectra. At the testing stage, beef jellies with/without modified cattle rumen were produced.

Raw materials were subjected to physico-chemical modification, which included preliminary mincing through the cutting plate (2 mm) of a grinder and treatment with a solution of edible salt (6% concentration) and sodium hydroxide with different concentrations: 3% (pH of the solution = 12.05) — sample MR-1; 5% (pH = 12.30) — sample MR –2; 7% (pH = 12.78) — sample MR –3, where MR is modified rumen.

At the stage of the alkali-salt treatment, the hydromodule was 1:2.

Neutralization was carried out using the acetic acid solution with the concentration of 7% to specified pH values (Table 2). After obtaining collagen hydrolysates, the samples were dried using the laboratory test stand SVP-0.36 (Figure 1), which scheme is shown in Figure 2. The principle peculiarity of the construction is the presence of two systems for evacuation and cold supply. The first of them enables realizing the traditional vacuum freeze-drying with the phase transition "ice-steam", the second allows dehydration in the mode of vacuum evaporation.

**Figure 1.** General appearance of the test stand SVP-0.36 [12]

Working parameters of classical freeze-drying:

— Beginning of evacuation: T desubl.= minus 26 °C; $T_{pr1}$ = –10.5 °C; $T_{pr2}$ =11.7 °C; $T_{heat}$ = 22.4 °C;

— Beginning of drying: T desubl. = minus 28 °C; $P_{cham}$ = 83 Pa; $T_{pr1}$ = minus 25 °C; $T_{pr2}$ = minus 24 °C,

where T desubl. is a temperature of the desublimator;

$T_{pr1}$ is a practical temperature 1;

$T_{pr2}$ is a practical temperature 2;

$T_{heat}$ is a temperature of heating.

When determining indicators of the nutrition value, the following methods were used: moisture mass fraction by GOST 9793–2016[3]; protein mass fraction using the semi-automated unit Kjeltec System 1002 «Tecator» (FOSS, Denmark); fat mass fraction by GOST 23042–2015[4]; ash mass fraction by GOST 31727–2012 (ISO 936:1998)[5].

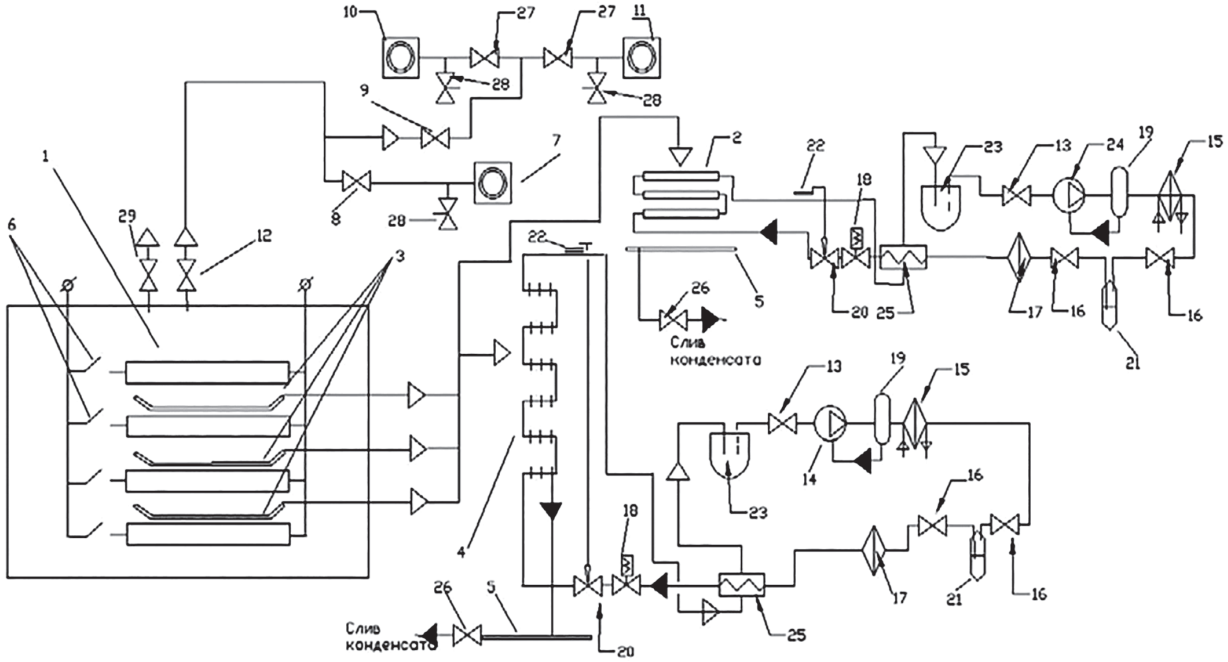



**Figure 2.** Principle scheme of the test stand of vacuum freeze-drying:
1 — electric heaters, 2 — desublimator, 3 — trays with a product, 4 — enclosed condenser, 5 — collection trays for condensate drain, 6 — tumbler switchers on/off of the electric heaters, 7 — vacuum aggregate NVM-5, 8 — valve SK26013–020, 9 — valve SK26013–010, 10 — vacuum aggregate 2NVR-5DM, 11 — vacuum aggregate 2NVR-01DM, 12 — valve SK26013–025, 13 — valve, 14 — compressor SC10G, 15 — air condenser, 16 — valve, 17 — filter-drier, 18 — solenoid valve, 19 — oil separator, 20 — electronic TEV, 21 — receiver, 22 — thermal bulb, 23 — liquid separator, 24 — compressor SC21CL, 25 — heat exchanger, 26 — valve, 27 — valve, 28 — leak valve, 29 — vacuum breaking valve.

The drying graph is presented as Table 1.

**Table 1. Freeze-drying graph**

| Time, h | Weight, kg | T desubl., minus °C | Pressure in chamber, Pa | $T_{pr1}$, °C | $T_{pr2}$, °C | $T_{heat}$ °C | Power, W |
|---|---|---|---|---|---|---|---|
| 10:00 | 17.98 | Beginning of evacuation | | | | | |
| 10:30 | 17.83 | 28 | 83 | minus 25 | minus 24 | — | 200 |
| 11:30 | 17.63 | 34 | 65 | minus 25,5 | minus 25 | 88 | 207 |
| 12:00 | 17.55 | 33 | 65 | minus 25 | minus 25 | 90 | 210 |
| 13:30 | 17.35 | 33 | 66 | minus 14 | minus 12 | 99 | 194 |
| 15:00 | 17.17 | 35 | 55 | 35 | 30 | 95 | 195 |
| 15:45 | 17.14 | 37 | 55 | 45 | 40 | 89 | 140 |
| 16:00 | 17.14 | 37 | 55 | 45 | 40 | 89 | 50 |

When studying a degree of *in vitro* protein digestibility, the method by Pokrovsky-Ertanov (the modified unit of MGUPB, Russia) was used.

