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Editorial
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Editor's column

The history of humankind pales into insignificance in comparison with the immense scale of the evolutionary history of life on Earth. However, the astounding rate of this change makes human species unique among other animals. Evolution is a process that knows no directions and does not always mean progress. Evolution occurs through natural selection, gene flow, genetic drift, and mutations. The milestones of human evolution include such dietary changes as meat-eating, cooking, and domestication of plants and animals. There was a time when animal protein helped people climb the evolutionary ladder. The problem with plant cells is that their cellulose cell wall requires strong chewing muscles and a massive visceral cranium. Regular consumption of cooked, or technologically processed, meat, decreased the size of human teeth and the entire masticatory system. High-protein food was rich in calories and encouraged a rapid brain development. As a result, ancient people began to produce tools, accumulate experience, and improve hunting methods.

In the XXI century, the agricultural sector has been developing so fast that it has progressed to a completely new stage. Now it has to face such global challenges as providing the Earth's growing population with food and fresh water, improving food security, reducing losses, and improving the ecological environment. The social progress of entire humankind and the very future of our civilization depend on how successfully the modern agribusiness will resolve these global issues. These dynamic problems arise in response to social development, and their solution requires combined efforts of all people.

In Russia, these issues are dealt with at the state level. Food security and food sovereignty are priority tasks. They receive much attention in such policy documents as the Food Security Doctrine of the Russian Federation (2020) and the Economic Security Strategy of the Russian Federation for the period until 2030. According to the Doctrine, it is food security "that ensures the national security of the country in the medium term. It maintains the Russian statehood and sovereignty, while being the most important component of demographic policy. Only food security can improve the quality of life of Russian citizens by guaranteeing high standards of livelihood, which is our strategic national priority".

Livestock farming is a major agricultural sector in many countries. It makes a significant contribution to the total gross domestic product, which contributes to the national economic growth. Global meat production is expected to grow by 13% over the next 10 years, according to the latest forecasts published by the Organization for Economic Cooperation and Development (OECD) and the United Nations Agricultural and Food Organization (FAO).

Vasiliy M. Gorbatov and Iosif A. Rogov were pioneers of modern Russian meat industry, and their contribution to the development of the domestic meat sector can hardly be overstated. Their teams were always at the forefront of the Soviet and Russian food industry.

Andrey B. Lisitsyn continues the legacy of Vasiliy M. Gorbatov. Over many years of work, he accumulated a wealth of experience and deep professional academic knowledge, which helped to solve a number of serious state issues. However, the real contribution made by Andrey B. Lisitsyn to Russian food science is the brilliant young scientists he helped to raise and the devoted fellow-scholars he shared his wisdom with. Together, they have created the national meat science and are working hard to make it even more successful. We congratulate Professor Lisitsyn on his birthday and wish him health and many years of active work for the benefit of Russian science!

Editor-in-Chief,
Corresponding Member of the Russian Academy of Sciences,
Professor A. Yu. Prosekov

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Russian methodology for designing multicomponent foods in retrospect

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Abstract: The article summarizes some scientific and practical prerequisites for creating multicomponent foods with desirable quality characteristics and consumer properties. Mathematical methods were used to model a multicomponent product according to the selected parameters of adequacy and quality, depending on the nutritional and biological value of raw materials. The Russian methodology of food design originated in the works of N.N. Lipatov. His six basic principles of designing balanced multicomponent foods are still relevant today. Further development was proposed by A.B. Lisitsyn who took into account individual protein digestibility of every component in the mixture and its effect on the amino acid composition of total protein. At the next stage, Yu.A. Ivashkin improved formulations using the methods of system analysis, modelling, and product range optimization. Modern food chemistry, food biotechnology, and information technologies allow for effective computer design and optimization of multicomponent food formulations for specific population groups. As a result, an increasing number of food scientists are engaged in improving food products. Literature analysis showed that the current stages of designing (modelling) multicomponent foods are mainly based on information and algorithms, using linear, experimental and statistical programming methods or an object-oriented approach. Russian food scientists still use the methodology developed by A.M. Brazhnikov, I.A. Rogov, and N.N. Lipatov. It allows for designing multicomponent foods with specified nutritional indicators and energy value. The Russian Academy of Sciences pointed to a need for "digital nutritiology" (Decree No. 178 of November 27, 2018 "On Current Problems of Optimizing the Population of Russia: Role of Science"). This new scientific direction could enable digital transformation of data on human physiological needs for nutrients, biologically active substances, and energy, as well as the chemical composition of basic foods. There is also a need for computer programs to give personalized recommendations for optimal nutrition.

Keywords: Design, multicomponent products, criteria, optimization

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INTRODUCTION

In designing multicomponent[1] food products, of great importance is an opportunity to model characteristics of the finished product and predict its quality, as well as functional and technological properties [1, 2].

Designing multicomponent products is based on the principle of food combinatorics. This process involves creating new formulations through a careful selection of raw materials, ingredients, as well as dietary and biologically active additives. Such combinations make the product balanced and ensure the required sensory and physicochemical properties, as well as nutritional, biological, and energy values [3, 4].

The information base created by many years of Russian scientific efforts is highly instrumental in improving food formulations through the use of design criteria and concepts.

This article offers a review of some theoretical and practical results achieved by the Russian science of "food combinatorics" from its foundation to the present day.

RESULTS AND DISCUSSION

A.M. Brazhnikov and I.A. Rogov were the first Soviet scientists who formulated the principles for mathematical design of multicomponent foods with a required set of consumer properties [5, 6].

Back then, food design meant developing models to govern all stages of creating a product of required quality. At the same time, it prioritized a need to express quality in quantitative terms.

[1] Multicomponent products are a combination of various types of raw materials, ingredients, food additives, etc.

A.M. Brazhnikov *et al.* classified food products into three groups to develop the analytical method [5]. Group I included those products (porridge, curd cheese, paste) whose components were interchangeable, both in terms of their relation to each other and their position in the general system. The relationships between the components were not taken into account. To describe the properties of those products, the authors used the general principles of thermodynamics.

Group II covered those products (minced meats, sausages, bread, butter, vegetable pastes, etc.) whose components could interact with each other in various ways without having a fixed position in the system. Their distinctive feature was that the physicochemical interaction of their components during processing could have highly significant effects on the quality of the finished product. The principle of superposition could not be applied to Group II in the same way as it could be applied to Group I. Thus, the authors concluded that designing Group II products required a greater awareness of the product characteristics and a quantitative expression of relationships between the components.

Finally, Group III included products (cakes, ready-to-eat foods, etc.) with interchangeable components and a rigidly fixed structure.

Thus, the authors set out the initial provisions of the analytical approach to designing meat products [5]. This approach was further developed by creating methods to determine specific quality indicators.

In 1980–1990, the most developed methods were those for designing binary systems[II]. It was difficult to achieve a specific amino acid profile in the protein systems of three or more components. In 1980, V.A. Shaternikov proposed the first analytical approach to designing food products with a binary composition [7].

The mass fraction of any *j*-th amino acid in the binary composition was calculated as:

$$A_j = X_1 A_{1j} + X_2 A_{2j} \quad (1)$$

where A_j is the content of the *j*-th essential amino acid, g/100 g protein;

A_{1j} and A_{2j} are the contents of the essential amino acid in the first and second components, g/100 g protein;

X_1 and X_2 are mass fractions of the first and second type proteins in the binary system ($X_1 + X_2 = 1$).

The scores of the *m*-th and *n*-th essential amino acids (used to optimize the binary composition) were calculated as:

$$C_m = \frac{X_1 A_{1m} + X_2 A_{2m}}{F_m};\ C_n = \frac{X_1 A_{1n} + X_2 A_{2n}}{F_n} \quad (2)$$

where X_1, X_2 are mass fractions of the first and second type proteins in the binary system ($X_1 + X_2 = 1$);

A_{1j} and A_{2j} are mass fractions of the *j*-th amino acid (including the *n*-th and *m*-th essential amino acids) in the first and second type proteins, g/100 g protein;

F_m and F_n are mass fractions of the *m*-th and *n*-th essential amino acids in the reference protein, g/100 g protein.

Below are proposed solutions for three typical situations.

First situation. If both proteins have a limited content of the same essential amino acid (given $C_m = C_n$), the composition protein score is a constant value equal to $C_m = C_n$, regardless of X_1 and X_2.

Second situation. If the first protein has a limited content of the *m*-th essential amino acid, while the second protein has it in excess (compared to the reference protein), the optimal ratio of X_1 and X_2 is determined by solving a system of linear equations:

$$\begin{cases} X_1 = 1 - X_2 \\ (1 - X_2) \cdot A_{1m} + X_2 A_{2m} = F_m \end{cases} \quad (3)$$

Another condition is needed for system (3) to determine the optimal ratio of X_1 and X_2, namely: $A_{1n} \geq F_n$ and $A_{2n} \geq F_n$.

Third situation. If the first component has a limited content of the *m*-th amino acid and an excessive content of the *n*-th amino acid ($A_{1n} > F_n$; $A_{1m} < F_m$), while the second component has a limited content of the *n*-th amino acid and an excessive content of the *m*-th amino acid ($A_{2m} > F_m$; $A_{2n} < F_n$), the optimal ratio between X_1 and X_2 in the binary system is determined by the graphical method (Fig. 1). This method allows a quick determination of the required values that ensure the maximum score of the limiting amino acid in the composition.

In 1983, this approach was approved by the USSR Ministry of Health within Guidelines No. 2688-83 for using milk and soy proteins in meat production.

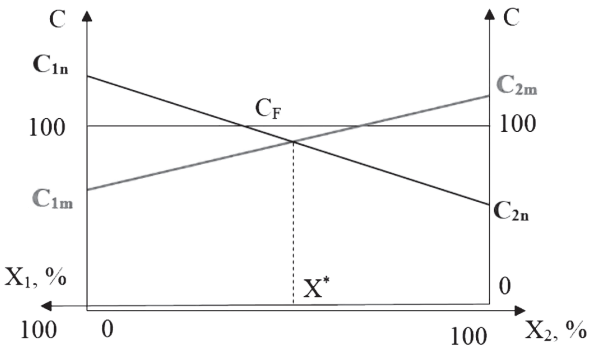

Figure 1 Changes in limiting amino acid scores in binary compositions depending on the X_1/X_2 ratio [5]. The $C_{2m} - C_{1m}$ line (red) is the changing score of the limiting *m*-th amino acid; the $C_{2n} - C_{1n}$ line (blue) is the changing score of the limiting *n*-th amino acid; C_F is the 'ideal' protein score; X* is the optimal ratio of components corresponding to C*, the maximum score of the composition

[II] Binary systems are protein systems made of two components.

In 1981, N.A. Mikhailov (whose research supervisors were I.A. Rogov, Doctor of Technical Sciences and V.G. Vysotsky, Doctor of Medicine) developed some basic analytical principles of designing combined products based on modelling the biological value of protein. He used those principles to create combined paste, as well as a number of cereals and diabetic protein-wheat bread with an increased biological value [8–12].

In addition, N.A. Mikhailov proposed a comprehensive statistical model of protein biological value to determine the optimal composition of ingredients in combined products or correct the initial ratio of ingredients to ensure a specific biological value after heat treatment.

N.N. Lipatov (Jr.) proposed a completely different classification of food products that is still used today for designing functional products [13–16]. In particular, it includes three generations of industrial foods:

– products that have sensory characteristics similar to traditional ones, with raw materials partially replaced with hydrated components equivalent in protein content;
– multicomponent products with a nutrient ratio close to a statistically sound standard that take into account the metabolism in specific population groups united by nationality, age or other characteristics; and
– products with a specially selected combination of components that can ensure their targeted use as functional products by certain population groups.

In addition, N.N. Lipatov developed six basic principles for formulating balanced foods and diets [13–16], namely:

– compliance with a rationally balanced formulation;
– compliance of an amino acid composition of protein-containing ingredients with a statistically sound reference protein;
– a possibility of changing the fatty acid composition by adding fat-containing ingredients;
– the nearest approximation to a desirable ratio of saturated, monounsaturated, and polyunsaturated fatty acids in any combination of fat-containing ingredients;
– taking into account the composition of other dishes and foods in the diet; and
– a balanced multicomponent composition for a single or daily ration in terms of energy value, macro- and micronutrients, and ballast agents.

These principles are still used as a foundation for research in the field of food combinatorics.

N.N. Lipatov *et al.* conducted several studies to develop methods for creating foods with a specified nutritional value [13–16]. In doing so, they assumed that the mechanical processing of raw materials to ensure the required level of dispersion or structural and mechanical characteristics did not violate the principle of superposition with respect to their biologically valuable nutrients. They used this hypothesis in making logical constructions about deterministic formalized approaches to measuring the quantity of individual ingredients. As a result, the authors made valid and reliable conclusions about formulating products with a specified nutritional value and formalized the qualitative and quantitative conceptions about the rationality of using essential amino acids in the technology of adequate exotrophy. Formalization takes into account the mutual balance of essential amino acids.

The scientists formulated the main principle and criterion for the rational use of essential amino acids in new types of foods. The principle gives preference to such combinations of *n*-protein-containing components with mass fractions X_i^p in which the maximum proportion of assimilable essential amino acids in the protein, given equal provision of the body with anabolic material, can be used for anabolic purposes without degradation in the biosynthesis of nonessential amino acids, let alone biological oxidation in compensating for the energy expenditure of the body [13].

Taking into account a possibility of $C_{min} \geq 1$ (where C_{min} is the minimum score of essential amino acids in the protein of the designed product in relation to the reference protein) or a possibility of $C_{min} < 1$, the selection criterion X_i^p can be symbolically presented as

$$\left\{ A_\phi^\Sigma\left(X_i^p\right) - A_r^\Sigma;\ \ \frac{A_\phi^\Sigma\left(X_i^p\right) - A_\alpha^\Sigma\left(X_i^p\right)}{C_{\min}\left(X_i^p\right)} \right\} \to \min \qquad (4)$$

where A_α^Σ is the total mass fraction of assimilated essential amino acids that can be used by the body for anabolic purposes without further degradation;

A_ϕ^Σ is the actual sum of assimilated essential amino acids.

The authors transformed the above formula introducing p_i as a mass fraction of digestible protein in the *i*-th component (%) and a_{ij} as a mass fraction of the j-th essential amino acid in the protein of the *i*-th component (g/100 g). Criterion (4) for finding a preferable ratio of the mass fractions X_i^p of these components in the designed product with regard to rational use of the *k* essential amino acids can be presented as:

$$\left\{ \frac{\sum_{j=1}^{k}\sum_{i=1}^{n} X_i^p p_i a_{ij}}{\sum_{i=1}^{n} X_i^p p_i} - \sum_{j=1}^{k} A_{rj};\ \ \frac{\dfrac{\sum_{j=1}^{k}\sum_{i=1}^{n} X_i^p p_i a_{ij}}{\sum_{i=1}^{n} X_i^p p_i} - C_{\min}\left(X_i^p\right)\sum_{j=1}^{k} A_{rj}}{C_{\min}\left(X_i^p\right)} \right\} \to \min \qquad (5)$$

$$\frac{\sum_{j=1}^{k}\sum_{i=1}^{n} X_i^p p_i a_{ij}}{\sum_{i=1}^{n} X_i^p p_i} = A_j$$

where is the mass fraction of *j*-th essential amino acid in the protein of the designed product with the fixed j, g/100 g protein;

A_{rj} is the reference mass fraction of the *j*-th essential amino acid, g/100 g protein.

N.N. Lipatov (Jr.) developed the following methodological approaches to designing foods with the required set of nutritional indicators.

The first stage involves modelling the amino acid composition of protein in the designed product and selecting X_i^p that provide the minimum functional values (4).

The second stage involves modelling the fatty acid composition, given that the mass fractions of components $X_i^{L(p)}$ containing protein, as well as fat, are constant and predetermined by the first stage. Based on the modelling results, mass fractions X_i^L are selected that together with $X_i^{L(p)}$ provide the required approximation to the physiologically determined ratio of saturated, mono- and polyunsaturated fatty acids.

The third stage involves calculating the energy value Q_p of the designed product, taking into account only those $X_i^{c(p,L)}$ which are sources of protein and/or fat. The result is then compared with the required Q. If the estimated energy value is less than Q, the product is supplemented with additional technologically permissible carbohydrate-containing components in quantities that ensure the required Q. If Q_p is greater than Q, X_i^L are recalculated. If necessary, X_i^L with excessively high L_i values can be replaced with those with lower L_i values.

Using the Mitchell-Block principle, N.N. Lipatov developed a number of indicators, namely: the utilization coefficient for essential amino acids; the utilization coefficient for the amino acid composition of the product, g/100 g protein; the ratio of amino acids as a balance of essential amino acids in relation to the physiologically determined norm (standard); and the indicator of excess in the content of essential amino acids as the total amount of essential amino acids that are not used for anabolic purposes [17, 18].

Thus, we can conclude that the main studies of N.N. Lipatov were devoted to the trophological, mathematical, informational, and algorithmic aspects of food design. He supervised the creation of ordinary and specialised products for baby and gerodietetic nutrition. Finally, he established a scientific school to improve the quality of foods considered as objects of a single exotrophic chain of production, consumption, and assimilation of nutrients by the human body.

A.B. Lisitsyn combined the mathematical methods of I.A. Rogov and N.N. Lipatov for calculating the amino acid composition and total protein digestibility in multicomponent mixtures [19–21].

Protein digestibility is one of the most important indicators of the product's biological value, along with its amino acid balance. A.B. Lisitsyn understood the need to take into account individual protein digestibility of all components when estimating the product's biological value and study their effect on the amino acid composition of total protein. The mathematical interpretation of his concept can be presented as follows:

$$A_j = \frac{\sum_{i=L+1}^{m} X_i \sum_{l}^{L} X_i \pi_i p_i a_{ij} + \left(\sum_{i=L+1}^{m} X_i - Y \right) \sum_{i=L+1}^{m} X_i \pi_i p_i a_{ij} + \sum_{i=L+1}^{m} X_i \sum_{l}^{L} X_i \pi_i p_i + \left(\sum_{i=L+1}^{m} X_i - Y \right) \sum_{i=L+1}^{m} X_i \pi_i p_i + Y \sum_{i=L+1}^{m} X_i \sum_{i=m+1}^{n} X_i \pi_i p_i a_{ij}}{+ Y \sum_{i=L+1}^{m} X_i \sum_{i=m+1}^{n} X_i \pi_i p_i}, \quad (6)$$

$$\sum_{i=1}^{m} X_i = 1; \sum_{i=m+1}^{n} X_i = 1; Y \leq \sum_{i=L+1}^{m} X_i$$

where A_j is the content of j-th amino acid, g/100 g protein;

X_i is the mass fraction of the i-th component in the mixture, unit fraction;

π_i is the dimensionless characteristic (coefficient) of protein digestibility of the i-th component;

p_i is the mass fraction of protein in the i-th component, % or unit fraction;

a_{ij} is the mass fraction of j-th amino acid in the protein of the i-th component, g/100 g protein;

n is the total number of ingredients in the formulation;

(n-m) is the number of replacement ingredients during modelling;

L is the number of ingredients that are not replaced during modelling;

(m-L) is the number of ingredients varying (replaced) during modelling;

Y is the total amount of varying ingredients in the formulation.

Thus, A.B. Lisitsyn substantiated the principles of designing meat products with a given biological value, taking into account individual protein digestibility and the amino acid composition of every ingredient in the formulation. His mathematical formulas allow us to devise the amino acid composition of multicomponent systems, taking into account individual protein digestibility of every component.

Yu.A. Ivashkin combined the structural and the parametric optimization approaches in his works. Structural optimization is the determination of optimal structural parameters of the formulation. Parametric optimization involves calculating optimal deviations from the norm. Yu.A. Ivashkin *et al.* suggested using structural and parametric optimization for every criterion with pairwise comparison and quality assessment of the resulting product using an independent quality functional and desirability scales [22, 23]. The multicriteria optimization of the combined product (nutritional and biological values) consists in building its model according to the specified adequacy and quality parameters, depending on the composition of initial components.

For this, a parametric model of the product is devised, taking into account:

– the required chemical composition (protein, fat, carbohydrates, etc.);
– mass fractions of the main components (key ingredients, fiber, biologically active additives, enzymes, etc.); and
– structural relationships of biological value indicators (amino and fatty acid compositions) according to various compliance criteria.

Consideration is also given to what makes a balanced diet for a certain population group.

An objective function is the minimum deviation from the given structural group of nutritional and biological indicators [22, 23], namely the criteria below.

(1) The optimization criterion for chemical elements that determine the nutritional value $P(z)$ of the designed product:

$$P(z) = \sum_{i=1}^{n} \left(z_i^0 - \sum_{j=1}^{m} b_{ij} x_j \right)^2 \to \min \qquad (7)$$

where z_i^0 is the reference content of the i-th element of nutritional value;

b_{ij} is the specific content of the i-th element of chemical composition in the j-th component of the designed product;

x_j is the mass fraction of the j-th component.

(2) The criterion of the minimum deviation from the given structural indicators of biological value $P_i(A)$, for example, the monostructure of essential amino acids ($i = 1$) and fatty acids ($i = 2$):

$$P_i(A) = \sum_{k=1}^{n} \left(A_k^0 - \frac{\sum_{j=1}^{m} a_{ki} b_{ij} x_j}{\sum_{j=1}^{m} b_{ij} x_j} \right)^2 \to \min; \quad i = 1,2 \qquad (8)$$

where A_k^0 is the reference content of the k-th monostructural indicator of biological value;

a_{ki} is the specific content of the k-homonostructural ingredient in the i-th element of chemical composition;

b_{ij} is the specific content of the i-th element of chemical composition in the j-th component of the designed product;

x_j is the mass fraction of the j-th component.

(3) The criterion of the minimum deviation from the given structure $P_i(V)$ of the vitamin composition ($i = 1$), minerals ($i = 2$), and carbohydrates ($i = 3$):

$$P_i(V) = \sum_{k=1}^{n} \left(V_k^0 - \frac{\sum_{j=1}^{m} b_{kj} x_j}{\sum_{j=1}^{m} x_j} \right)^2 \to \min; \quad i = 1,2,3 \qquad (9)$$

where b_{kj} is the specific content of the k-th ingredient in the j-th element of chemical composition;

x_j is the mass fraction of the j-th component.

Yu.A. Ivashkin proposed to use simulation modelling to solve the problem of structural and parametric optimization of a multicomponent product in various combinations of linear and non-linear criteria and restrictions. It involves "playing out" all possible combinations of initial ingredients with subsequent verification of restrictions and calculation of criteria.

Noteworthily, any problem of the NP[III] class can be solved by simulation modelling. The complexity depends on the number of ingredients in the formulation. If the space of problem solutions is very large, this method may take longer than a "reasonable" time to produce results.

A.E. Krasnov *et al.* used new information technologies to produce foods of given quality. In particular, they created sausage meat formulations under varying conditions of uncertainty. Their study showed how to transform the problem of stochastic programming with uncertain target criteria into the linear programming problem with stochastic constraints [24].

Considerable attention is now paid to modelling interactions between food components based on the laws of equilibrium statistical thermodynamics. For the first time, a polynomial dependence was found between the properties of food mixtures and the mass fractions of their ingredients. Its relationship with macroscopic thermodynamic parameters of the mixtures was also shown.

Scientific modelling of multi-component food products with a specific set of nutritional and energy indicators is still relevant worldwide. An ever wider circle of researchers are engaged in various aspects of improving food technology.

E.I. Muratova *et al.* proposed an object-oriented approach to designing multicomponent food products (for example, confectionery). A distinctive feature of this approach is presenting a formulation as a hierarchical structure (the Saati method) [25, 26].

Each vertex of this structure is an object (raw materials – semi-finished product – finished product). Each level is a certain stage of food technology that can have its own number of vertices located lower in the hierarchy. The algorithm for calculating a multicomponent product begins with the lowest level in the longest branch of the hierarchical structure (Fig. 2).

Figure 2 shows a three-level hierarchy of formulation, where the first index is the level number and the second is the number of a component in the formulated mixture. When several semi-finished products are used at the same level, their first index becomes a composite and is indicated as a list (i, j), where i is the level number and j is the serial number of the semi-finished product at the i-th level. This composite index is used lower in the hierarchy (shown by the dashed arrow).

The algorithm for calculating the multiphase formulation begins with the lowest level in the longest

[III] In the theory of algorithms, the NP (non-deterministic polynomial) class refers to a multitude of decision problems whose solutions can be verified on a Turing machine within a certain input polynomial time, if there is some additional information (the so-called solution certificate) [39].

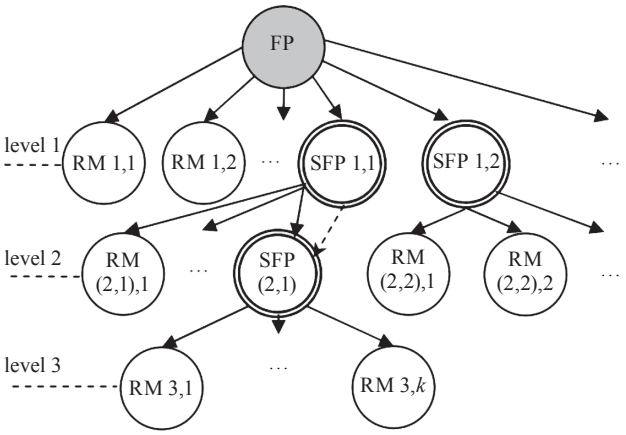

Figure 2 Hierarchical structure of the product formulation: FP – finished product; RM – raw materials; SFP – semi-finished product [23]

branch of the hierarchical structure. According to Fig. 2, the calculation of the formulation begins with the semi-finished product FP (2, 1), since the path to its components is the longest in the hierarchy. The initial data for calculating the lowest level include the loading of all types of raw materials and semi-finished products, loss of dry matte, and a given amount of finished products equal to 1 t.

According to the authors, the main advantage of this approach is the object-oriented representation. It allows for inheriting properties and methods while adding new calculation formulas that take into account new raw materials, production features, as well as technical and economic indicators of the processes.

O.N. Musina and P.A. Lisin proposed a methodology for system modelling of multicomponent food products [27–29]. They defined system modelling as a strategy for studying and creating biosystems, particularly food products, their formulations, and production technologies.

The basic principle of system modelling is the decomposition of a complex biosystem into simpler subsystems. This is a principle of the system hierarchy. In this case, the mathematical model of the system is based on the block principle: the general model is divided into blocks which can have relatively simple mathematical descriptions. All subsystems interact with each other and constitute a common unified mathematical model.

Figure 3 shows a visual interpretation of the basic principles of system modelling of multicomponent products.

System modelling principles allow for the decomposition of the production system at the stage of formulating composite mixtures using linear models. In such models, mathematical dependencies (equalities or inequalities) are linear with respect to all variables in the model. Problems of this kind are used to select the optimal option from a set of possible formulations according to a given criterion. In 1939, the Russian mathematician L. Kantorovich and the American scientist G. Danzig began to develop what was later called “the simplex metho”. It became a universal method of linear programming used in solving optimization problems.

A.A. Borisenko proposed a methodology for optimizing multicomponent food mixtures using universal mathematical methods. His methodology allows for the development of foods with a given nutrient composition [30, 31]. Taking into account certain restrictions and permissible deviations of nutrient mass fractions from the reference amounts, the author proposed to use the Lagrange function and the system

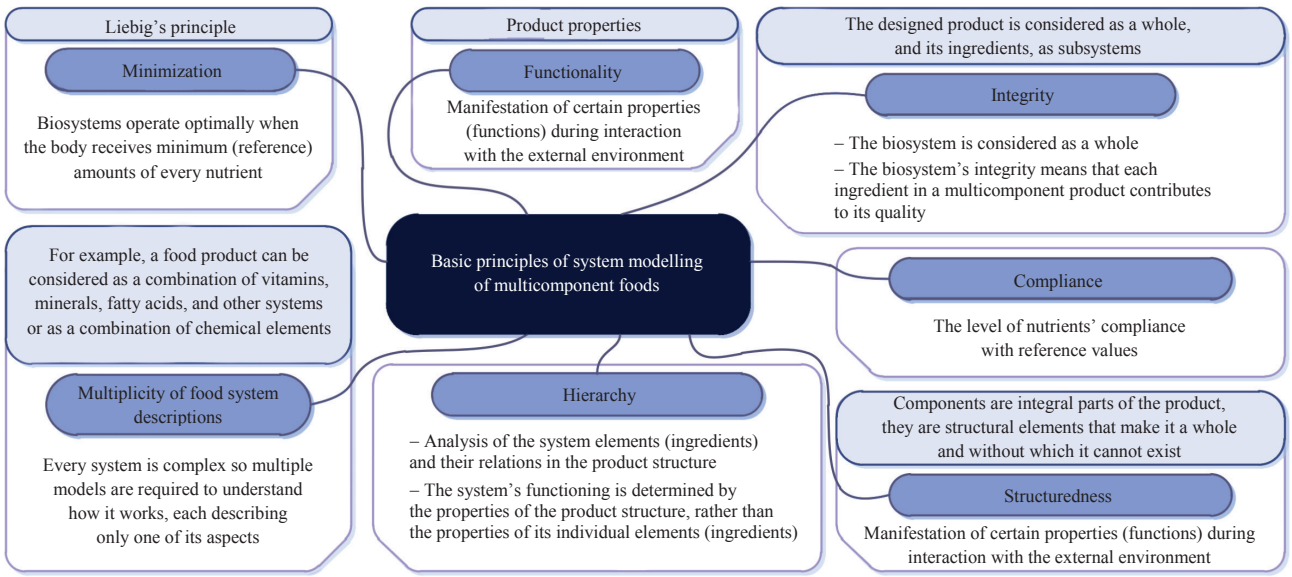

Figure 3 Basic principles of system modelling of multicomponent products [27]

of equations in the form of conditions of the Kuhn-Tucker theorem for convex programming. Solving these problems produces a vector of component mass fractions to ensure the most balanced nutrient composition.

The author concluded that the most balanced formulation cannot always guarantee the highest quality of the finished product. Therefore, in most cases, there is a need for a fairly wide range of formulated options. To achieve that, he proposed to optimize formulations in two stages. The first stage of modelling a formulation involved determining all possible quantitative ratios of the ingredients. The second stage was a qualitative assessment and selection of several most optimal variants. The author used Harrington's desirability function as a general criterion for quality assessment.

A.Yu. Prosekov developed the principles of forming dispersed food systems and designing functional products from modern perspectives [32–34].

T.V. Sanina and Yu.S. Serbulov proposed a differentiated approach to a comprehensive assessment of highly nutritional bakery products. The authors believe that consumers should select key quality indicators for foods with increased nutritional value to make their assessment objective. In addition, quality assessment should check if the product satisfies certain needs consistent with its purpose [35].

A.A. Zaporozhsky *et al.* formulated new gerodietetic products with specified qualitative characteristics based on natural raw materials. For this, they used a methodological approach and the principles of modern nutrition, qualimetry, food combinatorics, and neural network approximation of theoretical (estimated) and experimental data [36, 37].

T.Yu. Reznichenko *et al.* substantiated an integrated technological approach to the development of functional foods enriched with biologically active substances and dietary fibre. They studied the factors that determine the quality of specialized products and critical control points that identify their functional character at the stages of production and distribution. They also developed a range of consumer properties that included functional indicators in addition to sensory and physicochemical characteristics. Finally, the authors developed an algorithm to examine a functional cereal breakfast bar [38, 39].

V.M. Kiselev and E.G. Pershina looked at the production and consumption of functional foods as a multi-factor system subjected to comprehensive assessment. They used the methods of food combinatorics, parity of needs, and the vital concept, taking into account modern requirements of nutrition. With this approach, the authors studied a possibility of evolutionary development of functional food design based on food combinatorics. They identified consumer preferences for functional foods and systematized them in a model of consumer value [40].

O.N. Krasulya *et al.* considered the design of multicomponent foods based on the functional and technological properties (FTP) of their main raw materials and ingredients. They also took into account the kinetics of biochemical and colloidal processes, as well as analytical and empirical relations characterizing the main patterns of heterogeneous disperse systems with varying physicochemical factors [42, 43].

In the age of digital (information) technologies, the design of multicomponent food formulations can be improved by using linear, experimental and statistical programming methods, or an object-oriented approach. M.S. Koneva *et al.* proposed using neural network technologies [44]. The relationship between sensory criteria and the quantitative composition of the formulation was identified by neural network and regression analysis of the ranking score of sensory characteristics. The model parameters were obtained with Statistica software. The convolution of the balancing index and sensory evaluation was proposed as a multiplicative desirability function. MathCAD scripts were used to optimize the composition of antianemic smoothie for pregnant women.

N.A. Berezina *et al.* developed a program in Object Pascal for designing gerodietetic bread compositions [45]. The technological adequacy of the flour mix, which ensured a stable quality of the final product, was modelled by introducing the flour technological indicator ("falling number") calculated using the Perten formula.

The mathematical foundations of solving single-criterion optimization problems are quite well studied today. However, various areas of engineering, research and management have multicriteria problems in which several criteria need to be simultaneously optimized. M.A. Nikitina and I.M. Chernukha proposed using the Pareto method for multicriteria optimization [46].

The informational aspects of modelling and evaluating the nutritional adequacy of raw materials and finished products are very important in improving the quality and technology of specialized multicomponent food products.

CONCLUSION

The analysis of literature on the principles and methods of designing balanced foods showed that the initial stage in this process involved formalizing qualitative and quantitative assumptions about the rational use of essential amino acids in the adequate exotrophy technology. N.N. Lipatov's contribution to designing balanced formulations in Russia cannot be underestimated. His principles of creating multicomponent foods and balanced diets are still relevant today. Further development of food combinatorics was related to informational and algorithmic aspects of food design.

The conceptual approaches to computer-aided food design proposed by N.N. Lipatov (Jr.) are used to model functional products with specified qualitative characteristics. Based on the optimal choice of raw materials and ratios of ingredients, they result in formulations whose nutrients (essential amino acids,

unsaturated fatty acids, macro- and microelements, and vitamins) are consistent with the medical and biological requirements in terms of quantity and quality.

The computer systems and software products actively used in Russia to automate technological calculations for food and diet formulations include Etalon, Generic 2.0, Food & Life, CheesePro 1.0, ShkoOptiPit, and others. They are based on the databases of foods and raw materials, scientific research and industrial experience, as well as mathematical methods of modelling and designing food covered in the works of I.A. Rogov, A.M. Brazhnikov, N.N. Lipatov (Jr.), and other scientists. With the help of those systems, new types of products were developed by Moscow State University of Applied Biotechnology, Gorbatov All-Russia Meat Research Institute, Research Institute of Baby Food, All-Russia Research Institute of Dairy Industry, and other institutes. These products had an improved composition of chemical elements, amino and fatty acids, as well as better energy values, quality indicators, etc. The experimental and theoretical (mathematical) data were 98% reliable.

The foreign software solutions (DietPlan, Nutri-Survey, NutriBase, NUT, MyFitnesspal, and 8fit) are based on calculating the individual's daily energy intake and their need for basic nutrients.

Designing foods in the digital age, we need to take into account not only nutritional and biological values, but also medical, technological, economic, social, and other factors. Computer technologies allow us to address problems with numerous parameters, alternatives, and criteria, as well as restrictions and conditions. By processing and formalizing data, they help us find optimal solutions based on complex optimization models and objective assessment of options.

A need for "digital nutritiology", a new scientific field, was highlighted in Decree of the Presidium of the Russian Academy of Sciences No. 178 dated November 27, 2018 "On the Current Problems of Optimizing the Population of Russia: Role of Science" (paragraph 11). This new direction is supposed to translate into the language of numbers our physiological needs for energy, nutrients, biologically active substances, and balanced diets, on the one hand, and the chemical composition of foods and general diets, on the other.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Multi-criteria food products identification by fuzzy logic methods

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Abstract: The paper deals with the theory of fuzzy sets as applied to food industry products. The fuzzy indicator function is shown as a criterion for determining the properties of the product. We compared the approach of fuzzy and probabilistic classifiers, their fundamental differences and areas of applicability. As an example, a linear fuzzy classifier of the product according to one-dimensional criterion was given and an algorithm for its origination as well as approximation is considered, the latter being sufficient for the food industry for the most common case with one truth interval where the indicator function takes the form of a trapezoid. The results section contains exhaustive, reproducible, sequentially stated examples of fuzzy logic methods application for properties authentication and group affiliation of food products. Exemplified by measurements of the criterion with an error, we gave recommendations for determining the boundaries of interval identification for foods of mixed composition. Harrington's desirability function is considered as a suitable indicator function of determining deterioration rate of a food product over time. Applying the fuzzy logic framework, identification areas of a product for the safety index by the time interval in which the counterparty selling this product should send it for processing, hedging their possible risks connected with the expiry date expand. In the example of multi-criteria evaluation of a food product consumer attractiveness, Harrington's desirability function, acting as a quality function, was combined with Weibull probability density function, accounting for the product's taste properties. The convex combination of these two criteria was assumed to be the decision-making function of the seller, by which identification areas of the food product are established.

Keywords: Fuzzy logic, Harrington's desirability function, identification criteria of food products, identification areas

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INTRODUCTION

In the food industry, the task of identification – that is, determining the attribution of a food product to a particular class in terms of condition, quality and taste characteristics – stands alone. For the solution of this task there exist: a set of criteria both measurable and expert; typical characteristics that product clusters must meet; and stratifying borderline values [1–5].

At the same time, all the obtained relations are empirical. Besides, as discriminatory criteria are construed, product clusters often intersect according to some measured parameters, so it makes sense to introduce a characteristic of attribution [6]. The latter would be a unit ("the sample certainly belongs to this product cluster") in cluster centers and would decrease at the borders ("the sample belongs to some extent to one cluster and to some extent to the neighboring one"). This would allow making product identification more transparent and applicable to real food applications [7, 8].

The method of fuzzy sets theory application to the problems of the food industry, proposed in this paper, will create lax regulatory restrictions on the composition, quality and sanitary characteristics of the product, taking into account the varied errors of methods and measurements. The purpose of this research was to provide food industry experts with a tool that allows building a robust multiparameter identification criteria based on empirical product data.

STUDY OBJECTS AND METHODS

The concept of fuzzy sets as applied to the food industry. In order to define fuzzy set A for elements of $\mathbb{R}^n$, enter the indicator membership function[1]:

$$\chi_A(x) \in [0,1], x \in \mathbb{R}^n \quad (1)$$

[1] Hereafter: the indicator function and the membership function are interchangeable concepts

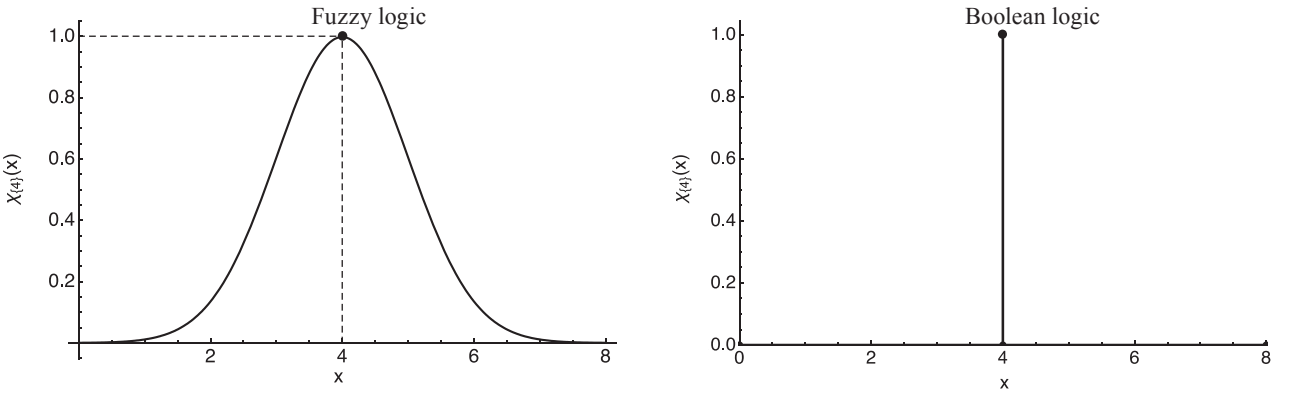

Figure 1 Fuzzy and Boolean approaches to the definition of a set consisting of an element {4}

Concurrently, the set in the classical sense of, defined in this way, is a special case of A^*, a fuzzy set:

$$\chi_{A^*}(x) \in \{0,1\}, x \in \mathbb{R}^n \quad (2)$$

Thus, fuzzy logic extends the Boolean one with two values {0,1} to the continuum of values in the interval of [0,1]. The difference between the approaches is shown in Fig. 1. Most often, the value $\chi_A(x)$ is interpreted as a subjective assessment of *x* as attributed to *A*, for example $\chi_A(x) = 0.9$ means that *x* is 90% of *A* [9].

The example of interpretation contains the word "subjective", which presupposes the possibility of each subject having an opinion concerning the relationship of each specific set attribution on the basis of their own indicator function. For food industry, this means the need of using a consensus membership function for each criterion; the function based on a particular food industry experts' consolidated opinion, as well as confirmed experimental data [10].

The subjectivity of assessment also implies the existence of a method of translating psycholinguistic conclusions about the considered attribution to the digital domain of the indicator function.

The concept of a linguistic variable includes the object under study, as well as a set of natural language phrases (linguistic lexemes) that the variable can take in a fuzzy sense. The method of establishing the relevance is individually selected for each industry and case of study. Common sense is one of the primary factors, since the number of linguistic lexemes used by experts and intended for digital transformation is extremely diverse, for example: "true", "false", "almost false", "almost true", "unknown", "possible", "sometimes", "may be", etc. (Fig. 2).

The main prerequisite for the use of fuzzy logic as applied to the food industry is the inability to build clear relations and criteria that link the quality and performance of products and are not subject to multiparameter, unamenable to expression, factors of influence and measurement errors [11].

In a way, the definition of a fuzzy set via the indicator function contains neither lack of focus nor ambiguity, so it is possible to use the fuzzy logic framework for setting standards and identification methods in the food industry.

Basic operations with fuzzy sets. Let us examine in more detail the possible fuzzy sets manipulations and highlight the most common operations in terms of the food industry (in fuzzy logic it is impossible to identify a finite set of basic functions, through which all the others could be expressed; besides, operations on sets become "blurred") [12–14].

Consider the sets $A, B \subset \mathbb{R}^n$. The relation of inclusion of the *A* set *B* into:

$$A \subset B \Leftrightarrow \chi_A(x) \leq \chi_B(x), \forall x \in \mathbb{R}^n \quad (3)$$

The most practical option for constructing fuzzy negation $\bar{A}$ is:

$$\chi_{\bar{A}}(x) = 1 - \chi_A(x) \quad (4)$$

There is an unlimited number of simple fuzzy negations; besides, this method is convenient for constructing linguistic expert models, for example, the negation for "unknown" ($\chi_A(x) = 0.5$) will also be "unknown".

The expansion of conjunction (operation "AND") for fuzzy sets is called the t-norm (or triangular norm), and the expansion of disjunction (operation "OR") is called the s-norm. In practice, most commonly used are:

The logical product of $A \cap B$ and sum $A \cup B$:

$$\chi_{A\cap B}(x) = \min(\chi_A(x), \chi_B(x))$$
$$\chi_{A\cup B}(x) = \max(\chi_A(x), \chi_B(x)) \quad (5)$$

The algebraic product of $A*B$ and sum $A+B$

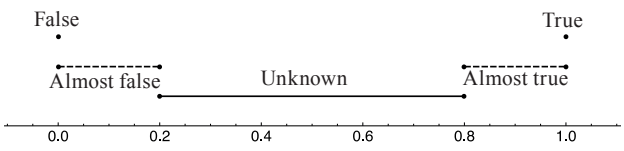

Figure 2 An example of relation between the linguistic "attribution" variable and the intervals of the indicator function

$$\chi_{A*B}(x) = \chi_A(x) \cdot \chi_B(x)$$
$$\chi_{A+B}(x) = \chi_A(x) + \chi_B(x) - \chi_A(x) \cdot \chi_B(x) \quad (6)$$

The presented pairs of t- and s- norms are called dual, since when using the above negation, de Morgan's laws are implemented in a fuzzy form, which makes their application practically convenient in calculations.

Failure of the law of complementarity in the general case must be noted as an important feature of fuzzy logic. Denoting t-norm as &, s-norm as |, we have:

$$\chi_{A\&\bar{A}}(x) \geq 0$$
$$\chi_{A|\bar{A}}(x) \leq 1 \quad (7)$$

The postulate of Boolean algebra "some criterion and its negation are simultaneously unjust" violates the introduction of intermediate variants. In particular, that of the lexeme "unknown", since it and its negation are assumed to be simultaneously and equally fair. This fact demonstrates the coexistence of the property and its negation.

With multi-criteria identification of food products it is often necessary to assign weight numbers for each individual criterion while obtaining the aggregate indicator quality function. To do this, convex integration with λ coefficient (denoted as $(A+B)^{\lambda}$ is used:

$$\chi_{(A+B)^{\lambda}}(x) = \lambda\chi_A(x) + (1-\lambda)\chi_B(x) \quad (8)$$

This formula is easily generalized for the case of criteria. Supposing there are fuzzy sets A_1, A_2, ... , A_m, where $A_i \subset \mathbb{R}^n$ their convex integration will have the form:

$$\chi_S(x) = \sum_{i=1}^{m} \lambda_i \chi_{A_i}(x)$$
$$S = \left(\sum_{i=1}^{m} A_i\right)^{\Lambda} \quad (9)$$
$$\Lambda = \{\lambda_1, \lambda_2, \dots, \lambda_m\}, \sum_{i=1}^{m} \lambda_i = 1$$

Constructing indicator functions, it is useful to control the smoothness and speed of the transition of one linguistic concept to another. To do this, we use a power function that defines A^{α} as follows:

$$\chi_{A^{\alpha}}(x) = \chi_A(x)^{\alpha}, \alpha > 0 \quad (10)$$

If $\alpha < 1$, the function reduces the requirements for membership to the set A^{α} with respect to A, at $\alpha > 1$, the function clarifies it.

Linear fuzzy classification. From the standpoint of the probability theory the indicator function can be interpreted as conditional probability

$$\chi_A(x) = P(X \in A \mid X = x) \quad (11)$$

that is, the probability of membership to the set of a random variable *X*, provided that it was implemented by *x* value. It should be noted that this is the basic difference between the approaches: fuzzy logic operates by the degree of membership to a particular set. While probability theory (and "probabilistic" logic) indicates the probability of occurrence of mutually exclusive events.

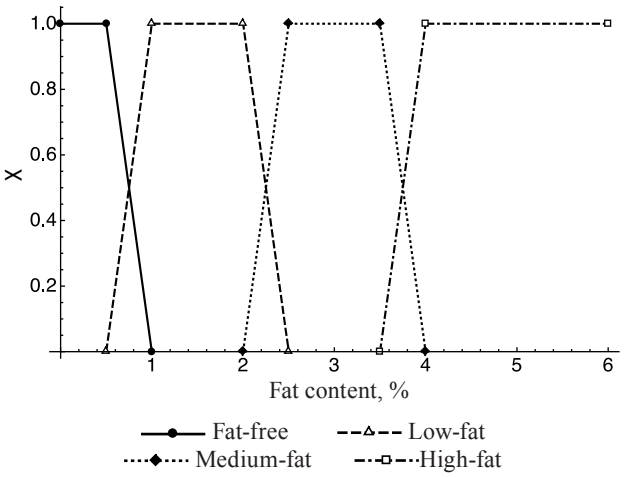

Figure 3 Fuzzy classification of drinking milk by fat content

As an example, consider the fuzzy classification of drinking milk by fat content (Fig. 3)[II]. According to this classification, milk with a fat content of 3.75% is both 0.5 medium-fat and 0.5 high-fat. We consciously give no percentages here, because it is not a matter of probability (otherwise, in a batch of milk with the same fat content of 3.75%, half of the bottles would be recognized as "medium-fat", and the other half – as "high-fat", which makes no sense). Fuzzy sets exist in superposition with each other, this being their main advantage in food identification. Continuing the example on the same classifier, 0.8 milk of average fat content is actually the same as 0.2 of extra fat content, and this has a direct interpretation since two linguistic postulates describing different degrees of one measurable criterion are associated. At the same time, it should be noted that combining probabilistic and fuzzy methods has its own scope; besides, probability distributions can be used as indicator functions, as will be shown below.

In the example with milk fat linear functions are used to determine the degree of membership, being the most practically applicable for the food industry due to the simplicity of construction and linguistic explanation of the result [15, 16]. In order to construct a linear characteristic function $\chi_A(x)$ for some criterion *A* on the domain $R \in \mathbb{R}$ there are three steps to follow:

(1) Determination of the intervals (x_i, y_i), $i = 1 \dots n$, where $\chi_A(x) = 1$ that is, belonging to such intervals is characterized by the lexeme "certainly Yes";

(2) Determination of the intervals (x_j', y_j'), $j = 1 \dots m$, where $\chi_A(x)$, that is, their linguistic characteristics is "certainly No";

[II] State Standard 31450-2013. Drinking milk. Specifications. Moscow: Standartinform; 2014. 9 p.

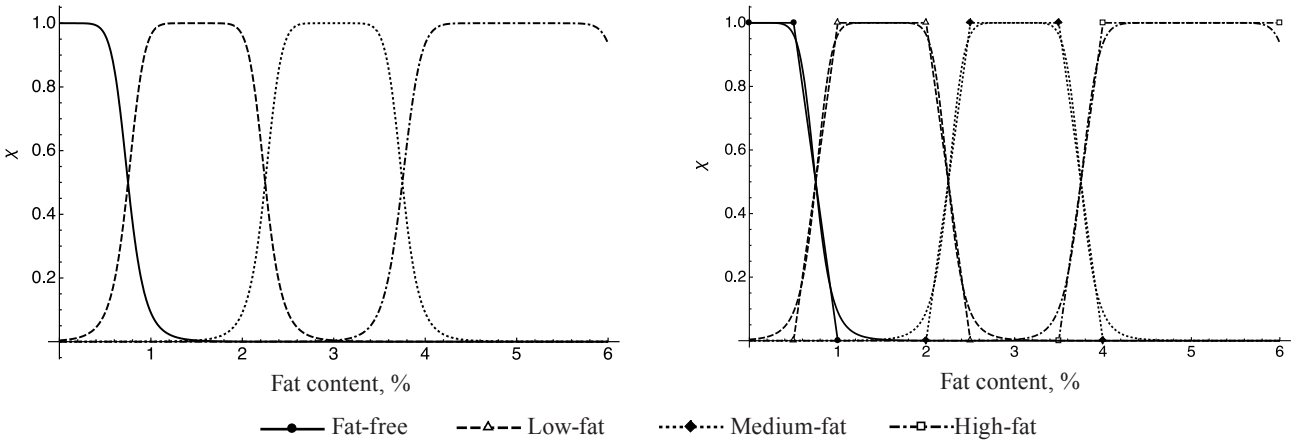

Figure 4 Approximation of fuzzy classification of drinking milk by fat content

(3) Combination of the intervals into one list with the length of $k = n + m$ and sort them in ascending order of the left border. Since in the resulting list the intervals with the characteristics "certainly Yes" and "certainly No" will alternate, it remains only to connect the boundaries by linear function.

(a) For the sequence (x_i, y_i); (x'_{i+1}, y'_{i+1}) the function will look like:

$$\chi_A(x) = -\frac{1}{x'_{i+1} - y_i}x + \frac{y_i}{x'_{i+1} - y_i} + 1, x \in [y_i; x'_{i+1}] \quad (12)$$

(b) For the sequence (x'_i, y'_i); (x_{i+1}, y_{i+1}) the function will look like:

$$\chi_A(x) = \frac{1}{x_{i+1} - y'_i}x - \frac{y'_i}{x_{i+1} - y'_i}, x \in [y'_i; x_{i+1}] \quad (13)$$

Of course, in practice the most common case is that with one truth interval, and the function takes the form of a trapezoid, as seen in the graph of milk classification.

For one truth interval the convenient approximation is:

$$\chi_A(x) = \frac{1}{1 + \left(\frac{x - c}{l}\right)^p} \quad (14)$$

where c is the center of the interval, l is its range, p is the smoothing fit. In the context of the example, the indicator functions for dairy products will take the form:

$$\frac{1}{1 + \left(\frac{x}{0.75}\right)^8}, \frac{1}{1 + \left(\frac{x - 1.5}{0.75}\right)^8}, \frac{1}{1 + \left(\frac{x - 3}{0.75}\right)^8}, \frac{1}{1 + \left(\frac{x - 5}{1.25}\right)^{12}} \quad (15)$$

for fat-free, low fat, medium fat and high fat products, respectively. The patterns of these functions, as well as comparison of the two approaches are shown in Fig. 4.

RESULTS AND DISCUSSION

To determine the value of the indicator function $\chi_A(x)$ at a particular point, it is sometimes necessary to resort to nested fuzzy sets. This happens, for example, when the values of the linguistic variables of the expert group differ for the same criterion at a point. When a indicator function of a set is realized not by a specific number, but by another indicator function, it is called a second order fuzzy set. In practice, it is very difficult to use such items, and they are absolutely unsuitable for establishing legal relations between contractors of the food industry, in particular, producers and consumers. In this case, instead of the nested indicator function at a point, its integral value is considered, for example, the consensus of experts or the probability value, if the function was represented by the probability density [17–19].

As an example, consider a criterion of a food product, which according to regulatory documents should fall into the interval [–1,1] (we consider it as a fuzzy set I) with a measuring device error of ± 0.5 The error was deliberately taken as comparable to the length of the interval for a more visual demonstration of the behavior of the indicator function at the boundary.

Suppose that the measurement error $\varepsilon \sim N(0, \sigma^2)$ is a normally distributed random variable with zero expectation and dispersion, whose value can be determined from the instrument error. If we assume that 95.6% (which corresponds to the probability of a normally distributed random variable falling within the range $\pm 2\sigma$ relative to the mean value) of measurements of x fall within the range $x \pm 0.5$ (the assumption can be strengthened or weakened depending on the conditions and the nature of the error), it means that:

$$0.5 = 2\sigma, \sigma^2 = \frac{1}{16} \quad (16)$$

To construct the indicator function of the criterion, let us ask: "what probability does the product satisfy the criterion with if its measurement showed the result of?". Obviously, a second order fuzzy set emerges: for each x there is an error probability density that can serve (after some manipulations) as a nested indicator function. As previously stated, it is more convenient to assume the integral value as the value at the point, that is, from a probabilistic point of view, to calculate the conditional probability $\mathbb{P}(x_r \in I \mid x)$, where $x_r = x + \varepsilon$ is the real value of the indicator. In this case, $\chi_I(x)$ is the possible real value $x + \varepsilon$ on the interval [–1,1] probability density

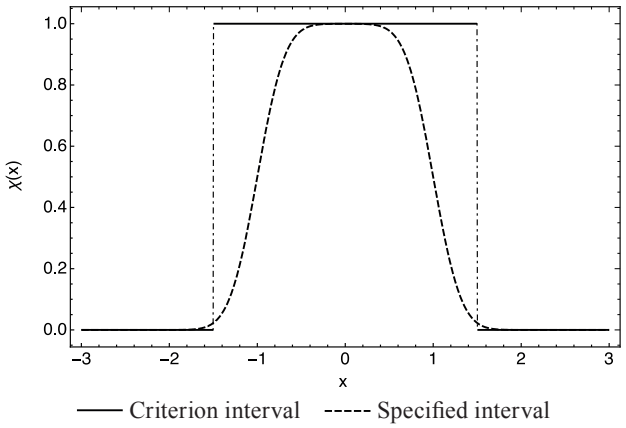

Figure 5 Indicator functions of the interval criterion in strict and fuzzy approaches

integral (i.e., the probability of $x + \varepsilon$ falling into the specified interval):

$$\mathbb{P}(-1 \le x + \varepsilon \le 1) = \mathbb{P}(x + \varepsilon \le 1) - \mathbb{P}(x + \\ + \varepsilon \le -1) = \mathbb{P}(\varepsilon \le 1 - x) - \mathbb{P}(\varepsilon \le -1 - x) \quad (17)$$

The last expression is nothing but the difference between the distribution functions $\Phi_\varepsilon(\cdot)$ of the random variable ε. The formula is:

$$\chi_I(x) = \Phi_\varepsilon(1 - x) - \Phi_\varepsilon(-1 - x)$$
$$\Phi_\varepsilon(x) = \frac{1}{2}\left(1 + \operatorname{erf}\left(2\sqrt{2}x\right)\right) \quad (18)$$
$$\operatorname{erf} x = \frac{2}{\sqrt{\pi}} \int_0^x e^{-t^2} dt$$

erf x is the error function, included for convenience of calculations in many packages of mathematical data processing, in particular, MS Excel.

In the classical approach to identification, any measurement that falls within the interval [–1,1] ± 0.5 will be recognized as corresponding to the criterion (to simplify the example, the questions of additional and outlier measurements are omitted here), while already at the values –1 and 1 the level of belonging to the criterion in the fuzzy approach will be equal to only 0.5 (Fig. 5), and when approaching the boundaries of a large interval –1.5 and 1.5, there is no chance for the criterion. Moreover, to provide the characteristic "most likely the product has a criterion" (function value 0.8), the measurement value must fall within the range [–0.79, 0.79].

This approach should be taken into account specifically at the boundaries of the interval identification. For example, when establishing a boundary for foods of mixed composition with milk fat content the following definition is proposed: if milk fat content exceeds 51% of the total fat phase, the product is called milk-based. If it makes less than 50% – milk-containing, respectively, with a measurement error of ± 0.5%. In this case, products containing milk fat in the range of [50.25%, 50.75%] will not belong to any specified class with a sufficiently high level of confidence.

Despite measurement errors, the boundary of identification classes should be set without taking them into account. Regardless of the nature (except for the assumption of distribution symmetry) and the type of error at the point of the boundary, the indicator function of both classes will be equal to 0.5. This is a logical assumption to refer the product to a particular class if the measurement gave a boundary indicator. In the above example, this boundary will be the point 50%.

However, if indicator functions of two identification classes, being adjacent linguistic characteristics of the same criterion, take the same value of 0.5 at a point, it makes sense setting a boundary between these classes at this point. For the multidimensional case, the boundary will be represented by a hyperplane, but in practice the dimension exceeding two is rarely considered.

Harrington's function as an example of indicator function. One of the applied tools in the qualitative assessment of the developed food industry identification methods is Harrington's desirability function [20].

The idea of Harrington's function is to transform the values of the criteria into a dimensionless desirability scale that allows comparing and combining the characteristics of products of different nature. It establishes compliance between experts' psycho-linguistic assessments and natural indicators of criteria. In addition, it has all the necessary practical properties of the indicator function, which allows using it actively in fuzzy logic applications.

Generally, Harrington's function is of the form of:

$$d(x) = e^{e^{-Y(x)}}, x \in \mathbb{R} \quad (19)$$

where $Y(x)$ is a function that establishes a relation between the values of the experimental variable and the dimensionless scale [21]. In practice, it is almost always linear, being accountable for the shift and steepness of Harrington's function curve in accordance with application needs. It is so as to correspond to the well-established mapping of the function value intervals to the linguistic variable of desirability: "very good" – 0.8, 1; "good" – 0.63, 0.8; "satisfactory" – 0.37, 0.63; "bad" – 0.2, 0.37; "very bad" – 0.0.37.

If there are n criteria with corresponding desirability functions $d_i(x)$, the consolidated estimate is expressed as a weighted geometric mean:

$$\bar{d}(x_1, \ldots, x_n) = \left(\prod_{i=1}^{n} d(x_i)_i^{w_i}\right)^{\frac{1}{\sum_{i=1}^{n} w_i}} \quad (20)$$

The useful property of the function is insensitivity within the range of 0 to 1 values (estimates "very bad" and "very good", respectively). It can be used in the construction of criteria linked to the product's shelf life.

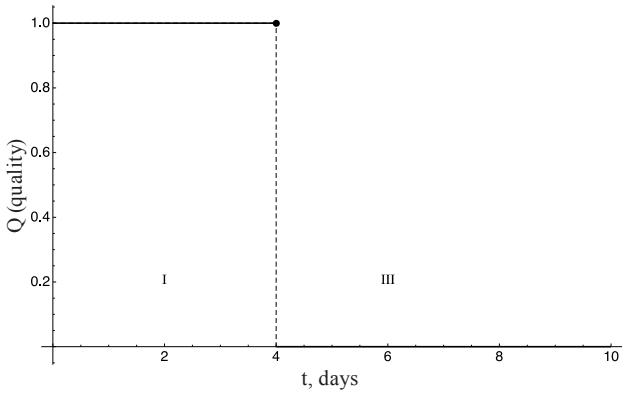

Figure 6 Product quality indicator function, classically

In food products – complex biological systems – quality deterioration is often subject to the exponential law, and one of the key factors is the change in microbiological parameters. So, Harrington's function can be considered as $\chi_Q(t)$ indicator function of Q fuzzy set – i.e., the products corresponding to public health regulations. Here storage time $t > 0$ is used as a product characteristic.

Supposing a product has 4 days' shelf-life. Let us first consider its validity indicator function without the use of fuzzy logic (Fig. 6).

Identification areas I "the product meets the standards and is ready for consumption" and III "the product must be disposed of" are shown, respectively. Within strict logic, at point {4}, it is expected that the function has a gap of the first kind. As an applicable rule, the function reflects rather Cinderella's carriage qualitative characteristics before and after midnight than those of the actual food product.

Since shelf life usually has a margin of 20–25%, consider the following indicator function:

$$\chi_Q(t) = 1 - e^{e^{5-t}} \quad (21)$$

The function is a fuzzy negation of Harrington's function, but this is natural when smaller values are assumed to have a larger desirability value. Its graph is shown in Fig. 7.

In addition to the identification areas I and III, whose linguistic characteristics remain the same, there appears area II – "the product is safe for use, but already undergoing degrading qualitative changes". In this sense, products from identification area II are no longer suitable for end-users and must be sent for extending shelf life processing (sterilization, canning, etc.). At the same time, it should be noted that, for example, when canning, a fuzzy logic device must also be used to establish the final shelf life of the product from raw materials within the boundaries of identification area II.

As it was mentioned above, changes in microbiological parameters have a direct impact on the quality of the product. Logically the phases of microbiological cultures' development can be compared with Harrington's function identification areas; in particular, area I corresponds to the lag phase, area II – to acceleration and exponential growth of microorganisms phase, and area III – to deceleration and stationarity phase. At the same time, substrate and other biotechnological characteristics of change in the population of microorganisms are calculated for each specific product, which may lead to diversities in the general matches given.

Combination of the product's quality characteristics. The construction of indicator functions is inextricably linked with decision-making systems. In the case of one criterion (for example, safety, as described above), the fuzzy logic apparatus gives no clear advantage over a strict approach. In the end, all the contractors of the food industry (consumers, manufacturers, law enforcement agencies, etc.) make a binary decision whether a particular product sample complies with a criterion [22, 23]. Due to the fact that the criterion is unique (for example, expiration date) they identify the above decision with the function of the ultimate goal ("buying" vs. "not buying", "recalling" vs. "not recalling", "fining" vs. "not fining").

In the example with the fety function, three clear identification areas can be introduced. For them, for instance, the seller will have a system of specific actions (I – "selling", II – "reselling for recycling", III – "recycling").

However, even when the second criterion in the decision-making system is engaged, it is much harder to establish the precise boundaries of identification classes.

Consider the instance with the safety criterion with an additional indicator "consumer quality" – a characteristic that demonstrates the taste and overall satisfaction from the consumption of the product – added. In the fuzzy logic the unction of this indicator decreases faster than the safety function. For example, for baking and confectionery products, taste profiles degrade much earlier than the products become unfit for use. The taste of "fresh bread" is of great value to the consumer and has its impact on their purchase preferences, but it is not unique or decisive, as shelf life is also taken into account.

To construct an example of the consumer quality function $\chi_T(t)$, let us use the probability theory apparatus, assuming that fresh $(t = 0)$ product has some taste profile lost on expiry [24]. In practice, it makes sense to put an experiment to determine the distribution histogram of the moment of fresh taste degradation. However, for the purposes of exemplification, it will be simulated with the help of Weibull distribution, used in survival analysis, giving a good approximation in the study of products' storage stability [25]. The density of this distribution $f_{W(k,\lambda)}(t)$ has the form:

$$f_{W(k,\lambda)}(t) = \frac{k}{\lambda^k} t^{k-1} e^{-(t/\lambda)^k}, t > 0 \quad (22)$$

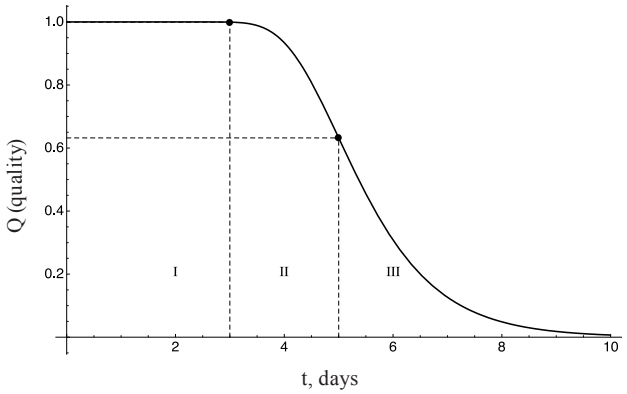

Figure 7 Product quality indicator function based on Harrington's desirability function

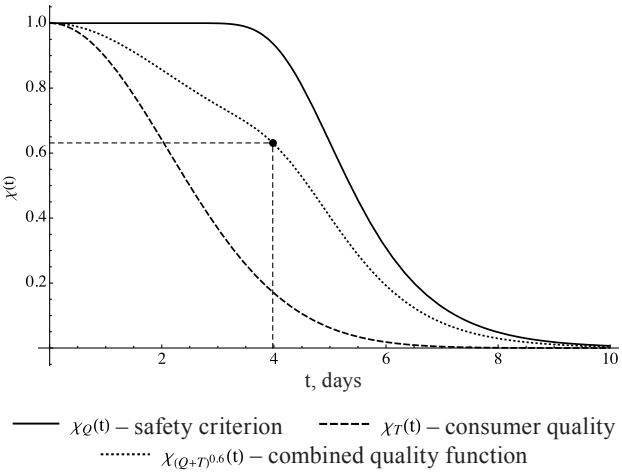

Figure 8 Convex combination of two consumer functions

As a quality function we take the survival function $S_{W(k,\,\lambda)}(t)$ for the given distribution. It is equal to the probability that the value of the random variable under study will exceed *t*, in this case, the probability that the taste has not yet been lost by *t*. For the Weibull distribution, it has a convenient expression:

$$S_{W(k,\lambda)}(t) = e^{-(t/\lambda)^k}, t > 0 \qquad (23)$$

Supposing that for a product with the safety function described by formula (23) the average taste profile is lost on the second day, an approximation of distribution parameters with $\lambda = 2$, $k = 2$ can be derived.

The problem of food products' seller is to establish the time when the product should already be sold at the residual price (the time of entering area III) taking into account the safety and consumer quality criteria.

If the weight of safety indicator is set at 0.6, and the weight of consumer quality indicator is set at 0.4, respectively, a convex criteria combination (8) will take the form:

$$\chi_{(Q+T)^{0.6}}(t) = 0.6\left(1 - e^{e^{5-t}}\right) + 0.4\, e^{-(t/2)^2} \qquad (24)$$

Solving the equation (0.63 being Harrington's function upper exponent for "satisfactory"):

$$\chi_{(Q+T)^{0.6}}(t) = 0.63 \qquad (25)$$

we obtain $t \cong 4$. This means that after four days the product must be sold in traditional or alternative ways. Guided by rate expiry date only, the seller would get the value of 5, thus having no time left for operational maneuvers. The type of function graphs and their convex combination is shown in Fig. 8.

CONCLUSION

Thus, the apparatus of fuzzy logic allows building multi-criteria decision-making systems in the food industry. They help effectively make decisions about products' quality and safety and, in the case of violations and arbitral bodies' involvement, differentiate the administrative impact on the contractors of the food industry.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to this article.

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Specialized hypocholesterolemic foods: Ingredients, technology, effects

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Abstract:
Introduction. Overweight and obesity are leading risk factors for metabolic syndrome (MS). From 20 to 35% of Russian people have this condition, depending on their age. MS is a precursor of cardiovascular disease, diabetes mellitus, diabetic nephropathy, and non-alcoholic steatohepatitis. Specialized foods (SFs) with hypocholesteremic effects are an important component of the diet therapy for MS patients. Creating local SFs to optimize the nutritional status of MS patients and prevent related diseases is a highly promising area of research. The aim of our study was to develop the formulation and technology of SFs and evaluate their effectiveness in MS treatment.
Study objects and methods. The objects of the study were food ingredients and SFs. Safety indicators and micronutrient contents were determined by standard methods, whereas nutritional and energy values and amino acid contents were determined by calculation.
Results and discussion. Based on medical requirements, we selected functional ingredients and developed a formulation and technology of SFs with an optimized protein, fat, and carbohydrate composition. The formulation included essential micronutrients and biologically active substances with a desirable physiological effect. Clinical trials involved 15 MS patients aged from 27 to 59. For two weeks, they had a low-calorie standard diet with one serving of SFs in the form of a drink instead of a second breakfast. The patients showed a significant improvement in anthropometric indicators. Blood serum tests revealed decreased contents of total cholesterol (by 16.9%), low-density lipoprotein cholesterol (by 15.3%), and triglycerides (by 27.9%).
Conclusion. We developed technical specifications and produced a pilot batch of SFs. The trials showed an improvement of lipid metabolism in the MS patients who were taking SFs as part of their diet therapy.

Keywords: Metabolic syndrome, specialized food, food ingredients, diet therapy

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INTRODUCTION

The key factors leading to metabolic syndrome are increased visceral fat and decreased sensitivity of peripheral tissues to insulin resulting in compensatory hyperinsulinemia. These conditions are associated with disorders of carbohydrate, lipid, and purine metabolism and arterial hypertension. External contributors to metabolic syndrome include perinatal development, nutrition structure, level of physical activity, bad habits, stress, and others [1, 2]. Genetic factors also play a role [3]. Almost all metabolic syndrome conditions are risk factors for cardiovascular diseases, and a combination of them significantly increases the risk of their development. Metabolic syndrome is a precursor of socially significant diseases such as type 2 diabetes, diabetic nephropathy, non-alcoholic steatohepatitis, etc. [4].

According to the International Diabetes Federation (IDF), abdominal obesity is the key criterion for metabolic syndrome diagnosis, whereas arterial hypertension and lipid and carbohydrate metabolism disorders are additional criteria [5].

In Russia, the first unified criteria for metabolic syndrome diagnosis were proposed by the Russian Society of Cardiology (RSC) in 2008 and revised in 2009. They consider the central (abdominal) type of obesity to be the main component of metabolic

syndrome. It is diagnosed at a waist circumference of over 80 cm for women and over 94 cm for men. Other metabolic syndrome criteria include high blood pressure (> 130/95 mm Hg), high triglycerides (> 1.7 mmol/L), high low-density lipoprotein cholesterol (> 3.0 mmol/L), low high-density lipoprotein cholesterol (< 1.0 mmol/L for men; < 1.2 mmol/L for women), fasting hyperglycaemia (fasting plasma glucose ≥ 6.1 mmol/L), and impaired glucose tolerance (plasma glucose 2 h after glucose loading within 7.8–11.1 mmol/L). Patients with central obesity and two additional criteria are diagnosed with metabolic syndrome [6].

World statistics for metabolic syndrome depend on the diagnostic criteria. According to numerous studies conducted in various countries, metabolic syndrome is diagnosed in 10–30% of the world adult population. The metabolic syndrome rate in Russia varies from 20 to 35%, depending on the age group (higher in old age). It is 2.5 times as common in women as it is in men [7].

Obesity is a leading risk factor for diet-related diseases, including metabolic syndrome [8]. Over the past three decades, the world rate of overweight and obesity has grown by 30% among adults and by 50% among children. By 2025, 40% of men and 50% of women will be obese [9]. According to the World Health Organization (WHO), overweight and obesity lead to type 2 diabetes (44–57% of cases), coronary heart disease (17–23%), arterial hypertension (17%), gallstone disease (30%), osteoarthritis (14%), malignant neoplasms (11%), as well as impaired reproductive function [10–12].

In Russia, the overweight and obesity rates have seen a significant growth in the last decade, reaching 60% and 24% among adults and 20% and 5.65% among children, respectively [13–15].

An integrated approach to metabolic syndrome treatment involves combining pharmacological agents and dietetic nutrition. Metabolic syndrome patients should have a low-calorie diet (1200 kcal for women and 1500 kcal for men). The amounts of fats, carbohydrates, and proteins should not exceed 25%, 55%, and 20% of the daily calorie intake, respectively [1, 2, 4]. The main objective is to lower the risk of developing MS-related diseases by reducing body weight and increasing tissue sensitivity to insulin [1].

Metabolic syndrome treatment can be made more effective by enriching the diet with specialized foods that have an improved chemical composition. These foods contain functional ingredients and biologically active substances that meet modern safety requirements and have hypolipidemic and hypoglycaemic effects. As a result, they supply the patient's body with nutrients, including essential polyunsaturated fatty acids, vitamins, macro- and microelements.

Taking into account the biological role of food proteins and their beneficial effect on lipid and carbohydrate metabolism, it is advisable to use ingredients containing complete, easily digestible proteins. Milk whey proteins have a balanced amino acid composition and a high biological value. Compared to other proteins of animal and plant origin, they have a higher content of essential amino acids (lysine, tryptophan, methionine, threonine) and branched-chain amino acids (valine, leucine and isoleucine), which are involved in synthesizing muscle protein [16, 17].

Among plant proteins, soy proteins have been traditionally used in diet correction and prevention of lipid metabolism disorders and related diseases. They are isolated from unmodified soy with modern water extraction technology. This technology preserves native amino acids and active isoflavones while removing proteolytic enzyme inhibitors, lectins, urease, lipoxygenase, and some other compounds. The highly purified soy isolates contain over 80% protein, are easily digestible, have a balanced amino acid composition, as well as hypocholesterolemic and antiatherogenic effects [18–22].

According to modern scientific literature, the soy protein hypocholesterolemic effect can be explained by cholesterol interacting with peptide fractions in the small intestine. Peptide fractions are formed during protein digestion in the gastrointestinal tract. This interaction impairs the micellar solubility of cholesterol and its absorption, changes the enterohepatic circulation of bile acids, and thus lowers cholesterol in the liver and reduces the expression of certain genes of lipid transport protein mediators [23, 24]. Soy protein has a high content of glutamine, an amino acid necessary for glutathione to form. Glutamine protects cells from damage by free radicals and plays an important role in the functioning of the immune system [25]. Soy protein has a limited content of three essential amino acids: threonine, methionine, and cysteine. Therefore, it is advisable to use it in combination with milk whey protein, whose amino acid composition is closest to that of the "ideal" protein.

Fats are an important supplier of energy in the diet and a source of sterols, fat-soluble vitamins. However, excessive consumption of fats contributes to the development of metabolic syndrome and related complications. General recommendations for treating lipid metabolism disorders are to reduce total cholesterol and saturated fatty acids, while increasing the proportion of monounsaturated fatty acids to 10–15% and polyunsaturated fatty acids to 7–9% of the total caloric intake [26–29]. In recent years, scientists have taken greater interest in the functional role of monounsaturated fatty acids in the human body. According to literature, they reduce the level of atherogenic low density lipoproteins and free radical oxidation in the body, as well as prevent insulin resistance [30–32].

Recent studies confirm the importance of omega-3 polyunsaturated fatty acids in the treatment of lipid metabolism disorders. They also have a beneficial

effect on the lipid profile of blood and reduce the risk of developing cardiovascular diseases [33–35].

The carbohydrate profile should be modified by excluding mono- and disaccharides, which cause a sharp increase in blood glucose, and by introducing slowly digested and absorbed carbohydrates, which cause a gradual increase in postprandial glycemia.

Intensive sweeteners of natural or synthetic origin and sugar substitutes from the polyol family (xylitol, sorbitol, maltitol, lactitol, isomaltitol, and erythritol) are widely used in the food industry to form the sensory profile characteristic of traditional sweet products, in particular drinks. Intensive sweeteners (such as aspartame, saccharin, cyclamates, potassium acesulfame, sucralose, etc.) are sweeter than sugar dozens or even hundreds of times. However, they barely cause any hyperglycemic or insulinemic effect. Polyols, which are polyhydric alcohols in chemical structure, have a lower calorie content and sweetness rate than sucrose (except xylitol with a sweetness rate of one). Polyols cause a more gradual increase in postprandial glycemia compared to carbohydrates. They do not require insulin for absorption, which makes them suitable for in low-calorie and diabetic foods. Mixing sweeteners often produces a synergistic effect, which makes it possible to achieve a sweetness profile close to sucrose [36, 37]. Excessive intake of sweeteners can have an adverse effect on the gastrointestinal tract causing increased bowel sounds, a feeling of bloating or heaviness, and diarrhea. Therefore, some polyols have upper permissible levels of daily intake, for example 40 g for xylitol and sorbitol, 45 g for erythritol, and 3 g for mannitol [38].

Although dietary fiber is not an essential nutrient, its deficiency is a risk factor for many diseases. Dietary fiber is known to normalize the motor-evacuation function of the large intestine and have a prebiotic effect. Mostly soluble dietary fibers (alginates, pectin, inulin, β-glucans, gum arabic, some hemicelluloses, and modified celluloses) have a beneficial effect on lipid and carbohydrate metabolism. Their hypocholesterolemic effect is due to their ability to bind and excrete bile acids and slow down cholesterol absorption in the small intestine. They also reduce lipids absorption by increasing their excretion and inhibit cholesterol synthesis in the liver caused by the formation of short-chain fatty acids during dietary fiber fermentation in the large intestine. The hypoglycemic effect of soluble dietary fibers is caused by slowing gastric emptying, decreasing availability of starch for digestive enzymes, and reducing glucose absorption in the small intestine. As a result, dietary fibers lower postprandial glycemia [39, 40].

Minerals and vitamins are essential food components that perform important physiological functions in the body. There is a problem of micronutrient deficiency in Russia, which is a risk factor for many nutrition-related diseases. Therefore, it is advisable that metabolic syndrome patients enrich their diet with vitamins (groups B, C, E, A, D, beta-carotene), minerals (potassium, magnesium, calcium), and trace elements (chromium, zinc) [41, 42].

Trivalent chromium (Cr) is vital for normal carbohydrate metabolism in humans and animals [43]. Chromium stimulates glucose delivery into cells, inducing genes of intracellular signalling systems. There is evidence of direct interaction of chromium with insulin. In particular, it interacts with its dimers, thus stabilizing the hormone structure or enhancing its binding to the receptor [44]. The biochemical and physiological effects of zinc in mammals are determined by its ability to regulate the chronic inflammatory status by reducing inflammatory cytokines, reduce the effects of oxidative stress, and participate in lipid and carbohydrate metabolism.