A pH value was determined using a pH meter Testo-205 with a pH measurement range of 0.5÷14 (Testo, Germany).

Rheological properties were studied using a rotary viscometer "Polimer RPE-1M" with the system of sensing elements of the rotor-cylinder type T1-V1 (NPO "Chimavtomatika", Russia). A penetration level was measured using a

[3] GOST 9793–2016. "Meat and meat products. Methods for determination of moisture content". Moscow: Standartinform, 2018. — 9 p. (In Russian)

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

[5] GOST 31727–2012 (ISO 936:1998) "Meat and meat products. Determination of total ash". Moscow: Standartinform, 2019. — 11 p. (In Russian)

semi-automated penetrometer PN10 with a conic indenter with a weight of 70 g and a 60-degree angle 2 α; then, the values were recalculated into the ultimate shear stress by Rebinder's equation. Structural-mechanical properties of products, in particular, shear stress and shear strain, were determined on a universal testing machine "Instron-1140" using a Kramer shear press (Instron, USA).

Microstructural examination was performed according to GOST 19496–2013[6] using a light microscope AxioImaiger A1 (Carl Zeiss, Germany), video camera AxioCam MRc 5 and computer system for image analysis AxioVision 4.7.1.0 [13].

In addition, the Fourier transform infrared spectroscopy (FTIR) was applied using a Fourier Transform Infrared Spectrophotometer ALPHA (Bruker, USA) with a module of single attenuated total reflection with the diamond crystal intended for the universal basic spectral analysis in the mid-IR region of 375 to 7500 $cm^{-1}$. Sample preparation consisted in the following: powder-like samples of MR (modified rumen) were applied on the diamond crystal of the interferometer and fixed with a holding-down device; after that, a spectrum was obtained in the automated mode.

The obtained results were processed using the conventional methods of the analysis of variance. Differences in indicators were considered significant at a level of significance interval ≤ 0.05.

## 3. Results and discussion

Cattle rumen in the native state had pH 6.99.

**Table 2. Parameters of cattle rumen modification and physicochemical indicators of samples**

| Indicator | Sample | | |
|---|---|---|---|
| | MR-1 | MR-2 | MR-3 |
| pH of solution for akali-salt hydrolysis | 12.05 | 12.30 | 12.78 |
| pH after acid treatment | 4.50 | 4.51 | 4.68 |
| pH after additional washing | 5.50 | 5.70 | 5.80 |
| Penetration degree (I), units of apparatus, after the main stages of alkali-salt treatment | 23.0 ± 2.0 | 60.0 ± 6.0* | 36.0 ± 4.0 |
| Ultimate shear stress (Θ) after the main treatment stages, kPa | 27.79 | 4.08 | 11.34 |
| I, units after additional washing | 45,0 ± 4,0 | 32,5 ± 3,0* | 46,7 ± 3,0* |
| Θ, kPa, after additional washing | 7.3 | 13.9 | 6.7 |
| Moisture mass fraction after freeze-drying,% | 1.5 | 1.8 | 1.5 |
| Ultimate shear stress after freeze-drying, kPa | $2.8 \cdot 10^3$ | $0.76 \cdot 10^3$ | $1.3 \cdot 10^3$ |

* — P ≤ 0,05 (error probability)

Based on the results presented in Table 2, it is possible to see differences between the samples in rheological indicators (a penetration degree and ultimate shear stress) caused by differences in the hydration properties, interactions of the substrate and moisture according to the principle of swelling. The values of other determined indicators differed insignificantly.

To ensure long-term storage of the modified collagen-containing raw materials and to obtain samples in the powder-like form extending technological possibilities of their use in food product recipes, vacuum freeze-drying was used [14,15,16].

The process was performed to the ultimate moisture of 2%. When studying the indicators of microbiological spoilage, the shelf-life of 1.5 years was established for the obtained freeze-dried products. Preliminary rehydration (1:3) for 30 min. is recommended in meat product manufacture.

When assessing an effect of freeze-drying temperature on the complex of quality indicators of dried products, it was concluded that the most optimal freeze-drying temperature was minus 20 °C. The applied parameters of freeze-drying can be realized in the commercial freeze-drying units. The processes of preliminary freezing and subsequent vacuum dehydration with the phase transition "ice-steam", which are used in freeze-drying, inevitably lead to changes in the structure of capillaries and fibers. These changes decisively influence rehydration of dried samples and preservation of the protein structure.

As a result of freeze-drying, a firm fixed capillary porous structure of samples (xerogel) is formed. The value of ultimate shear stress after drying samples sharply increased (Table 1), which also correlates with the data of the histological studies.

Firm large bundles of collagen fibers arranged tangentially were revealed in the control sample (Figure 3a). Tinctorial properties of sections were comparatively pronounced.

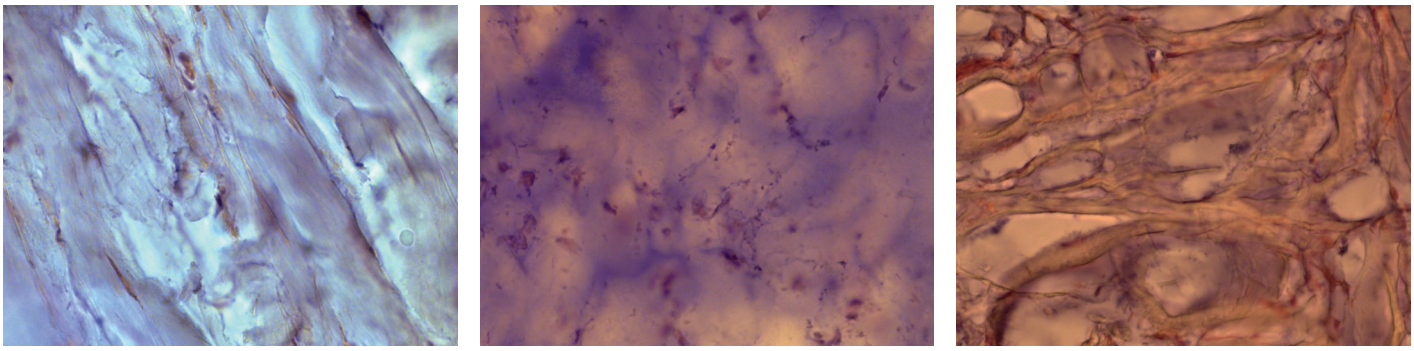
The results of the microstructural analysis proved the hydrolytic destruction of collagen fibers (Figure 3b); the configuration of the section changed, for example, fiber bundles were swollen, transformed into the vitreous state, which correlates with the process of thermotropic gelation upon heat treatment [17,18,19,20].