Zinc deficiency can be an important risk factor for type 2 diabetes. Plasma zinc levels are inversely correlated with glycated hemoglobin levels in diabetes [45]. Zinc improves glucose metabolism and insulin sensitivity in diabetics. It plays an important role in the synthesis, deposition, and secretion of insulin in pancreatic β-cells. Zinc deficiency has a negative effect on insulin sensitivity and glucose tolerance [46–49]. In addition, zinc stimulates glycolysis, inhibits gluconeogenesis, and is involved in glucose transport in adipocytes [50]. The metabolic effect of zinc in obesity is associated with its impact on adipokines, hormones of adipose tissue (interleukin 6, tumour necrosis factor, leptin, adiponectin, and others) [51–53]. In particular, experimental studies show that an adequate level of zinc in adipose tissue is important for the normal functioning of adipocytes and leptin synthesis [52]. Complexes of chromium and zinc with enzymatic hydrolysates of various food proteins can be effectively used to obtain new food sources of these trace elements in an organically bound and highly bioavailable form. Using such complexes in human nutrition is physiologically justified [54].

The Russian market of dietetic foods for the prevention and treatment of nutrition-related diseases (including metabolic syndrome) is quite limited. This situation creates a need for studies aimed to develop new foods that meet modern safety and clinical efficacy requirements.

Powdered specialized foods are most suitable for a clinical setting. They can be used to make drinks and cocktails or added to ready-made cereals and dairy products (kefir, fermented baked milk, yogurt, and curdled milk). In addition, dry products are easy to transport and store, are microbiologically stable, and have a long shelf life. Their production technology ensures a wide range of products with various sensory profiles.

In connection with the above, our study aimed to develop and evaluate the clinical efficacy of specialized foods intended for dietetic treatment of lipid metabolism disorders in metabolic syndrome patients.

STUDY OBJECTS AND METHODS

The following ingredients were used to develop specialized foods for metabolic syndrome patients:
– Supro Plus 221 D IP soy protein isolate with 80% protein (Solae, USA);
– Lacprodan 80 whey protein concentrate with 80% protein (Arla Foods Ingredients SF, Argentina);
– MD1925 QS maltodextrin with 18.9% dextrose equivalent (DE) (Syral, France);
– Cegepal 03-C microencapsulated rapeseed oil with 68% fat (BASF Personal Care and Nutrition GmbH, Germany);
– Crystalline maltitol with 99.5% main component (Shandong Lujian Biological Technology Co., LTD, China);
– Genu DZ citrus pectin with 58–62% esterification (CP Kelco Germany GmbH, Germany);
– Life, DHA S17-P100 docosahexaenoic acid (DSM Nutritional Products Europe Ltd, Switzerland);
– Karnipur Crystalin L-carnitine with 99% main component (Lonza Ltd, Switzerland);
– EM28304 vitamin premix (DSM Nutritional Products Europe Ltd, Switzerland): vitamins A, D_3, E, K_1, C, B_1, B_2, B_6, B_{12}, PP, calcium D-pantothenate, folic acid, biotin, maltodextrin;
– 2-aqueous lactic acid magnesium (PURAC biochem B.V., Spain);
– Carbonic calcium (Mineraria Sacilese S.P.A, Italy);
– Potassium citrate 3-substituted monohydrate (V.A.G. Chemie GmbH, Germany);
– Zinc chloride (analytic grade, State Standard 4529-78[I]);
– 6-aqueous chrome chloride (analytic grade, State Standard 4473-78[II]);
– Sodium hydroxide (analytic grade, State Standard 4328-77[III]);
– Apple natural food flavoring (Givaudan Schweiz AG, Switzerland);
– Apricot natural food flavoring (Givaudan Schweiz AG, Switzerland);
– Stevilia E mixture of sweeteners: erythritol (E968), stevia extract (E960) (TU 9197-002-49929776-13 (Aspasvit, Russia); and
– Powdered beta-carotene dye (DSM Nutritional Products Europe Ltd., Switzerland).

All the ingredients met safety requirements established by the Technical Regulations of the Customs Union, namely 021/2011[IV], 033/2013[V], and 029/2012[VI]. The food additives were used within the amounts established in Technical Regulations 029/2012.

The specialized food physicochemical parameters were determined by standard methods, namely:
– moisture mass fraction: according to State Standard 29246-91[VII];
– vitamin A: according to State Standard R 54635-2011[VIII];
– vitamin E: according to State Standard R 54634-2011[IX];
– vitamins C, B_1, B_2, B_6, minerals (calcium, magnesium, potassium, chromium, zinc), mono- and disaccharides, L-carnitine: according to Regulation 4.1.1672-03[X];
– water activity: by a mirror-cooled dew point sensor on an AquaLab 4TE analyser (Decagon Devices, USA);
– amino acid composition of the milk and soy protein component: by calculation using the manufacturers' specifications;
– nutritional and energy values, percentage of average daily requirement for nutrients and energy: by calculation using the handbook on chemical composition and caloric content of food ingredients, taking into account recommended daily intake of nutrients and energy according to Technical Regulations 022/2011[XI], the Uniform Sanitary Epidemiological and Hygienic Requirements for the Goods Subject to Sanitary and Epidemiological Supervision (Control), as well as manufacturers' specifications [55].

RESULTS AND DISCUSSION

Producing specialized foods that meet the biomedical requirements for metabolic syndrome patients involves selecting ingredients with a desirable chemical composition and hypocholesterolemic effect.

[I] State Standard 4529-78. Reagents. Zinc chloride. Specifications. Moscow: Izdatel′stvo standartov; 1990. 10 p.
[II] State Standard 4473-78. Reagents. Chromic (III) chloride hexahydrate. Specifications. Moscow: Izdatel′stvo standartov; 1992. 15 p.
[III] State Standard 4328-77. Reagents. Sodium hydroxide. Specifications. Moscow: Izdatel′stvo standartov; 2001. 19 p.
[IV] TR TS 021/2011. Tekhnicheskiy reglament Tamozhennogo soyuza "O bezopasnosti pishchevoy produktsii" [TR CU 021/2011. Technical regulations of the Customs Union "On food safety"]. 2011.
[V] TR TS 033/2013. Tekhnicheskiy reglament Tamozhennogo soyuza "O bezopasnosti moloka i molochnoy produktsii" [TR CU 033/2013. Technical regulations of the Customs Union "On milk and dairy products safety"]. 2013. 107 p.
[VI] TR TS 029/2012. Tekhnicheskiy reglament Tamozhennogo soyuza "Trebovaniya bezopasnosti pishchevykh dobavok, aromatizatorov i tekhnologicheskikh vspomogatel′nykh sredstv" [TR CU 029/2012. Technical regulations of the Customs Union "Safety requirements for food additives, flavours and processing aids"]. 2012.
[VII] State Standard 29246-91. Dry canned milk. Methods for determination of moisture. Moscow: Izdatelstvo standartov; 2001. 6 p.
[VIII] State Standard R 54635-2011. Functional food products. Method of vitamin A determination. Moscow: Standartinform; 2013. 12 p.
[IX] State Standard R 54634-2011. Functional food products. Method of vitamin E determination. Moscow: Standartinform; 2013. 15 p.
[X] Regulation 4.1.1672-03. Guidelines on quality and safety control methods for biologically active food additives. Moscow: Federal Center for State Sanitary and Epidemiological Supervision of the Ministry of Health of Russia; 2004. 240 p.
[XI] TR TS 022/2011. Tekhnicheskiy reglament Tamozhennogo soyuza "Pishchevaya produktsiya v chasti ee markirovki" [TR CU 022/2011. Technical regulations of the Customs Union "Food labelling"]. 2011. 29 p.

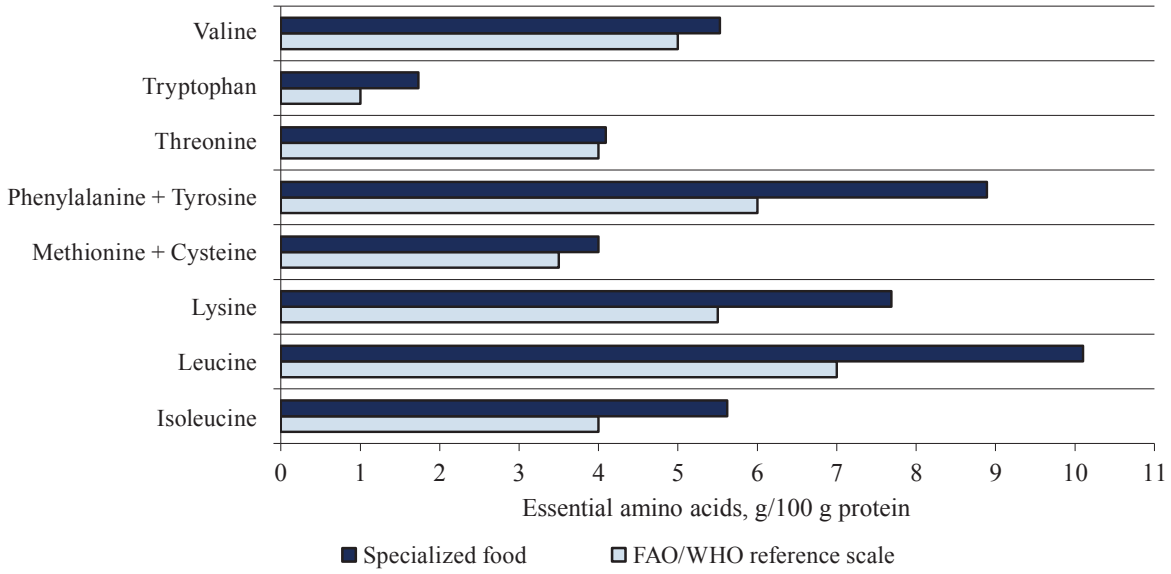

Figure 1 Essential amino acids in the specialized food protein component (compared to a reference scale)

Consumer properties of the product (physicochemical and sensory indicators) and its safety depend primarily on the ingredients, their technological and sensory compatibility.

The Russian market offers a wide range of functional ingredients and biologically active substances that meet modern safety requirements. In addition to satisfying human needs for food and energy, they also have a health-beneficial effect due to their physiological action. The amount of a functional ingredient in the product should be physiologically significant. It means that it should meet the physiological need for it and, at the same time, ensure adequate consumer properties (appearance, taste, aroma, texture, etc.).

A 1:1 ratio of soy protein isolate and milk whey protein was used as a protein component in the specialized food formulation. Lacprodan 80, a whey protein concentrate available on the Russian market, is produced using membrane technology that does not have a denaturing effect on proteins. Whey protein concentrate contains about 80% of complete easily digestible protein with a high amino acid score compared to the FAO/WHO reference scale (1985). Its minerals (mg/100 g) include calcium (365 mg), sodium (246 mg), magnesium (52 mg), and potassium (524 mg) [56]. The low lactose content makes this protein source suitable for people with lactase deficiency.

Supro Plus XT 221D IP, an isolated soy protein used in the specialized food formulation, contains 85% protein, about 3% fat, and 1% carbohydrates. Its minerals (mg/100 g) include calcium (50 mg), phosphorus (900 mg), magnesium (34 mg), potassium (1300 mg), sodium (780 mg), iron (12 mg), and copper (1.6 mg).

This combination of milk and soy proteins allowed us to optimize the amino acid composition of the protein component and ensure a high score of essential amino acids compared to the FAO/WHO reference scale (1985) (Fig. 1).

The specialized food fat component is a mixture of rapeseed oil microcapsules and docosahexaenoic acid (DHA). A source of monounsaturated and polyunsaturated fatty acids, rapeseed oil contains about 60% oleic acid, 20% linoleic (ω-6) acid, and 10% α-linolenic (ω-3) acid. DHA is a powder with a slight fishy odor that contains 17–21.5% of DHA isolated from microalgae.

Maltodextrin, a product of incomplete hydrolysis of corn starch, was used as a source of carbohydrates. These are so-called “complex” carbohydrates with a sweetness rate of 0–0.3 and DE 18.9%.

Maltitol (a polyhydric alcohol obtained by hydrogenating starch-based maltose) and Stevilia E (a mixture of erythritol and stevia extract) were used to form the taste profile of the rehydrated beverage. Maltitol had a sweetness rate of 0.8, and Stevilia E was five times as sweet as sugar.

The widespread use of various types of pectin as a source of soluble dietary fiber is due to the chemical structure of its molecules. Differences in physicochemical properties (solubility, gelling and complexing ability) are determined by the degree of esterification and the molecular weight of pectin molecules. The ability of pectin to dissolve in water and form colloidal systems is important for its use in food production and for its physiological effect on the human body. The FAO/WHO experts recommend pectin for treating cardiovascular diseases, hyperlipidemia, diabetes, impaired glucose tolerance, obesity, and hypomotor dyskinesia of the colon and the gallbladder [37]. Knowing that highly esterified pectins have an increased solubility in water, we used citrus pectin with a 58–62% degree of esterification.

L-carnitine (levocarnitine) is a biologically active substance whose effectiveness is clinically proven. This compound plays a major role in the transport of fatty acids into mitochondria. In clinical practice, L-carnitine is successfully used in treating a wide range of diseases, such as anorexia, chronic fatigue syndrome, cardiovascular pathology, hypoglycemia, male infertility, and kidney disease [57]. The studies conducted by the Clinic of Nutritional Treatment at the Federal Research Centre of Nutrition and Biotechnology demonstrated the effectiveness of L-carnitine in the diet of patients with metabolic disorders accompanied by obesity [58]. According to physiological needs for minor and biologically active food substances, the average L-carnitine requirement for adults is 300 mg/day. The maximum daily intake may reach 900 mg/day [38].

To improve the vitamin status of patients, specialized foods were enriched with vitamins A, D_3, E, K_1, C, B_1, B_2, B_6, B_{12}, PP, calcium D-pantothenate, folic acid, and biotin in the form of a special water-soluble premix.

In view of the importance of minerals in physiological processes that ensure normal functioning of the body, we used salts rich in magnesium, potassium, and calcium, namely lactic magnesium, potassium citrate, and calcium carbonate. We also used protein-chelate complexes of zinc and chromium ions with peptides of soy protein hydrolysate. The preparation of an enzymatic soy protein hydrolysate is described in [59].

Thus, the specialized food formulation included the following ingredients based on the requirements for metabolic syndrome dietary therapy: whey protein concentrate, soy protein isolate, microencapsulated rapeseed oil, maltodextrin, docosahexaenoic acid, maltitol, pectin, potassium citrate, magnesium lactic acid, calcium carbonate, a mixture of sweeteners (stevia extract, erythritol), vitamin premix (vitamins A, E, C, D_3, B_1, B_2, B_6, B_{12}, PP, folic acid, pantothenic acid, K_1, biotin), L-carnitine, organic sources of zinc and chromium, beta-carotene dye, and natural flavoring agents.

The specialized food technology included the following main stages: enzymatic hydrolysis of soy protein isolate, obtaining protein-chelate complexes of zinc and chromium, preparing a mixture of minor ingredients (pre-mix), obtaining specialized foods, packaging, and labelling.

The protein-chelate zinc complex was obtained by mixing a pre-prepared 10% aqueous solution of enzymatic soy protein hydrolysate and a 25% aqueous solution of zinc chloride in the ratio of 10:1, adding a solution of sodium hydroxide to reach pH 7.0–7.1, and then thermostating for 60 min at room temperature with constant stirring. To remove sediment and mechanical impurities, the resulting solution was microfiltered in a tangential flow with a pore diameter of under 5.0 μm. Those zinc ions which were not related to the peptide-amino acid matrix were removed by nanofiltration. The filtrate was pasteurized at 75°C for 30 s and freeze-dried.

The protein-chelate chromium complex was obtained by mixing a 10% aqueous solution of enzymatic hydrolysis of soy protein isolate and a 10% aqueous solution of chromium chloride in the ratio of 100:1. The nanofiltration stage was excluded from the process. The obtained protein-chelate complexes were ground in a knife mill and sieved through a sieve with a 1.0 mm mesh diameter.

The protein-chelate complexes were fine powders with the following characteristics: beige color, 2.3% moisture, specific smell, bitter-salty taste, and high solubility in water. Zinc complex had a zinc content of 46.1 mg/g and chromium complex had a chromium content of 4.7 mg/g.

The main objective of dry mixing is to achieve uniform distribution of minor ingredients in the product. We used the technology of phased mixing, taking into account the $1:10^{-2}$–$1:10^{-3}$ ratio of the main ingredients (sources of proteins, fats, and carbohydrates) and micro additives (macro- and microelements, vitamins, and biologically active substances).

At the first stage, a premix was obtained using docosahexaenoic acid, calcium carbonate, flavoring agents, vitamin premix, beta-carotene, protein-chelate chromium complexes of zinc and chromium, L-carnitine, and 10% of the formulated amount of microencapsulated rapeseed oil for more even distribution. The ingredients were mixed in a turbulent mixer at 40 rpm for 35 min. At the second stage, the resulting premix was mixed with the rest of the ingredients at 40 rpm for 30 min. The fill factor of the mixing chamber was 0.7. Using a complex trajectory of mixing under the influence of gravity with a specified multidirectional spatial movement of the mixing chamber minimized the negative effect of centrifugal forces and prevented so-called "dead zones" and heating of the product. Direct filling batchers were used to package 30 g portions of the finished product in film bags.

The above technology allowed us to obtain a homogeneous powdery mixture with evenly distributed minor ingredients, which ensured a recommended intake of all the nutrients with every portion of the product. With a moisture content of 3.35 ± 0.04% and a water activity indicator (A_w) of 0.2304 ± 0.0009, the specialized food is a low-moisture product. This characteristic ensures the stability of its properties, as well as of quality and safety indicators throughout its shelf life.

Powdered specialized foods can be added to ready-made cereals, desserts, and fermented dairy products. When rehydrated, they can be used as a drink or a cocktail. For this, the contents of a package (30 g) must be poured into a glass and stirred vigorously with 100–150 mL of hot water (60–80°C) until the product is homogeneous, or beaten in a blender. The amount of water can vary, depending on the desired consistency. One serving (30 g) is recommended per day.

Table 1 Contents of specialized food nutrients per 100 g, one serving and % of the average daily requirement (ADR)

Nutrient	100 g	Serving (30 g)	% of ADR (30 g)*
Protein, incl.	20.9	6.3	8
animal protein, g	11.7	3.5	
plant protein, g	9.2	2.8	
Fat, incl.	14.3	4.3	5
oleic acid (monounsaturated), g	10.6	3.2	21**
docosahexaenoic (ω-3) acid, mg	600	180	26**
α-linolenic (ω-3) acid, mg	1770	530	76**
Carbohydrates, incl.	28.4	8.5	2
lactose, g	1.14	0.34	
Soluble dietary fiber (pectin), g	4.5	1.35	68*
Maltitol, g	9.5	2.85	
L-carnitine, mg	360	108	36**
Minerals			
Calcium, mg	1075	323	32
Magnesium, mg	406	122	30
Potassium, mg	2270	680	19
Zinc, mg	13.0	3.9	26
Chromium, μg	57.0	17	34**
Vitamins			
C, mg	196	59	100
B_1, mg	2.23	0.54	39
B_2, mg	1.98	0.67	42
B_6, mg	2.2	0.66	33
B_{12}, μg	3.0	0.9	90
PP, mg	18.0	5.4	30
Folic Acid, μg	700	210	105
Pantothenic acid, mg	5.4	1.62	27
A, μg	800	240	30
D_3, μg	10.0	3.0	60
E, mg	12.1	3.63	36
K_1, μg	90.0	27.0	23**
Biotin, μg	30.0	9.0	18**
Energy value/calorie content, kJ/kcal	1500/358	450/107	4

* Technical Regulations of the Customs Union 022/2011

** Uniform Sanitary Epidemiological and Hygienic Requirements for the Goods Subject to Sanitary and Epidemiological Supervision (Control)

Table 1 shows the contents of food components and biologically active substances in 100 g of the specialized food and its 30 g serving, as well as a percentage of the average daily requirement for macro- and micronutrients in one serving.

The content of macro- and micronutrients in one specialized food serving meets the medical recommendations for metabolic syndrome treatment.

Based on our studies, we developed Technical Specifications 10.86.10-007-01897222-2018 "Specialized food for dietetic preventive and dietetic therapeutic nutrition – an instant drink". Valetek Prodimpex, a Russian research and production company produced a pilot batch of specialized foods. The sanitary-chemical and microbiological tests confirmed the products' compliance with the current regulatory requirements established by Technological Regulations of the Customs Union 021/2011 and 027/2012[XII].

The clinical efficacy of specialized foods was assessed by the Department of Metabolic Diseases at the Federal Research Centre of Nutrition and Biotechnology. The study involved 15 metabolic syndrome patients aged 27 to 59. For two weeks, they had a 1500 kcal hypocaloric standard diet with one specialized food drink instead of a second breakfast. During the treatment, the patients showed a decrease in body weight, body mass index, waist volume, and body fat mass by an average of 3.6, 3.9, 3.9, and 4.4%, respectively. Their blood serum tests featured a decrease of 16.9% in total cholesterol, 15.3% in low-density lipoprotein cholesterol, and 27.9% in triglycerides, compared to the initial level.

CONCLUSION

Based on the requirements of modern nutritional science, we developed a formulation of specialized foods for metabolic syndrome patients, including ingredients and biologically active substances with a hypolipidemic effect. Our technology ensures uniform distribution of minor ingredients and, therefore, a desirable content of nutrients in each serving of the product according to medical and biological requirements. Further, we developed Technical Specifications 10.86.10-007-01897222-2018 "Specialized food for dietetic preventive and dietetic therapeutic nutrition – an instant drink". The clinical trials of a pilot batch of specialized foods within a standard hypocaloric diet showed their effectiveness for metabolic syndrome patients.

CONTRIBUTION

Concept development – A.A. Kochetkova, V.K. Mazo; data collection and processing, writing a manuscript – V.M. Vorobyeva, I.S. Vorobyeva, Kh.Kh. Sharafetdinov, S.N. Zorin; text editing – A.A. Kochetkova, V.K. Mazo.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Biological value of semi-smoked sausages with cedar oil cake

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Abstract:
Introduction. Development of novel meat products with better quality and biological value remains one of priority objectives of modern food industry. New meat products correspond with the principles of healthy diet due to their improved fatty acid profile and lower sodium content. The present research featured semi-smoked sausages with 15% of cedar oilcake and a low-sodium curing mix. The cedar nut oilcake is as a source of highly unsaturated fatty acids and high-grade protein. In addition to the physiological effect, the low-sodium curing mix increases the resistance of the combined fat phase to deterioration during storage.
Study objects and methods. Sausages of control and experimental formulations were made in laboratory conditions. The control formulation included raw second-grade beef, semi-fat pork (30% of fat), and traditional curing ingredients, i.e. sodium chloride and nitrite curing mix. In the experimental formulation, 15% of the semi-fat pork was replaced by cedar oil cake, and 30% of sodium chloride – by magnesium chloride. The samples were tested for fatty and amino acid composition, biological value of the lipid and protein phase, chemical composition, as well as physical, chemical, and sensory properties. Other research data included water activity, as well as acid, peroxide, and thiobarbituric value during storage at a temperature of 4 ± 2°C for 15 days.
Results and discussion. The study of fatty acid composition showed significant differences in the ratio of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA, $P < 0.05$) in the samples. When 15% of pork was replaced by cedar oilcake, the amount of SFA decreased by 19.8%, while the content of MUFA and PUFA increased by 10.2% and 24.9%, respectively. These changes improved the indices of atherogenicity and thrombogenicity. The quality of the protein component also improved, as the utility coefficient of amino acids increased from 0.83 to 0.87, and the coefficient of comparable redundancy decreased from 7.2 g/100 g of protein to 5.35 g/100 g of protein. The sausages with cedar oil cake and low sodium chloride content received a high consumer evaluation. The hydrolysis of the lipid fraction was the same in both samples. The process of lipid oxidation was inhibited, which can partially be explained by a lower water activity.
Conclusion. The biological value, consumer quality, and storage stability of semi-smoked sausages could be improved by replacing fat-containing meat raw materials with cedar oil cake and reducing sodium chloride content. The new product demonstrated a better nutrition quality and can be recommended for mass production.

Keywords: Meat products, formulations, fatty acid composition, sodium, lipid oxidation, water activity

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INTRODUCTION

Contemporary domestic and foreign studies prove that diet can significantly improve health and quality of life. The specific character of raw materials and processing technology makes meat one of the most important food products [1, 2]. A healthy diet can prevent cardiovascular diseases, obesity, and hypertension. As a rule, a healthy diet implies reducing or eliminating the content of cholesterol, saturated fatty acids, and sodium, as well as enriching food products with biologically active components of plant origin.

Gorbatov All-Russia Meat Research Institute summarized and structured materials on the fatty acid composition of meat obtained from various farm animals and poultry. The study indicated a significant difference in their fat profile and a general tendency to an increased content of saturated fatty acids [3].

Semi-smoked sausages are meat products with a high content of fat component and sodium. However, they are extremely popular with consumers. As a result, domestic meat industry produces them in a large quantity and assortment [4]. Semi-smoked sausages are

made from comminuted meat raw materials subjected to roasting, cooking, and smoking. The simple production technology, as well as their appearance and sensory indices, make semi-smoked sausages suitable for programmed formation of sensory properties, as well as nutritional and biological value.

The selection and justification of the fat component is extremely important when developing a new technology for semi-smoked sausages of improved quality and nutritional value. The fat component, its type and amount determine the total fat content, fatty acid composition, and flavor of the final product. A high content of saturated fatty acids in the fat component may decrease the biological value and digestibility of the fat, while a big amount of unsaturated acids increases the risk of oxidation during heat treatment and storage. The latter may lead to the formation of various organic compounds, e.g. aldehydes, ketones, hydroxy acids, etc. They possess a specific intense smell, which can affect the results of sensory evaluation and reduce product safety [5–8]. The specific smell of meat products also appears when lipids enter the Maillard reaction during heat treatment [9]. However, lipids are able to retain and enhance pleasant smells formed by other components of the formulation. Therefore, lipids can develop both programmed and unwanted tastes of the final product.

Domestic and foreign studies of the fat component in semi-smoked sausages feature various ways of reducing the mass fraction of fat and improving the fatty acid composition. As a rule, researchers propose two solutions. The first solution is to use low-calorie ingredients. The second solution is to replace animal fat with highly unsaturated vegetable oils or fat raw materials of plant origin. The second approach is more advantageous, since fat is involved in the formation of plastic consistency and aromatic properties of meat products.

Due to the differences in the physical properties of raw meat and vegetable oils, the latter cannot be directly used in sausage production. The problem is that direct replacement can misbalance the meat system and result in fat pockets in the final product [10–13]. This problem can be solved by changing the physicochemical properties of vegetable oils, e.g. by microencapsulation or emulsification. The changes should be aimed either at increasing the stability of the oils to oxidation, or at improving the water- and fat-holding properties of ground meat [14, 15].

Muguerza *et al*. replaced 20% of pork backfat with pre-emulsified olive oil in the formulation of Chorizo de Pamplona, a traditional Spanish dry fermented sausage. This reduced the total fat content by 53% and increased the proportion of MUFA. Similar results were obtained when pork fat was substituted by soybean oil. Olive and soybean oils reduced cholesterol content by 12.92% and 5.65%, respectively [16–18].

Korean scientists studied emulsified rice bran oil. Its fatty acid profile fully meets the requirements of the World Health Organization (WHO). The research in question used a combined fat phase that included 55% of bacon and 45% of rice bran oil. Such combination provided an optimal ratio of PUFA and SFA and did not reduce the stability of the meat emulsion [19, 20]. Linseed, rapeseed, perilla, canola, camellia, and grape seed oils also proved to have a positive effect on the ratio of SFA and USFA in various sausage products [11, 12, 21, 22].

There have been a lot of studies related to the use of plant materials to reduce the mass fraction of fat in meat products while enriching them with dietary fiber and USFA. By adding 3–6% of flax flour, it was possible to increase the amount of α-linolenic acid and improve the ratio of PUFA and SFA in minced beef cutlets [7]. When used in meat product formulations, raw materials with a high content of dietary fiber, e.g. bran of rice, oat, or wheat, decreased the mass fraction of fat and SFA [23–26]. The same effect was obtained by using soy, lemon, tiger nuts, and pea fibers [27–32].

Nuts and nut derivatives, e.g. oil, flour, oil meal, or oil cake, can serve as an alternative source of unsaturated fats. Oil cake is rich in fat and contains complete protein and antioxidant vitamins. There have been studies that featured fortifying meat products with hazelnuts and walnuts [24, 33]. Nuts of Siberian cedar pine and their products demonstrated an especially high biological value [35, 37].

The present research featured the biological value of semi-smoked sausages with cedar oilcake (CO) and a lower sodium content achieved by partial replacement of sodium chloride with magnesium chloride. The product with the combined fat phase was tested for stability during refrigerated storage.

STUDY OBJECTS AND METHODS

The study featured semi-smoked sausages (for formulations – see Table 1). The control and the experimental formulations differed in the basic and auxiliary raw materials. In the experimental formulation, 15% of semi-fat pork with a fat content of ≤ 30% was replaced by 15% of cedar oil meal. The chemical composition of cedar oil cake makes it a balanced complex of proteins, lipids, nutrients, vitamins, and dietary fibers. Oil cake proteins possess a high biological value and all essential acids, including high amounts of tryptophan, lysine, and sulfur-containing amino acids [36]. Oil cake looks like a cream-colored powder, free of foreign matters; its structure flakes and is easy to deform. Oil cake is oily to the touch and has a sweet taste and a light smell of cedar nuts. The amount of cedar oil cake in the formulation and the choice of the final product were based on the available data on the use of nuts in various meat products.

Nitrite and edible salt (sodium chloride) were used as curing ingredients for the control sample. In the

Table 1 Formulations of semi-smoked sausage

Ingredients	Samples	
	without oil cake (control)	with oil cake (experimental)
Critical ingredients, kg		
Trimmed second-grade beef	50.0	50.0
Semi-fat trimmed pork	50.0	35.0
Cedar oil cake	–	15.0
Auxiliary ingredients, kg/100 kg		
Nitrite salt	1.250	1.062
Edible salt (sodium chloride)	1.450	1.146
Magnesium chloride	–	0.492
Granulated sugar	0.100	0.100
Black pepper	0.120	0.120
Allspice	0.060	0.060
Red pepper	0.060	0.060
Nutmeg	0.050	0.050
Fresh garlic	0.100	0.100

experimental formulation, the curing mix included nitrite salt and edible salt, 30% of which was replaced with magnesium chloride. This replacement was aimed at reducing the sodium in the product. According to WHO, its consumption exceeds the physiological need, thus leading to nutritionally dependent diseases [38]. From the technological point of view, the replacement of edible salt with sodium chloride makes the fat component resistant to oxidation, especially if the proportion of unsaturated acids is high.

In both the experimental and the control formulations, nitrite salt was added in an amount that provided the standard content of sodium nitrite, i.e. 0.075% from the weight of the raw meat.

The sausages were produced from chilled second-grade trimmed beef and semi-fat pork. The raw meat was ground using a meat grinder plate with the bore diameter of 2–3 mm for beef and 8–12 mm for pork. The ground meat was mixed with 2.7% of curing ingredients (from the weight of the raw material). The resulting materials were stored for ripening at 0–4°C for 24 h. The ripened raw meat was used to prepare sausages. Cedar oil cake was subjected to no prior preparation. It was stored at –12°C and briefly heated at room temperature before being introduced into the formulation.

The formulations of the experimental and the control samples were combined in the mixer in the following order: beef, pork, cedar oil cake (in the experimental sample), sugar, spices, and garlic. The components were mixed for 6–8 min until smooth. The temperature of the ground meat after the mixing did not exceed 12°C.

The ground meet was molded into an artificial protein sausage casing. The sausages were kept in a hanging room for 4 h at 2–4°C. After that, the sausages underwent heat treatment: first, they were dried at 60°C for 30–40 min, then cooked at 70 ± 2°C, and, finally, smoked at 72–74°C until the surface obtained the required reddish-brown color. Subsequently, the sausages were cooled to ≤ 6°C and stored at 2–6°C for 15 days.

A comparative analysis of the parameters of the biological value and quality was performed based on the sensory properties and the chemical, fatty acid, and amino acid compositions. During storage, a set of experiments was performed to study the oxidative damage to the lipid fraction by determining acid, peroxide, and thiobarbituric values, as well as water activity.

Research methods. The sensory evaluation was done by tasting. It involved a nine-point scale, as required by State Standard 9959-2015[I].

As for the chemical composition of the sausages, the mass fraction of protein was determined by the Kjeldahl method (State Standard 25011-2017[II]), the mass fraction of fat – by the Soxhlet method (State Standard 23042-2015[III]), the mass fraction of ash – by the mineralization of the batch weight (State Standard 31727-2012 (ISO 936: 1998)[IV]), the mass moisture content – by roasting the sample to constant batch weight (State Standard 33319-2015[V]).

The fatty acid composition was determined by gas chromatography using an Agilent 7890A chromatograph. The mass fraction of methyl ethers of fatty acids was defined in relation to their total amount, as required by the State Standard 51483-99[VI]. High purity nitrogen was used as the carrier gas, while grade A hydrogen was used as auxiliary.

The obtained results made it possible to calculate the ratios of SFA, MUFA, and PUFA, as well as the indices of atherogenicity and thrombogenicity using the Wilbrich and Southhein formula [39, 40].

The amino acid composition was determined by capillary electrophoresis using a Kapel'-105M system. The balance of the amino acid composition was established by the method of test values: the total of the essential amino acids (EAA), the utility coefficient of the essential amino acids (U), and the coefficient of comparable redundancy.

The peroxide value (PV) was determined by direct titration of peroxides formed during the oxidation of the fat fraction with a sodium thiosulfate solution.

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[II] State Standard 25011-2017. Meat and meat products. Methods for determining protein. Moscow: Standartinform; 2018. 14 p.
[III] State Standard 23042-2015. Meat and meat products. Methods for determining fat. Moscow: Standartinform; 2016. 9 p.
[IV] State Standard 31727-2012. (ISO 936:1998). Meat and meat products. Method for determining the mass fraction of total ash. Moscow: Standartinform; 2013. 12 p.
[V] State Standard 33319-2015. Meat and meat products. Method for determining the mass fraction of moisture. Moscow: Standartinform; 2016. 6 p.
[VI] State Standard 51483-99. Vegetable oils and animal fats. Using gas chromatography to determine the mass fraction of methyl ethers of individual fatty acids to their total. Moscow: Standartinform; 2008. 11 p.

Table 2 Fatty acid composition of semi-smoked sausages

Acid	Notation	Mass fraction, % of total fatty acids	
		without cedar oil cake (control)	with cedar oil cake (experimental)
Saturated fatty acids (SFA)			
Capric	$C_{10:0}$	0.05 ± 0.001	–
Lauric	$C_{12:0}$	0.62 ± 0.040	0.18 ± 0.010
Myristine	$C_{14:0}$	1.73 ± 0.080	1.48 ± 0.020
Palmitic	$C_{16:0}$	25.5 ± 0.980	20.1 ± 1.290
Margarine	$C_{17:0}$	0.61 ± 0.040	0.53 ± 0.060
Stearin	$C_{18:0}$	11.68 ± 1.09	10.1 ± 0.830
Arachic	$C_{20:0}$	0.27 ± 0.030	0.17 ± 0.070
Geneukosan	$C_{21:0}$	0.16 ±	–
Monounsaturated fatty acids (MUFA)			
Palmitoleic	$C_{16:1,\ \omega\text{-}7}$	3.77 ± 0.040	3.11 ± 0.060
Heptadecene	$C_{17:1}$	0.33 ± 0.020	0.30 ± 0.010
Elaidic	$C_{18:1,\ \omega\text{-}9,\ trans}$	0.10 ± 0.001	0.18 ± 0.003
Oleic	$C_{18:1,\ \omega\text{-}9,\ cis}$	39.64 ± 0.73	44.33 ± 0.61
Gadolein	$C_{20:1}$	0.17 ± 0.003	0.61 ± 0.050
Polyunsaturated fatty acids (PUFA)			
Linoleic	$C_{18:2,\ \omega\text{-}6}$	7.96 ± 0.250	9.61 ± 0.540
Eicosapentaenoic	$C_{20:2}$	–	0.26 ± 0.005
α- Linolenic	$C_{18:2,\ \omega\text{-}3}$	0.20 ± 0.030	–
γ- Linolenic	$C_{18:3,\ \omega\text{-}6}$	–	0.85 ± 0.040
Arachidonic	$C_{20:5,\ \omega\text{-}6}$	1.11 ± 0.030	0.86 ± 0.040
USFA, %	–	40.62 ± 0.56	32.56 ± 1.11
MUFA, %	–	44.01 ± 0.32	48.53 ± 0.64
PUFA, %	–	9.27 ± 0.330	11.58 ± 0.59
USFA/ MUFA	–	0.92	0.67
USFA/PUFA	–	4.38	2.82
PUFA/USFA	–	0.23	0.36
Atherogenicity index	–	0.62	0.44
Thrombogenicity index		1.46	1.05

A sample of semi-smoked sausage was crashed to extract fat with chloroform in the presence of anhydrous sodium thiosulfate. The resulting extract was dissolved in glacial acetic acid and titrated with a 0.01N sodium thiosulfate solution in the presence of a saturated solution of potassium iodide and starch. The PV value was expressed in mmol ½ O/kg [41].

The acid value was determined by direct titration in a neutral alcohol-ether mix of free fatty acids with a 0.1N sodium hydroxide solution in the presence of phenolphthalein. The extraction of fat from the crashed samples was performed similarly to the method that was used to determine PV, i.e. extraction from a crashed sample with chloroform [41].

The thiobarbituric value (TBV) was determined by a modified distillation method: a colored complex formed as a result of the interaction of malondialdehyde with 2-thiobarbituric acid. The crushed sample was heated in distilled water where hydrochloric acid was added. The resulting distillate was mixed with a solution of thiobarbituric acid and heated in a water bath to develop a color reaction. The color intensity of the resulting solutions was measured using a spectrophotometer at a wavelength of 538 nm (green filter) [41].

The mass fraction of chlorides was established by argentometric titration. The method is based on the determination of chlorine ions by titration of an aqueous extract from the sample with a solution of silver nitrate in the presence of chromic acid potassium.

The content of sodium ions in the finished product was determined with the help of an ELIS-112Na ion-selective electrode (Russia). The range of determination of Na^+ ion activity equaled 1.0–3.5 pNa. The test involved a 150-MI pH meter.

Water activity (A_w) was determined by the cryoscopic method using an AVK-4 water activity analyzer (Russia). To determine the water activity, the test sample was cooled, while its temperature was measured using the precision meter. Then, a special program was used to analyze the process thermogram and determine the cryoscopic temperature, which was converted into values of the water activity indicator. The results were processed using a personal computer [42].

The values were obtained after triplicate tests of homogeneous sausage material. The arithmetic mean and standard deviation were used to define the standard error of the mean and the confidence limits. The calculation took into account the Student's coefficient t (n, p) at the confidence level of 95% (P = 0.05) and the number of measurements.

RESULTS AND DISCUSSION

The biological value was assessed by comparing the fatty acid and the amino acid compositions of the semi-smoked sausages with and without cedar oil cake. Table 2 shows the fatty acid composition.

The fatty acid composition in the control formulation had the following ratio of fatty acids: USFA:MUFA:PUFA – 40.62%:44.01%:9.27%

When 15% of semi-fat pork was substituted by cedar oil cake, it led to a significant decrease in the total content of USFA, which was 19.8% relative to the control formulation. MUFA and PUFA increased by 10.2% and 24.9%, respectively. SFA decreased in the following manner: palmitic acid – by 21%, lauric acid – by 70%, and arachinic acid – by 37%, if compared with the control formulation. The experimental sample revealed neither capric nor genicosanoic USFA.

The experiment revealed a significant increase in MUFA, which has a beneficial effect on blood lipoproteins and prevents coronary heart disease. Therefore, an increase in MUFA means a higher biological value of the semi-smoked sausages with cedar oil cake [3]. The total increase in MUFA occurred after the cis-oleic acid increased from 39.64% in the control formulation to 44.33% in the experimental formulation.

Table 3 Content of essential amino acids in semi-smoked sausages

Essential amino acids	Ideal protein, FAO/WHO scale	Samples			
		without cedar oil cake (control)		with cedar oil cake (experimental)	
		Protein, g/100 g	Amino-acid score, %	Protein, g/100 g	Amino-acid score, %
Valine	5.0	5.78	115.60	5.37	107.40
Isoleucine	4.0	4.48	112.00	4.38	109.50
Leucine	7.0	6.88	98.20	6.64	94.80
Lysine	5.5	8.05	146.30	7.68	139.60
Methionine + Cysteine	3.5	3.89	111.40	3.44	98.30
Threonine	4.0	4.48	112.00	4.19	104.70
Tryptophan	1.0	1.18	118.00	1.41	141.00
Phenylalanine+ Tyrosine	6.0	7.56	126.00	5.80	96.60

The tests also demonstrated a change in the ratio and composition of PUFA. When 15% of semi-fat pork was replaced with cedar oil cake, the content of linoleic acid (omega-6) increased by 20.7%. In addition, long-chain eicosapentaenoic (omega-3) and γ-linolenic (omega-6) acids were registered in the product, the latter being the precursor of dihomo-γ-linolenic, or eicosatrienoic, acid. The fatty acid composition of sausages with cedar oil cake demonstrated a bigger total amount of long chain UFA. These fatty acids have a lower melting point compared to SFA of a similar chain length.

The comparison of the indices of atherogenicity and thrombogenicity was in favor of the experimental formulation, which makes the final product anti-atherosclerotic.

The sausage formulation did not fully correspond with the modern concept of balanced nutrition in terms of SFA:MFA:PUFA ratio (30:60:10) [43]. However, a greater amount of essential PUFA and a decrease in SFA is one of the main arguments in favor of using cedar oil cake as the main raw material.

Similar results were obtained by replacing beef fat with walnuts, walnut pasta, and hazelnuts [44, 45]. These studies also showed that a decrease in SFA improved the fatty acid composition of meat products.

Table 3 demonstrates the amino acid composition of the sausages under study.

Therefore, cedar oil cake makes it possible to obtain a product of high biological value. The scores for essential amino acids were high: lysine – 139.6%, tryptophan – 141.0%, and sulfur-containing amino acids – 98.3%. The total content of essential amino acids in the sausage with cedar oil cake was 38.91 g/100 g protein. This amount was lower than in the product with only meat raw materials, which was 42.3 g/100 g protein. However, it was higher than in ideal protein (36 g/100 g protein). Leucine appeared the first limiting amino acid in the experimental sample: its score was 94.8%.

Table 4 Biological value of protein in semi-smoked sausages

Indicatiors	Samples	
	without cedar oil cake (control)	with cedar oil cake (experimental)
Total essential amino acid, g/100 g protein	42.30	38.91
Utility coefficient, unit fraction	0.83	0.87
Comparable redundancy coefficient, g/100 g protein	7.20	5.35

In addition, the biological value of the new semi-smoked sausages proved that cedar oil cake improved the amino acid ratio (Table 4). As a result, the utility coefficient of amino acid composition increased, and the proportion of amino acids not used for anabolic purposes decreased. In terms of the content of potentially utilizable essential amino acids, the amount of protein in the product was equivalent to their amount in 100 g of reference protein.

The obtained data showed that cedar oil cake can be recommended for semi-smoked sausage production. The analysis of fatty acid and amino acid composition proved that such replacement increased the biological value of the final product. A comprehensive assessment of the quality of the semi-smoked sausages was based on sensory and physicochemical parameters (Table 5 and 6).

The nine-point panel evaluation of the semi-smoked sausages involved such parameters as appearance, inner color, smell, taste, and texture. Each of nine panelists evaluated randomly encoded samples of sausages in triplicate. The score of each sensory property was calculated based on the opinion (evaluation) of each panelist (Table 5).

The obtained data proved that cedar oil cake and a lower amount of sodium chloride had a positive effect on the smell and taste of the semi-smoked sausage. The panelists noted that the usual meaty smell was accompanied with a faint smell of cedar nuts. In general, they evaluated the taste as milder and pointed out a specific pleasant aftertaste, which made them give the sample a higher score. The new curing mix decreased the salty flavor. The fine structure of the oil cake resulted in its better distribution in the meat,

Table 5 Sensory evaluation of semi-smoked sausages

Samples	Appearance	Inner color	Smell	Taste	Texture	Total
Without cedar oil cake (control)	7.4 ± 0.3	7.4 ± 0.1	6.5 ± 0.3	6.3 ± 0.5	7.2 ± 0.2	6.9 ± 0.7
With cedar oil cake (experiment)	7.9 ± 0.6	8.0 ± 0.5	8.8 ± 0.2	8.5 ± 0.6	7.8 ± 0.1	8.2 ± 0.2

Table 6 Physicochemical characteristics the semi-smoked sausages

Indicator	Samples	
	without cedar oil cake (control)	with cedar oil cake (experimental)
Mass fraction of protein, %	16.2 ± 0.37	17.4 ± 0.34
Mass fraction of fat, %	23.4 ± 0.29	25.2 ± 0.27
Mass fraction of moisture, %	56.8 ± 0.24	52.2 ± 0.21
Mass fraction of chlorides, %	2.8 ± 0.05	2.8 ± 0.08
Mass fraction of sodium, %	1.13 ± 0.07	0.76 ± 0.04
Energy value, kcal	248	284

thus preserving the texture of the sausage even when the percentage of cedar oil cake was relatively high. The panelists described the texture as dense. A slight decrease in the inner color intensity did not affect the score for this indicator. The total score for sausages with cedar oil cake was 8.2 points, which corresponded with "excellent" on the nine-point scale. The sausages made without cedar oil cake received 6.9 points and were evaluated as "good". According to the results of the sensory evaluation, the semi-smoked sausages with cedar oil cake and low salt content received high consumer characteristics.

The obtained results were consistent with other studies. For instance, almond and walnut had a positive effect on the sensory properties of various meat products, e.g. chopped semi-finished products and emulsified sausages. However, if the percentage of the new component exceeded 25%, the final product acquired a specific taste, while the structure became heterogeneous, especially in case of coarse-ground nuts [46–49].

Table 6 shows the physicochemical parameters of the sausages. The nutritional values of both samples conformed to the requirements of regulatory documents for this group of products. The analysis revealed no significant differences in the chemical composition of the samples. However, the sausage with cedar oil cake had a higher mass fraction of protein and fat than the control sample.

The partial replacement of sodium chloride with magnesium chloride led to a decrease in the sodium. The daily reference intake for sodium is 2 g per day for an adult. In the control sample of the semi-smoked sausage, the sodium content was 1.13%, which corresponds to 56.5% of the daily reference intake. In the experimental product, it was 0.76%, or 38% of the daily requirement. The obtained experimental data confirmed the high nutritional value of the developed formulation.

Ground meat used in semi-smoked sausages is a complex system with a high content of pro-oxidants, which contributes to the formation of free radicals. Free radicals, in their turn, are most active against MUFA and PUFA, the amount of which increased in the formulation with cedar oil cake. However, cedar oil cake contains natural antioxidants that can inactivate free radicals. Tocopherol is one of the most significant antioxidants. According to our previous study, its content in cedar meal is 11.43 mg/100 g [36]. The smaller amount of sodium chloride is one more protective mechanism in the developed formulation. The effect of competing factors on the lipid oxidation process of the combined fat phase requires further research when it goes about semi-smoked sausages.

The amount of primary and secondary oxidation products was determined on days 1 and 15 of refrigerated storage at 2–6°C and 70–80% of relative humidity. The hydrolysis of fat facilitates the development of oxidation processes. As a result, assessment of acid value had to be performed simultaneously (Table 7).

The hydrolysis process in both samples revealed a similar development pattern. After the expiry date, the acid value increased by 2.3 times in the control sample and by 2.2 times in the experimental sample. In both cases, the acid value was significantly lower than the standard. In addition, the intensity of the hydrolysis process decreased in the experimental sample.

The oxidative changes in the combined lipid fraction of the experimental formulation vs. the control formulation were determined according to accumulation of primary oxidation products. By the end of storage, the peroxide value increased by 28.3% in the experimental

Table 7 Indicators of oxidative damage in semi-smoked sausages

Samples	Storage time, days	Acid value, mg KOH	Peroxide value, mmol½ O/kg	Thiobarbital value, mgMA/kg
Without cedar oil cake (control)	0	0.75 ± 0.063	2.36 ± 0.130	0.250 ± 0.009
	15	1.73^{a} ± 0.046	3.4^{a} ± 0.350	0.298^{a} ± 0.007
With cedar oil cake (experimental)	0	0.68 ± 0.036	2.29 ± 0.110	0.246 ± 0.006
	15	1.54^{ab} ± 0.024	2.94^{ab} ± 0.120	0.282^{ab} ± 0.008

The a–b values in the columns differed significantly ($P < 0.05$)

Table 8 Water activity in semi-smoked sausages

Samples	Storage time, days	Water activity (A_w), units
Without cedar oil cake (control)	0	0.9652 ± 0.0031
	15	0.9694 ± 0.0030
With cedar oil cake (experimental)	0	0.9461 ± 0.0033
	15	0.9437 ± 0.0032

sample and by 44.0% in the control sample. Therefore, cedar oilcake and low sodium chloride content slowed it down without reversing it. The results must have been caused by the effect of cedar tocopherols, which are most active against radicals attacking double bonds of USFA. Lipid peroxidation is triggered by the removal of a hydrogen atom from a free PUFA or an acid within phospholipids. When heme iron becomes non-heme, reactions start branching, and the process is reinitiated. Stabilization of heme-containing meat proteins is caused by a decrease in sodium chloride, which inhibits the oxidation process [50].

However, in spite of the fact that the samples differed significantly in peroxide values, the obtained results do not guarantee that the lipid oxidation rate reduced in the formulations with cedar oil cake and low sodium content. Peroxides and hydroperoxides are unstable intermediate reaction products, which quickly turn into the products of secondary oxidation. Hence, peroxide value is a variable value and does not fully reflect the degree of oxidative changes [51].

Thiobarbital value is a more objective indicator of oxidative spoilage. It describes the amount of malonic aldehyde that is formed during storage. The process of accumulation of secondary oxidation products was less intensive in the sausages with cedar oil cake and lower salt content over the entire storage period. The thiobarbital value increased by 19.2% in the control sample without cedar oil cake and by 14.6% in the experimental sample with cedar oil cake. This increase could be associated with more intense hydrolytic processes and lipid peroxidation during storage, as well as with aerobic storage conditions [52].

The obtained results indicated the stabilization of the lipid fraction of the semi-smoked sausages with cedar oil cake and low sodium content, since no extraneous rancid taste and aroma were registered.

Water activity is one of the product stability parameters. Water activity values for the semi-smoked sausages under study during storage are presented in Table 8.

The decrease in the water activity in the experimental sample compared with control could be explained by lower moisture content. In addition, magnesium chloride has a more pronounced effect on the moisture retention in the product, as described in [53, 54].

CONCLUSION

The research showed that 15% of cedar oil cake introduced into the traditional formulation of semi-smoked sausages to substitute 15% of pork increased the biological value of the product. Its fatty acid composition improved due to a decrease in saturated fatty acids, including palmitic acid. The mono- and polyunsaturated fatty acids increased, including long-chain eicosapentaenoic and γ-linolenic acids. The parameters of hydrolytic and oxidative changes in the combined fat phase demonstrated a greater stability during storage. This improvement could be explained by two facts. First, the composition of cedar oil cake had natural antioxidants. Second, sodium chloride was partially replaced with magnesium chloride (30%) in the curing mix. This replacement also decreased the amount of sodium in the composition of the final product.

According to the sensory evaluation, cedar oil cake and lower content of sodium chloride had a positive effect on the taste, smell, and texture of the sausage. The new sausages contribute to a healthy diet while their prospective production can be of practical use to meat industry.

CONTRIBUTION

G.V. Gurinovich supervised the project. All the authors took part in research, data processing, writing, and updating the article: I.S. Patrakova, S.A. Seregin, A.G. Gargaeva, O.Ya. Alekseevnina, O.M. Myshalova, M.V. Patshina.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to the publication of this article.

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Effects of encapsulated green coffee extract and canola oil on liquid kashk quality

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Abstract: In this study, we used a water-in-oil (W/O) emulsion encapsulation technique to enhance green coffee extract in the novel kashk product and protect it against hot filling. Green coffee extracts (GCE) in free (1%, 0.5%, and 0.25%) and encapsulated form (EGCE) (5%, 2.5%, and 1.25%) were added to kashk during hot filling, and their physicochemical and sensory properties were investigated. The EGCE kashk had higher oxidative stability (0.43 h) than the control due to the extract's high phenolic content and DPPH radical scavenging activity (74%). Although a high concentration of GCE caused a higher pH (4.02), the latter declined in all the samples during the storage period. Further, the size of droplets in the emulsion varied from 3.20 to 8.51 µm, confirming the well-encapsulated GCE by Fourier transform infrared. In addition, palmitic acid and oleic acid were detected in GCE by gas chromatography as the main saturated and unsaturated fatty acids, respectively. All the treatments had similar rheological properties and the highest flow index was observed in the samples with EGCE 5% on day 60. The sensory evaluation showed that the assessors preferred the kashk formulated with 1% GCE. Finally, GCE encapsulation protected the color of the samples, and the b* value remained unchanged, whereas the lightness (L*) increased. We suggest that a W/O emulsion is a successful technique for GCE encapsulation in kashk and can offer the latter to consumers as an alternative type of flavored dairy product with a better shelf life and health benefits.

Keywords: Antioxidant activity, encapsulation, green coffee extract, kashk, rheological properties

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INTRODUCTION

Fortified dairy products appeal to a wide variety of consumers and help them increase their intake of bioactive components. Kashk is a local name for a traditional low-fat dried yogurt in Iran. It is obtained from boiled and concentrated yogurt and is available in a semi-liquid or dried form. Liquid kashk contains 20–25% nonfat solids, 1% fat, 3% salt, and at least 13% protein [1].

Coffee is one of the most consumed and commercialized food products in the world and one of the most traded commodities, second only to petroleum [1]. It has various biological and pharmacological properties, such as antioxidant, anti-inflammatory, and antimicrobial properties, as well as other health benefits [2].

Natural therapy is a modern approach to preventing common diseases. Therefore, coffee bean extract or powder can be used in cosmetic products and numerous functional foods [1, 3–5]. However, natural antioxidants are usually heat sensitive and susceptible to oxidation, which limits their application in food industry [6].

Microencapsulation is a promising technique that protects bioactive materials and controls the release of entrapped ingredients [1]. Over the last few years, encapsulation has attracted much attention in food, pharmaceutical, and cosmetic industries due to its wide application in the design of functional products [1]. Encapsulation techniques are often based on drying processes, such as spray-drying or freeze-drying, due to the liquid nature of extracts containing bioactive compounds [7]. Several studies have investigated the protective role of these techniques against adverse conditions to which extracts can be exposed. They found that encapsulation promoted better volatile retention

and increased the shelf life of bioactive components and extracts [8–11]. There are numerous studies about encapsulation of coffee extract and its antioxidant compounds [12–14].

The incorporation of green coffee extract (GCE) in kashk has not been reported so far. Since kashk is prepared using hot filling, and heating causes a loss of antioxidants, GCE was encapsulated using a water-in-oil (W/O) emulsion technique. Thus, our aim was to investigate the physicochemical and sensory characteristics of kashk incorporated with free and microencapsulated GCE during the storage period.

STUDY OBJECTS AND METHODS

Materials. Green coffee (Robusta coffee) was supplied from the local market of Shiraz (Fars, Iran). Starter culture (-CH1-DVS-50U) was purchased from Christian Hansen (Denmark), and all chemical reagents were obtained from Merck Co. (Germany).

The research was conducted at the Fars Agricultural and Natural Resources Research and Education Center.

Green coffee extraction. According to the slightly modified method of Upadhyay and Ramalakshmi, 10 g of ground coffee was added to 100 mL of distilled water and held in a hot water bath for 30 min [2]. Then the slurry was cooled at room temperature and filtered to obtain a clear extract for analysis.

Microencapsulation. GCE was microencapsulated using a W/O emulsion technique based on the Tran *et al*. method with slight modifications [15]. Glycerol monostearate (GMS) with HLB 3.8 (1.5 wt%) was added to canola oil and shaken at 4000 rpm at 70°C. Then, the aqueous solution containing the GCE was heated to 40°C. The W/O emulsion (10:90) was prepared by blending the GCE-containing aqueous phase and the GMS-containing canola oil phase at 27000 rpm and 70°C for 2 min. Then, the suspension was cooled while stirring with a magnetic at 1000 rpm for 2 h and left for 30 min for microcapsules to precipitate. Finally, the suspension was centrifuged at 350 g and 4°C for 10 min. The precipitate was washed twice with saline and filtered. The obtained microcapsules were stored in a refrigerator until usage.

GCE kashk production. Liquid kashk was prepared according to the method described in [1]. Then, free and encapsulated green coffee extracts in different amounts (0.25–5%) were added to kashk. The samples were named GCE 1%, GCE 0.5%, GCE 0.25%, EGCE 5%, EGCE 2.5%, and EGCE 1.25%. The sample without GCE was used as a control.

Total phenolic content. The amount of total phenol in different concentrations of the extracts was determined according to Folin-Ciocalteu as described by Ballesteros *et al*., with some modifications [12]. Briefly, the samples (0.1 mL) were introduced into test tubes containing 0.75 mL of Folin–Ciocalteu's reagent and 0.75 mL of 2% sodium carbonate. The tube was mixed and kept for 1 h in the darkness at room temperature. The absorbance was measured at 765 nm using a UNICO 2100 UV–vis spectrophotometer. Phenolic compounds were measured in triplicate, and the results were averaged. A calibration curve of gallic acid (ranging from 25 to 100 mg mL^{-1}) was prepared in methanol. The results, which were determined by the regression equation of the calibration curve (y = 0.000245x – 0.0377; correlation coefficient r = 0.998), were expressed as gallic acid (GA) mg equivalents g^{-1} sample.

Antioxidant activity. The free radical scavenging activity of GCE was measured by using the 1,1-diphemyl-2-picryl-hydrazyl (DPPH) following the method of Ribeiro *et al*. with a slight modification [16]. A DPPH solution was added to the extract and mixed. Then, the mixture was kept at room temperature in the darkness for 1 h. The absorbance of resulting solutions was measured at 515 nm. The blank sample was prepared in the same manner except that methanol was used instead of the DPPH solution. A standard curve was prepared using TBHQ (tertiary butylhydroquinone) at different concentrations. The percentage of scavenging activity was calculated as below:

$$\% \text{ of scavenging} = [(A_0 - A_1)\,(A_0)^{-1}] \times 100 \qquad (1)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample turbidity factor. Finally, IC_{50} (an absorbance value of 0.5 in the reducing power assay) was calculated.

Oxidative stability. The 892 Professional Rancimat (Metrohm, Herisau, Switzerland) was used to determine the oxidation stability of the samples. Three grams of the sample was heated at 110°C under a purified air flow rate of 20 L h^{-1}.

pH value. The pH of kashk enriched with GCE was measured using a pH meter (Greisinger electronic, Germany).

Particle size distribution. The mean particle size of the microcapsules was measured using a dynamic light scattering technique at ambient temperature (Nano Particle Analyzer SZ-100, Horiba, Germany).

Fourier transform infrared spectroscopy (FTIR). The FTIR analysis of GCE and EGCE was recorded by a Perkin-Elmer Spectrum RXI spectrometer (USA) in the transmit mode in the range of 400–4000 cm^{-1} in KBr pellets at a resolution of 4 cm^{-1}. A DLaTGS (Deuterated Triglycine Sulphate Doped with L-Alanine) detector was used to perform the measurements at room temperature (25 ± 0.5°C) at 24 scan/min to find possible functional groups.

Fatty acid composition. The fatty acid composition was determined according to the Golmakani *et al*. method [17].

Rheological measurements. Viscosity was measured using a Brookfield rotational viscometer (Model LVDV I+, Version 3.0, Stoughton, MN, USA)

with a spindle C30 and a heating circulator. The kashk samples were mixed for 5 min at room temperature at 60 rpm. Flow behavior was described using the Power law, Bingham, and Casson models according to equations:

$$\tau = k\gamma n \text{ (Power law model)} \quad (2)$$

$$\sigma - \sigma_0 = \eta\gamma \text{ (Bingham model)} \quad (3)$$

$$\sigma_{0.5} = k_0 c + kc(\gamma)_{0.5} \text{ (Casson model)} \quad (4)$$

where τ is the shear stress, Pa; γ is the shear rate, s^{-1}; k is the consistency coefficients, $Pa \cdot s^n$; η is Bingham plastic viscosity; kc is Casson plastic viscosity; n is the flow index, and σ_0 and $k_0 c$ are yield stress of Bingham and Casson models, respectively.

Color analysis. Changes in kashk color were measured using a Choroma CR-400 meter (Japan). L, a and b values were expressed as L* (black to white), a* (green to red), and b* (blue to yellow) [18].

Sensory analysis. Sensory properties of the samples were determined by 30 panelists. Sensory analysis included aroma, color, taste, and overall acceptability. It used a five-point hedonic scale, with 5 indicating "like extremely" and 0 "dislike extremely", compared to the control sample. The analysis lasted 7 consecutive days [19].

Statistical analysis. A one-way analysis of variance (ANOVA) was performed at a confidence level of 0.05 (SPSS version 16.0). The means were compared using the Duncan's multiple range at a significance level of 0.05. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Total phenolic content. The total phenolic content of GCE is presented in Table 1.

The content of phenols in a 1200 ppm concentration of GCE was 39.08 mg GA g^{-1}, confirming its antioxidant activity due to the polyphenolic compound [12]. These results were close to those found by Siva, Rajikin [20]. They reported total phenolic contents of GCEs obtained by isopropanol and methanol to be 30.65 mg GA g^{-1} and 16.26 mg GA g^{-1}, respectively. Similarly, Naidu *et al.* reported 32.19% and 31.71% for arabica and robusta isopropanol/water extracts, respectively [21]. However, the total phenolic content of coffee depends on its variety [6]. Bidchol *et al.* and Ballesteros *et al.* also found that extracts of spent coffee grounds had 19.99 ± 3.56 mg 100 mL^{-1} chlorogenic acid and 350.28 ± 11.71 mg GAE 100 mL^{-1} [7, 12].

The most common polyphenols in coffee are phenolic acids, mainly caffeic acid, a type of trans-cinnamic acid, and its derivative, chlorogenic acid [22]. Chlorogenic acid is able to directly interact with reactive oxygen species (ROS), making it an effective OH• scavenger [12]. However, the content of chlorogenic acid in green coffee beans varies depending on genes, species, climate, nutrient state of soil, processing techniques such as decaffeination, degree of ripeness, and also roasting. Since phenolic acid is heat sensitive, green coffee beans have a higher content of chlorogenic acid [8]. In addition, it was found that coffee extract exhibited an antioxidant activity similar to grapes and pomegranates [12].

Antioxidant activity. According to Table 1, the IC_{50} of GCE is 1.95 mg mL^{-1}, which is higher than that of

Table 1 Total phenolic content and IC_{50} in green coffee extract (GCE) and TBHQ

Sample	Total phenol, mg GA g^{-1}	IC_{50}
GCE	39.08	1.95
TBHQ	–	1.04

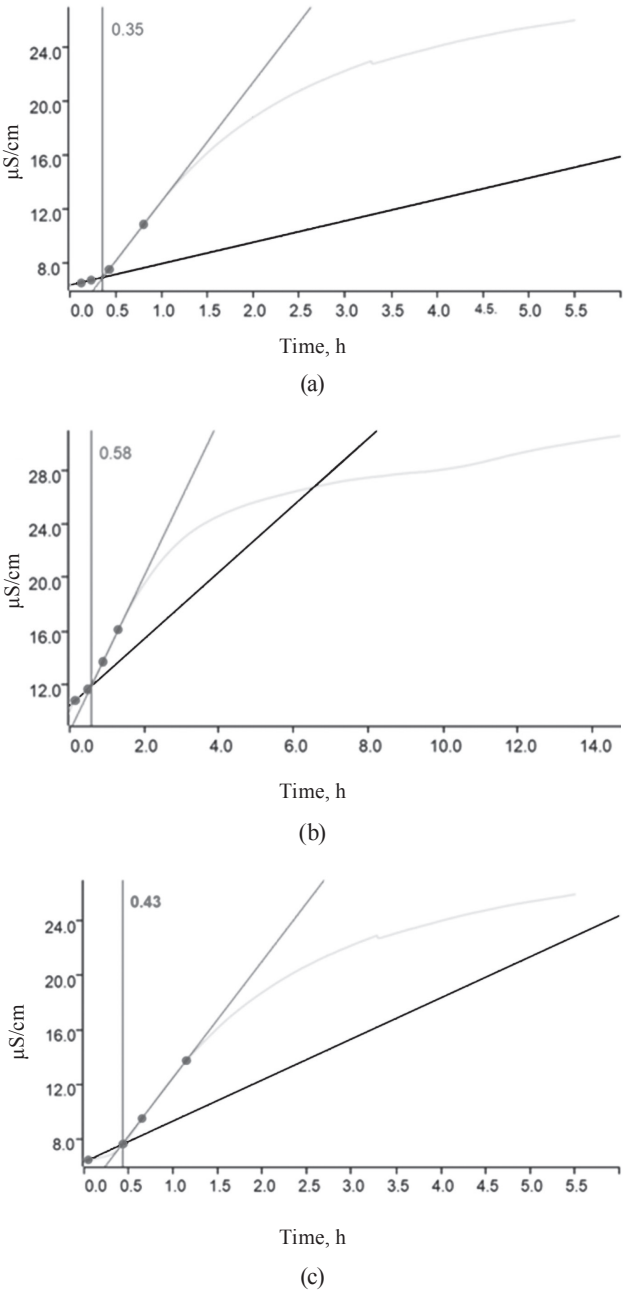


Figure 1 Oxidative stability of: (a) control, (b) GCE 1% and (c) EGCE 5%

Table 2 pH of kashk samples supplemented with GCE during storage

	Storage period, days				
Sample	1	15	30	45	60
Control	4.04 ± 0.06^{aA}	4.02 ± 0.04^{aB}	3.98 ± 0.03^{aC}	3.86 ± 0.07^{aD}	3.81 ± 0.01^{aE}
GCE 1%	4.02 ± 0.01^{bA}	4.04 ± 0.02^{bB}	4.06 ± 0.05^{bC}	3.99 ± 0.06^{bD}	3.94 ± 0.06^{bD}
GCE 0.5%	3.98 ± 0.02^{cA}	4.02 ± 0.03^{cB}	4.04 ± 0.06^{cC}	3.96 ± 0.05^{bD}	3.91 ± 0.01^{bE}
GCE 0.25%	3.95 ± 0.03^{dA}	3.98 ± 0.07^{dA}	4.01 ± 0.03^{dA}	3.94 ± 0.01^{bB}	3.88 ± 0.03^{cC}
EGCE 5%	4.03 ± 0.06^{bA}	4.05 ± 0.01^{bA}	4.08 ± 0.01^{bA}	4.01 ± 0.03^{bA}	3.97 ± 0.01^{bB}
EGCE 2.5%	4.00 ± 0.02^{cA}	4.02 ± 0.02^{cA}	4.05 ± 0.01^{cC}	3.98 ± 0.01^{bA}	3.92 ± 0.05^{bB}
EGCE 1.25%	3.97 ± 0.08^{dA}	4.00 ± 0.05^{dB}	4.03 ± 0.06^{cC}	3.95 ± 0.06^{bD}	3.89 ± 0.07^{cE}

Small letters in each column and capital letters in each row show a significant difference between samples ($P < 0.05$)
Results are reported as means ± SE, for three replicates of each sample

TBHQ (1.04 mg mL^{-1}). Based on the data, we calculated the DPPH radical scavenging activity of GCE (74%) [23]. The high total phenolic content of GCE found in our study confirmed its high radical scavenging activity and antioxidant potential. In addition, the technique we used and the coating material had a great impact on the retention of phenolic compounds and antioxidant activity of encapsulated samples [12].

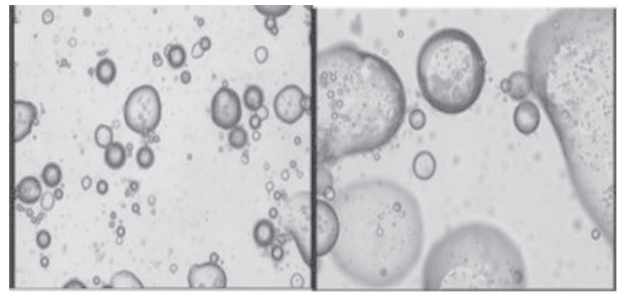
Similar results were revealed by Naidu *et al*. who reported 92, 87, and 76% antioxidant activity for arabica and 88, 82, and 78% for robusta at 60:40, 70:30, and 80:20 isopropanol/water ratios, respectively [21]. Jeszka-Skowron *et al*. also found that the antioxidant capacities of arabica and robusta green coffee averaged 56.3% [24]. Interacting with DPPH, antioxidants transfer an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character [25]. The degree of discoloration indicates the scavenging potential of the antioxidant extract. This radical scavenging ability is mostly related to the types and amounts of antioxidative components in the extract and their ability to donate a hydrogen group [7]. The antioxidant constituents in green coffee are chlorogenic, ferulic, caffeic, and coumaric acids [26]. Chlorogenic and caffeic acids are considered the most relevant markers in coffee samples [27]. However, other compounds such as caffeine, trigonelline, and phenylalanines (formed during coffee roasting) have antioxidant properties as well.

Oxidative stability. The oxidative stability curve and the induction time for different treatments of kashk samples are shown in Fig. 1.

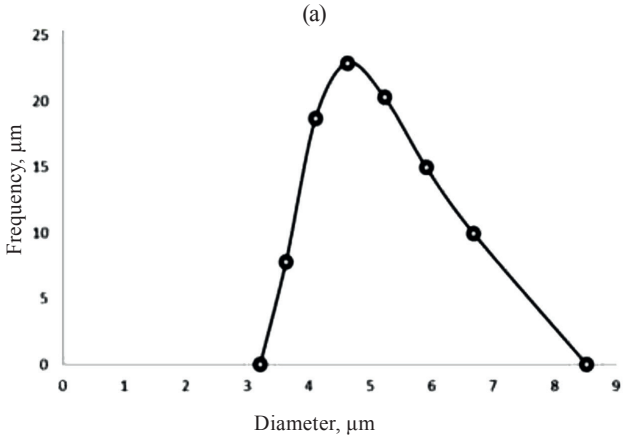
As we can see, the longer the induction time, the greater the oxidative stability. The induction time of the control, GCE, and EGCE were 0.35, 0.58, and 0.43 h, respectively. The kashk samples containing GCE (free or encapsulated) showed a longer induction time than the control due to GCE's antioxidant activity. Noteworthily, food products supplemented with extracts rich in polyphenols have an increased antioxidant potential [2, 4, 28]. However, encapsulation shortened induction time, which was in contrast to [2, 29, 30]. Many studies that used microencapsulation managed to avoid the deterioration of unsaturated fatty acids by oxidation. Indeed, the wall materials surrounded droplets and protected them from environmental conditions [31–33]. As expected, canola oil is sensible to oxidation due to a high amount of unsaturated fatty acids [34].

pH. According to Table 2, the initial pH of the control was significantly higher than that of other samples ($P < 0.05$). It decreased from 4.04 to 3.81 during storage. However, the GCE kashk showed the reverse trend for 30 days, followed by a pH decrease ($P < 0.05$). We also found that higher GCE concentrations led to higher pH of the samples, even at the end of the storage period. According to Carvalho *et al*., the pH of GCE is approximately 5.77–5.95 [31].

The acidity of all kashk samples containing GCE microencapsules also increased throughout the



(a)



(b)

Figure 2 (a) Optical microscopy of EGCE (100×, 400×) and (b) Droplet size distribution in initial emulsion

whole storage period, which was in agreement with Lee *et al*. [35]. This phenomenon was due to the production of lactic acid during storage. The activity of lactic acid bacteria, hydrolysis, and lipid oxidation resulted in lactic acid accumulation. Thus, the reduction of pH occured [36].

On the other hand, increased acidity during storage, called post-acidification, is attributed to the activity of kashk starter cultures at refrigerated temperature. They include *Streptococcus thermophilus* and *Lactobacillus delbruekii subsp. bulgaricus* that produce small amounts of lactic acid by fermenting lactose [37]. In addition, the positive effect of GCE on probiotic bacteria found in our study was confirmed by Marhamatizadeh *et al*. [38]. They reported that adding coffee extract at 0.4%, 0.8%, and 1.2% (w/v) had a positive effect on the fermentation and survival of probiotic bacteria in milk and yoghurt. According to Lee *et al*., the reduction of pH in all kashk samples containing GCE microencapsules was related to the production of lactic acid during storage [35].

Particle size distribution. The average particle size of the initial emulsion droplets is presented in Fig. 2. According to the curves, the droplets size was in the range of 3.20 to 8.51 μm.

These results confirm a microscopic size of encapsulated emulsion droplets. Particle size has a great influence on such features as the surface oil and the final content of the ultimate encapsulated powder [39]. For instance, particles larger than 30 μm may create a sandy

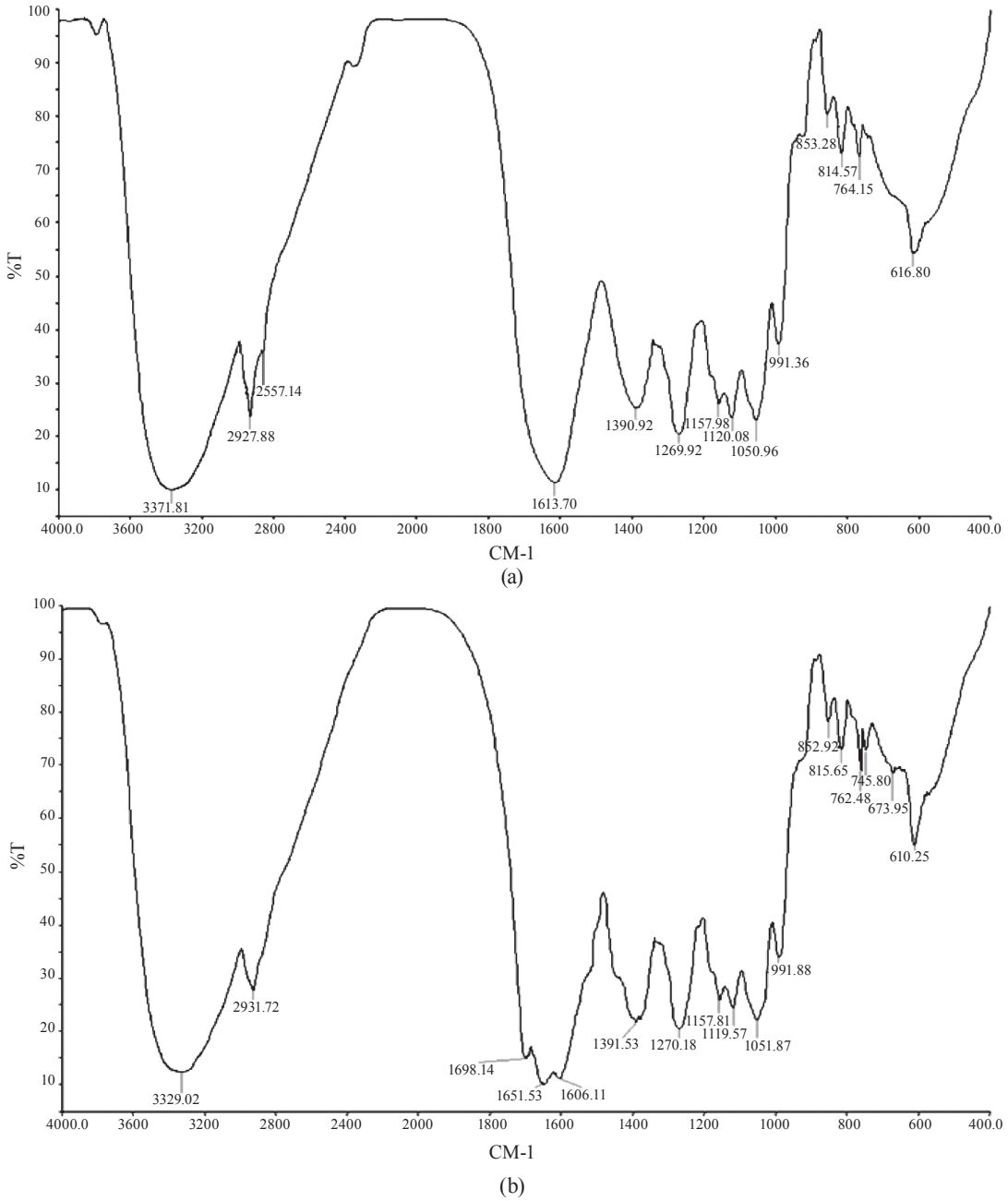


Figure 3 Fourier transform infrared spectra of: (a) GCE 1% and (b) EGCE 5%

Table 3 Fatty acid composition of control, GCE 1% and EGCE 5% kashks, %

Fatty acid	Control	GCE 1%	EGCE 5%
Butyric acid	0.93	2.25	0.77
Caproic acid	0.63	3.97	1.78
Caprylic acid	1.51	4.26	1.65
Capric acid	4.37	3.67	3.83
Lauric acid	4.27	5.56	2.63
Myrisric acid	15.23	12.31	5.89
Myristoleic acid	1.34	1.24	0.54
Pentadecanoic acid	0.72	0.37	0.20
Palmitic acid	39.21	28.01	17.99
Palmitoleic acid	1.02	1.10	0.97
Margaric acid	0.68	0.50	0.35
Heptadecenoic acid	0.67	0.78	0.15
Stearic acid	3.24	2.26	2.02
Oleic acid (cis)	21.95	14.67	43.93
Elaidic acid (trans)	2.09	10.79	13.99
Linoleic acid (cis)	0.06	0.51	2.59
Behenic acid	0.06	0.42	0.05
			100

feel in the mouth. The average diameter of vegetable oil droplets was 32 μm in the initial emulsion and 1–4 μm in D-limonene primary emulsion [40, 41]. Our results were in agreement with Karim *et al*. and Silva *et al*. [29, 31].

FTIR analysis. The average values of typical FTIR spectra for GCE and EGCE are depicted in Fig. 3.

There, we can see characteristic broad low-frequency absorption bands at 3371 and 3329 cm^{-1} respectively, in the hydroxyl region (4000–3000 cm^{-1}). These bands represented the stretching vibrations of O–H in the constituent sugar residues and adsorbed water [36]. We also observed three sharp peaks in the range of 3000 to 2800 cm^{-1} (2927, 2931, and 2852 cm^{-1}) for both arabica and robusta roasted coffee samples, but no identification was attempted. Nonetheless, FTIR analysis of caffeine in soft drinks revealed two sharp peaks at 2882 and 2829 cm^{-1}, which were attributed to the asymmetric stretching of C-H bonds of methyl (-CH_3) group in the caffeine molecule and the peak region.