As one of the tasks was to substantiate a possibility to include vacuum freeze- drying, which forms specific microdisperse systems of food systems, into the technological process of raw material processing, the structure after drying was studied (Figure 3c). Xerogel-type structures with cells of the disperse phase and capillaries, in which liquid was retained, were revealed in different layers.

Peculiarities of the microstructure suggest the manifestation of the fibrillar structure, presence of capillaries, which can facilitate quick rehydration of samples, and, when necessary, the use of comminution to the powder-like or fiber-like state and more rational use as a component — a source of a dietary fiber analog.

At the next stage of the work, the task of studying changes in the samples at the molecular structure level was solved by the infra-red spectroscopy using Fourier Transform Infrared Spectrophotometer ALPHA-P.

[6] GOST 19496–2013 "Meat and meat products. The method of histological study" Moscow: Standartinform, 2019. — 12 p. (In Russian)



a b c

**Figure 3.** Histological structure of samples: native cattle rumen (a, ×200), rumen after physico-chemical modification (b, ×200), freeze-dried modified rumen (c, ×200)

As a result of the analysis of the IR-spectra (Figure 4 and Figure 5), it was established that the studied samples (control, MR-2) corresponded to the proteinoids of the connective tissue, the control to proteins of the gelatin group, according to the database incorporated into the computer of the spectrometer.

It is known that peaks characteristic of valence and deformation vibrations of different groups (–OH, $CH_n$, >C=O, ether groups and several others) can be found in infrared spectra.

The obtained IR-spectra in the control and modified collagen-containing raw materials (for example, MR-2) were similar; however, the peak heights varied. Therefore, taking into account the special features of the initial raw materials, we consider it expedient to compare the results with those obtained for the known protein structures. It is reasonable to compare the results of IR-spectroscopy with several domestic and foreign analogs. It is known that IR-spectra of polypeptides, proteinoids and products of their modification contain several intensive absorption bands. With that, changes linked with vibrations of amide CONH-groups, general structural elements of proteinoid molecules, were revealed.

Several absorption bands are known: amide-A (absorption band ≈ 3300 $cm^{-1}$), amide-B (≈3100 $cm^{-1}$), amide-I (1600–1700 $cm^{-1}$), amide-II (≈1500–1600 $cm^{-1}$) and so on. In principle, the corresponding peaks of absorption of IR-radiation in these ranges of wave numbers were observed in the MR samples. However, shift of several peaks to the right was observed for the samples from the studied series upon intensification of raw material processing (Figure 4 and Figure 5).

For example, in the range of 600–800 $cm^{-1}$, it is possible to reveal an interaction with sulfates upon "soft" acid hydrolysis. In our case, the complex processing with alkali and then acid was used, which apparently increased the polarity of protein groups and the corresponding height of the absorption band.

For comparison, we obtained the IR-spectrum for foreign food-grade gelatin (Figure 6) represented by more smoothed line, apparently due to both purification and fractional melting of proteins on the automated line of an enterprise for production of high-grade gelatin.

IR-spectra, several strong absorption bands linked with the state of peptide bonds were visualized. The character of the bonds was associated with the complex of valence and deformation vibrations of N–H and C–H types. It is believed that IR-spectra reflect well the conformation in the protein secondary structure; consequently, it is possible to expect the preservation of the properties of the tropocollagen particle or collagen molecule [21,22].