These findings were successfully used by Chemat *et al*. to develop predictive models for quantitative analysis of caffeine [42]. In their study, the wave number in the region between 1400–900 cm^{-1} was identified by vibrations of several types of bonds, including C-H, C-O, C-N, and P-O. The stretching of the cis = C-H and cis -C=C- at 1651 cm^{-1} was an indicator of unsaturated fatty acids in vegetable oils [1]. Alcohols, saturated aldehydes and α, β-unsaturated aldehydes are major secondary oxidation products. In the region between 1730–1680 cm^{-1}, all aldehydes exhibit C=O stretching bands [2]. It is evident that carbonyl stretching around 1698 cm^{-1} may represent acetone [27]. The disappearance of peaks at 2857 cm^{-1} and the appearance of two peaks at 1698 and 1651 cm^{-1} in the EGCE treatment was due to the presence of canola oil and indicated well-encapsulated GCE.

Fatty acid composition. As can be seen in Table 3, the samples containing encapsulated GCE had a lower content of fatty acids than the others. The results indicated the protective role of encapsulation against the oxidation reaction. This finding was in agreement with the results of Fantoni *et al.* and Sun-Waterhouse *et al*. [19, 43]. In addition, EGCE treatment provided higher amounts of linoleic, oleic, and elaidic acids. The significant difference found in the contents of oleic and linoleic acids was due to the presence of canola oil in the encapsulated samples. Indeed, oleic acid and linoleic acid are two main fatty acids found in canola oil [44]. Dubois *et al.* claimed that oleic acid is the principal ingredient of various vegetable oils, including olive and rapeseed oils, and is a major dietary monoenoic acid [45]. Among PUFAs (polyunsaturated fatty acids), linoleic acid is the only one that reduces LDL-cholesterol. Consequently, encapsulation of canola oil has health benefits, as well as protective properties.

According to the fatty acid profile, the control had a slightly higher content of total saturated fatty acids than the GCE kashks, while the EGCE samples had the lowest. The EGCE had the largest content of PUFAs and MUFAs due to the presence of canola oil.

Rheological characteristics. The rheological parameters of the three kashk samples (control, GCE 1%,

Table 4 Effects of storage on some model parameters of control, GCE 1%, and EGCE 5% samples

		Power Law			Bingham			Casson		
Day	Sample	*n*	*k*, Pa·s^n	r^2	σ_0	η, Pa·s^n	r^2	k_{0C}	k_c, Pa·s^n	r^2
1	control	0.07 ± 0.00^a	15.04 ± 0.15^a	97.33 ± 0.15^a	18.41 ± 0.07^a	0.017 ± 0.00^a	94.27 ± 0.40^a	4.25 ± 0.01^a	0.04 ± 0.00^a	95.83 ± 0.25^a
	GCE 1%	0.07 ± 0.00^a	14.94 ± 0.27^a	97.10 ± 0.20^a	18.36 ± 0.12^a	0.017 ± 0.00^a	93.80 ± 0.40^a	4.24 ± 0.02^a	0.04 ± 0.00^a	95.50 ± 0.30^a
	EGCE 5%	0.07 ± 0.01^a	14.72 ± 0.97^a	96.90 ± 0.72^a	18.25 ± 0.44^a	0.018 ± 0.00^a	93.33 ± 1.55^a	4.22 ± 0.07^a	0.04 ± 0.01^a	95.16 ± 1.14^a
60	control	0.06 ± 0.01^a	15.29 ± 1.06^a	97.30 ± 0.69^a	18.51 ± 0.45^a	0.016 ± 0.00^a	94.23 ± 1.44^a	4.26 ± 0.06^a	0.04 ± 0.01^a	95.83 ± 1.09^a
	GCE 1%	0.07 ± 0.03^a	15.30 ± 2.36^a	97.23 ± 1.49^a	18.47 ± 0.99^a	0.016 ± 0.01^a	93.96 ± 3.28^a	4.25 ± 0.14^a	0.04 ± 0.02^a	95.60 ± 2.36^a
	EGCE 5%	0.09 ± 0.01^a	13.53 ± 0.71^a	96.03 ± 0.60^a	17.71 ± 0.34^a	0.022 ± 0.00^a	91.33 ± 1.35^a	4.14 ± 0.05^a	0.05 ± 0.01^a	93.67 ± 1.01^a

Results are reported as means ± SE, for three replicates of each sample
^{a} $P < 0.05$

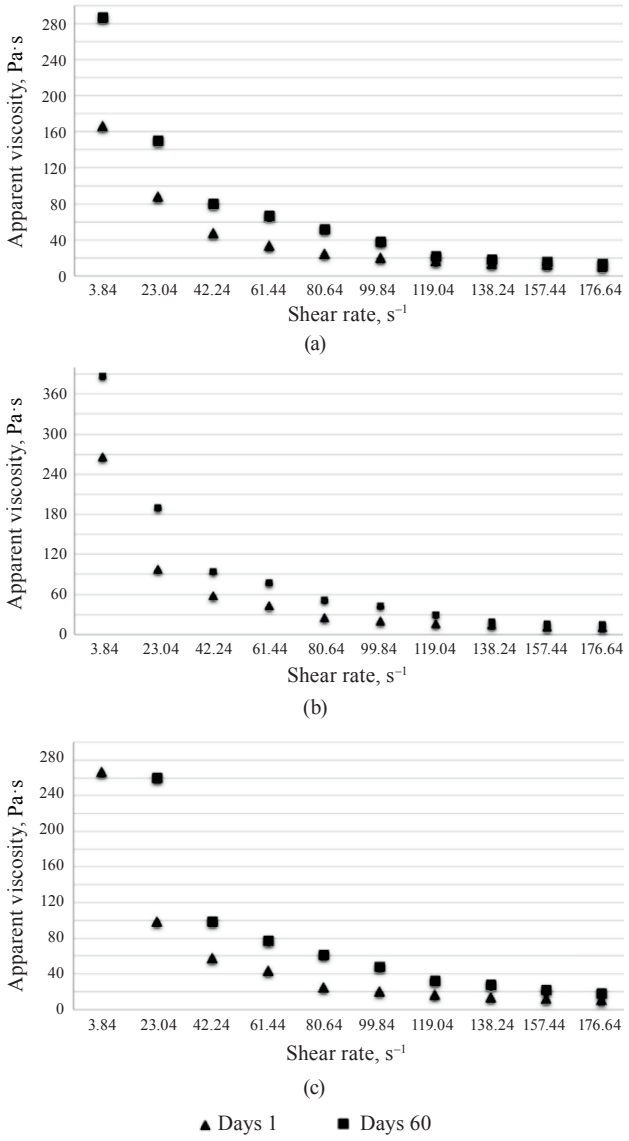


Figure 4 Apparent viscosity of the kashk samples during storage: (a) control; (b) GCE 1%; and (c) EGCE 5%

and EGCE 5%) on days 1 and 60 of the storage period are presented in Table 4. We found no significant differences in the viscosity of the treatments. As shown in Fig. 4, the samples' viscosity increased after 60 days of storage. Noteworthily, post-acidification caused a decline in the negative electric charge of casein micelles by dissolving calcium and inorganic phosphate. It attenuates colloidal stability and subsequently casein becomes insoluble near its isoelectric pH (about 4.6). This phenomenon, strengthening protein-protein complexes and protein-polyphenol interaction, enhances serum released from the gel matrix and, at the same time, increases viscosity [46]. To explain the consistency coefficient, flow index, and yield stress of the samples, we used different rheological models, namely Power law, Bingham, and Casson models. According to R^2 of the samples (higher than 0.97), the data fitted the Power Law model more than the others (Table 4). As can be seen, there were no significant differences in flow index values between all the samples at the beginning and at the end of storage ($P > 0.05$). The same trend was observed in the other model parameters throughout storage.

According to the Power law equation, the kashks containing GCE 1% and EGCE 5% had the highest and the lowest consistency coefficient on the first day of storage, respectively. At the end of storage, however, the control and the EGCE 5% kashk had the lowest (0.064) and the highest (0.090) flow index.

In Bingham's equation, the control had the lowest (0.016) and the EGCE 5% sample had the highest (0.018) consistency coefficient at the beginning of storage. The same trend was revealed on day 60. The highest yield stress (18.51) was observed in the control and the lowest (17.71) in the EGCE 5% kashk on day 60. Further, no significant differences were seen among the samples.

The Casson equation showed the same trend at the end of storage. However, we recorded the lowest yield stress (4.14) in EGCE 5% and the highest (4.26) in the control on day 60. Values within this range were found in kashk with and without the addition of gum tragacanth [1]. A higher consistency coefficient and a lower flow index can be considered appropriate to achieve a high viscosity and a clean mouthfeel [1]. Overall, all the samples showed a plastic-shear thinning behavior because of lower flow index (n) values (< 1) and a higher consistency coefficient.

Color. Table 5 summarizes the color characteristics of the kashk samples supplemented with GCE on days 1 and 60.

Increasing the concentration of GCE (microencapsulated or free) led to higher b* and L* values. We found that the highest b* value was related to the brownish yellow color of coffee extract. Among all the samples, those incorporated with microencapsulated GCE had higher b* and L* values compared with the others ($P < 0.05$). The higher L* value might be due to the reflection properties of lipid droplets or microencapsulated particles.

In general, we noticed no differences in the a* value of the kashk samples incorporated with GCE (free or microencapsulated). The L* and a* values of all the treatments increased throughout storage (60 days) ($P < 0.05$). However, the b* value did not change significantly in the microencapsulated GCE samples, while increasing considerably in the control ($P < 0.05$) due to the brownish yellow color of coffee extract. This result was in agreement with the studies of Lee *et al*. and Alavi *et al*. who reported enhanced L* and a* values in yoghurt enriched with powder peanut sprout extract microcapsules during 16 days [35, 36]. However, the b* value remained almost unchanged.

Sensory analysis. Sensory attributes of the samples were evaluated on the first and last days of storage (Fig. 5).

We found significant differences between the color of the kashk samples at the beginning ($P < 0.05$). The GCE 1% sample had a higher score, whereas the EGCE 0.25%

Table 5 Color characteristics of kashk samples with GCE on day 1 and day 60

	Day 1			Day 60		
Sample	L*	a*	b*	L*	a*	b*
Control	56.50 ± 0.50^{Bb}	-6.20 ± 0.21^{Ba}	11.37 ± 0.70^{Bc}	63.55 ± 0.09^{Ab}	-3.55 ± 0.19^{Ab}	14.34 ± 0.95^{Aa}
GCE 1%	55.06 ± 0.90^{Bc}	-6.18 ± 0.30^{Ba}	12.30 ± 0.02^{Bb}	69.68 ± 0.42^{Aa}	-2.22 ± 0.08^{Aa}	13.62 ± 0.43^{Ab}
GCE 0.5%	56.27 ± 0.85^{Bb}	-6.13 ± 0.54^{Ba}	12.60 ± 0.32^{Bb}	62.33 ± 0.14^{Ab}	-3.24 ± 0.11^{Ab}	10.26 ± 0.15^{Bc}
GCE 0.25%	54.41 ± 0.56^{Bb}	-6.08 ± 0.26^{Ba}	11.44 ± 0.84^{Ac}	62.37 ± 0.74^{Ab}	-4.85 ± 0.42^{Ac}	11.65 ± 0.71^{Ac}
EGCE 5%	57.44 ± 0.19^{Ba}	-6.71 ± 0.40^{Ba}	13.57 ± 0.42^{Aa}	62.24 ± 0.06^{Ab}	-2.24 ± 0.11^{Aa}	13.22 ± 0.48^{Ab}
EGCE 2.5%	56.72 ± 0.85^{Bb}	-6.51 ± 0.99^{Ba}	12.37 ± 0.59^{Ab}	60.34 ± 0.08^{Ac}	-3.12 ± 0.36^{Ab}	11.97 ± 0.35^{Ac}
EGCE 1.25%	55.42 ± 0.80^{Bc}	-6.95 ± 0.09^{Ba}	11.61 ± 0.64^{Ac}	60.55 ± 0.29^{Ac}	-4.34 ± 0.65^{Ac}	11.25 ± 0.31^{Ac}

Different small letters in each column and capital letters in each row show a significant difference between samples ($P < 0.05$). Results are reported as means ± SE, for three replicates of each sample

had a lower score ($P < 0.05$). The color of all the samples improved throughout storage. The GCE 1% had a better score, which was attributed to the light greenish yellow color of the coffee extract. However, it seems that GCE encapsulation protected the color of the kashk samples. As expected, the product with GCE 1% (free form) had better odor, taste, consistency, and overall acceptancy than the others.

Among all the samples, the EGCE 1.25% kashk obtained a lower score ($P < 0.05$). As predicted, the odor of kashk containing a free form of GCE improved with the concentration increased. This finding confirms a positive effect of GCE on kashk products. Nevertheless, a decline in odor was observed during storage due to the loss of some volatile compounds. We found that the samples' consistency improved with the extract

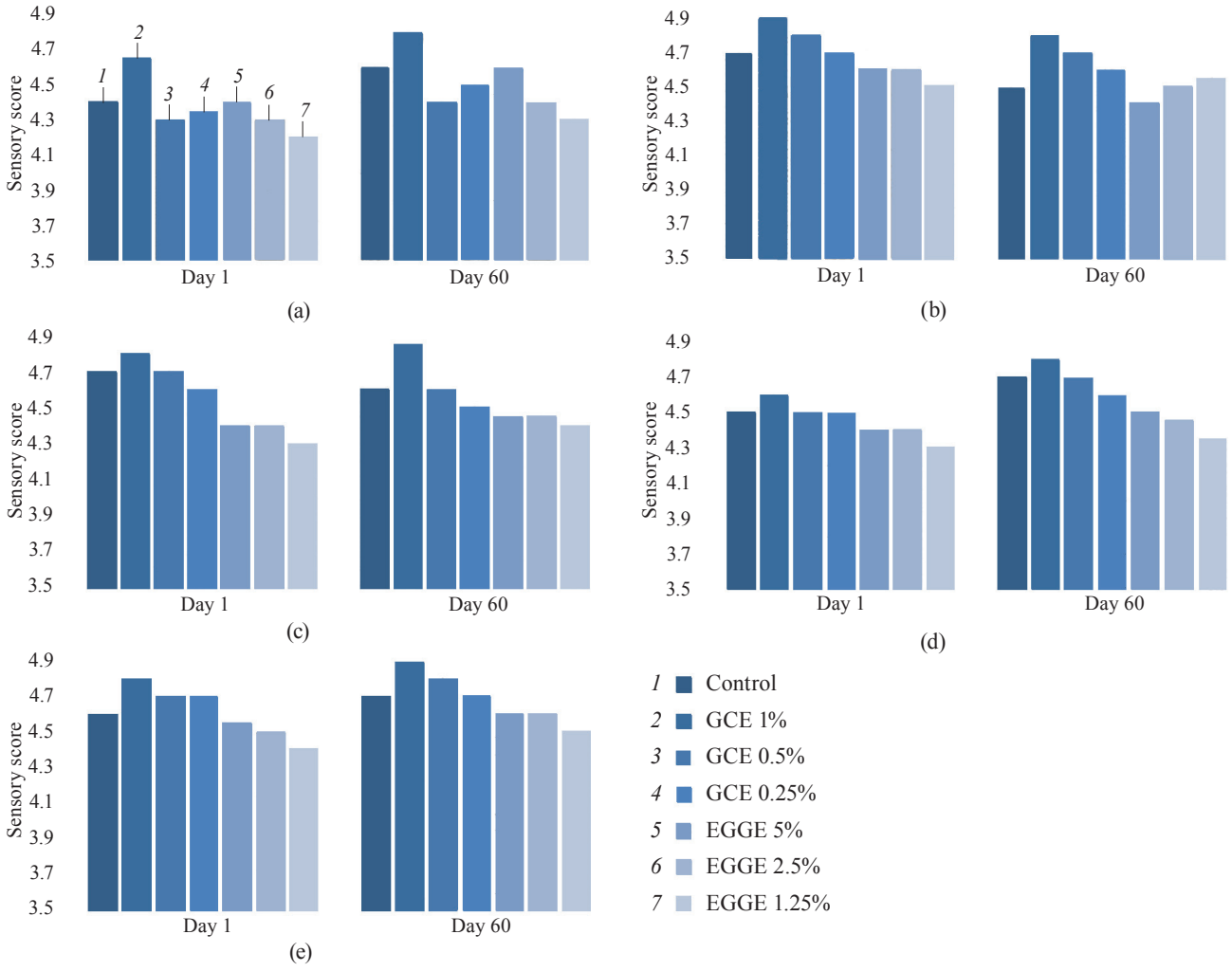


Figure 5 Sensory attributes of kashk samples supplemented with GCE on day 1 and day 60: (a) Color; (b) Odor; (c) Taste; (d) Consistency; (e) Total acceptability

concentration (free form) increased. Overall, the kashk fortified with GCE 1% was favored by the assessors, possibly due to its oily appearance.

Nonetheless, the type of microencapsulation technique influences the protective effects of odor/flavor components. For instance, Rodrigues *et al.* used cashew gum and Arabic gum as a wall material for microencapsulation of coffee extract during spray drying [47]. Both Arabic gum and cashew gum had similar aroma protective effect and showed no differences between the control and experimental samples. Furthermore, there is some dependence between the product's sensory properties and the type of herbal extract. Ribeiro *et al.* evaluated the color parameter of cottage cheese incorporated with free and encapsulated mushroom extract and revealed no changes in cottage cheese color [16]. However, the samples' color improved after 7 days ($P < 0.05$). Also, Gurkan *et al.* analyzed the taste and flavor characteristics of yogurt enriched with basil (*Ocimum basilicum* L.) powder or extract during three weeks of storage at 4°C [48]. They identified 49 volatile compounds which enhanced the sensory score of yogurts. The presence of some volatile carboxylic acids gave yogurt an acceptable acidic taste.

CONCLUSION

We employed a W/O emulsion to encapsulate GCE in kashk at different concentrations. Among all the samples, the kashk incorporated with a high amount of GCE had a higher pH. However, pH reduced throughout storage due to the production of a small amount of lactic acid. Furthermore, GCE possessed antioxidant activity, mainly due to its high phenolic content, and the samples containing GCE and EGCE had high oxidative stability. In addition, we confirmed a protective effect of encapsulation against the oxidation reaction. The EGCE sample had higher contents of only three free fatty acids (linoleic, oleic, and elaidic) due to the presence of canola oil in its composition. The control had the highest content of total saturated fatty acids.

The FTIR spectra indicated that the GCE was well encapsulated. The rheological behavior of the samples, a plastic-shear thinning behavior, fitted the Power Law model more than the others. Since particles larger than 30 μm may create a sandy feel in the mouth and the emulsion droplets in our study had a micrometer size lower than 30 μm, we could claim that the EGCE kashk had the best texture. Adding GCE (free and microencapsulated) to kashk affected its sensory properties. The kashk with 1% of GCE was preferred by the assessors. In addition, the coffee aroma was felt at the end of storage, which is a positive effect of using GCE in dairy products. Finally, GCE encapsulation protected the color of the samples and the b* value remained unchanged, while lightness (L*) increased. Overall, using a W/O emulsion can be successfully employed as a technique for GCE encapsulation in kashk and the resulting product can be offered to consumers as an alternative type of flavored dairy product.

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Processing cottage cheese whey components for functional food production

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Abstract:
Introduction. The study offers a new rational approach to processing cottage cheese whey and using it as a highly nutritional functional ingredient in food production. We proposed a scientifically viable method for hydrolyzing cottage cheese whey with enzyme preparations of acid proteases from *Aspergillus oryzae* with an activity of 400 units/g and a pH range of 3.0 to 5.0.
Study objects and methods. Pre-concentrated whey was enzymatically hydrolyzed at 30°C, 40°C, and 50°C for 60 to 180 min (pH 4.6). Non-hydrolyzed whey protein concentrates were used as a control. The amount of enzyme preparation was determined by calculation. All hydrolysate samples showed an increase in active acidity compared to the control samples. Further, we conducted a full-factor experiment with three levels of variation. The input parameters included temperature, duration of hydrolysis, and a substrate-enzyme ratio; the output parameters were the degree of hydrolysis and antioxidant capacity.
Results and discussion. The experiment showed the following optimal parameters for hydrolyzing cottage cheese whey proteins with the enzyme preparation of proteases produced by *Aspergillus oryzae*: temperature – 46.4°C; duration – 180 min; and the amount of enzyme preparation – 9.5% of the protein content. The antioxidant capacity was 7.51 TE mmol/L and the degree of hydrolysis was 17.96%.
Conclusion. Due to its proven antioxidant capacity, the whey protein hydrolysate obtained in the study can be used as a functional food ingredient.

Keywords: Cottage cheese whey, protein, enzymatic hydrolysis, functional ingredient, *Aspergillus oryzae*, concentration factor

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INTRODUCTION

Processing whey, including cottage cheese whey, is highly relevant today due to several urgent problems. Among them are deficiencies in nutrients and raw materials, as well as low social and environmental efficiency. According to analytical data, 59% of the whey produced in Russia is fed to livestock and only 21% is processed for further use. The remaining 20% is discharged into fields or wastewater, exacerbating the existing environmental problems [1]. When discharged into the environment, whey acts as a biochemical contaminant. It is characterized by high biological oxygen consumption (50–60 g O_2 per one liter annually) and high chemical oxygen consumption (50.5–54 g O_2 per one liter) [2]. Thus, whey entering sewage systems or, in emergency cases, water bodies can cause serious environmental problems. Simple calculations show that the oxidation of organic compounds contained in 25 tons of whey (an output of a medium-sized cheese factory) needs as much oxygen as the oxidation of household wastewater in a city with a population of

40000 people. Wastewater has a high concentration of readily oxidizable organic compounds. Therefore, whey can cause a decrease in dissolved oxygen concentration in water bodies. Moreover, the presence of suspended protein particles can lead to the accumulation of bottom sediments and rotting processes [3].

A positive scenario suggests a growth in production from using highly efficient technologies for the deep processing of raw materials, creating "smart" storage and logistics systems, as well as minimizing losses and waste. For this, we need to focus on the "intravital" formation of the composition and properties of raw materials. It is a prerequisite for modern food technologies and "smart" agriculture. Only this approach can lead to potential progress in technologic development and consistently contribute to positive trends in the nutrition of the population [4].

A healthy lifestyle requires new ways of increasing the nutritional value of foods. Intensive food production often leads to raw materials losing essential micronutrients at all stages of processing (refining, pasteurization, etc.) [5].

Another possible cause of nutritional deficiency is excessive consumption of medicines for chronic diseases, including gastrointestinal disorders, acute respiratory viral infections and flue epidemics, etc. These drugs cause "pharmacological" malabsorption, contributing to the deficiency of essential nutrients supplied with food [6, 7]. As a result, they affect adaptive, compensatory, and regulatory capabilities of the body, change its physiological functions, and lead to chronic diseases of not only the digestive system, but also other organs and systems. These include atherosclerosis, hypertension, type 2 diabetes, metabolic immunosuppression, alimentary obesity, autoimmune pathology, etc. Moreover, the lack of proteins, polyunsaturated fatty acids, vitamins, and minerals in the diet leads to impaired immunoreactivity and resistance to natural and anthropogenic environmental factors [8].

The current situation dictates a need for new directions and technologies for producing healthy foods, including dairy products. However, one of the problems here is introducing functional ingredients. The development of functional foods often involves enriching foods with functional ingredients and/or eliminating those substances which cause negative reactions (food hypersensitivity). For this, we need adequate scientific data on healthy nutrients used as ingredients and their effect on the product's taste and aroma profile [9, 11].

Controlled biocatalysis with specific enzymes can undoubtedly help food formulators develop functional food products [12].

Many researchers suggest using whey protein hydrolysates as functional ingredients. Due to bioactive peptides, they enhance the beneficial effect of traditional foods on public health [13, 15]. According to many authors, milk proteins modified by enzymatic hydrolysis have both technological properties (moisture binding, emulsifying, and foaming abilities) and functional properties (antioxidant, immunomodulating, hypotensive, etc.) [16–19].

Whey protein hydrolysates are used in the production of specialized products, for example, in sports nutrition [20].

In addition, whey treated with modern methods can increase the biological value of the end-product and improve functional and technological properties of raw materials and meat systems. For example, introducing whey into the meat system can regulate certain bio- and physicochemical processes by activating the biotechnological potential of natural systems in the ingredients [21]. In one study, hydrated protein preparations of Belcon Alev I and Lactobel ED were used to produce high-quality cooked sausages with a high biological value, digestibility, and prebiotic properties [22].

Whey protein is successfully used in the production of sausages as it not only creates a gelatinous mass that replaces fat, but also retains moisture [23]. This means that the yield of end-products can be increased without reducing the content of valuable animal protein or using additives. In addition, the end-products have better functional and technological properties, as well as improved taste characteristics [24].

Another benefit of using concentrated whey proteins in meat production is improved absorption of the end-product by the human body, which, together with a reduced calorie content, contributes to the physiological value of the product. Thus, replacing the fat component of the meat product with a protein fraction of dairy origin can be a fundamentally new solution to the global problem of obesity [25].

Cottage cheese whey is currently the main source of protein hydrolysates. Despite high volumes of whey produced in Russia and its obvious benefits, there are insufficient data on its use as a raw material for functional ingredients [10, 26].

Our study aimed to prove a possibility of using cottage cheese whey proteins subjected to biocatalysis as functional food ingredients.

STUDY OBJECTS AND METHODS

The objects of the study included cottage cheese whey and enzyme preparations – acid proteases from *Aspergillus oryzae* with an activity of 400 units/g and a pH from 3.0 to 5.0.

The initial samples of whey, concentrate, and hydrolysate were analyzed for active acidity (pH) potentiometrically according to State Standard 32892-2014[1] and for a mass fraction of total protein according

[1] State Standard 32892-2014. Milk and dairy products. Method of pH determination. Moscow: Standartinform; 2015. 13 p.

to State Standard 23327-98[II]. The assays were performed in triplicate.

Whey protein concentrate was obtained on an AL 362 pilot ultrafiltration unit (Altair, Russia) with a concentration factor of 5.0.

Enzymatic hydrolysis was carried out as follows. An enzyme preparation was introduced into cottage cheese whey preheated to the hydrolysis temperature (namely 30°C, 40°C, and 50°C) for 60–180 min. Active acidity was 4.6. Whey protein concentrates were used as a control. The enzyme amount was calculated by the formula:

$$M_E = \frac{P \cdot \alpha}{A} \times 1000 \quad (1)$$

where M_E is the amount of the enzyme preparation per 1 g protein;

P is the protein content in 100 g whey;

α is the required enzyme activity; and

A is the initial enzyme activity.

The hydrolysis was carried out in a thermostatic water bath with constant stirring. At the end, the samples were heated to 85°C and held for 15 min to inactivate the enzyme preparation.

The samples were then cooled and poured into sterile dishes.

The degree of hydrolysis was determined spectrophotometrically according to the method of Spencer *et al*. [27]. In particular, we took 2 mL of the sample into a 15 mL plastic falcon and added 10 mL of a 1% aqueous solution of sodium dodecyl sulfate. For this, we used an automatic pipette (Eppendof, Germany) with a measurement range of 500–5000 μL. The resulting reaction mixture was incubated in a water bath at 75 ± 1°C for 15 min.

A series of dilutions of L-leucine in a 1% SDS aqueous solution with concentrations of 0.15–3.0 mmol/dm^3 were used as standards for determining the degree of hydrolysis according to the Spencer *et al.* method. In particular, the falcons with different standard concentrations were successively filled with 2 mL of 0.2125 M sodium phosphate buffer (pH 8.20) and 250 μL of a standard solution, as well as 50 μL of a hydrolysate sample in the first case, 250 μL of a UV concentrate sample in the second case, and a blank sample in the third case. In addition, 2 mL of a 0.1% solution of 2,4,6-trinitrobenzenesulfonic acid was added to all the falcons. The falcons were tightly closed and shaken. The samples were then incubated in a water bath at 50°C for one hour. At the end of the incubation, 4.0 mL of a 0.1 M hydrochloric acid solution was added to each falcon to stop the reaction. The falcons were tightly closed, shaken, and kept for 30 min at room temperature for cooling. The optical density of the solutions was determined on a Synergy 2 microplate photometer-fluorometer (BioTek, USA) at a wavelength of 340 nm.

To determine the amount of leucine equivalents, we took 0.75 mL of the hydrolysate with the maximum degree of hydrolysis (100%) and transferred it into a 5.0 mL microreaction vessel. Then, we added 0.75 mL of distilled water and 2.4 L of concentrated hydrochloric acid. The vessel was incubated in an oven at 120 ± 2°C for 23 h. After incubation, the samples were cooled for one hour at room temperature and filtered under vacuum in a funnel with a glass filter. The contents of the microreaction vessel were quantitatively transferred to the filter and rinsed with distilled water. The pH of the wash water entering the Bunsen flask was monitored using Lach-Ner universal paper. The contents of the Bunsen flask were quantitatively transferred into a 100 mL laboratory glass beaker. The active acidity of the filtrate was adjusted to 7.00 ± 0.02 pH by adding a 40% aqueous solution of sodium hydroxide. The neutralized filtrate was quantitatively transferred into a 100 mL volumetric flask and the volume was adjusted to the mark with a 1% SDS aqueous solution. The contents of the flask were thoroughly mixed.

After foam collapse, a 0.25 mL sample was taken from the volumetric flask and analyzed. The degree of hydrolysis of the hydrolysate protein was calculated according to the equation:

$$DH = \frac{\left(\frac{(D^{340}_{hydrolysate} - D^{340}_{blanksample}) \cdot 30}{K} - \frac{(D^{340}_{UV\text{-}concentrate} - D^{340}_{blanksample}) \cdot 6}{K} \right)}{\left(\frac{(D^{340}_{AH} - D^{340}_{blanksample}) \cdot 133.33}{K} - \frac{(D^{340}_{UV\text{-}concentrate} - D^{340}_{blanksample}) \cdot 6}{K} \right)} \times 100 \quad (2)$$

where

$D^{340}_{hydrolysate}$ is the optical density in the hydrolysate sample at 340 nm;

$D^{340}_{blanksample}$ is the optical density in the blank sample at 340 nm;

$D^{340}_{UV\text{-}concentrate}$ is the optical density in the UV - concentrate sample at 340 nm;

30 is the hydrolysate dilution factor;

6 is a dilution factor for raw materials to obtain a hydrolysate;

K is the slope of the calibration graph showing the dependence of the optical density of the solution at 340 nm on the concentration of the standard in the sample (0.1733 L/mol);

[II] State Standard 23327-98. Milk and milk products. Determination of mass fraction of total nitrogen by Kjeldahl method and determination of mass fraction of protein. Moscow: Standartinform; 2009. 11 p.

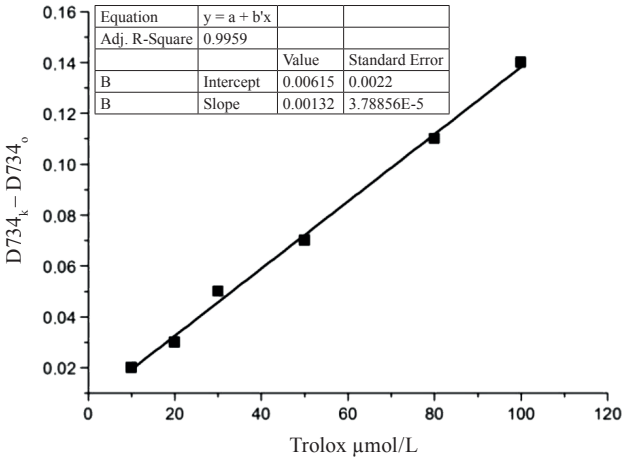


Figure 1 Decrease in optical density of ABTS radical cation solutions vs. Trolox concentrations in the samples

D_{AH}^{340} is the optical density in the acid hydrolysate sample (100% hydrolysis) at 340 nm; and

133.33 is the acid hydrolysate dilution factor.

The *in vitro* antioxidant capacity (TEAC) was measured using the ABTS radical cation.

The ABTS radical cation was obtained according to the Re *et al.* method by incubating a solution of 7 mM ABTS and 2.45 mM potassium peroxodisulfate in the dark at room temperature for 12–18 h [28]. The concentrated solution of ABTS radical cation was diluted with a 50 mM phosphate-buffered saline (with 100 mM sodium chloride), pH 7.4, to OD_{734} = 0.70 ± 0.02. This value corresponded to the final concentration of ABTS radical cation = 47 µM (ε_{734} = 1.5×10^4 mol^{-1}·L·cm^{-1}).

To determine the antioxidant capacity (AOC), 20 µL of the test samples or a Trolox solution and 180 µL of the ABTS radical cation solution were added to the wells of 96-well non-absorbing polystyrene microplates with a flat bottom. The control was 180 µL of the ABTS radical cation solution and 20 µL of a 50 mM phosphate-buffered saline (with 100 mM sodium chloride), pH 7.4. The reaction was recorded as OD_{734} decreased during 40.5 min with a measurement interval of 60 s at 25°C on a Synergy 2 photometer-fluorimeter (BioTek, USA). The assays were performed in quadruplicate.

The calibration curve of decreased optical density versus Trolox concentrations varying within 1–10 µM can be seen in Fig. 1. Equivalent concentrations of antioxidants in the samples were determined in relation to the decrease in optical density of the reaction medium in the presence of the studied compounds. The AOC of the samples was expressed in µM TE. When testing the antioxidant activity of hydrolysate samples with respect to the ABTS radical cation, the working range of dilution factors for a 50 mM phosphate-buffered saline (pH 7.4) was 150.

Sensory analysis described by Spellman was used to determine bitterness in enzymatic hydrolysates [29].

To optimize the conditions for enzymatic hydrolysis of cottage cheese whey, we conducted a full-factor experiment with three variables: temperature (X_1),

Table 1 Variation levels of independent parameters in multifactorial experiments to optimize the hydrolysis of cottage cheese whey

Factor	Variable	Level of variation		
		–1	0	+1
Temperature, °C	X_1	30	40	50
Hydrolysis duration, min	X_2	60	120	180
E/S, %	X_3	0.5	4.5	9.5

Table 2 Enzyme amounts per 1 g protein

Enzyme-substrate ratio, %	Enzyme amount per 100 g whey, mg
0.5	4.225
4.5	38.025
9.5	80.275

Table 3 Results of full-factor experiments to optimize enzymatic hydrolysis of cottage cheese whey proteins

Sample	Bitter taste	Degree of hydrolysis (DH), %	TEAC, TE mmol/L
control samples			
Control 1	–	0	5.30
Control 2	–	0	4.17
Control 3	–	0	2.65
fermented samples			
4	–	7.82	4.17
5	–	10.02	7.58
6	+	12.68	7.20
7	–	8.41	4.55
8	–	11.29	5.68
9	+	13.07	6.74
10	–	8.59	5.19
11	+	11.92	6.36
12	++	13.06	8.71
13	–	7.31	7.58
14	–	11.43	6.06
15	–	13.13	8.71
16	–	8.21	5.30
17	–	11.88	9.09
18	–	15.35	9.81
19	–	8.24	1.14
20	–	12.08	8.71
21	+	15.75	4.55
22	–	8.43	3.41
23	–	11.28	5.30
24	–	15.08	4.55
25	+	9.01	3.03
26	–	13.07	4.92
27	–	18.01	4.55
28	–	9.59	3.79
29	–	14.96	5.68
30	–	19.66	8.71

Table 4 Effects of variable factors on the hydrolysis degree of cottage cheese whey proteins

Factor	Effect	Std. dev.	Student t-test	*P*	–95, %	+95,%
	11.95086	0.098745	121.0273	0.000000	11.74253	12.15920
Temperature, °C (L)	2.54302	0.241874	10.5138	0.000000	2.03271	3.05333
Temperature, °C (Q)	–0.51056	0.209255	–2.4399	0.025942	–0.95204	–0.06907
Duration, min (L)	1.88078	0.241874	7.7759	0.000001	1.37047	2.39109
Duration, min (Q)	0.30944	0.209255	1.4788	0.157487	–0.13204	0.75093
E/S (L)	6.68667	0.241627	27.6735	0.000000	6.17688	7.19645
E/S (Q)	0.61926	0.209685	2.9533	0.008897	0.17686	1.06166
1L by 2L	1.06167	0.295931	3.5875	0.002269	0.43731	1.68603
1L by 3L	1.97152	0.295324	6.6758	0.000004	1.34844	2.59460
2L by 3L	0.77102	0.295324	2.6108	0.018268	0.14795	1.39410

duration of hydrolysis (X_2), and enzyme-substrate ratio E/S (X_3). Each of the parameters varied at three levels (Table 1). The output parameters were the degree of hydrolysis (DH) and antioxidant capacity (TEAC).

The variation levels of independent parameters in the multifactorial experiments conducted to optimize the hydrolysis of cottage cheese whey are shown in Table 1.

The results of the multifactorial experiments were statistically processed using the DOE block of Statistica 10.0 (StatSoft Inc., USA).

RESULTS AND DISCUSSION

The protein content was 0.56% in the initial whey and 1.35% in the concentrate. The enzyme amounts per 1 g of protein are shown in Table 2.

The results of the full-factor experiments conducted to optimize enzymatic hydrolysis of cottage cheese whey proteins are demonstrated in Table 3.

The experiments showed an increase in the degree of hydrolysis and antioxidant activity of cottage cheese whey proteins with larger amounts of enzyme preparations and longer fermentation at 30°C and 50°C. The maximum degree of hydrolysis (19.66%) was recorded at 50°C, 180 min fermentation, and 9.5% enzyme. However, higher temperatures led to a noticeable, almost two-fold decrease in antioxidant activity in the control samples, which were not hydrolyzed. Thus, temperature had a significant effect on this indicator (Table 4).

According to sensory evaluation, the most bitter taste was registered in the sample that was hydrolyzed at 30°C with the maximum duration and enzyme amount (13.06% degree of hydrolysis). However, sample No. 30, which was obtained at the maximum temperature, duration of hydrolysis, and enzyme amount, did not taste bitter. It means that these conditions make the process more directional, producing hydrolysates that do not contain peptides with bitter amino acids at the end of the chain. At the same time, this sample had the highest degree of hydrolysis.

Table 4 shows the statistical analysis of effects that variable factors have on the degree of hydrolysis of cottage cheese whey proteins. As we can see, all the variable factors, except for the quadratic duration factor, have a significant ($P < 0.05$) effect on the degree of hydrolysis.

The relation between the degree of whey protein hydrolysis and variable parameters is graphically

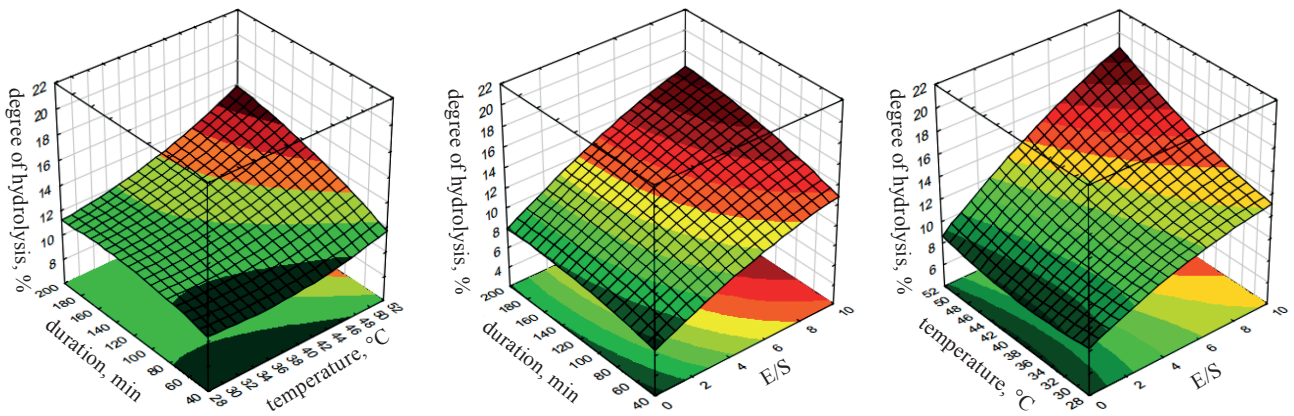
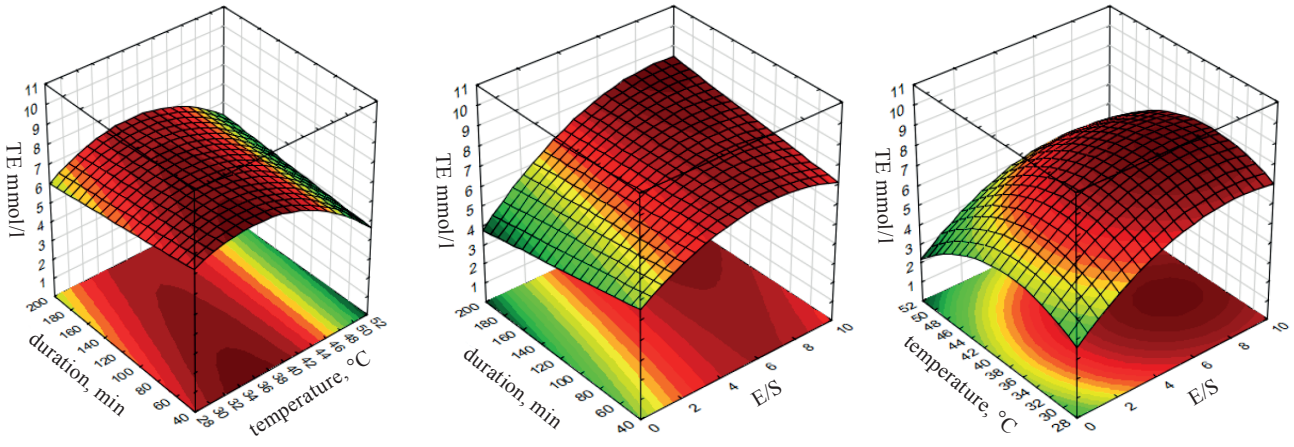


Figure 2 Degree of hydrolysis in UV concentrate hydrolysates of cottage cheese whey versus variable parameters with an average third factor: (a) Degree of hydrolysis versus duration and temperature; (b) Degree of hydrolysis versus duration and enzyme-substrate ratio; (c) Degree of hydrolysis versus temperature and enzyme-substrate ratio

Table 5 Effects of variable factors on antioxidant activity of cottage cheese whey proteins

Factor	Effect	Std. dev.	Student t-test	*P*	–95, %	+95,%
	6.01776	0.350932	17.14794	0.000000	5.27736	6.758159
Temperature, °C (L)	–1.36738	0.859600	–1.59071	0.130097	–3.18098	0.446221
Temperature, °C (Q)	1.21000	0.743674	1.62706	0.122116	–0.35902	2.779015
Duration, min (L)	–0.15247	0.859600	–0.17738	0.861309	–1.96607	1.661126
Duration, min (Q)	–0.00333	0.743674	–0.00448	0.996476	–1.57235	1.565682
E/S (L)	2.81889	0.858721	3.28266	0.004391	1.00715	4.630632
E/S (Q)	1.10494	0.745203	1.48273	0.156446	–0.46730	2.677179
1L by 2L	0.60167	1.051714	0.57208	0.574754	–1.61726	2.820590
1L by 3L	–0.19918	1.049557	–0.18978	0.851732	–2.41355	2.015191
2L by 3L	1.04324	1.049557	0.99398	0.334171	–1.17113	3.257609



(a) (b) (c)

Figure 3 Antioxidant capacity of UV-concentrate hydrolysates versus variable parameters with an average third factor:
(a) Antioxidant capacity versus duration and temperature; (b) Antioxidant activity versus duration and enzyme-substrate ratio;
(c) Antioxidant activity versus temperature and enzyme-substrate ratio

illustrated in Fig. 2. The graphs show no local maxima or minima, suggesting that the degree of hydrolysis rises with an increase in each of the parameters.

Table 5 shows the statistical analysis of effects that variable factors have on the antioxidant activity of cottage cheese whey proteins. As we can see, only the linear factor of enzyme amount has a significant ($P > 0.05$) effect on the antioxidant capacity.

The surfaces of equal response of hydrolysates TEAC versus variable parameters of whey concentrate hydrolysis are presented in Fig. 3. We can clearly see the presence of a local maximum of antioxidant activity in Figs. 3a and 3b.

Finally, we correlated the key factors in the multifactorial experiments in order to select optimal conditions for the enzymatic hydrolysis of the UV-concentrate of cottage cheese whey using the enzyme preparation from *Aspergillus oryzae*.

CONCLUSION

Based on the statistical analysis, we selected the following optimal conditions for the hydrolysis of cottage cheese whey proteins: temperature – 46.4°C; duration – 180 min; and the enzyme amount – 9.5% of the protein content. These conditions provided the antioxidant capacity of 7.5 TE mmol/L with a 17.96% degree of hydrolysis.

The given data open up new prospects for processing acid cottage cheese whey and using whey proteins as potential functional components with increased antioxidant activity. We showed that targeted biocatalytic conversion can make whey proteins more functional. The obtained hydrolysate of cottage cheese whey proteins can be used to develop new functional foods, including meat and dairy products.

CONTRIBUTION

E.Yu. Agarkova led the research. A.G. Kruchinin statistically processed the data. N.A. Zolotarev developed and analyzed the test samples. N.S. Pryanichnikova checked the data reliability. Z.Yu. Belyakova systematized the data. T.V. Fedorova summarized the data.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Absorption of iodotyrosine from iodized milk protein in animals

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Abstract:
Introduction. One of the ways to the solve iodine deficiency problem is the addition of iodine to farm animal feed. It allows producing iodized livestock products. Promising sources of organic iodine are iodotyrosine-containing iodized milk proteins. Organic iodine accumulation in organs and tissues has not been sufficiently studied.
Study objects and methods. We determined iodotyrosine content in rat blood plasma and in pig muscle tissue. For this purpose, high performance liquid chromatography with mass spectrometric detection and cathodic stripping voltammetry were used.
Results and discussion. At the first stage of the study, we examined iodotyrosines in rat blood plasma after a single administration of iodized milk protein or potassium iodide (30 µg I/kg weight) at specific time intervals. A significant increase in the concentration of monoiodotyrosine and diiodotyrosine was recorded 4 and 24 h after the administration. At the second stage, we studied the accumulation of iodotyrosines in the muscle tissue of pigs during their fattening period (104 days). The diet of the control animal group included potassium iodide (0.6 mg I/kg of feed). The experimental groups A and B got iodized milk protein (0.3 and 0.6 mg I/kg of feed, respectively). Monoiodotyrosin content in the muscle tissue of pigs of the experimental groups was 3.0 and 5.2 times higher than that in the control group. Diiodotyrosine content was 4.9 and 8.2 times higher. In the experimental group A, iodine content in muscle tissues was 26% higher than that in the control group, in the experimental group B it was 72% higher. Calculations of iodine intake balance and its accumulation in muscle tissues showed that in animals whose diet included iodized milk protein, the iodine assimilation was much higher (0.70 and 0.53%) than in the control group (0.21%).
Conclusion. Iodotyrosines from iodized milk protein are absorbed by the gastrointestinal tract in an unchanged form and accumulate in muscle tissues. The findings give more clear understanding of physiological and biochemical mechanisms of organic iodine absorption in animals.

Keywords: Iodine, iodotyrosines, plasma, muscle tissue, iodized milk protein, absorption

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INTRODUCTION

Among non-metals, iodine is the most important biologically active microelement in human and animal nutrition. The main role of iodine in the body is synthesis of thyroid hormones, triiodothyronine and tetraiodthyronine, or thyroxine. The importance of these hormones is enormous, as they take part in various metabolic processes and influence the tissue growth and differentiation [1, 2]. Iodine deficiency leads to morphological and functional changes in the thyroid gland, to decreased thyroid hormones production and, as a result, to pathological conditions in humans and animals [3, 4]. Iodine deficiency has remained a problem for many countries, including Russia [5–7]. More than half of the regions in the Russian Federation are iodine deficient, and 60% of the population suffers from iodine deficiency [8].

A decisive role in iodine deficiency prevention is given to the production of iodine-enriched foods of mass consumption (salt, milk, bread, meat products) [9–11]. One of the ways to produce iodized livestock products is addition of iodine to farm animal feed. The iodine content in milk can reach 500 µg/kg and more, in chicken eggs – up to 60 µg/egg [15–18]. Considering the accumulation of significant amounts of iodine in milk and eggs, the European Food Safety Agency (EFSA) has set a maximum level of iodine in feed: for dairy cows and small dairy ruminants – 2 mg/kg, for laying hens – 3 mg/kg of feed dry matter [18].

Table 1 Experiment scheme (stage I)

Group	Number of animals	Diet
Control	20	Balanced common diet (CD)
Experimental group A	16	CD + iodized milk protein (1500 μg/kg body weight)
Experimental group B	16	CD + potassium iodide (39 μg/kg body weight)

The iodine content in meat is directly related to its content in feed. However, even with significant concentrations of the trace element in the diet its content in muscle tissue of animals and poultry is much lower than in milk and eggs [19–21]. According to Flachowsky, the proportion of iodine absorbed from feed is 0.3% for pork and less than 1% for beef compared to 30–40% for milk and 10–20% for eggs [22]. Significant concentrations of iodine in milk and eggs are explained by the fact that not only the thyroid gland, but also exocrine glands, such as salivary, gastric, cervical uterine, and lacteous glands, can uptake iodine from the blood [23, 24].

The accumulation of iodine in the muscle tissue of animals and poultry when entering the body in an inorganic form is negligible. Iodine in the form of iodide ion is almost completely absorbed in the gastrointestinal tract, most part of it is used by the thyroid gland, some is captured by exocrine glands and leukocytes, and the rest is excreted from the body [25].

Currently, there is a growing interest in the study of organic forms of minerals. Iodine in organic molecules is more stable during feed storage. A number of studies have found a positive effect of organic iodine compounds on iodine accumulation in the body and the productivity of farm animals [26, 27]. He *et al*. showed that *Laminaria digitate* alga in the diet of pigs led to a 45% increase of iodine content in muscle tissue compared to the control group whose diet included iodine in the form of potassium iodide [28]. Promising sources of organic iodine are iodized milk proteins containing iodotyrosines. Iodotyrosines of milk proteins are analogues of natural compounds produced by the thyroid gland and involved in the metabolism of iodine. Iodine accumulation in organs and tissues entering as part of organic compounds has not been sufficiently studied.

The purpose of the work was to study the mechanism of absorption and accumulation of iodotyrosines, which are part of milk iodized protein, in the animal muscle tissue.

STUDY OBJECTS AND METHODS

The objects of the study were iodized milk protein ("Chemical Technologies", Russia) and potassium iodide ("Iodobrom", Russia). In iodized milk protein, the matrix for iodization is whey proteins, unlike in "Iodcasein", where the matrix is milk casein.

Iodized milk protein is obtained by the Lublinskij *et al*. method [29], which involves mixing protein raw materials with an aqueous solution of inorganic iodine and enzyme treatment. A mixture of skimmed fresh milk and proteins of different natural origin is used as a protein raw material. In iodized milk protein, iodine is present in the form of mono- and diiodotyrosines, which are full analogues of natural organic iodine compounds. The content of total iodine in iodized milk protein is 2%, monoiodotyrosine – 1.32%, and diiodotyrosine – 0.64%.

The study included two stages. At the first stage, we studied the mechanism of iodotyrosine absorption. For that, mono- and diiodotyrosine content in rat blood plasma was studied after a single injection of iodized milk protein or iodide potassium. The study used Wistar rats aged 8–10 weeks obtained from a licensed source (Andreevka branch of Scientific Centre of Biomedical Technologies, Moscow, Russia). The experiments were performed in the vivarium of Gorbatov Federal Research Center of Food Systems of Russian Academy of Sciences, Moscow. All manipulations with the rodents were carried out in strict accordance with the protocol of research and the current regulatory documentation[III] [30]. After five-day adaptation the animals were divided into groups (six animal units in each) and placed in plastic cages ("Tecniplast", type IV S) on a fine wood chips litter. The rats had unlimited access to tap water and food. We used complete feed (by "Laboratorkorm").

After the adaptation the animals randomly were divided into three groups. The scheme of the experiment is presented in Table 1.

The drug aqueous solutions were injected once with an intragastric probe. Dosages of drugs were 30 μg iodine/kg of body weight. After the injections, the animals were subjected to food deprivation for no more than 24 h.

Four animals from each group were subjected to euthanasia in a CO_2 chamber (VetTech, UK) 1 h, 4 h and 24 h after administration. Blood plasma was taken and stored at –30°C for future experiments.

Mono- and diiodotyrosine content in rats' blood plasma was determined by liquid chromatography with mass spectrometry detection [31]. Samples were dried, degreased, and subjected to enzymatic hydrolysis using *Streptomyces griseus* proteases. The extraction and purification of iodotyrosines from the samples was performed by solid-phase extraction, followed by derivatization of the extract. Identification of the analytes was carried out according to the absolute retention time of chromatographic peaks of iodthyrosine recorded in the mode of monitoring of multiple reactions. The iodotyrosine concentration was determined based on the area of chromatographic peaks.

[I] State Standard 31886-2012. Principles of Good Laboratory Practice (GLP). Application of the GLP principles to short term studies. Moscow: Standartinform; 2013. 10 p.

[II] State Standard 33044-2014. Principles of good laboratory practice. Moscow: Standartinform; 2015. 12 p.

Table 2 Experiment scheme (stage II)

Groups	Diet	Monoido-tyrosine content, mg/kg feed	Diiodty-rosine content, mg/kg feed
Control	CD + potassium iodide (0.6 mg I/kg)	–	–
Experimental group A	CD + iodized milk protein (0.3 mg I/kg)	0.2	0.097
Experimental group B	CD + iodized milk protein (0.6 mg I/kg)	0.4	0.194

CD is balanced common diet

The results of the research were processed by parametric methods of variational statistics using the Student t-criterion for unrelated groups ($P < 0.05$) [32]. Arithmetic mean M, mean square deviation m, and the mean error of arithmetic mean σ were determined to calculate the reliability of the differences between the two samples.

At the second stage of the research we studied iodotyrosines accumulation in muscle tissue of large white pigs at the age of 4 months. Three groups of 20 animals each were formed. The selection of groups was carried out on the analogue principle. The duration of the experiment was 104 days. All the animals were kept on a balanced diet, fed twice a day. The diet included wheat, barley, corn, soybeans, peas, wheat meal, fish forage flour, yeast, as well as minerals and vitamins. The chemical composition of the feed was balanced by the main nutrients and depended on the fattening period. Access to water was free.

The control group received potassium iodide in the amount of 0.6 mg I/kg of feed, while the experimental groups A and B received iodized milk protein in the amounts of 0.3 and 0.6 mg I/kg of feed, respectively. The scheme of the experiment is presented in Table 2.

During the experiment we recorded average daily feed consumption, as well as initial and final weight of animals. At the end of the study, three pigs from each group were slaughtered and butchered. We calculated weight before the slaughter, kg; weight of hot carcass, i.e. weight after slaughter and visceration, kg; carcass yield, i.e. the ratio of the hot carcass weight to the weight before slaughter, %; chilled carcass weight (after 24 h storage at 4 ± 2°C), kg; and mass of muscle tissue, kg. *M. longissimus dors*i was sampled for chemical analysis. Iodotyrosine content in muscle tissue was determined by high performance liquid chromatography with mass spectrometric detection according to [31].

Iodine content was assessed on a TA-Lab voltammetric analyzer ("Tomanalit", Russia). The method includes mineralization of samples and subsequent analysis of their aqueous solutions with the help of cathodic stripping voltammetry. During the mineralization process and subsequent ultraviolet irradiation of the solution of the mineralized sample solution all forms of iodine are transformed into iodide ions. Iodide ions are concentrated on silver modified or mercury-film electrodes in the form of low-soluble sludge followed by cathodic reduction of the sludge with linear change of potential. The resulting cathodic peak at the potential minus (0.4 ± 0.05) B for the modified silver electrode and minus (0.3 ± 0.05) B for the mercury film electrode is an analytical signal. The content of iodide ions in the solution of the prepared sample is determined by the method of standard additives of the certified mixture of iodide ions.

The amount of iodine absorbed from feed (%) was calculated as a ratio of iodine accumulated in muscles during fattening to an amount of iodine consumed with feed. The amount of accumulated iodine in muscle tissue was determined by subtracting the amount of iodine in muscles at the beginning of the experiment from the amount of this trace element at the end. The initial amount of iodine was estimated based on the iodine content established at the consumption of inorganic iodine, taking into consideration the initial mass of muscle tissue. The latter was calculated based on the muscle tissue yield determined at the end of the experiment.

Statistical processing of results was carried out using the method of dispersion analysis ($P < 0.05$) [32]. The data are presented as arithmetic mean M and standard square deviation m.

RESULTS AND DISCUSSION

The mechanism of inorganic iodine compounds absorption is studied quite well. Iodine in the form of iodide ion is absorbed in the stomach and upper intestine for 30 min. The thyroid gland takes from 5 to 30% of iodine, some part is used by leukocytes and exocrine glands [25]. Organic iodide is believed to detach from the organic molecule in the liver and enters the blood in the form of iodide ion [33]. However, in the process of presystemic metabolism of iodized amino acids, in particular iodotyrosines, iodine detachment may not occur, and they enter the systemic blood flow unchanged. Absorption of organic selenium in the form of selenomethionine carries in a similar manner [34, 35].

To define the features of iodotyrosine metabolism in animals, we determined the concentration of monoidotyrosine and diiodotyrosine in rats' blood plasma. We tested control sample (with no iodide), experimental sample A (with iodized milk protein), and experimental sample B (with potassium iodide). The results of the study are presented in Table 3.

Table 3 shows that the monoiodotyrosine concentration in the blood plasma of intact animals (control group) did not change throughout the experiment. After administration of iodized milk protein, the concentration of monoiodotyrosine did not differ from the control group in 1 h, but was

Table 3 Iodotyrosine concentration in rat blood plasma, ng/mL (n = 4)

Time, h	Groups								
	Control			Experimental group A (iodized milk protein)			Experimental group B (potassium iodide)		
	M	m	σ	*M*	m	σ	*M*	m	σ
Monoiodthyrosine concentration									
0	0.093	0.01	0.01	–			–		
1	0.098	0.01	0.01	0.11	0.01	0	0.078	0.01	0.01
4	0.08	0.02	0.01	0.72*	0.07	0.04	0.1	0.02	0.01
24	0.085	0.01	0.01	0.31*	0.03	0.02	0.115*	0.01	0.01
Diiodotyrosine concentration									
0	0.05	0.01	0	–			–		
1	0.048	0.01	0.01	0.043	0.01	0.01	0.035	0.01	0.01
4	0.04	0.02	0.01	0.27*	0.03	0.02	0.04	0.01	0.01
24	0.04	0.01	0	0.155*	0.01	0.01	0.05	0.01	0

* statistically significant differences ($P < 0.05$) from the indicator of the animals in the control group

significantly higher 4 and 24 h later ($P < 0.05$). In 4 h and 24 h monoiodotyrosine content in the sample A exceeded that in the control sample by 8 and 3.6 times, respectively.

In the sample B, a 35% increase in monoiodotyrosine concentration was recorded only after 24 h ($P < 0.05$). This can be explained by a more active synthesis of thyroid hormones after an increased iodine intake into the thyroid gland. Iodized tyrosines are formed in the gland during thyroglobulin proteolysis and can flow into the bloodstream along with hormones.

The content of diiodotyrosine and monoiodotyrosine in the control sample was at the same level during the experiment (Table 3). 4 and 24 h after administration of iodized milk protein, the concentration of diiodotyrosine in rats of the experimental group A exceeded that ibn rats of the control group by 6.8 and 3.9 times, respectively ($P < 0.05$). Introduction of potassium iodide did not cause an increase in diiodotyrosine content in rats of the experimental group B.

According to the finding, iodized milk protein increased significantly iodotyrosine content in rats' blood plasma. Concentrations of monoiodotyrosine and diiodotyrosine were maximal 4 h after administration of iodized milk protein. This is probably due to the fact that milk whey protein digestion takes 2–3 h. In the later period, the concentration of amino acids in the blood reaches maximum, and then it decreases, which is confirmed by our findings. It is also should be noticed that the content of monoiodotyrosine in blood was twice as much as that of diiodotyrosine. Such ratio of iodized amino acids consists with their content in iodized milk protein.

Thus, an increased concentration of iodized amino acids in rat blood plasma after taking iodized milk protein may indicate that monoiodotyrosine and diiodotyrosine are able to enter into systemic blood stream unchanged, without being deodized in the liver during presystemic metabolism.

Table 4 Performance parameters of test pigs ($M \pm$ m, n = 20)

Indicator	Groups		
	Control	Experimental group A	Experimental group B
Initial body weight, kg	47.55 ± 7.52	49.65 ± 7.77	48.95 ± 8.98
Final body weight, kg	113.25 ± 6.63	118.25 ± 7.41	115.59 ± 8.07
Average daily weight gain, g/day	631.69 ± 19.96	659.60 ± 18.27	640.81 ± 35.37
Average daily feed intake, kg/day	2.3 ± 0.19	2.3 ± 0.17	2.3 ± 0.12

Getting into the systemic blood stream, amino acids begin to be distributed to various organs and tissues of the body. At the next stage, we studied the accumulation of iodtyrosines and the degree of iodine absorption in the muscular tissue of the pigs.

We did not find statistically significant differences between the control and experimental groups (Table 4).

The results of the research showed that iodine in organic form in the diet of pigs did not have a significant impact on the slaughter parameters of the animals (Table 5).

The yield of carcasses was the same in pigs of the control and experimental groups and amounted to 70%. The content of muscle tissue in pig carcages varied according to the iodine-containing supplement consumed. Muscle tissue yield of the animals in the experimental groups exceeded that in the control by 0.45% ($P < 0.05$), which indicates the positive effect of iodized milk protein on this parameter.

Table 5 Slaughter parameters and muscle yield of test pigs ($M \pm m$, n = 3)

Parameter	Groups		
	Control	Experimental group A	Experimental group B
Pre-slaughter weight, kg	110.09 ± 3.10	111.68 ± 2.79	110.21 ± 3.52
Hot carcass weight, kg	77.09 ± 2.07	78.18 ± 2.00	77.12 ± 2.50
Chilled carcass weight, kg	75.76 ± 2.07	76.87 ± 2.00	75.79 ± 2.49
Muscle tissue weight, kg	63.49 ± 1.82	64.76 ± 1.62	63.85 ± 1.95
Muscle tissue yield, %	83.80 ± 0.11	84.25 ± 0.12*	84.25 ± 0.21*

* statistically significant differences ($P < 0.05$) from the indicator of the animals in the control group

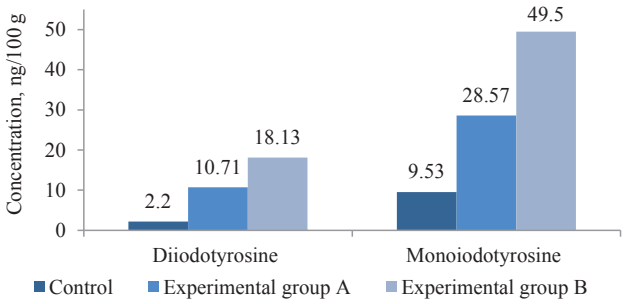

Figure 1 Iodotyrosine content in pig muscle tissue

When determining iodotyrosines in the muscle tissue of pigs (Fig. 1), it was found that contents of monoidotyrosine and diiodotyrosine in the animals from the experimental groups were significantly higher than those from the control group. The content of monoiodotyrosine in the muscle tissue of pigs from the experimental groups A and B was by 3.0 and 5.2 times, and diiodotyrosine – by 4.9 and 8.2 times higher than in the control group, respectively. In the experimental groups, with the increase in the content of iodtyrosines in feed, their concentration in meat also increased, but not directly proportional to the increase in the amount of iodized amino acids consumed. The results recorded during this stage were obtained for the first time. For the last five years there have been no available data confirming or refuting our findings.

The content of total iodine in animals of the experimental groups was higher compared to that in pigs from the control group (Table 6).

At the same time, even in the experimental group A, where iodine content in the diet was twice less than in the control group, the concentration of iodine in muscles was 26% higher. In the experimental group B, with the equal content of iodine in the feed, the concentration of iodine in muscle tissue was 72% higher than in the control group.

Calculations of iodine intake balance and its accumulation in muscle tissue showed that in animals receiving iodine in the form of iodized milk protein, the degree of iodine absorption was much higher than that in the control group. According to Franke *et al*., inorganic iodine absorption in the muscle/fat fraction is not more than 0.24%, which corresponds to our result recorded in the control group [36]. Besides, the researchers found a tendency of iodine absorption decreasing with an increase in its content in feed. This pattern was also found in our study. In the experimental group A, where iodine content in the diet was twice less, iodine absorption was higher than that in the experimental group B.

The data obtained confirm the assumptions of some authors about better absorption and more intensive accumulation of organic iodine compounds in animals. For example, Banoch *et al*. observed a similar effect when adding iodine-rich algae *Chlorella spp.* compared to potassium iodide. A noticeable effect of iodine introduced into the feed on the pork quality was not established [37].

Table 6 Iodotyrosine content in pig muscle tissue

Parameter	Groups		
	Control	Experimental group A	Experimental group B
Total iodine, µg/100 g	8.83 ± 0.72	11.20 ± 0.98*	15.20 ± 0.62*
Amount of absorbed iodine in muscle tissue, % of injected amount	0.21 ± 0.03	0.70 ± 0.09*	0.53 ± 0.03*

* statistically significant differences ($P < 0.05$) from the indicator of the animals in the control group

CONCLUSION

The results of the study showed that iodotyrosines entering the body of animals in the form of iodized milk protein can be absorbed in the gastrointestinal tract in an unchanged form without iodine detachment and can accumulate in the muscle tissue. At the same time, there was a significant increase in the concentration of monoiodthyrosine and diiodotyrosine in the blood plasma of experimental animals. The content of iodized tyrosines in the muscle tissue of animals whose diet included iodine in the form of iodized milk protein significantly exceeded that in animals whose diet included inorganic iodine. In addition, it should be noted that the proportion of absorbed iodine from organic compounds is much higher than the absorption degree of inorganic iodine.

The findings can provide more clear understanding about physiological and biochemical mechanisms of organic iodine absorption in animals.

CONTRIBUTION

Concept and research design, statistical processing, and editing – L.S. Bolshakova. Collection and material processing, text writing – L.S. Bolshakova, D.E. Lukin.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Xanthan-based biodegradable packaging for fish and meat products

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Abstract: Nowadays, the development of environmentally-friendly packaging materials is relevant worldwide. Biodegradable packaging materials are promising due to their safety and ability to extend shelf life of food products. This study aimed to investigate the properties of biodegradable film based on a bacterial exopolysaccharide (xanthan) with the view to extend the quality and shelf life of chilled meat products. We studied pork and carp samples packed in biodegradable film and stored at 0–2°C. Biodegradable packaging had positive effects on sensory, physicochemical, and microbiological parameters, as well as on ecological safety of the raw materials. During storage of packed chilled pork, its mass loss decreased from 2.16 to 0.21% (norm to 0.30%), and water activity reduced from 0.985 to 0.960, which had a positive effect on the microbiological resistance of pork during storage. The use of biodegradable film contributed to the preservation of quality and freshness of carp, which was confirmed by sensory and microbiological indicators. Total microbial contamination in carp packed in biodegradable film was significantly lower than that in unpacked samples, which extended its shelf life for one day compared to control. Biodegradable packaging also allowed mass loss and pH value to decrease during storage and inhibited oxidation processes in the samples under study. Free fatty acid content decreased by a factor of two, and peroxides, by 7%. Thus, biodegradable films can be effective film coatings to use in the food industry. This method of packaging not only preserves the functional and technological properties of food products, lowers their mass loss, and extends their shelf life, but also reduces costs and is environmentally friendly.

Keywords: Biodegradable packaging, film coating, xanthan, shelf life, food quality, meat products

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INTRODUCTION

Among the fundamental principles of raw materials and foods quality are their safety, sustainability, and long-term nutritional value [1, 2, 14].

One of the promising directions in addressing the global pollution of human habitat by polymer waste is to create environmentally safe packaging [3, 4]. Much attention is paid to the development of biodegradable and edible packaging materials which simplify product dosing and portioning without polluting the environment [5–7].

Using natural polymers – polysaccharides – as a film-forming basis is highly promising in the production of biodegradable coatings. Polysaccharide-based films protect raw materials and food products from mass loss (due to reduced moisture evaporation rate) and from the penetration of oxygen and other substances. As a result, it slows down the changes in the product quality [8, 9].

Films based on microbial polysaccharides are not yet sufficiently used in national economies. They have lower barrier and mechanical properties (resistance to high product and environment moisture) than polymeric films. But their main advantage is that they do not pollute the environment because they are biodegradable [10].

In this regard, it is highly relevant to develop environmentally safe biodegradable coating for meat raw materials using exopolysaccharides of bacterial origin. Our aim was to study sensory, physicochemical, and microbiological parameters, as well as environmental safety and storage time of meat and fish raw materials packed in biodegradable film based on exopolysaccharide of bacterial origin (xanthan).

STUDY OBJECTS AND METHODS

The objects of our study were xanthan (France), chilled pork, and pond carp. Pigs were slaughtered at "Products of the Volga Region" meat processing plant in accordance with the requirements of the Technical Regulations of the Customs Union on safety of meat and meat products (TR CU 034/2013[I]). Carcasses were cut in accordance with State Standard 31778-2012[II]. Pork meat (*m. longissimus dorsi*) was cut into portions (20–40 g) and packed in biodegradable xanthan-based film. Unpackaged raw materials were used as control samples. As for fish, we used freshly dead pond carp yearlings (90–110 g) grown at the Fish Cultivation Laboratory at Saratov State Agrarian University. Experimental fish samples were packed in a biodegradable film, while unpacked fish was used as a control. A film coating was made according to the method described in the Patent of the Russian Federation No. 2662008C1 "Biodegradable food film coating" [11].

Mesophilic aerobic and facultative anaerobic microorganisms in pork and carp were determined according to State Standard 10444.15-94[III] on meat-and-pepton agar (MPA). Coliform bacteria were determined according to State Standard 31747-2012[IV] on Kessler and Endo media. Salmonella was determined according to State Standard 31659-2012[V], using non-selective enrichment medium (buffered pepton water), selective enrichment medium (RVS broth), and differential diagnostic media (bismuth-sulfite agar and Endo agar). *L. monocytogenes* lysteria were determined according to State Standard 32031-2012[VI], using PBL1, PBL2 (Listeria enrichment broth), and agar listeria Ottaviani-Agosti (ALOA-agar). *Proteus* bacteria in pork were determined according to State Standard 28560-90[VII] on agar for *Proteus* release, and staphylococcus in carp, according to State Standard 31746-2012[VIII], using sodium chloride broth and salt-egg yolk agar.

To determine pork freshness, we selected experimental samples (packed in biodegradable film) and control (unpacked) samples stored at 0–4°C. The selection was carried out according to State Standard 7269-79[IX]. The pork samples were sent to the production laboratory. Each sample was wrapped in parchment paper and numbered. Chilled raw materials were stored at 0–2°C. Changes in acid and peroxide numbers, as well as thiobarbituric value, indicated the processes occurring in the lipid fraction during storage.

Toxic lead and cadmium were determined by the method of the Scientific Council on Analytical Methods 450xs (Methodological Guidelines 4.1.986-00[X]).

Mass fraction of antibiotics was determined by an express-method based on antibiotic suppression of dehydrogenase activity of testing cultures in a liquid nutrient medium (Methodological Guidelines 4.2.026-95[XI]).

Water-binding capacity (WBC) was determined by pressing method on filter paper by Grau-Hamm modified by Volovinska-Kelman [12].

Acid and peroxide numbers were determined according to standard methods of Gorbatov All-Russia Meat Research Institute to assess the quality and safety of meat and meat products.

Thiobarbituric index was determined according to the Sidwell method modified by Turner.

Concentration of hydrogen ions was determined by potentiometric method at a 2696 contact pH meter with automatic compensation in the range of 0 to 40°C for pH and temperature measurements of aqueous solutions.

Water activity (A_w) of raw materials was determined by a cryoscopic method based on the determination of the freezing temperature of the sample and its conversion into the indicator of water activity.

Fats were extracted from fish raw materials by an extraction-weight method according to State Standard 54053-2010[XII]. The content of individual fatty acid methyl esters in relation to total fatty acid content was determined by gas chromatography according to State Standard R 51486-99[XIII] and State Standard R 51483-99[XIV] using a Crystal 2000M gas chromatograph. The acid number of extracted fats was determined according

[I] TR TS 034/2013. Tekhnicheskiy reglament Tamozhennogo soyuza "O bezopasnosti myasa i myasnoy produktsii" [TR CU 034/2013 Technical regulations of the Customs Union "On safety of meat and meat products"]. 2013. 108 p.

[II] State Standard 31778-2012. Meat. Dressing of pork into cuts. Specifications. Moscow: Standartinform; 2014. 16 p.

[III] State Standard 10444.15-94. Food products. Methods for determination of quantity of mesophilic aerobes and facultative anaerobes. Moscow: Standartinform; 2010. 6 p.

[IV] State Standard 31747-2012. Food products. Methods for detection and quantity determination of coliformes. Moscow: Standartinform; 2013. 16 p.

[V] State Standard 31659-2012. Food products. Method for detection of Salmonella spp. Moscow: Standartinform; 2014. 21 p.

[VI] State Standard 32031-2012. Food products. Methods for detection of Listeria monocytogenes. Moscow: Standartinform; 2014. 28 p.

[VII] State Standard 28560-90. Food products. Method for detection of bacteria of Proteus, Morganella, Providencia genera. Moscow: Standartinform; 2010. 6 p.

[VIII] State Standard 31746-2012. Food products. Methods for detection and quantity determination of coagulase-positive staphylococci and Staphylococcus aureus. Moscow: Standartinform; 2013. 22 p.

[IX] State Standard 7269-79. Meat. Methods of sampling and sensory methods of freshness test. Moscow: Standartinform; 2006. 7 p.

[X] MUK 4.1.986-00. Metodika vypolneniya izmereniy massovoy doli svintsa i kadmiya v pishchevykh produktakh i prodovol′stvennom syr′e metodom ehlektrotermicheskoy atomno-absorbtsionnoy spektrometrii [Methodological Guidelines 4.1.986-00. Method of measurement of mass fraction of lead and cadmium in food products and food raw materials by electrothermal atomic absorption spectrometry]. Moscow: Federal Center of State Sanitary and Epidemiological Surveillance Department, Ministry of Health; 2000. 32 p.

[XI] MUK 4.2.026-95. Ehkspress-metod opredeleniya antibiotikov v pishchevykh produktakh [Methodological Guidelines 4.2.026-95. Express- method for determining antibiotics in food products]. Moscow: Institute of Nutrition of RAMS; 1995. 14 p.

[XII] State Standard 54053-2010. Confectionery. Methods for determination of fat fraction. Moscow: Standartinform; 2013. 15 p.

[XIII] State Standard R 51486-99. Vegetable oils and animal fats. Preparation of methyl esters of fatty acids. Moscow: Standartinform; 2008. 6 p.

[XIV] State Standard R 51483-99. Vegetable oils and animal fats. Determination of individual fatty acid methyl ester fraction to total fatty acid content by gas chromatography. Moscow: Standartinform; 2008. 7 p.

Table 1 Environmental safety indicators for pork packed in xanthan-based film

Toxic elements	Content, mg/kg (M ± m)
Lead	0.054 ± 0.003
Cadmium	0.025 ± 0.0013

to State Standard R 52110-2003[XV]. The peroxide number was determined by the Golovkina and Perkel method. Peroxides in fish fats were determined by pH titration.

Sensory analysis of pork and fish was carried out according to State Standard 23670-79[XVI] and State Standard 814-96[XVII], respectively. The analysis included appearance, color, aroma, flavor, and texture.

The results were statistically processed using Microsoft Excel 2010 (Microsoft Corp. USA) and StatPlus 2009 Professional 5.8.4 for Windows statistical analysis package (StatSoft Inc., USA). The Student *t*-criterion was used to assess the validity of differences between samplings.

RESULTS AND DISCUSSION

We aimed to develop and investigate eco-safe packaging, namely bactericidal biodegradable film based on bacterial exopolysaccharide (xanthan) to extend the shelf life of animal raw materials.

At the initial stage, we assessed the sanitary and hygienic state of the raw materials (Tables 1 and 2).

The content of toxic elements (lead and cadmium) met the requirements of the Technical Regulations of the Eurasian Economic Union "On safety of meat and meat products"[XVIII]. We did not detect any antibiotics (levomycetin, grisin, bacitracin) or their traces in experimental pork samples packed in xanthan film (TR CU 034/2013[I]).

Further, we examined carp for parasitological parameters (Table 2).

The hygienic parameters of carp are presented in Table 3.

The results of sanitary and hygienic analysis showed that the fish raw materials under study met the requirements of the TR EAEU 040/2016[XVIII] (Tables 2 and 3).

The quality of chilled meat during storage and moisture loss are known to depend on temperature and cooling rate. Meat mass loss due to moisture evaporation during cooling is not only a quantitative characteristic. The product's porous surface and thermal burns result in deteriorated marketable conditions. De-iced pores are filled with air, which accelerates oxidative processes reducing the quality and marketability of pork [13].

In our study, the mass loss of chilled pork packed in bio-degradable film decreased from 2.16% to 0.21% during storage (Table 4).

These results confirm that the biodegradable film is tightly attached to the surface of the raw material. This ensures reliable sealing of the packaging and prevents moisture exchange [19].

In our study, the storage of pork packed in biofilm in cardboard containers significantly weakened temperature fluctuations and had a positive effect on mass loss reduction. Biodegradable packaging not only ensures the microbiological stability of meat products, but also improves their sensory properties due to an increased meat water-binding capacity. The mass loss of pork packed in biodegradable film was lower and the meat was more dense than unpackaged pork.

Table 2 Parasitological indicators of carp

Living helminth larvae	Content
Trematodes	
Opistorchis	nd
Clonorchis	nd
Pseudamphistomum	nd
Metagonimus	nd
Nanophietus	nd
Echinochazmus	nd
Metorchis	nd
Rossikotrema	nd
Apophallus	nd
Nematodes	
Dioctophyme	nd

nd – not detected

Table 3 Hygienic indicators of carp

Indicator	Level
Nitrosamines (N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA)	–
Dioxins	–
Polychlorinated biphenyls	–

Table 4 Mass loss of cooled pork packed in biodegradable film (experimental sample) and unpackaged pork (control sample) during storage

Duration of storage, days	Mass loss, g	
	Control sample	Experimental samples
1	184.14	199.02
2	178.54	195.63
4	170.23	190.21
6	168.12	186.02
8	163.05	181.37
11	158.78	176.56
13	150.01	168.60

[XV] State Standard R 52110-2003. Vegetable oils. Methods for determination of acid value. Moscow: Izdatel′stvo standartov; 2003. 8 p.

[XVI] State Standard 23670-79. Cooked sausage goods and meat loaves. Specifications. Moscow: Izdatel′stvo standartov; 2003. 25 p.

[XVII] State Standard 814-96. Iced fish. Specifications. Moscow: Izdatel′stvo standartov; 2001. 6 p.