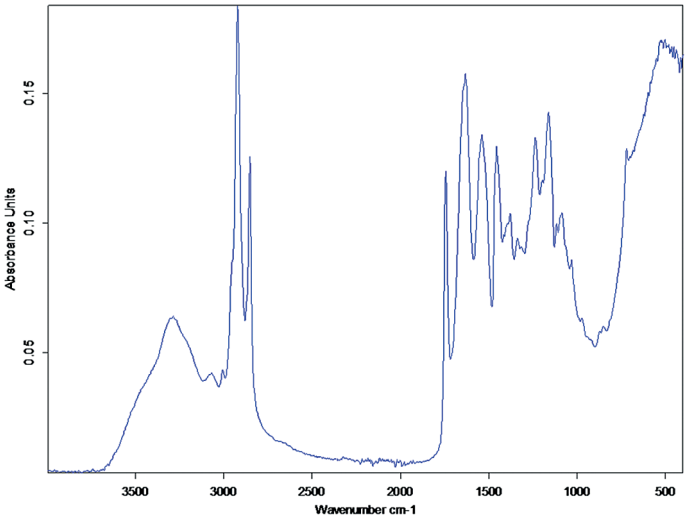



**Figure 4.** Infrared absorption spectrum of cattle native rumen (control)

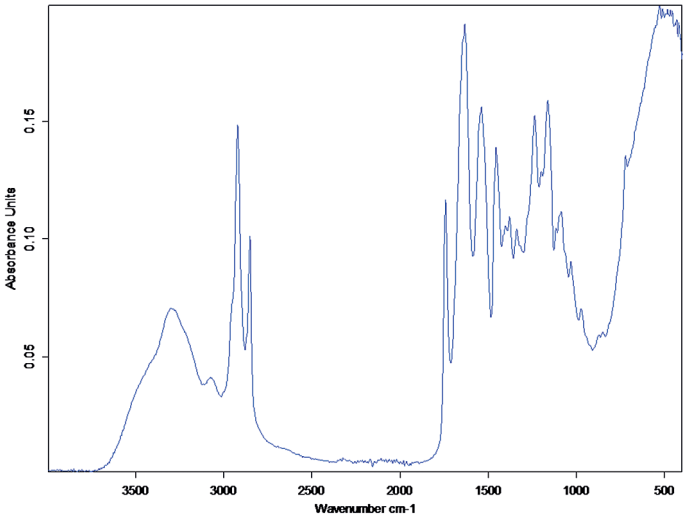



**Figure 5.** Infrared absorption spectrum of sample MR-2

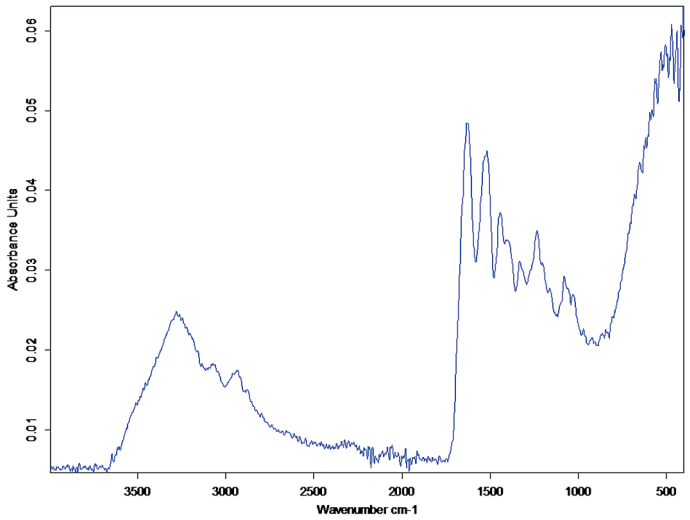



**Figure 6.** Infrared absorption spectrum of foreign food-grade gelatin

Differences found in comparison with the gelatin food additive were linked with more complex chemical composition of the studied raw materials and supramolecular-oriented architectonics of fibrils and bundles of collagen fibers. However, the comparison of the results of investigations and literature data show that, in general, the spiral conformation of proteinoids retained, which allows using the whole potential of functional and technological properties of the raw material [23].

Therefore, positive changes were established in raw materials, in particular, an increase in the lyophilic properties, defatting, bleaching, "opening" of the chemical bonds of protein. As a result of using freeze-drying with the regimes recommended by us, the longest shelf-life of the protein module is ensured. In this work, MR-2 was considered the most rational option for raw material processing (Table 2).

As fibrillar animal proteins and products of their modification are able to form jellies with significant strength, testing was carried out by producing jellies (Table 3). As a control sample, we used the beef jelly recipe; the experimental sample contained the obtained freeze-dried modified rumen instead of food-grade gelatin, which enables intensifying gelation process and enriching a product with dietary fiber analogs due to an increase in the activity of the functional groups of biopolimers (collagen) as a result of its modification.

**Table 3. Recipes of processed jellies**

| Name | Indicators of raw material input for model systems, g | |
|---|---|---|
| | Control | Experiment |
| Beef | 350/175* | 350/175 |
| Food-grade gelatin | 12 | – |
| Modified cattle rumen | – | 34 |
| Carrot | 20 | 20 |
| Onion | 20 | 20 |
| Black pepper | 0.25 | 0.25 |
| Aromatic composition | 0.25 | 0.25 |
| Water | According to the recipe collection | |
| Total, g | 500 | 500 |

* Meat weight before and after heat treatment

The technology included cooking of the meat raw material (beef) for 3–3.5 hours. Vegetables (carrot, onion and spices) specified by the recipe were added an hour before the end of cooking. Then, meat was minced on a grinder and mixed with a broth, 2% edible salt was added and the mixture was brought to the boil with intermittent stirring. The rehydrated sample of the modified cattle rumen was added after meat mincing [24,25,26].

The obtained solutions were poured into molds and placed into a refrigeration chamber for thermotropic gelation. The recipes are variable in regard to spice addition; for example, garlic, parsley, aromatic compositions and so on can be added.

It was noticed that gelation occurred in a temperature range of 15.7–16.5 °C, which approximately corresponds to the data of A. Veis, who studied gelatins of different types [27,28]. The experimental jelly was formed faster also at a temperature of 16.5 °C, while the control was formed less intensively at 15.7 °C. This was especially obvious for the thin jelly layer (≈10 mm).

The histological structure of jelly represented a firm mass with sufficient electronic density. Pores with insignificant sizes (about 35 μm in diameter) were seen on the surface (Figure 7). In our opinion, generation of the porous structure is linked with the molecular structure of the disperse phase. As is well known, gelation is initiated by formation of a biopolymer molecule framework, which cells can contain water, solutions, fat, minor food compounds, which can be used for product enrichment with bioactive substances [29,30,31,32].

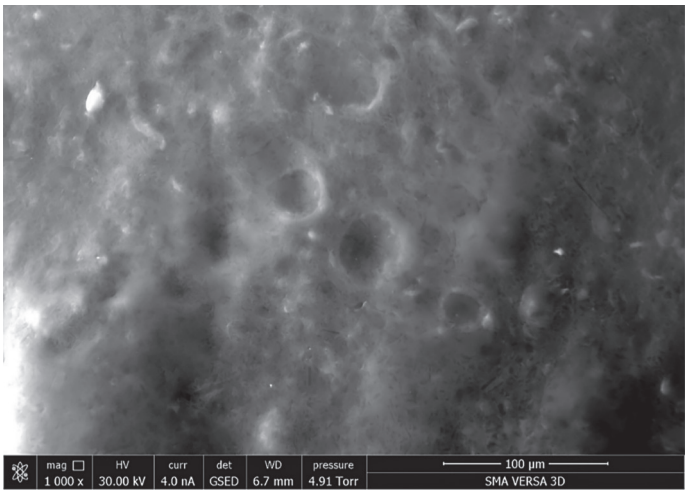



**Figure 7.** Electron diffraction pattern of jelly × 1000

Light microscopy images of the same samples show that the main part is represented by a homogeneous basophilically stained mass of hydrolyzed collagen, in which particles of connective tissue that more or less retained the structural organization of fibers (cellular elements were not revealed, fibers were considerably loosened), as well as individual fragments of muscle fibers were observed. At the same time, the elements of connective and muscle tissues were visualized better in the control sample compared to the experimental one.

Chemical composition and physico-chemical indicators of the produced jellies are presented in Table 4.

**Table 4. Chemical composition and physico-chemical indicators of jellies**

| Indicator | Samples | |
|---|---|---|
| | Control | Experimental |
| Moisture mass fraction, % | 78.0 ± 3.9 | 74.0 ± 3.7* |
| Protein mass fraction, % | 9.6 ± 1.0 | 11.3 ± 1.1* |
| Fat mass fraction, % | 10.2 ± 1.0 | 10.6 ± 1.1* |
| Ultimate shear stress, kPa | 1.2 ± 0.1 | 1.5 ± 0.2 |
| Gelation temperature, °C | 15.7 ± 0.5 | 16.5 ± 0.5 |

* — P ≤ 0,05 (probability of an error between the control and experimental samples)

Indicators of digestibility are shown in Figure 8.

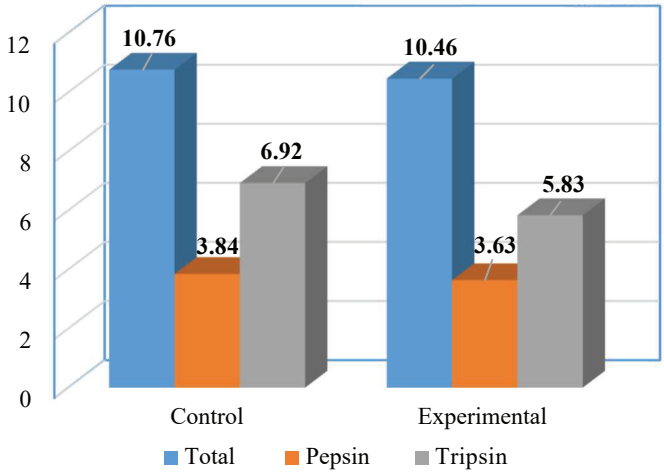



**Figure 8.** Indicators of *in vitro* digestibility of the samples, mg tyrosin/100 g protein

The energy value was 130.2 kcal/100 g in the control sample and 140.6 kcal/100 g in the experimental one. It is necessary to note that the developed jellies are a source of components (modified collagen) which exert features of dietary fibers imparting dietetic properties to products. Ultimate shear stress corresponded to the similar indicator for meat systems, structured with animal protein; the experimental sample was superior in terms of this indicator.

## 4. Conclusion

The complex analysis of the experimental results including the data from physical, chemical, histological, spectral methods for animal raw material investigation enables making a conclusion about formation of disperse food systems that corresponds to the set tasks.

The performed experiments prove conclusively a possibility and expediency of using secondary products from the meat industry to obtain biopolymer components of the multifunctional purpose. Taking into consideration formation of quite a firm, monolithic disperse system of the modified collagen-containing raw material that fixes species and other ingredients, as well as a complex of its physico-chemical indicators, it is possible to make a conclusion about a possibility to develop experimentally a broad range of foods, including meat-based products.

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# RESEARCH OF COMPOSITIONS OF AMINO ACIDS, FATTY ACIDS AND MINERALS IN MEAT PATE WITH ADDITION OF MEAT-AND-BONE PASTE

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**Keywords:** *pate, meat-and-bone paste, chicken bones, nutritional value, calcium*

***Abstract***
*This article analyses the nutritional value of meat pate produced with the addition of meat-and-bone paste obtained from chicken bones. In the test samples of the pate, 20% of the poultry meat was replaced with the meat-and-bone paste. The comparative characteristic of the chemical, amino acid, fatty acid and mineral compositions of meat pate is given in the article. The comparative analysis of the nutritional value of meat pate showed that the addition of meat-and-bone paste decreases the moisture content by 0.23%, fat content by 1.22%, and increased the protein content (by 0.52%). In the test sample of the product the proportion of minerals increased significantly from 1.3% to 2.23% compared to the control sample. In terms of amino acid composition, the addition of meat-and-bone paste up to 20% instead of poultry meat significantly increases the content of amino acids like isoleucine (from 196 mg/100 g to 661 mg/100 g), leucine (from 807 mg/100 g to 1083 mg/100 g), threonine (from 454 mg/100 g to 610 mg/100 g). The test samples of pate, compared with the control samples, contain a higher amount of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, including oleic (39.698%) and linoleic (21.546%) acids. The content of the saturated fatty acids (SFA) in the control sample are 37.8%, in the test sample it accounts to 32.9%. According to the mineral composition: the content of calcium is significantly increased in the test sample, (from 268.0 mg/100 g to 480.0 mg/100 g). In general, the addition of meat-and-bone paste made of chicken bones allows fortification the pate with the essential amino acids, mono- and polyunsaturated fatty acids and calcium.*

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## Introduction

Pate is a homogenized food product with a predominant content of meat or liver. The principle of pate manufacturing is based on combining of various types of products, as well as methods of their processing (boiling, blanching, sauteing, frying, homogenization, etc.) depending on the recipe [1]. The composition of meat pates can provide a significant impact on their nutritional characteristics. The specialized literature describes a wide variety of meat pate recipes where chicken offal is added. These types of pate are sold on the world market.

In the market of meat pate kinds, chicken pates are becoming more and more popular due to the increase in the global production of poultry meat. Along with the increase in consumption of poultry meat, the production of by-products obtained after the poultry slaughter is increasing simultaneously. The range of by-products include feathers, blood, bones, skin, viscera, offal, glands, limbs and various fatty tissues [2]. From chicken meat, in the production of pate, chicken breasts, chicken meat are used [3.4.5.6.7.8.9.10], secondary products obtained during slaughter and primary processing of poultry — muscular stomachs, liver, heart, heads, legs (up to 30%) [11,12,13,14,15,16,17,18,19]. However, the meat-and-bone offal obtained from the slaughter of poultry still remains unclaimed. The main barriers to the use of bone raw materials for food purposes in production is the lack of a proven technology for processing bone raw materials and lack of special equipment.

Nevertheless, in terms of nutritional value, first of all raw bone is a rich source of minerals. The main components of bone tissue are mineral substances, which make up to ¼ of the volume, or ½ of the tissue mass, which mineral substances are mainly represented by calcium salts of carbonic and phosphoric acids, magnesium salts of phosphoric acid and even less calcium fluoride are found in a smaller amount [20]. About 99% of all calcium is found in the skeleton. The elemental composition of the ash elements of the bone tissue is characterized by the following data (in %): CaO — 52; MgO — 1.2; $P_2O_5$ — 40.3; $Na_2O$ — 1.1, $K_2O$ — 0.2; Cl — 0.1; F — 0.1; $CO_2$–5.0 [21]. In addition, food bone is high in fat,

protein and phosphorus-calcium compounds. The chemical composition of the bone is as follows: water — 13.8–44.4%, protein (collagen) — 32.0–32.8%, minerals — 28.0–53.0%, fat — 1.3–26, 9% [22]. The fat obtained from various types of bone differs in the content of lecithin and unsaponifiable substances (they are found in the greatest amount in pork bone fat). In terms of the content of saponifiable substances (0.2–0.3%), edible bone fat is much superior to fats obtained from soft fat raw materials from the same types of livestock [23]. The main organic part of the bone is the bone collagen (ossein); it accounts for 93% of the total amount of bone proteins, the rest is glycoproteins, lipids and glucosaminoglycans (chondroitin sulfate, keratan sulfate and other glucosamines and galactosamines). The composition of ossein (%): moisture 70, proteins 25–28, minerals 3, fats 0.2 [24].

The use of bone raw materials for food purposes is one of the key areas of sustainable saving of raw materials in the processing of animal food products. The processes of fine and ultrafine grinding of bones make it possible to obtain a pasty mass without either large and small fractions of bone particles [25,26].