[XVIII] TR EAEHS 040/2016. Tekhnicheskiy reglament Evraziyskogo ehkonomicheskogo soyuza "O bezopasnosti ryby i rybnoy produktsii" [TR EAEU 040/2016. Technical regulation of the Eurasian Economic Union "On safety of fish and fish products"]. 2016. 140 p.

Table 5 Sensory indicators of carp during six-day storage

Indicator	Characteristic	
	Experimental samples	Control samples
	24 h (day 1)	
Appearance	Clean surface of natural coloring, glossy due to film coating. Pink gills. No external damage (Fig. 6)	Clean surface of natural coloring. Pink gills. No external damage
Texture	Dense, elastic	Dense, elastic
Odor	Characteristic of fresh carp, without off-odors.	Characteristic of fresh carp, without off-odors.
	48 h (day 2)	
Appearance	Clean matte surface, dried crust. Pink gills. No external damage	Clean surface of natural coloring. Pink gills. No external damage
Texture	Dense, elastic	Dense, elastic
Odor	Characteristic of fresh carp	Characteristic of fresh carp
	72 h (day 3)	
Appearance	Clean matte surface, dried crust. Pink gills. Without external damage	Clean surface of natural coloring. Dark red gills. Fish without external damage
Texture	Dense, elastic	Less dense
Odor	Characteristic of fresh carp	Characteristic of fresh carp
	96 h (day 4)	
Appearance	Clean surface, with dried crust. Pink gills. Fish without external damage	Clean surface of natural coloring. Dark-colored gills Fish without external damage
Texture	Dense, elastic	Infirm, Inelastic
Odor	Characteristic of fresh carp	Off-odor
	120 h (day 5)	
Appearance	Clean surface with dried crust. Pink gills. Fish without external damage	Clean surface of natural coloring. Dark-colored gills Fish without external damage
Texture	Dense, elastic	Infirm, Inelastic
Odor	Off-odor	With signs of spoilage
	144 h (day 6)	
Appearance	Clean surface with dried crust. Pink gills Fish without external damage	Mucous surface of unnatural coloring. Dark-colored gills. Fish without external damage
Texture	Dense, elastic	Infirm
Odor	Off-odor	With signs of spoilage

In addition, biodegradable films slow down oxidation processes. The decreased rate of oxidation processes in the experimental samples correlates with their sensory indicators. Sensory evaluation showed that all packaged pork samples were fresh throughout the storage period, namely seven days. Packing in biodegradable film improves the sensory properties of raw materials, which could not be achieved when storing without packaging. The experimental samples had a more attractive appearance and preserved their flavor during the shelf life. The samples packed in biodegradable film had a brighter color, as the proposed packaging prevented oxidation of heme pigments. The control samples had a specific drying crust on the meat surface. The film did not degrade the taste, consistency, or color of pork.

The sensory evaluation of carp packed in biodegradable film showed that the fish met all the criteria for this type of raw materials within the first four days, followed by a decline in the sensory indicators. The control (unpackaged) samples deteriorated as early as day 3 (Table 5).

A possible duration of refrigerated storage for fish raw materials is determined by their initial properties, as well as refrigeration and storage conditions. For example, sealed packaging eliminates the need to regulate the air humidity and prevents microbial contamination. As a result, sealed packaging can, to some extent, compensate for the lowering of the storage temperature by a few degrees.

We found that the use of biodegradable film contributed to the preservation of the quality and freshness of carp, keeping its sensory indicators at the required level for quite a long time and reducing the natural loss of product mass during storage.

The analysis of Tables 5 and 6 showed the positive effect of biodegradable film packaging on the carp quality during short-term storage in a cold chamber without special conditions. The analysis of pork's physicochemical indicators revealed that biodegradable film packaging inhibited the microbial and enzymatic activity, which reduced structural and chemical changes in the raw materials under study.

The activity of water is known to have a great impact on the growth of microorganisms. The initial water activity value of pork A_w was 0.985. Packing in biodegradable film based on exopolysaccharide of

Table 6 Change in fish mass during storage

Duration of storage, days	Control sample	Experimental sample
	Mass loss	
1	104.54	109.16
2	98.34	105.39
3	95.13	100.67
6	86.47	96.81
Average weight for 4 days	96.12	103
Average deviation		+6.89

Table 7 Functional and technological indicators of pork and carp

Sample	pH		A_w		Moisture, %	
	Pork					
	day 1	day 7	day 1	day 7	day 1	day 7
Control	6.30	6.10	0.985	0.982	69.2	66.8
Experiment	6.30	5.78	0.985	0.960	69.2	71.9
	Carp					
Control	6.42	6.39	0.9819	0.9816	68.98	71.39
Experiment	6.42	6.35	0.9819	0.9569	68.98	71.5

bacterial origin reduced this value to the level of 0.96 for 7 days. Thus, biodegradable film had a positive effect on microbiological stability of pork and carp during storage.

The data characterizing the functional and technological characteristics of the raw materials are presented in Table 7.

According to the data from Table 7, biodegradable film packaging decreased pH to 5.78, as a result of accelerating glycolysis, which contributed to the inhibition of bacterial growth on meat surface. In addition, pH values in the experimental samples were lower due to the biodegradable film's pH (7.5). This contributed to an increase in moisture by 5.1% and improved hydration properties of muscle fibers during storage. The same trend was observed for the water-binding capacity of experimental and control samples. Based on the data presented in Table 7, we can conclude that biodegradable film packaging showed an identical effect on both meat and fish raw materials; the mass loss and pH values of carp reduced during storage.

Thus, the packaging of animal raw materials in biodegradable film based on exopolysaccharides before cooling preserved the quality and increased their stability during storage by slowing chemical, microbiological, and enzymatic processes which cause spoilage. Microbiological characteristics of pork stored at 0–2°C for 10 days are presented in Tables 8 and 9.

Table 9 Microbial contamination of pork during 10-day storage

Sample	Coli form bacteria						*Proteus* bacteria					
	Storage duration, days						Storage duration, days					
	1	2	3	5	7	10	1	2	3	5	7	10
Control	+	+	+	+	+	+	–	–	+	+	+	+
Experimental	+	+	+	+	+	+	–	–	–	+	+	+

On days 1 and 2 of meat storage, we observed a gradual increase in the number of mesophilic aerobic and facultative anaerobic microorganisms, as well as coli form bacteria, both in the control and experimental samples. Three days later, the number of mesophilic aerobic and facultative anaerobic microorganisms decreased in the experimental samples, while in the control ones this indicator increased, compared to day 1. *Proteus* bacteria were detected in the control samples on day 3. This bacterial growth indicated the beginning of meat spoilage (rotting processes). In the experimental samples, *Proteus* was found only on day 5. *Proteus* bacteria count in the control samples was significantly higher than in the samples packed in biofilm. Pathogenic bacteria, including salmonella and *L. monocytogenes,* were not found in the samples.

Obviously, the packaging reduces oxygen access to raw materials and almost completely inhibits the growth of aerobic microorganisms. As a result, the shelf life of chilled pork in biodegradable film increased to 5 days.

We also studied the influence of biodegradable film packaging on the microbiological processes occurring in carp meat. It was revealed that the biofilm significantly reduced the total contamination of fish on days 1 and 2, thereby increasing the storage duration. Microbiological characteristics of fish stored at 0–2°C for 2–6 days are presented in Tables 10 and 11.

Pathogenic bacteria, including salmonella and *L. monocytogenes* and staphylococci, were not found in the samples under study. The data allowed us to conclude that the film contributed to extending carp shelf life. Thus, the microbiological results closely correlated with the sensory characteristics of carp.

The oxidation processes in lipids are important in the storage of raw meat. Lipids are relatively unstable because they contain unsaturated fatty acids that are

Table 8 Total bacterial count in pork during 10-day storage

Sample	Mesophilic aerobic and facultative anaerobic microorganisms					
	Storage duration, days					
	1	2	3	5	7	10
Control	$2.0\times10^6 \pm 0.02$	$2.0\times10^7 \pm 0.20$	$1.0\times10^7 \pm 0.40$	$2.0\times10^7 \pm 0.20$	$3.5\times10^7 \pm 0.20$	$1.0\times10^8 \pm 0{,}20$
Experimental	$1.0\times10^7 \pm 0.20$	$2.5\times10^7 \pm 0.20$	$5.0\times10^6 \pm 0.04$*	$1.0\times10^7 \pm 0.40$*	$2.0\times10^7 \pm 0.20$*	$5.0\times10^7 \pm 0.80$*

*$P \leq 0.05$

Table 10 Total bacterial count in carp during storage

Sample	Mesophilic aerobic and facultative anaerobic microorganisms	
	Storage duration, days	
	1	2
Control	$1.0\times10^7 \pm 0.080$	$4{,}0\times10^4 \pm 0{,}4$
Experimental	$1.0\times10^3 \pm 0.080$*	$2{,}0\times10^3 \pm 0{,}2$*

* $P \leq 0.05$

Table 11 Microbial contamination of carp during storage

Sample	Coli form bacteria			
	Storage duration, days			
	1	2	3	6
Control	+	+	+	+
Experimental	+	+	+	+

easily oxidized. The oxidation of lipids, as well as the pigments of muscle tissue, depends on oxygen partial pressure. Oxidative changes in lipids under aerobic conditions have a limited rate of hydroperoxic radical formation [15, 16].

We studied oxidation processes of raw meat packed in biodegradable film and their effect on meat shelf life. The shelf life of pork to a large extent depends on the resistance of the lipid fraction to oxidation, which, in its turn, depends on the content of radicals of unsaturated fatty acids and the degree of their unsaturation. To evaluate the rate of oxidative processes occurring in cooled meat during storage, we determined peroxide, acid, and thiobarbituric values in the experimental samples (packed in biodegradable film based on exopolysaccharide of bacterial origin) and in the control samples (Table 12).

As one can see from Table 12, the control samples showed a more intensive enzymatic hydrolysis rate of triglycerides and phospholipids in the control samples.

The accumulation of secondary oxidation products depends on the initial thiobarbituric value and has a negative effect on the sensory parameters and shelf life of products. By the end of storage (day 7), the value was 0.056 for unpackaged meat and 0.026 for pork packed in biodegradable film, which confirms the prospects of the proposed packaging. The analysis of lipid parameters of pork stored at 0–2°C showed that by day 7, the thiobarbituric value in pork packed in biodegradable film was 2.2 times lower than in the control samples. We found that the duration of induction increased and the rate of peroxides accumulation decreased with reduction of air oxygen access to raw materials.

A slight increase in peroxide value indicated the inhibition of oxidative processes. At the final stage of pork storage (day 7), the growth of peroxide values for packaged and control samples was 0.034 and 0.038%, respectively. The slower oxidation process in packaged pork can be explained by a low gas permeability of the packaging film combined with the ability of muscle tissue to absorb oxygen. A low amount of oxygen in the package inhibits oxidative processes and, in combination with low temperature, creates favorable conditions for raw materials.

A determining factor of the fish shelf life is often lipid oxidation, which causes negative changes in its sensory properties (taste, flavor, color, texture) and nutritional value, as well as possible formation of toxic oxidation products. The processes of lipid change are quite complex since they occur as a result of chemical, biological, and enzymatic transformations. These processes often occur simultaneously, but lead to the formation of the same intermediate end products such as peroxides, free fatty acids, aldehydes, ketones, as well as products of their polymerization. The oxidizing properties of fats depend on the degree of fat unsaturation and factors inhibiting oxidation, namely heat, light, traces of heavy metals, etc.

In addition, the degree of lipolysis is of importance, since this process is the first stage of degradation of the product. Enzymes (e.g. lipoxygenases) catalyze lipid oxidation, interacting mainly or exclusively with free fatty acids. The stability of food material in relation to lipolytic decomposition is an indicator of biochemical activity of enzymes, cofactors, and lipid substrates. Water-insoluble lipids tend to aggregate, forming a boundary layer. Thus, the sensitivity to lipolysis and subsequent lipid oxidation is determined by the physicochemical properties of this unique two-dimensional medium. In the case of fish, of great importance is its physiological condition, which affects its quality and shelf life.

The fatty acid composition of carp fat phase showed that its lipids were characterized by high biological efficiency, but at the same time by instability during storage due to their unsaturation (Table 13).

Since peroxide and acid values are indicators of the product's safety, we determined these parameters in the control and experimental carp samples (Table 14).

Table 13 demonstrates that the content of free fatty acids in the experimental samples was almost half as large as in the control samples on day 1 of storage. This

Table 12 Changes in lipid fraction of pork packed in biodegradable film based on xanthan

Sample	Storage, days	Acid value, mg KOH	Peroxide value, % iodine	Thiobarbituric value, N/mol/mL
		M	M	M
Experimental	0	0.300	0	0
	2	0.430	0.003	0
	5	0.460	0.020	0.031
	7	0.650	0.034	0.026
Control	0	0.300	0	0
	2	0.600	0.007	0
	5	0.670	0.026	0.031
	7	0.780	0.038	0.056

Table 13 Fatty acid composition of carp fat phase

Fatty acid notation	Fatty acid	Test results	Scientific documentation	Method error
C12:0	Lauric	0.1	State Standard R 51483-99[XIV]	at the content of the required substances: less than 5–0.28%; equal to or more than 5–1.42%
C14:0	Myristic	1.2		
C16:0	Palmitic	15.4		
C16:1	Palmitoleic	3.8		
C18:0	Stearic	3.8		
C18:1	Oleic	31.5		
C18:2	Linoleic	41.9		
C18:3	Linolenic	0.2		
C20:0	Arachidic	2.0		

Table 14 Peroxide and acid values in carp during storage

Indicator	Measure	Test results		Method error
		Control sample	Experimental sample	
24 h (day 1)				
Acid value	mg KOH/g	10.3	6.4	± 7% Rt.
Peroxide value, % iodine	meq active oxygen/kg	2.92	2.78	± 14% Rt.
144 h (day 6)				
Acid value	mg KOH/g	27.5	23.5	± 7% Rt.
Peroxide value, % iodine	meq active oxygen/kg	13.6	12.7	± 14% Rt.

suggests that hydrolytic processes in the tissues of the experimental carp samples occurred more slowly during storage. Nevertheless, the content of free fatty acids exceeded the current norm six to seven times at the final stage of research.

According to the data presented in Table 14, the content of peroxides on day 1 of storage corresponded to the norms (less than 10 meq/kg) in both groups and was higher in the control compared to the experimental samples. By the end of the experiment, it reached the levels that did not meet the safety requirements.

Thus, the change in the fat fraction in carp packed in biodegradable film slowed down the oxidation of its lipids. However, it should be noted that the carp samples under study had an increased initial content of free fatty acids and peroxides that did not meet the requirements of technical regulations[XVIII], which may be due to the diet used in carp cultivation.

The results of our research are largely comparable with numerous studies on the influence of various biodegradable films on the quality and shelf life of animal raw materials. Jeevahan *et al.* showed a positive effect of starch-based nanocellulose film on food products [17]. Another work was devoted to the use of biodegradable nanocomposite pigment films for food conservation [18].

Wang *et al*. and Pavlath revealed that biodegradable cellulose composite film based on corncob lignin and made by an anti-solvent precipitation method is effective in food technologies [19, 20]. In addition, water-soluble Vivos (MonoSol) film has been recently studied. However, its use is limited, as it dissolves only in hot water [9]. Promising are film coatings based on polysaccharides (chitosan, alginates), as well as calcium or magnesium salts, which were developed by Harvard University researchers [21].

CONCLUSION

This paper suggested the use of bacterial exopolysaccharides (xanthan) as the main ingredient for food film coatings. We revealed that this packaging reduced oxygen access to raw materials and almost completely inhibited the growth of aerobic microorganisms, resulting in extended shelf life resistance of meat and fish during storage. Under such a coating, myoglobin retained its native state, so the meat had a richer color, which appeals to consumers. Biodegradable film packaging increased not only the microbiological stability of meat, but also its water-binding capacity and sensory properties. Pork packed in the biodegradable film has higher juiciness and denser texture compared to unpacked samples. In addition, we observed the significant inhibition of aerobic and coliform bacteria growth in experimental samples at 0–2°C.

The storage temperature of 0–2°C for pork and carp, relative air humidity of 85–90%, and air speed of 0.2–0.3 m/s provided high quality for 10 days for pork and 2 days for fish. It should be noted that the chosen temperature did not prevent the development of microflora, enzymatic processes, and processes of fat fraction oxidation. Nevertheless, biodegradable film extended the shelf life of chilled meat products and protected it from microbial and oxidative damage.

The packaging of meat raw materials in biodegradable film can be very promising to use in the food industry. This method of packaging not only preserves the functional and technological properties of food products, lowers their mass loss, and extends their shelf life, but also reduces costs and is environmentally friendly.

The findings were presented at the 20th Russian agro-industrial exhibition “Golden Autumn-2018” and awarded a diploma and a bronze medal.

In addition, we developed technical documentation for the production of pork cuts 9213-012-00493497-18 (“Pork cuts packed in biodegradable film”). The technology was tested and adopted at “Products of the Volga Region” meat processing plant (Engels, Saratov region).

CONTRIBUTION

The authors were equally involved in the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Improved technology for new-generation Kazakh national meat products

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Abstract:
Introduction. Extract of goji berries (*Lycium barbarum* L.) and buckwheat flour (*Fagopýrum esculéntum* L.) possess antioxidant and antimicrobial properties. As a result, they can be used to improve traditional Kazakh horse-meat formulations to obtain functional cooked and smoked meat products. These natural biologically active substances can improve the oxidative stability of pigments, lipids, and proteins of finished products. The research objective was to assess the potential of goji extract and buckwheat flour as additives that can improve the oxidative stability and general quality of Kanagat, a national Kazakh cooked and smoked horse-meat product. Goji extract and buckwheat flour were used in two concentrations – 0.5% and 1.0%.
Study objects and methods. The research featured sensory evaluation of taste, smell, color, determination of color parameters (L∗, a∗, b∗), pH, free amine nitrogen, total carbonyl proteins, acid value, peroxide value and thiobarbituric acid reactive substances (TBARS), as well as a histological analysis.
Results and discussion. When 1.0% of goji extract and 1.0% of buckwheat flour were added to the traditional formulation, it improved the oxidative stability and quality of the modified horse-meat product while preserving its sensory properties and color parameters. A set of microstructural studies showed that the processing of meat products with 1.0% of goji extract and 1.0% of buckwheat flour had a destructive effect on most fibers. The affected fibers showed multiple decays of myofibrillar substance which turned into a fine-grained protein mass. The abovementioned concentration caused effective inhibition of hydrolytic changes, as well as oxidation of proteins and lipids.
Conclusion. The new technology made it possible to produce a new national horse-meat product fortified with 1.0% of goji extract and 1.0% of buckwheat flour. The specified amount of biologically active additives improved the oxidative stability and quality of the product, while maintaining its sensory and color characteristics.

Keywords: Meat industry, meat products, hydrolysis, oxidation, goji, buckwheat flour

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INTRODUCTION

The socio-technological development of meat industry pursues two main goals. First, meat industry enterprises and research institutions should satisfy consumer demands. Second, they should develop and produce high-quality functional products that are environmentally safe and beneficial for human health in biomedical terms.

To function properly, human body needs a healthy, nutritious, and well-balanced diet. It is becoming more and more difficult to provide food that would satisfy this requirement because of constantly decreasing resources, modern lifestyle, environmental pollution, and overall degradation of food quality [1–3].

Horse meat has a high nutritional value. Its protein content is 18–25%, which is quite high. In addition, the proteins of horse muscle tissue are rich in essential amino acids, which are represented in the optimal ratio. Horse meat provides vitamins B. It is a source of such major mineral elements as magnesium and chlorine. These minerals are known to improve blood buffering and regulation of blood pressure. Almost all vitamins

and minerals found in meat are more easily digestible than those found in products of plant origin. Horse meat is rich in vital vitamins and mineral elements that help to improve metabolism in patients with obesity, atherosclerosis, and hypertension. Horse meat is also good for people suffering from cardiovascular, hepatic, and pancreatic diseases [1].

In addition, horse-meat fat has a unique fatty acid composition [1]. Adding functional ingredients, e.g. natural antioxidants, is one of the strategies of developing functional national meat products [4]. This strategy has already provided a number of functional meat foods [5]. Improved horse-meat products injected with multicomponent curing solution have already been in the focus of some studies [6]. However, the process of obtaining national meat products from horse meat remains largely understudied.

There are many natural extracts and flours that can be used in functional food production. Goji berries (*Lycium barbarum* L.) have recently become one of the most popular plants with such properties [7]. Goji berries contain free amino acids, e.g. proline, taurine, and betaine with its anti-aging effect, as well as gamma-aminobutyric acid, phenylpropanoids, flavonoids, and polyphenolic compounds. They are also rich in vitamins, primarily thiamine, riboflavin, and ascorbic acid (vitamin C). Unfortunately, dry berries contain much less ascorbic acid than fresh ones. In addition, goji berries contain zinc, iron, copper in trace amounts, and some oil. Goji juice is known to contain seven different flavonols. Most of them have isohamnetin 3-O-glycosides, but they are poor radical absorbents. Quercetin, 3-O-glycosides, catechins, and hydroxybenzoic acids with catechin structure are strong antioxidants. Unfortunately, their concentration in the juice proved insignificant. It is ascorbic acid that proved to be the main antioxidant in goji berry juice [7–10].

Goji berries demonstrate antioxidant and antibacterial activities against *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Listeria monocytogenes*, and *Yersinia enterocolitica*. Goji extract also exhibits immunomodulating properties and can inhibit chromium-induced production of free radicals, apoptosis, and DNA fragmentation. In addition, goji extract has pronounced cytoprotective properties and can restore the antioxidant status of cells [11, 12].

Goji berries are a powerful hepaprotector. They contain cerebrosides, i.e. natural organic compounds from the group of complex lipids that protect liver cells from toxic chemicals. They are even more beneficial for human liver than such well-known hepatoprotector as milk thistle (*Silybum marianum* L.). Pyrrole is another hepatoprotective compound found in goji berries. Its rather unusual molecules contain a nitrogen atom in their central ring. Pyrrole proved superior to goji cerebrosides in hepaprotection [13].

The list of the most famous antioxidants involves tocopherols (vitamin E), carotenoids (vitamin A), and ascorbic acid (vitamin C). Vitamin C is believed to be the most important of them. As it was mentioned above, goji berries are rich in these vitamins. Some studies showed that goji antioxidants are five times stronger than those found in prunes and more than 25 times stronger than antioxidants found in broccoli. Surprisingly, broccoli was considered the undisputed record holder among antioxidant plants until very recently. Broccoli is still on the list of the so-called superfoods.

European scientists have compiled a table of the ORAC index, i.e. Oxygen Radical Absorbance Capacity. This is an indicator of the ability of antioxidants to absorb free radicals. According to this table, goji berries are the most powerful antioxidant in the world. The daily human need is about 5000 ORAC units, whereas 100 g of goji berries contains 25300 ORAC units [14].

Buckwheat flour (*Fagopýrum esculéntum* L.) is another interesting component that can be used in formulations of functional national meat products. Its popularity in food science is associated with flavonoids. Buckwheat flour flavonoids prevent the development of malignant tumors, protect human body from aging and disease, and boost immune system. Buckwheat grains, and hence buckwheat flour, do not contain gluten, which means that buckwheat products can be consumed by patients with celiac disease. Bakery from buckwheat flour helps to make their diet diverse [15].

The chemical composition of buckwheat flour also contains rutin, which is a very useful flavonoid. It gives buckwheat useful properties for the cardiovascular system. This fragrant flour lowers blood pressure by expanding blood vessels. Ground buckwheat prevents excessive platelet formation, lowers cholesterol, and saturates blood with oxygen. Buckwheat flour is good for blood circulation, as it decreases the permeability of blood vessels. In addition, buckwheat flour is rich in rutin, which makes it useful for people with varicosis and gout, as well as for those who have undergone radiation treatment [15].

Buckwheat prevents development of gallstones and regulates bile acid secretion. This product is known for its ability to strengthen and cleanse intestines; it also helps against chronic diarrhea and dysentery. Buckwheat flour improves the absorption of calcium, thus strengthening bone tissue and preventing osteoporosis. It is very good for nervous system and improves brain function. In addition, it boosts immune system and metabolism. Buckwheat flour is rich in vitamins, which makes is good for hair, nails, and skin. Finally, this product improves food absorption and has a beneficial effect on the pancreas [15].

As it was already mentioned, buckwheat is rich in rutin, which cannot be produced by human body. Rutin enters the body with food products and improves the elasticity and strength of blood vessels, thereby reducing the risk of hypertension. Regular consumption of buckwheat flour products can significantly lower blood

sugar levels. Buckwheat flour is also rich in high-grade proteins and complex carbohydrates that provide body with energy [16].

Buckwheat flour is rich in vitamins, minerals, and plant proteins. It contains vital amino acids, natural antioxidants, and dietary fiber. Buckwheat flour contains neither harmful carbohydrates nor gluten. Other beneficial effects of buckwheat flour manifest themselves in that it removes wastes, toxins, and other harmful substances, produces a powerful general tonic effect on human health, activates metabolism, improves cardiovascular system, and lowers blood sugar [16].

Our research objective was to establish the potential of goji extract and buckwheat flour for improving oxidative stability and general quality of meat products. The substances were used as additives in the amounts of 0.5% and 1.0% to produce a functional Kazakh horse-meat product called Kanagat.

STUDY OBJECTS AND METHODS

Kanagat is a national Kazakh horse-meat product of new generation. It was produced in the processing department of the limited liability partnership AF Kaynar (Almaty, Kazakhstan) from first-category chilled horse meat. The upper layer of muscle tissue was trimmed from the hip part of the carcass together with the superficial fat layer. The first-category chilled horse meat was cut into pieces of ≤ 0.4 g and about 10 cm thick.

15% of curing solution was injected into the meat pieces by weight of the raw material with a special injector intended for pickle pumping. The amount of curing ingredients in the curing solution corresponded to the addition of 2.5 kg of salt and 150 g of sugar per 100 kg of raw meat. 2.5–5.0 kg of goji extract or buckwheat flour was added to the curing solution meant for test samples. The cured meat was massaged in a TUZ-KZ tenderizer of ETDU brand for 40 min at 0–4°C. After massaging, the meat was cut into 0.1 kg pieces with a thickness of ≤ 5 cm and coated with a waterproof material. After that, the meat underwent heat treatment in a multi-purpose heat chamber. The product was then boiled at 74–75°C for 2–2.5 h until the temperature in the center of each piece reached 72°C. The cooked product was cooled and then smoked for 30 min at 40°C. The finished Kanagat was cooled to 10–12°C, vacuum-packaged, and stored for 21 days at 0–4°C.

The research featured five samples. For the control sample, 15% of curing solution was introduced into pieces of horse meat, as described above. The test samples were injected with 15% of curing solution that contained 2.5 kg of goji extract per 100 kg (which was equivalent to 0.5%-concentration in the finished product), 5.0 kg of goji extract (1.0%), 2.5 kg of buckwheat flour (0.5%), and 5.0 kg of buckwheat flour (1.0%).

The goji extract (*Lycium barbarum* L.) was supplied by Dannie Chen Shaanxi Jintai Biological Engineering Co., Ltd. (Xi'an, Shaanxi, China). The buckwheat flour (*Fagopýrum esculéntum* L.) was produced by the Scientific Developpment and Production Center "Kudesnitsa" of the company "Aladushkin Grupp" (St. Petersburg, Russia).

The sensory properties of the samples were determined by five panelists with certified tasting abilities. The panelists passed a triangular test to differentiate the aroma, smell, and color of fresh and rancid sausage. The samples were evaluated using a 1-to-5 scale [17].

A Konica Minolta CR-410 colorimeter (Konica Minolta Holding, Inc., Ewing, NJ, USA) was used to estimate lightness (L∗), redness (a∗), and yellowness (b∗) [17].

Free amine nitrogen was determined using a modified Serensen titration method [18].

Protein oxidation was measured by evaluating the formed carbonyl groups [19].

As a standard for fat hydrolysis rate, the acid value of extracted lipids was measured as specified in ENISO 660:2001 [19].

The standard IDF method was used to determine the peroxide values of the meat. The test used all lipids extracted from the samples [18].

As for the substances of 2-thiobarbituric acid reagent, TBARS were determined by the method described by Botsoglou *et al*. [17]. The research employed a UV-VIS Camspec M550 dual-beam spectrophotometer (Camspec Ltd, Cambridge, UK). The pH of the samples was determined using a Microsyst MS 2004 pH-meter (Mikrosist, Plovdiv, Bulgaria). The pH-meter was equipped with a combined pH electrode and a Sensorex S450CD combined recorder (Sensorex pH electrode station, Garden Grove, California, USA) [20].

High performance liquid chromatography (HPLC) with a coulometric electrochemical detector was used to analyze oil-soluble antioxidants extracted from the goji berries and the buckwheat flour and their concentrations in the horse meat [21, 22].

The method of ISO 4833:2003 was used to prepare the samples for microbiological analysis and total microscopic count of facultative anaerobic mesophilic microorganisms [23].

The data obtained from different samples were independently analyzed using SAS software [17]. Multiple Student-Newman-Keuls tests were used to compare the differences between means. Mean values and standard mean errors were calculated. The significance of differences was determined at $P \leq 0.05$.

The histological studies of the Kanagat were performed in accordance with the classical microstructural analysis and standard methods. Histological sections were made using a MICROM HM-525 cryostat microtome (CarlZeiss, Germany) [24, 26].

Table 1 Sensory evaluation of the taste, aroma, and surface color of the cross-section of vacuum-packaged samples after 21 days of storage at 0–4°C

Sample	Sensory evaluation		
	Surface color of cross-section	Smell	Taste
Control	2.65 ± 0.09^{e}	2.90 ± 0.03^{e}	2.75 ± 0.10^{d}
goji extract (0.5%)	4.30 ± 0.07^{c}	4.90 ± 0.05^{b}	4.90 ± 0.01^{a}
goji extract (1.0%)	4.85 ± 0.02^{a}	5.00 ± 0.02^{a}	4.50 ± 0.05^{b}
buckwheat flour (0.5%)	4.70 ± 0.03^{b}	4.80 ± 0.03^{c}	4.55 ± 0.04^{b}
buckwheat flour (1.0%)	3.50 ± 0.08^{d}	4.70 ± 0.04^{d}	4.35 ± 0.08^{c}

The standard deviations presented in the table indicate that all statistical differences are significant: for the control sample (2.65 ± 0.09^{ebcd}), for the sample with 0.5% of goji extract (4.30 ± 0.07^{caed}), etc.

The following method of short-term additional fixation was used for the sections mounted on the slide. An 8% formalin solution was applied to the histological section for 30 min. After that, the section was thoroughly washed with water for 3 min, dried at room temperature, and stained with hematoxylin and eosin. The histological preparations were studied and photographed using an AxioImaigerA1 light microscope (CarlZeiss, Germany) and an AxioCamMRc5 video camera. Image processing and morphometric studies were performed using the AxioVision 4.7.1.0 computer-aided image analysis system adapted for histological studies. To obtain reliable results, the experiments were performed in triplicates with 3–5 replications of the analyses of each sample for all parameters.

RESULTS AND DISCUSSION

The samples were stored at 0–4°C for 21 days. On day 21, the concentrations of antioxidants extracted from the goji extract and buckwheat flour of Kanagat were determined as follows: the samples with 0.5 and 1.0% of goji extract – 4.78 ± 0.21 and 9.81 ± 0.26 mg/g and the samples with 0.5 and 1.0% of buckwheat flour – 4.73 ± 0.19 and 9.75 ± 0.20 mg/g, respectively.

Sensory evaluation. The samples with 1.0% of goji extract received the highest sensory indices for taste, smell, and color after 21 days of storage at 0–4°C (Table 1). The samples with 0.5% of goji extract and 0.5% of buckwheat flour gotalmost the same results (Table 1). The control sample showed the worst sensory properties. It scored significantly lower ($P \leq 0.05$) than the other samples. Therefore, 2.5% of goji extract added to the curing solution preserved the fresh color and especially aroma of the vacuum-packaged horse-meat product after 21 days of storage. A similar research also reported the positive effect of a mix of dried goji berries and pumpkin powder on the quality and storage stability of cooked and smoked beef tenderloin [8].

Color characteristics. Table 2 demonstrates the changes in lightness (L∗), redness (a∗), and yellowness (b∗). The samples with 0.5% and 1.0% of goji extract again showed the most significant changes. The obtained results were consistent with sensory evaluation. They proved that goji extract produced a better effect on the color characteristics of the restructured horse meat than buckwheat flour.

Oxidative stability and quality. After 21 days of storage, the modified horse-meat samples revealed the following changes. The content of free amine nitrogen in all test samples was significantly lower ($P \leq 0.05$) than in the control samples. The samples with 0.5% and

Table 2. Surface color characteristics (L∗, a∗, b∗) of the cross-section of the of vacuum-packaged samples during 21 days of storage at 0–4°C

Characteristics	Samples	Storage time		
		Day 1	Day 11	Day 21
L∗	Control	49.77 ± 0.10^{e}	52.68 ± 0.20^{j}	53.40 ± 0.15^{i}
	goji extract (0.5%)	48.34 ± 0.11^{d}	49.94 ± 0.12^{f}	52.62 ± 0.16^{j}
	goji extract (1.0%)	50.51 ± 0.16^{g}	51.44 ± 0.19^{h}	52.33 ± 0.18^{i}
	buckwheat flour (0.5%)	$47.67 \pm 0.12^{a,b}$	47.75 ± 0.14^{b}	$48.89 \pm 0.15^{e,f}$
	buckwheat flour (1.0%)	47.43 ± 0.15^{a}	$47.91 \pm 0.13^{b,c}$	48.28 ± 0.11^{d}
a∗	Control	17.38 ± 0.19^{d}	18.72 ± 0.13^{h}	19.45 ± 0.18^{i}
	goji extract (0.5%)	15.76 ± 0.14^{b}	16.77 ± 0.17^{e}	17.67 ± 0.16^{e}
	goji extract (1.0%)	19.21 ± 0.19^{i}	19.48 ± 0.20^{i}	19.52 ± 0.17^{j}
	buckwheat flour (0.5%)	15.73 ± 0.21^{b}	18.63 ± 0.17^{g}	19.21 ± 0.20^{i}
	buckwheat flour (1.0%)	12.23 ± 0.15^{a}	18.01 ± 0.12^{f}	18.32 ± 0.19^{g}
b∗	Control	7.05 ± 0.14^{a}	7.54 ± 0.13^{c}	7.87 ± 0.21^{d}
	goji extract (0.5%)	7.60 ± 0.10^{c}	$7.99 \pm 0.12^{d,e}$	$8.03 \pm 0.16^{d,e}$
	goji extract (1.0%)	$7.71 \pm 0.14^{c,d}$	8.17 ± 0.13^{e}	8.85 ± 0.11^{j}
	buckwheat flour (0.5%)	7.33 ± 0.18^{b}	7.67 ± 0.17^{d}	$8.08 \pm 0.10^{d,e}$
	buckwheat flour (1.0%)	7.46 ± 0.15^{b}	7.58 ± 0.19^{c}	8.29 ± 0.11^{f}

Values ± standard deviations. Different superscript suffixes (a, b, c, d, e, f, g, h, i, j) after standard deviations denote statistical differences between the samples for each of the color characteristics ($P \leq 0.05$) in lines and columns

Table 3. pH, free amine nitrogen, total carbonyl proteins, acid value, peroxide value, and TBARS in vacuum-packed samples before and after 21 days of storage at 0–4°C

Parameters	Control	goji extract (0.5%)	goji extract (1.0%)	buckwheat flour (0.5%)	buckwheat flour (1.0%)
Curing solution injected, %	20	20	20	20	20
Moisture, %	84	85	86	84	85
pH of curing solution	8.18 ± 0.03^{c}	6.90 ± 0.04^{b}	6.81 ± 0.02^{a}	$7{,}00 \pm 0.03^{d}$	$6{,}99 \pm 0.03^{e}$
pH of raw material	5.62 ± 0.02^{a}	5.59 ± 0.04^{a}	5.60 ± 0.02^{a}	5.61 ± 0.02^{a}	5.61 ± 0.03^{a}
pH of final product:					
day 1	6.34 ± 0.04^{b}	6.27 ± 0.02^{a}	6.21 ± 0.04^{a}	6.45 ± 0.01^{c}	6.66 ± 0.02^{d}
day 21	5.59 ± 0.03^{a}	6.44 ± 0.05^{c}	6.33 ± 0.03^{b}	6.57 ± 0.03^{d}	6.75 ± 0.04^{e}
Free amine nitrogen, mg/100 g:					
day 1	6.42 ± 0.19^{a}	$7.25 \pm 0.13^{b,c}$	7.07 ± 0.20^{b}	$7.30 \pm 0.10^{b,c}$	7.04 ± 0.19^{b}
day 21	18.81 ± 0.21^{c}	13.76 ± 0.18^{b}	13.68 ± 0.10^{b}	13.37 ± 0.15^{a}	13.45 ± 0.10^{a}
Carbonyl proteins, nmol/mg of proteins:					
day 1	0.58 ± 0.17^{a}	0.62 ± 0.18^{a}	0.59 ± 0.16^{a}	0.62 ± 0.16^{a}	0.63 ± 0.13^{a}
day 21	4.12 ± 0.23^{e}	3.03 ± 0.27^{c}	2.01 ± 0.24^{a}	3.28 ± 0.22^{d}	2.63 ± 0.23^{b}
Acid value, mg KOH/g of fats:					
day 1	0.49 ± 0.08^{a}	0.50 ± 0.09^{a}	0.47 ± 0.07^{a}	0.49 ± 0.09^{a}	0.52 ± 0.06^{a}
day 21	2.17 ± 0.11^{c}	1.65 ± 0.13^{b}	1.39 ± 0.11^{a}	$1.47 \pm 0.10^{a,b}$	1.30 ± 0.14^{a}
Peroxide value, mmol O_2/kg of fats:					
day 1	$0.40 \pm 0.05^{a,b}$	0.35 ± 0.04^{a}	0.30 ± 0.05^{a}	$0.38 \pm 0.06^{a,b}$	0.33 ± 0.07^{a}
day 21	1.78 ± 0.07^{c}	1.44 ± 0.06^{b}	1.33 ± 0.07^{a}	1.50 ± 0.05^{b}	1.39 ± 0.08^{a}
Thiobarbituric value,					
mg MA/kg:	0.27 ± 0.04^{a}	0.24 ± 0.03^{a}	0.23 ± 0.01^{a}	0.26 ± 0.02^{a}	0.25 ± 0.04^{a}
day 1	1.94 ± 0.11^{c}	1.08 ± 0.07^{b}	0.89 ± 0.08^{a}	1.10 ± 0.05^{b}	0.93 ± 0.06^{a}
day 21					

Values ± standard deviations. Different superscript suffixes (a, b, c, d, e) after standard deviations indicate statistical differences between the samples in each line ($P \leq 0.05$)

1.0% of goji extract had the lowest content of free amine nitrogen. The content of carbonyl proteins increased in all samples after 21 days of storage at 0–4°C. This process was significantly slower ($P \leq 0.05$) in the samples with 1.0% of goji extract and 1.0% of buckwheat flour, where the total content of protein carbonyls decreased by 51 and 36% (Table 3).

Acidity values increased significantly ($P \leq 0.05$) in all samples during 21 days of refrigerated storage. The lipolytic changes were lower by 38% in the samples with 1.0% of goji extract and 1.0% of buckwheat flour and by 28% in the samples with 0.5% of goji extract and 0.5% of buckwheat flour, if compared with the control sample. Similar changes were registered in peroxide value and TBARS. Primary products of lipid oxidation (lipid hydroperoxides) showed a significant decrease ($P \leq 0.05$) by 24% in the samples with 1.0% of goji extract and 1.0% of buckwheat flour and by 17% in the samples with 0.5% of goji extract and 0.5% of buckwheat flour. Secondary products of lipid oxidation (TBARS) decreased by 53% in the samples with 1.0% of goji extract and 1.0% of bucruheat flour and by 44% in the samples with 0.5% of goji extract and 0.5% of buckwheat flour.