The purpose of this article is to study the chemical, amino acid, fatty acid and mineral compositions of meat pate with the addition of chicken bone meat-and-bone paste.

## Materials and methods

*Obtaining a paste-like mass from chicken bones*

At the initial stage of the test research, a technological scheme for processing chicken meat-and-bone raw materials was developed. To conduct research on the grinding of meat-and-bone raw materials, chicken bones (bones of the neck, drumstick, wings, breast) obtained after deboning were used. The chicken meat-and-bone raw materials were deboned at the first stage. Chicken meat-and-bone raw materials were obtained from meat processing enterprises and large meat plants in Semey city, East Kazakhstan region of the Republic of Kazakhstan.

Next meat-and-bone raw materials are pre-frozen for 60 minutes at temperatures within minus 18 °C to minus 20 °C in the freezers. After that, the frozen raw material is fed into the hopper of the crusher with a hole diameter of the output grate of 5 mm, and crushed. After grinding the chicken meat-and-bone raw materials, the meat-and-bone minced mass was obtained. After that, the meat-and-bones minced mass is added into a meat mixer along with the ice water. After mixing, minced meat-and-bones is crushed on a micro grinder "Supermasscolloyder", where the gap between the grinding wheels is 0.1 mm. After grinding the mass in the micro grinder, a homogeneous chicken meat-and-bone paste without bones, coarse connective tissue, blood clots and films is obtained [25].

*Technology of the meat pate production*

The technological process of meat pate production with the addition of meat-and-bone paste consists of receiving raw materials, blanching, grinding, cutting, filling shells, boiling loaves, cooling loaves, packaging, labeling and storage. The formulation is presented in Table 1.

**Table 1. Recipe for meat pate**

| Raw material | Control sample | Test sample with 20% of meat-and-bone paste |
|---|---|---|
| Poultry meat | 60.70 | 40.70 |
| Beef liver | 17.60 | 17.60 |
| Meat-and-bone paste | — | 20 |
| Pork fat | 5.30 | 5.30 |
| Onion | 6.30 | 6.30 |
| Carrot | 5.70 | 5.70 |
| Parsley (dry root) | 0.60 | 0.60 |
| Ground black pepper | 0.05 | 0.05 |
| Cooking salt | 1.05 | 1.05 |
| Broth | 2.70 | 2.70 |
| Total | 100 | 100 |

Raw materials received for production are tested in accordance with the current technical conditions and standards. Next, the liver is trimmed, cover film, bile ducts and other inclusions are removed. After trimming, the raw materials are soaked in running water for 2 hours to remove blood clots. The raw veined liver is cut into slices and blanched at 105 °C (water to liver ratio is 3:1) for 15–20 minutes. Before cutting, the raw material is ground on a grinder with a grate opening diameter of 2–3 mm. The chopped meat is sent for cutting at a temperature of 10–12 °C for 3–4 minutes.

Pate preparation. Beef liver, meat-and-bone paste, bacon, spices are pre-weighed. The components of the pate are mixed in a cutter with a sequential adding of raw materials: first, poultry meat, beef liver, then salt and pepper are added. The duration of cutting is 3–7 minutes. The second stage provides for addition of the fatty meat raw materials, meat-and-bone paste, onions, carrots. At the end of cutting, the paste mass should be homogeneous, spreadable and pasty mass.

Filling shells with minced meat (forming loaves). The shells are filled with minced meat with vacuum fillers. After filling the pate masses are sent for heat treatment. The meat pate is cooked in the heat chambers at a temperature of 80–85 °C until the temperature in the center of the loaf reaches 72–75 °C. After cooking, meat pate are cooled in the cooling chambers with a temperature of 0 to 6 °C and a relative humidity of 95% until the temperature in the mid of the pate loaf reaches no higher than 6 °C.

*Determination of the chemical composition*

The total chemical composition was defined by the method of one portion of the test sample. The method consists in successive determination of moisture, fat, protein and ash content in one sample of the product using a de-

vice for determining the moisture and fat content of meat and dairy products via accelerated method [27].

*Determination of amino acid composition*

The amino acid composition was determined using high performance liquid chromatography[1]. The method is designed to determine the concentration of 18 amino acids in the food products, using high-performance liquid chromatography with the help of a computer system for information recording, processing and storing.

*Calculation of amino acid score*

Amino acid score was calculated by the following formula:

$$\text{AKC} = \frac{m_1}{m_2} \cdot 100\%, \quad (1)$$

where:

$m_1$ — is the content of essential amino acid in the test product, g/100 g of protein;

$m_2$ — content of an essential amino acid in an ideal protein, g/100 g of protein.

To assess the balance of essential amino acids relative to the reference protein, the coefficient of rationality *Rc* was calculated by the following formula:

$$Rc = \frac{\sum_{i=1}^{n} A_i K_i}{\sum_{i=1}^{n} A_i}, \quad (2)$$

where:

*Ai* — content of the essential *i-th* amino acid, mg/g of protein;

*Ki* — utility coefficient of the *i-th* amino acid.

*Determination of fatty acid composition*

The fatty acid composition was determined by the method of gas chromatographic determination of fatty acids and cholesterol in food and blood serum[2]. The technique is designed to determine the composition of fatty acids (myristic palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidonic acids) and total content of cholesterol in the food products, their mixtures (rations) and blood serum.

*Determination of the mineral composition*

Mineral composition were determined according to regulatory documents R4.1.1672–03[3] "Guidelines for quality control and safety of biologically active food additives", GOST 26928–86[4] "Food-stuffs. Method for determination of iron", GOST 33824–2016[5] "Foodstuffs and food ingredients. Stripping voltammetric method for determination of toxic elements (cadmium, lead, copper and zink)".

*Statistical processing*

The results of measurements were processed in Excel-2016 and Statistica software. The results of the analyzes were statistically significant at $p \leq 0.05$. Data are presented as mean value ± standard deviation.

**Results and discussion**

The chemical composition of meat pate is presented in the Table 2. The results show that the addition of meat-and-bone paste leads to a decrease in moisture content (by 0.23% and fat by 1.22%), an increase in protein (by 0.52%). In the test sample, the proportion of minerals increased significantly from 1.3% to 2.23% in comparison with the control sample.

**Table 2. Chemical composition of meat pate**

| Parameters | Control sample | Test sample with 20% meat-and-bone paste |
|---|---|---|
| Moisture, % | 59.76 ± 0.81 | 59.53 ± 0.94 |
| Protein, % | 17.46 ± 0.28 | 17.98 ± 0.31 |
| Fat, % | 21.48 ± 0.38 | 20.26 ± 0.33 |
| Ash, % | 1.30 ± 0.02 | 2.23 ± 0.03* |
| Energy value, kcal | 263.16 | 254.26 |

* $p < 0.001$

At the next stage the amino acid composition of meat pate was researched. The results are presented in the Table 3 below. From the Table 3 it can be seen that the content of essential and non-essential amino acids prevails in the test sample of meat pate, which pate was produced with addition of meat-and-bone paste, than in the control sample.

As it follows from the above given data, the total amount of essential amino acids in the control sample of the meat pate was 3,889 mg/100g, the test sample — 5,209 mg/100g. The predominant essential amino acids were as follows: leucine (807 and 1,083 mg/100 g); lysine (727 and 996 mg/100 g) and valine (584 and 764 mg/100 g). The total amount of amino acids in the control sample of meat pate was 10,486 mg/100g, in the test sample it was 13,793 mg/100g.

According to the amino acid composition, the addition of meat-and-bone paste up to 20% instead of poultry meat increases its content of all amino acids. Thus, the content of isoleucine increased (from 196 mg/100 g to 661 mg/100 g), leucine (from 807 mg/100 g to 1083 mg/100 g), threonine (from 454 mg/100 g to 610 mg/100 g).

[1] Measurement procedure MN1363–2000 "Method of determination of amino acids in food products using high-performance liquid chromatography". Approved by the Chief State Sanitary Doctor of the Republic of Belarus on July 14, 2000.

[2] Measurement procedure MN1364–2000 "Method of gas chromatographic determination of fatty acids and cholesterol in food and blood serum". Approved by the Chief State Sanitary Doctor of the Republic of Belarus on July 14, 2000.

[3] R4.1.1672–03 " Guidelines for quality control and safety of biologically active food additives". Retrieved from https://www.rospotrebnadzor.ru/upload/iblock/33e/r-4.1.1678_03.pdf Accessed December 11, 2020. (In Russian)

[4] GOST 26928–86 "Food-stuffs. Method for determination of iron". Moscow: Standartinform, 2010. — 6 p. (In Russian)

[5] GOST 33824–2016 "Foodstuffs and food ingredients. Stripping voltammetric method for determination of toxic elements (cadmium, lead, copper and zink)". Moscow: Standartinform, 2016. — 23 p. (In Russian)

**Table 3. Amino acid composition of meat pate, mg/100g of the product**

| Name | Meat pate (control) | Meat pate (test) |
|---|---|---|
| 1 | 2 | 3 |
| Essential amino acids | 3,889 | 5,209 |
| Valine | 584 ± 12 | 764 ± 25* |
| Isoleucine | 196 ± 3.4 | 661 ± 14* |
| Leucine | 807 ± 13 | 1083 ± 24* |
| Lysine | 727 ± 22 | 996 ± 29* |
| Methionine | 196 ± 4.2 | 277 ± 4.4* |
| Threonine | 454 ± 10 | 610 ± 10 * |
| tryptophan | 150 ± 4.9 | 196 ± 5.7* |
| Phenylalanine | 471 ± 11 | 622 ± 22* |
| Non-essential amino acids: | 6597 | 8584 |
| Aspartic acid | 1,099 ± 15 | 1,423 ± 30* |
| Glutamic acid | 1,792 ± 41 | 2,233 ± 58** |
| Serine | 572 ± 18 | 722 ± 21* |
| Histidine | 287 ± 4.6 | 368 ± 9.9** |
| Glycine | 545 ± 13 | 763 ± 14* |
| Arginine | 747 ± 15 | 963 ± 25* |
| Alanine | 529 ± 11 | 761 ± 19* |
| Tyrosine | 339 ± 6.6 | 467 ± 9.5* |
| Cysteine | 156 ± 3.4 | 195 ± 6.3** |
| Proline | 531 ± 11 | 689 ± 13* |
| Total amount | 10,486 | 13,793 |

* $p < 0.001$; ** $p < 0.01$

According to the amino acid composition of meat pate, the content of essential amino acids was researched by comparative analysis in the reference protein recommended by the FAO/WHO amino acid scale (Table 4), and the amino acid score of proteins was calculated, which determines the ratio of the content of each essential amino acid in the analyzed protein to their content in reference value.

**Table 4. Amino acid composition of the meat pate**

| Amino acid | FAO/WHO reference value (mg/100 g) | The content of essential amino acids, mg per 100 g of protein | | Amino acid score,% | |
|---|---|---|---|---|---|
| | | control sample | test sample | control sample | test sample |
| Isoleucine | 4,000 | 1,122.56 | 3,676.31 | 28.06 | 91.91 |
| Leucine | 7,000 | 4,621.99 | 6,023.36 | 66.03 | 86.04 |
| Lysine | 5,500 | 4,163.80 | 5,539.48 | 75.70 | 100.72 |
| Methionine + Cystine | 3,500 | 2,016.03 | 2,625.14 | 57.60 | 75.00 |
| Phenylalanine + Tyrosine | 6,000 | 4,639.17 | 6,056.73 | 77.32 | 100.94 |
| Threonine | 4,000 | 2,600.23 | 3,392.65 | 65.00 | 84.82 |
| Tryptophan | 1,000 | 859.10 | 1,090.10 | 85.91 | 109.01 |
| Valine | 5,000 | 3,344.78 | 4,249.16 | 66.89 | 84.98 |
| General content of essential amino acid | | 23,367.66 | 32,652.9 | | |
| Protein content, g/100 g | | 17.46 | 17.98 | | |

The calculation of the amino acid score showed that all essential amino acids in the control sample of the meat pate are limiting amino acids (LAA). In the test sample, all amino acids are limiting, except for lysine (100.72%), phenylalanine + tyrosine (100.94%) and tryptophan (109.01%).

The high amino acid score was recorded in a test sample of the meat pate. Thus, the highest amino acid score is calculated for tryptophan 109.01%.

At the next stage, the fatty acid composition of meat pate was studied. The biological value of new kinds of pate can be judged by the balance of the fatty acid composition of the lipid components in the product (Table 5).

**Table 5. Fatty acid composition of meat pate**

| Name of the acid | Meat pate (control) | Meat pate (test) |
|---|---|---|
| 1 | 2 | 3 |
| Saturated fatty acids, % | 37.865 ± 1.893 | 32.995 ± 1.650 |
| $C_{14:0}$ myristic | 1.014 ± 0.051 | 0.687 ± 0.034 |
| $C_{15:0}$ pendadecanoic | 0.147 ± 0.007 | 0.133 ± 0.007 |
| $C_{16:0}$ palmitic | 24.252 ± 1.219 | 20.884 ± 1.044 |
| $C_{17:0}$ margarine | 0.368 ± 0.018 | 0.309 ± 0.015 |
| $C_{18:0}$ stearic | 11.903 ± 0.595 | 10.747 ± 0.537 |
| $C_{20:0}$ arachidic | 0.180 ± 0.009 | 0.180 ± 0.009 |
| $C_{21:0}$ geneucosan | — | 0.025 ± 0.001 |
| $C_{22:0}$ beguine | — | 0.030 ± 0.002 |
| Monounsaturated fatty acids, % | 40.333 ± 2.017 | 43.308 ± 2.165 |
| $C_{14:1}$ (cis-9) myristoleic | 0.049 ± 0.002 | 0.046 ± 0.002 |
| $C_{16:1}$ (cis-9) palmitoleic | 3.029 ± 0.151 | 2.834 ± 0.142 |
| $C_{17:1}$ (cis-9) margarinoleic | 0.212 ± 0.011 | 0.163 ± 0.008 |
| $C_{18:1}$ (cis-9) oleic | 36.321 ± 0.151 | 39.698 ± 1.985 |
| $C_{20:1}$ (cis-9) eicosene | 0.484 ± 0.024 | 0.381 ± 0.019 |
| $C_{24:1}$ (cis-15) celacholic | 0.239 ± 0.012 | 0.254 ± 0.013 |
| Polyunsaturated fatty acids, % | 21.802 ± 1.090 | 23.625 ± 1.181 |
| $C_{18:2n6t}$ linoleidine | 0.057 ± 0.003 | 0.042 ± 0.002 |
| $C_{18:2n6c}$ linoleic | 19.692 ± 0.985 | 21.546 ± 1.071 |
| $C_{18:3n6}$ Y-linolenic | 0.088 ± 0.004 | 0.098 ± 0.005 |
| $C_{18:3n3}$ linolenic | 0.857 ± 0.043 | 0.842 ± 0.042 |
| $C_{20:2}$ eicosadiene | 0.325 ± 0.016 | 0.292 ± 0.015 |
| $C_{20:3n6c}$ (cis-8.14.17) eicosatriene | — | 0.020 ± 0.001 |
| $C_{20:3n3c}$ (cis-11.14.17) eicosotriene | 0.199 ± 0.010 | 0.196 ± 0.010 |
| $C_{20:4n6}$ arachidonic | 0.583 ± 0.029 | 0.587 ± 0.029 |

The important criterion for the biological and nutritional value of products is the qualitative and quantitative composition of lipids. Fatty acids are the main component of the lipids.

Analysis of the fatty acid composition of pate lipids showed that pate samples had a high content of oleic,

palmitic, and linoleic acids. The presence of these acids in large quantities is due to the use of pork fat in the pate recipe. Test pate samples, compared to control, contain a greater amount of monounsaturated and polyunsaturated fatty acids, including oleic (39.698%), linoleic (21.546%).

From the Table 5 it can be seen that saturated fatty acids (SFA) in the control sample amounts to 37.8%, in the test sample — to 32.9%. Among the SFAs palmitic acid is especially prominent (24.252% and 20.884%, respectively). The test sample also contains a small amount of heneicosanoic (09.025%) and behenic (0.030%) fatty acids.

EFAs are used by the body as an energy material. SFA are used by the body as an energy source and their excess in the diet leads to a violation of fat metabolism and an increase of blood cholesterol [28,29]. Monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), especially linoleic, linolenic and arachidonic acids, are of great importance. Among MUFAs, oleic acid occupies high share (36.321% and 39.698%). Oleic acid lowers plasma cholesterol levels and protects against cardiac arrhythmias. PUFAs are an essential component of cell membranes. They participate in the renewal of cells and intracellular metabolic processes in the body; they contribute to removal of cholesterol from the blood, which prevents the development of atherosclerosis [30,31]. Among the PUFAs in the control and test samples, linoleic acid occupies high share.

A slightly lower amount of saturated fatty acids in the test sample with chicken meat-and-bone paste is caused by the lower melting point of the chicken bone fat = 33 °C ± 0.5, compared to pork = 37 °C ± 0.5. Bone pastes feature high content of phospholipids in comparison with the other animal fats. It is explained by the presence of bone marrow in the paste. Lecithin is one of the main phospholipids, which is involved in cholesterol metabolism, promotes the removal of high-density cholesterol from the body. Bone fat has the highest degree (about 97%) of digestion in the body compared to other animal fats (pork — 90...96%, beef — 73...83%) [32].

In the next stage the mineral composition of the meat pate (Table 6) was investigate. As for the mineral composition, the calcium content is higher in the test sample (480 mg/100g) in comparison with the control sample (268 mg/100g).

Calcium, which comes into a body with food, is absorbed by only 20–30%, and the process of its digestion is quite complicated. The degree of digestion of this macro element depends on the form of its compounds, on the composition and properties of the food products, the pH value of gastric juice and a whole number of the other factors. Calcium in food is represented in the form of sparingly water-soluble or completely water-insoluble salts — mainly carbonates, oxalates, phosphates.

**Table 6. Mineral composition of meat pate**

| Mineral substances, mg/100 g | Meat pate (control) | Meat pate (test) |
|---|---|---|
| Calcium | 268 ± 7.2 | 480 ± 8.7* |
| Magnesium | 40 ± 1.2 | 13 ± 0.3* |
| Iron | 2.23 ± 0.05 | 1.65 ± 0.04* |
| Copper | 0.006 ± 0.001 | 0.008 ± 0.001 |

* $p < 0.001$

The inhibitory effect of phosphates on digestion and absorption of calcium is caused by non-observance of the optimal ratio of calcium and phosphorus (1:1) in most kinds of food, especially meat products. The introduction of calcium in its optimal amount into the recipe of meat pate will normalize the ratio of calcium and phosphorus, which is not physiologically balanced in the raw meat itself, and is even more violated being exposed to the action of phosphates.

## Conclusion

Thus, as a result of the conducted research, the technology and recipe for production of meat pate with the addition of chicken meat-and-bone paste was proposed. It was shown that the addition of meat-and-bone paste decreased the moisture and fat content and increased protein content. At the same time, the proportion of minerals, in particular calcium, increased significantly. According to the results of the analysis of the chemical substances, amino acids, fatty acids and mineral compositions, it was shown that the optimal amount of meat-and-bone paste introducing into the recipe of meat pate is 20%, instead of poultry meat. The production of new kinds of pate with meat-and-bone paste allows for a more complete and rational use of secondary raw materials, increase in the profitability of their industrial production and increase of sales profits.

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