As for the comparison of pH value, samples with 0.1% and 0.5% of goji extract and buckwheat flour showed a small (1.3–2.6%) but significant ($P \leq 0.05$) increase after 21 days of storage. Unlike the control samples, the test samples demonstrated a statistically significant decrease in pH by 11.8%. The conclusions were confirmed by the results obtained for the total count of facultative anaerobic mesophilic microorganisms in the vacuum-packaged samples after 21 days of refrigerated storage (Table 4).

Histological analysis is widely used to determine the condition of raw materials and products, as well as their real composition. The analysis makes it possible to study the structure of the product as a whole together with the changes in its parts and components. It detects the presence of various tissues and cellular structures and their quantity in the product [24, 25].

The method of histological analysis is widely used in biology and medicine. However, in this study it was

Table 4 Facultative anaerobic mesophilic microorganisms in vacuum packaged samples during 21 days of storage at 0–4°C

Samples	Facultative anaerobic mesophilic microorganisms, log CFU/g		
	Day 1	Day 11	Day 21
Control	2.04	5.14	6.47
goji extract (0.5%)	2.01	3.97	4.95
goji extract (1.0%)	2.00	3.21	4.36
buckwheat flour (0.5%)	2.03	4.05	5.00
buckwheat flour (1.0%)	2.02	5.33	4.52

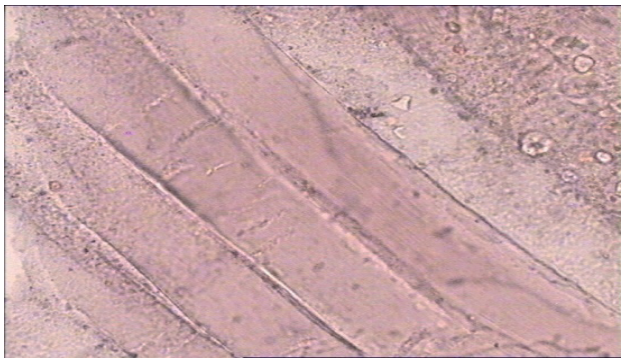

Figure 1 Microstructure of the control sample (340×magnification)

applied to the national cooked and smoked horse-meat product "Kanagat" after it had undergone thermal treatment and other types of technological impact [26].

In this research, histological analyses were performed in order to determine the effect of goji extract (*Lycium barbarum* L.) and buckwheat flour (*Fagopýrum esculéntum* L.) on the muscle and connective tissue of Kanagat. According to the microstructural analysis, the control sample consisted of large fragments of muscle, connective, and adipose tissue of 0.7–1.4 μm (Fig. 1).

The muscle fibers were straight, swollen, tightly adjacent to each other, and quite often fragmented. A fine-grained protein mass that formed as a result of mechanical action on muscle tissue during the grinding was spread between the coarse-grained structural elements. The fine-grained protein mass revealed particles of spices and fat drops of 12–100 μm in size, which were uniformly distributed over the mass of the sample. The surface coagulation layer adhered tightly to the coating. Bundles of muscle fibers that retained their integrity were so tightly adjacent to each other and swollen that the boundaries between them were difficult to detect. The transverse striation was wide and visible in occasional fibers. However, the bulk of muscle fibers had a homogeneous structure, with some disintegration and violation of the direction of myofibrils to one another. The nuclei of the fiber were homogeneous. Destructive changes were spotted in the form of individual microcracks.

As for the experimental samples, the coarse-grained structural components were in a fine-grained protein mass that included fragments of plant components, i.e. buckwheat flour, goji extract, and spices. The layout of the sample was dense, with no large cracks or cavities loosening the mass of the sample. The structural components of meat were closely interconnected. The fine-grained protein mass was penetrated by round-shaped microcapillaries of 250–350 μm in size (Fig. 2).

The fragments of muscle tissue that retained their microstructural features demonstrated swollen muscle fibers. The boundaries between them were hardly discernible. The transverse striation was either poor or not detected in some parts of the sample.

Destructive changes were multiple. The destruction degree of the fibers was greater than in the control samples. The fiber nuclei were homogeneous or shadow-like. Microflora was detected as a fine-grained protein mass in the form of small microcolonies of 0.2–0.3 μm. Microflora was diffuse between the fibers, under the sarcolemma, in the areas of fiber destruction, and in connective tissue layers. The layout of the structural elements was dense. The vacuoles were 70–300 μm in size, had clearly defined boundaries, and occasionally merged with each other.

1.0% of goji extract and 1.0% buckwheat flour accelerated the destructive changes in the main structural elements of meat, and, consequently, boosted its secondary structure formation. The samples with goji extract and buckwheat flour had a greater degree of swelling and destruction of muscle fibers. The destructive changes covered most fibers and were detected as multiple decays of myofibrillar substance, which turned into a fine-grained protein mass.

The intensive formation of the fine-grained protein mass contributed to the development of a compact monolithic mass of meat pieces, which formed a dense space framework after heat treatment. Unlike meat products developed according to traditional technologies, the pieces of meat in the test samples were more compact and less porous. There were fewer vacuoles, and they were smaller.

The microstructural studies showed that 1.0% of goji extract and 1.0% of buckwheat flour caused

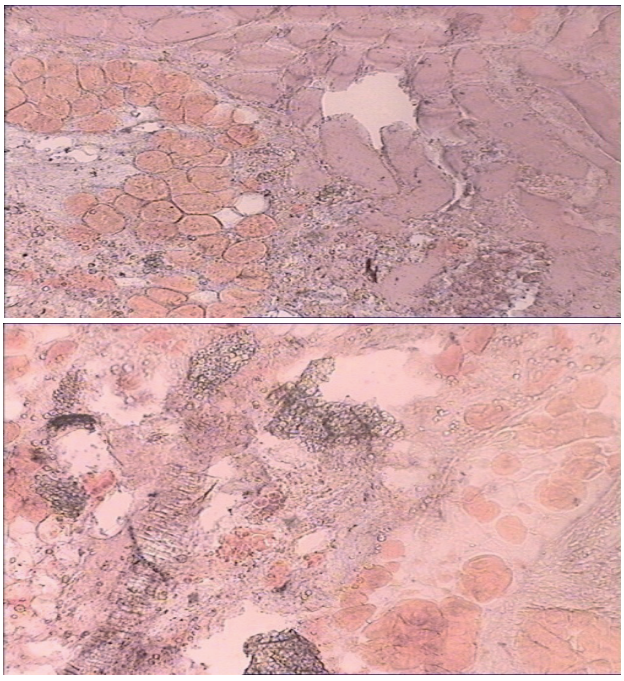

Figure 2 Microstructure of the national cooked and smoked meat product "Kanagat" (240×magnification)

destructive changes in most fibers. The affected fibers showed multiple decays of myofibrillar substance, which turned into a fine-grained protein mass. This, in turn, contributed to the development of monolithic structure.

CONCLUSION

Injecting 1.0% of buckwheat flour (*Fagopýrum esculéntum* L.) or 1.0% of goji extract (*Lycium barbarum* L.) into horse meat resulted in a functional national cooked and smoked horse-meat product with 1% of biologically active substances. This concentration inhibited lipolytic changes and oxidation of proteins and lipids. It also improved the oxidative stability and quality of the new national horse-meat product, while maintaining its sensory properties.

CONTRIBUTION

Ya.M. Uzakov developed the research concept and plan, as well as collected, analyzed, and interpreted data. M.A. Kaldarbekova was responsible for the accuracy and integrity of the research. O.N. Kuznetsova compiled and corrected the article.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of the present article.

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Antioxidant and antimicrobial properties of oregano extract (*Origani vulgaris herba* L.)

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Abstract:
Introduction. Some ingredients of plant origin possess both antioxidant and bacteriostatic properties. If used in the food industry, they can inhibit microbiological and oxidative damage, thus increasing the shelf life of meat products. Oregano extract is one of such substances, which means that it can be used as an antioxidant and preservative. Therefore, the study of this plant has a significant theoretical and practical potential for the food industry.
Study objects and methods. The present research featured ground trimmed beef. The samples with 20% of fat tissue were used to determine the microbiological parameters, while the samples with 30% of fat were used to obtain data on oxidative stability. The control sample contained no additional ingredients. The sample with food additives was pre-treated with acidity regulators and antioxidants, namely sodium acetate E262, ascorbic acid E300, sodium ascorbate E301, sodium citrate E331, and rosemary extract E392. The sample with oregano extract was pre-treated with oregano extract (*Origani vulgaris herba* L.) in the ratio of 5 g of extract per 1 kg of meat. The extract had been dissolved in 100 g of water. The samples were stored at 4 ± 2°C for 12 days. A CM5 spectrophotometer (Konica Minolta, Japan) was used to determine the color characteristics. The induction period of oxidative stability was determined using an Oxitest oxidative stability analyzer (Velp Scientifica, Italy). The studies were conducted in Austria, Linz.
Results and discussion. Oregano extract stabilized the redness rating. For the sample with oregano extract, the induction period of oxidative stability was twice as long as for the control sample and the sample with antioxidants. In addition, oregano inhibited the growth of aerobic and anaerobic microorganisms.
Conclusion. The antioxidant and antimicrobial properties of oregano extract prolong the shelf life of ground beef, which makes it possible to reduce the amount of food additives.

Keywords: Meat, *Origani vulgaris herba* L., antioxidants, colour stability, oxidative stability, antimicrobial properties

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INTRODUCTION

For many years specialists of the global meat industry have been trying to solve the problem of safety of meat products and to find optimal ways to preserve its consumer properties throughout the entire shelf life. They have come up with various methods of increasing the shelf life of the finished product, e.g. barrier packaging, low temperatures, preservatives and antioxidants, etc. Most damage during storage of meat products is of microbiological and oxidative nature. Microorganisms and oxidation lead to irreversible negative consequences. The biological value of the product deteriorates, so do its consumer properties, i.e. color, smell, and taste. Most importantly, such meat becomes unwholesome. Oxidative damage is caused by three different reactions: 1) enzymatic oxidation; 2) non-enzymatic, or free-radical (peroxide) lipid oxidation; 3) non-enzymatic, or non-radical oxidation. The resulting products of lipid degradation reduce the nutritional value of the finished product during storage [1–3]. The rate of oxidative changes is determined by the composition and quantity of pro-oxidants and antioxidants that are natural to the meat or were introduced during the production process.

Traditionally, meat industry exploits various food additives of bacteriostatic action, e.g. lactic or acetic acid and their salts, as well as antioxidants, e.g. ascorbic acid and its derivatives, tocopherols, etc. These additives either slow down the growth of microorganisms or reduce lipid oxidation. Therefore, the meat industry

needs complex mixes to extend shelf life and preserve the quality and safety of meat products.

However, current trends in the food industry demand that the amount of food additives should be reduced. This trend resulted in a whole new area in the food industry. This area develops ingredients from natural raw materials. They improve and preserve consumer properties of finished products, thus being able to substitute food additives [4]. Sources of such functional components range from vegetables, fruit, and berries, e.g. cabbage, grapes, plums, apples, pomegranates, wild rose, etc., to herbs and spices, e.g. thyme, cinnamon, rosemary, oregano, mint, etc. [5].

The antioxidant properties of plant extracts can be explained by various factors: the presence of natural ascorbic acid (vitamin C), alfatocopherol (vitamin E), beta-carotene (a precursor of vitamin A), flavonoids, and other phenolic compounds [6, 7].

The antimicrobial and antioxidant properties of plant extracts have long been the focus of scientific research. Grape seed extract was found to reduce total bacterial count in semi-finished chopped beef [8]. Rosemary extract with cloves slowed down the oxidation of chicken meat [9]. Meat oxidation could be inhibited by extracts of broccoli powder, lotus seeds, red grape husks, peanut skin, tomato processing by-products, olives, pomegranate, and other plant ingredients [10–15].

Lipid oxidation is not the only problem that might occur during production and storage of meat products. The process is also accompanied by protein oxidation, including myoglobin protein, which is responsible for the color of raw meat and meat products. In addition, oxidation processes of proteins and lipids can be interconnected, while heme/non-heme iron can cause oxidative changes in lipids [16]. According to Johns *et al*., heme iron has a greater prooxidised effect than free iron [17]. These data are consistent with those in [18], according to which the concentration of heme iron has a greater effect on the oxidation rate than the amount of non-heme iron. However, some other studies indicated that non-heme iron had a stronger catalyzing effect on the oxidation of meat products than heme iron [19, 20]. Plant extracts can inhibit both lipid oxidation and myoglobin oxidation, thus preserving the attractive color of meat and meat products.

Extracts of rosemary, garlic, ginger, onion, etc., have successfully been tested as antimicrobial plant components [21–24].

Oregano is a promising natural antioxidant. It is obtained by drying the leaves and flowers of common oregano (*Origanum vulgare* L.). This component is multifunctional: oregano extract can inhibit both oxidative and bacterial changes in meat [25–28].

The research objective was to justify the feasibility of using ethanol oregano extract in the production of chopped semi-finished products to substitute antioxidant and preservative food additives.

STUDY OBJECTS AND METHODS

The microbiological research featured samples of trimmed beef with 20% of fat tissue. To determine the oxidative stability and color characteristics, we used trimmed beef with a 30% fat content. The raw meat was ground using a meat grinder with the plate hole diameter of 2–3 mm.

Raw meat with no additional ingredients served as the control sample. The sample with food additives was pre-treated with acidity regulators and antioxidants that are traditionally used in meat industry to increase shelf life, i.e. sodium acetate E262, ascorbic acid E300, sodium ascorbate E301, sodium citrate E331, and rosemary extract E392. The additives were applied without prior preparation. The sample with oregano extract was pre-treated with oregano extract in the ratio of 5 g per 1 kg of meat. The extract had been dissolved in 100 g of water, as recommended by the manufacturer.

The prepared samples were stored at 4 ± 2°C for 12 days.

After production and throughout the whole storage period, we conducted studies to determine the color characteristics, microbiological parameters, and the induction period of oxidative stability.

Color characteristics included the indices of lightness, redness, and yellowness. They were determined using a CM5 spectrophotometer (Konica Minolta, Japan).

The induction period of oxidative stability was determined using an Oxitest analyzer (Velp Scientifica, Italy). The Oxitest analyzer monitors the change in absolute pressure in two autonomous thermostatically controlled chambers, which occurs during oxidation. The reaction proceeds at temperature = 90°C, pressure = 6 atm, oxygen purity = 99.9%.

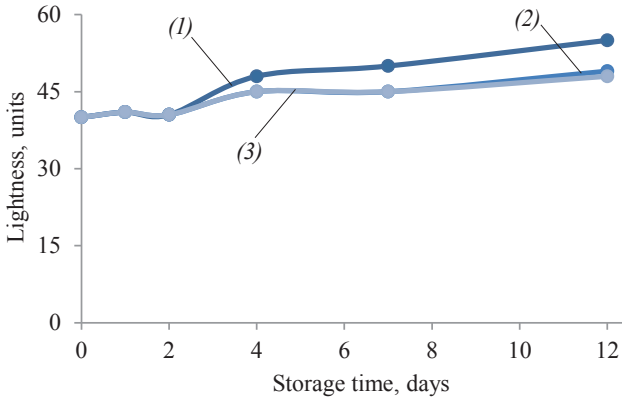
The microbiological analysis was performed using standard procedures described in the Bacteriological Analytical Manual (BAM) of the Food and Drug Administration (FDA).

RESULTS AND DISCUSSION

Figs. 1–3 present data on the change in color indices of beef during storage. The indices of lightness and yellowness showed no significant differences by the end of the shelf life. However, both food additives and oregano extract had a significant effect on the retention of redness. In addition, oregano extract made it possible to obtain the same effect as complex food additives that are based on acidity regulators and antioxidants.

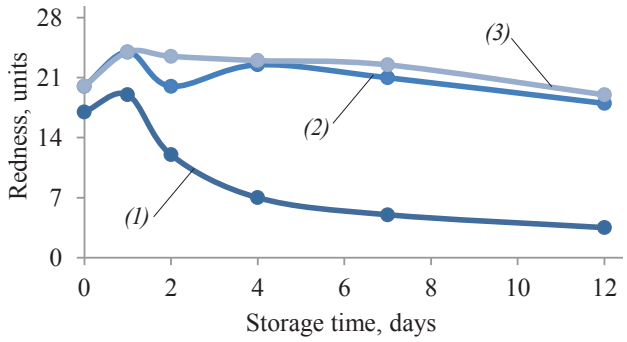
Oregano extract proved to be able to inhibit the oxidation of myoglobin in meat. Chemical changes in myoglobin are known to be associated with fat oxidation. The obtained data on color characteristics were consistent with the results of determining the antioxidant activity of oregano extract.

The antioxidant properties of oregano can be seen from the results of determining the induction period of



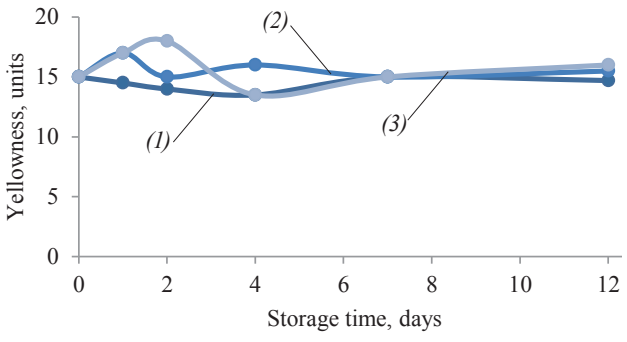
(1) control sample *(2)* sample with food additives
(3) sample with oregano extract

Figure 1 Dynamic pattern of lightness index during storage



(1) control sample *(2)* sample with food additives
(3) sample with oregano extract

Figure 2 Dynamic pattern of redness during storage



(1) control sample *(2)* sample with food additives
(3) sample with oregano extract

Figure 3 Dynamic pattern of yellowness during storage

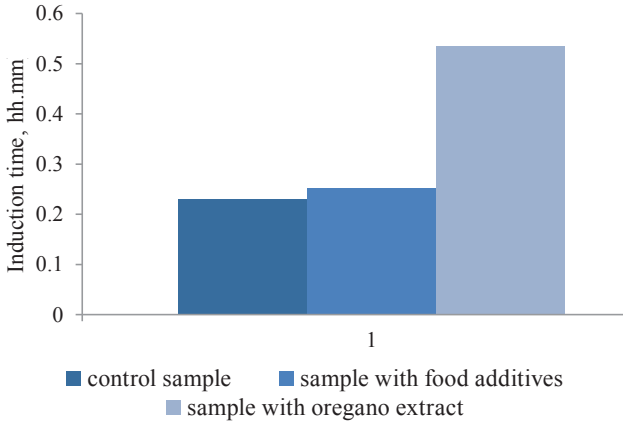
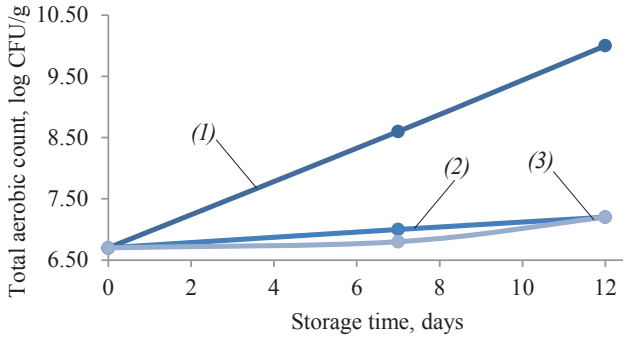
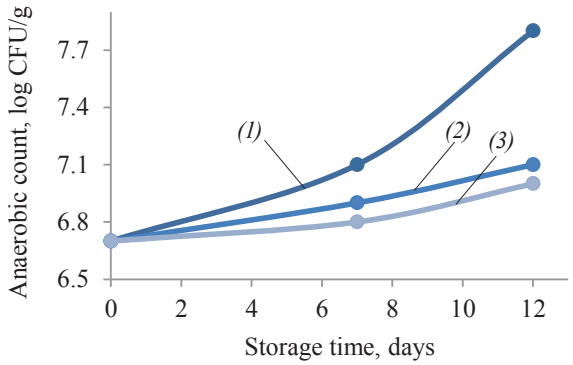


Figure 4. Induction period of oxidative stability of beef



(1) control sample *(2)* sample with food additives
(3) sample with oregano extract

Figure 5 Dynamic pattern of aerobic count in beef during storage



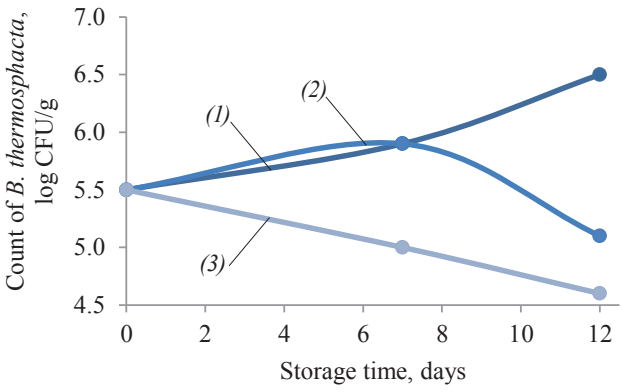
(1) control sample *(2)* sample with food additives
(3) sample with oregano extract

Figure 6 Dynamic pattern of anaerobic count in beef during storage

oxidative stability (Fig. 4). Oregano extract was able to double the oxidative stability of meat compared with the control sample and the sample pre-treated with additives.

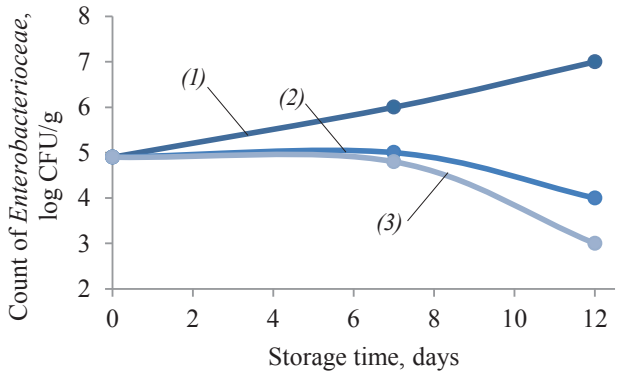
The obtained results were partially consistent with [29], where poultry was treated with a combination of clove, cinnamon, and oregano extracts, which decreased the total bacteria count and increased lightness and redness.

Similar data were obtained by Trindade *et al.*, who proved that 400 mg of oregano extract per 1 kg of meat reduced the amount of secondary decomposition products of fatty acids in beef burgers [26].



(1) control sample *(2)* sample with food additives
(3) sample with oregano extract

Figure 7 Dynamic pattern of *B. thermosphacta* count in beef during storage



(1) control sample *(2)* sample with food additives
(3) sample with oregano extract

Figure 8 Dynamic pattern of *Enterobacterioceae* count in beef during storage

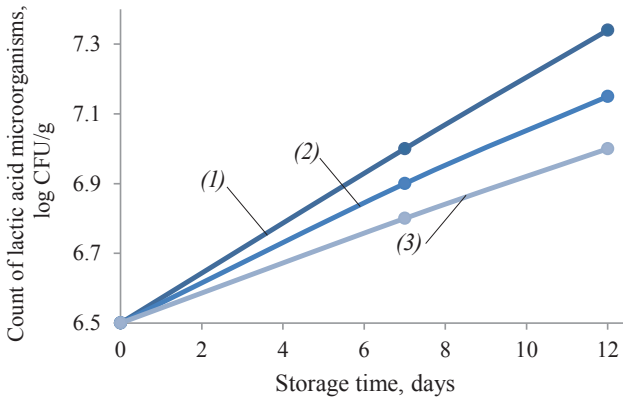
Another study showed that grape seed extract, rosemary oleoresin, and oregano extract had antioxidant properties when used in high fat meat products [30].

In addition to its antioxidant properties, oregano extract can be used in the meat industry for its bacteriostatic effect.

A set of experiments showed that oregano extract significantly slowed down the growth of both aerobic and anaerobic microorganisms (Figs. 5 and 6).

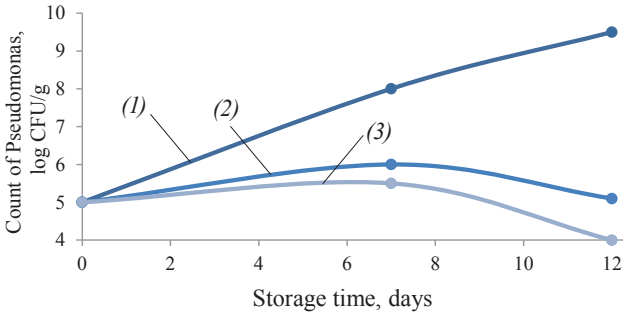
Similar data were obtained by Skandamis and Nychas, who revealed a decrease in the initial microflora of beef when 0.8% of oregano essential oil was added [27]. The same team of scientists also registered a reduction in the total bacterial count in ground beef by 1 log CFU/g when they added 1% oregano oil [28].

The obtained data are consistent with the results about the antibacterial properties of oregano described in [31]. Oregano was found to improve the permeability of *S. aureus* cell membranes [32].



(1) control sample *(2)* sample with food additives
(3) sample with oregano extract

Figure 9 Dynamic pattern in the count of lactic acid microorganisms in beef during storage



(1) control sample *(2)* sample with food additives
(3) sample with oregano extract

Figure 10 Dynamic pattern of *Pseudomonous* count in beef during storage

The antimicrobial properties of oregano essential oil appeared to have a bacteriostatic effect on *Salmonella Enteritidis* in mutton [33].

Cui *et al.* established the mechanism of the antibacterial properties of oregano essential oil [34]. They studied the effect of oregano essential oil on respiratory and energy metabolism of *Staphylococcus aureus*. The oil proved efficient against this methicillin-resistant microorganism.

The antimicrobial effect of oregano on *Salmonella* and *S. aureus* has been proven by a number of studies [31–34]. Therefore, the present research focused on the bacteriostatic effect of oregano on various microorganisms that cause meat spoilage (Figs. 7–10). According to the obtained data, oregano extract proved more effective in comparison with the control sample and the sample pre-treated with food additives. The antimicrobial properties of oregano extract, however, did not affect lactic acid microorganisms that can inhibit the development of putrefactive microflora (Fig. 9).

The antimicrobial properties of oregano were also confirmed by Agrimonti *et al.* [36, 37]. They put absorbent cellulose wipes saturated with oregano essential oil emulsion in packages with ground meat. The emulsion proved to have antimicrobial effect against psychrophilic microorganisms in ground beef. In addition, such oregano adsorbent wipes were efficient against certain types of microorganisms that can affect raw meat, namely *Pseudomonas putida*, *Pseudomonas fragi*, *Pseudomonas fluorescens*, *Enterococcus faecalis*, and *Lactococcus lactis*. It also decreased the count of some common foodborne pathogens, such as *Salmonella enterica*, *Campylobacter jejuni* and *Staphylococcus* [35]. The bacteriostatic properties of oregano are sometimes explained by the high content of thymol and carvacrol, i.e. compounds with documented antimicrobial activity [36, 37].

CONCLUSION

Thus, *Origani vulgaris herba* L. extract can be used in ground beef production to extend its shelf life, which makes it possible to reduce the amount of food additives. The results of the present study make a significant contribution to the justification of the antimicrobial and antioxidant effects of oregano extract. The multifunctional character of this ingredient was confirmed by its positive effect on the stability of color indexes, which helps to improve the consumer characteristics of the product during storage. Taking into account that the antioxidant effect largely depends on the dose, further studies are needed to determine the optimal amount of oregano extract for various meat products.

CONFLICT OF INTEREST

The author declares that there is no conflict of interests regarding the publication of this article.

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Changes of antioxidant activity and active compounds content in selected teas

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Abstract: Our study tested 45 tea infusions classified into five groups (white, green, red, black, and other teas) for the content of total polyphenols and flavonoids, as well as antioxidant properties, by the FRAP and DPPH methods. We examined these parameters after prolongation of the brewing time from 10 to 30 min and overnight storage. The results showed that the capacity of the teas to bind free radicals was differentiated and the amount of anti-oxidant compounds depended on their nature. In terms of antioxidant activity and total polyphenol content, the tested tea types were ranked in the following order: white > green > black > red > other teas (yerba mate > rooibos). Our experiment demonstrated a positive correlation between the polyphenol content and antioxidant activity of the analyzed teas. Also, the DPPH antiradical efficiency was comparable to their ability to reduce ferric ions. The extended brewing time had a significant effect on the antioxidant activity of the infusions and the polyphenolic compounds analyzed therein. In contrast, storage of the infusions for 24 h at room temperature changed their antioxidant activity and affected the total polyphenol content.

Keywords: Tea infusion, FRAP, DPPH, polyphenols, flavonoids, brewing time

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INTRODUCTION

Tea (*Camellia sinensis* L.) is considered to be the most popular beverage in the world [1, 2]. It has been estimated that the average daily consumption of tea is approximately 120 mL. Poland is among the first three European countries and ten world countries in terms of tea consumption. Of several species commonly referred to as tea, the most important is Chinese tea produced from the *Camellia sinensis* leaves [3]. The chemical composition of tea is highly diverse and may vary within a relatively broad range, depending on many factors (e.g. the position of leaves on the stem, growth conditions, processing methods, or brewing time) [4–6].

In recent years, of great interest has been the therapeutic activity of tea and, in particular, the antioxidant effects of its compounds, e.g. polyphenols and flavonoids [3, 6–10]. Due to differences in processing technology or species composition, each type of tea contains a different combination of biologically active substances, depending on the extraction time and temperature, as well as leaf fineness. Fresh green leaves are rich in isomeric flavan-3-ols (catechins), on average accounting for 30% of their mass, with epigallocatechin gallate (EGCG) being the most common component. During fermentation, some catechins undergo oxidation and condensation to high molecular weight compounds (3–6% theaflavins and 12–18% thearubigins) responsible for the characteristic flavor and aroma of infusions [4, 5, 9, 8, 11, 12].

Our study is an attempt to estimate the effect of infusions brewing time and storage on the content of bioactive compounds (polyphenols and flavonoids) and antioxidant properties of 45 loose-leaf and bagged teas available on the Polish market.

STUDY OBJECTS AND METHODS

Using the FRAP and DPPH methods, we compared the total content of polyphenols and flavonoids, as well as the antioxidant properties of infusions made from several types of loose-leaf (L) and bagged (E) teas available on the Polish market. Additionally, we assessed the effects of the brewing time and storage of infusions on the total content of polyphenols and antioxidant properties.

The research material consisted of 45 teas produced commercially by leading manufacturers

Table 1 Polyphenols content, flavonoids content, FRAP and DPPH antioxidant activity of tea after 10 min of brewing

Sample number	Polyphenols content (caffeic acid equivalent mg g^{-1} dry weight)	FRAP antioxidant activity (equivalent µmol $FeSO_4$ g dm^{-1})	DPPH antioxidant activity (% scavenging for 1g dry matter)	Flavonoids content, % g dry $matter^{-1}$ (calculated as quercetin)
1 BL	39.46	880.96	47.96	0.25
2 BL	83.40	982.64	91.76	0.47
3 BE	73.80	941.42	83.05	0.41
4 BL	71.40	930.43	68.63	0.42
5 BL	37.17	590.45	44.54	0.23
6 BL	81.08	578.28	66.53	0.49
7 BL	65.52	788.70	65.23	0.25
8 BL	57.56	645.41	60.49	0.28
9 BE	64.64	685.45	55.09	0.36
10 BE	75.72	515.12	3.76	0.08
11 BL	71.36	505.96	55.01	0.20
12 BL	32.16	461.53	72.54	0.06
13 BE	97.02	1035.66	57.15	0.39
14 BL	103.53	1317.66	66.01	0.44
15 BL	83.64	1148.91	53.39	0.21
Mean for B	**69.17**	**800.57**	**59.41**	**0.30**
16 GL	61.41	874.29	60.78	0.31
17 GL	121.50	1304.95	76.05	0.38
18 GE	114.32	1337.54	69.94	0.43
19 GL	78.69	1167.55	67.70	0.40
20 GE	85.60	1183.65	76.71	0.35
21 GE	107.03	899.82	37.38	0.24
22 GL	96.55	748.12	47.84	0.16
23 GL	86.67	379.59	24.35	0.30
24 GE	119.98	1033.55	30.12	0.14
25 GL	67.16	657.03	74.30	0.38
26 GL	126.35	2257.19	93.29	0.53
27 GE	94.55	1451.43	81.27	0.34
28 GL	126.04	1251.54	43.95	0.37
29 GL	114.23	1993.17	85.20	0.46
30 GL	107.69	1944.46	89.89	0.25
Mean for G	**100.52**	**1232.26**	**63.92**	**0.34**
31 WE	105.69	1388.97	79.16	0.35
32 WE	105.32	1300.64	70.92	0.40
33 WL	87.97	1311.89	64.42	0.23
34 WL	70.84	1400.81	61.26	0.19
35 WE	116.54	1630.40	81.27	0.36
Mean for W	**97.27**	**1406.54**	**71.40**	**0.30**
36 RE	38.99	676.42	52.24	0.17
37 RE	61.99	606.53	25.77	0.19
38 RL	42.85	707.69	25.34	0.18
39 RL	45.21	534.90	20.97	0.18
40 RL	46.29	622.39	29.08	0.18
Mean for R	**47.07**	**629.59**	**30.68**	**0.18**
41 RoE	45.81	723.54	54.88	0.14
42 RoE	29.06	603.01	29.62	0.09
43 YML	60.95	997.95	76.71	0.27
44 RoL	41.59	535.05	20.38	0.20
45 YML	75.19	895.75	46.15	0.26
Mean for RoY	**50.52**	**751.06**	**45.55**	**0.19**
$LSD_{0.05}$	**	**	**	**

B – black tea; G – green tea; W – white tea; R – red tea; Ro – Rooibos tea; YM – Yerba Mate, L – loose-leaf tea; E – bagged tea
***, **, * – significant at $P \leq 0.001$, 0.01 or 0.05

for the Polish market (Bastek Coffee & Tea, Mokate, Unilever Polska, Teekanne Polska, Roger Sp. z o.o., Amber Spark, William's Nature Products, Bio-active Sp. z o.o., Herbapol-Lublin S. A., Posti S.A., Himalaje – Najlepsze herbaty, Tata Global Beverages Polska Sp. z o.o.) and purchased in retail stores. In particular, we experimentally tested 5 white teas (W), 15 green teas (G), 5 red teas (R), 15 black teas (B), and 5 other teas, rooibos and yerba mate (Ro, YM). The names of the teas were assigned digital (chosen randomly in the five groups mentioned above) and letter designations.

Methodology of analyses. One gram of tea was mixed with 100 mL of boiling distilled water, covered, and brewed for 10 min. The extracts were filtered through a medium-sized filter. The infusions were assayed for the total polyphenol content (o-dihydroxyphenols expressed as an equivalent of caffeic acid) using the spectrophotometric method of Singleton and Rossi and the flavonoid content (expressed as an equivalent of quercetin) according to Polish Pharmacopoeia VIII [13, 14].

Furthermore, we determined the antioxidant activity of tea by using the FRAP method of Benzie and Strain (the capacity of reducing 1 mole of Fe (III) to Fe (II) expressed as µmoles of antioxidant compounds in 1 g of raw material) and the modified DPPH Brand-Williams *et al*. method using a free radical of 1,1-diphenyl-2-picrylhydrazyl [15, 16]. The results were presented as % of free radical scavenging.

Brewing time and freshness effects on antioxidant activity and polyphenols. The samples under analysis were brewed for 10 and 30 min and stored at room temperature for 24 h. Next, we assessed their antioxidant activity using the FRAP method and determined the total polyphenol content. All assays for each sample were performed in triplicate.

Statistical analysis. All the data were subjected to variance analysis. The significance of the differences between mean values were verified with Tukey's test (n = 3) at a significance level of 95%. Statistica 9.0 (StatSoft) program was employed for the calculations. Additionally, we used Excel to calculate the coefficients of simple correlations between the phytochemical features of the infusions and their antioxidant properties.

RESULTS AND DISCUSSION

The analyzed samples of teas available on the Polish market differed significantly in both the total polyphenol and flavonoid contents and their antioxidant properties evaluated after 10 min brewing (Table 1).

As we can see, the content of flavonoids in the infusions assayed with the methodology provided in FP VIII ranged from 0.06% for sample 12 BL (loose-leaf black tea) to 0.53% for sample 26GL (loose-leaf green tea) [14]. In general, the lowest values of the flavonoid content were found for red teas (0.17–0.19%) and rooibos (0.09–0.2%), whereas the average value in the other

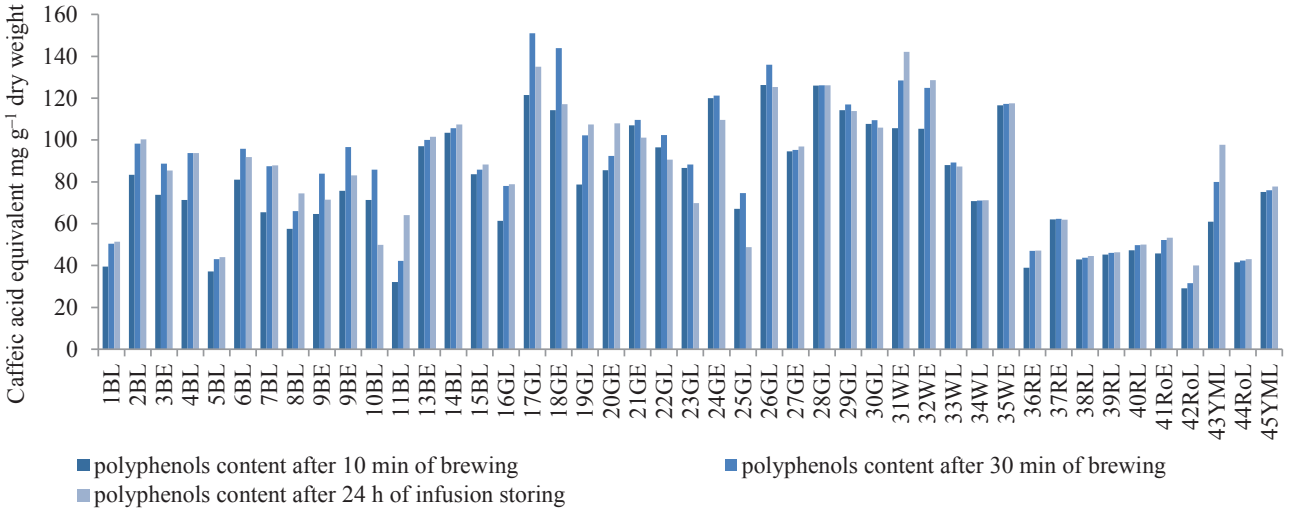

Figure 1 Changes in polyphenol content (caffeic acid equivalent mg·g^{-1} dry weight)

types of tea was about 0.3%. The flavonoid content in the analyzed teas highly correlated with their antioxidant activity, determined with the FRAP and DPPH methods (r^2 = 0.548–0.666), and with the polyphenol content (r^2 = 0.582). These results were in agreement with those reported by Castiglioni *et al*., as well as Fernando and Soysa [17, 18].

The total polyphenol content determined with the Folin-Ciocalteu method in the 45 analyzed tea infusions ranged from 29.1 (sample 42 RoE – loose-leaf rooibos tea) to 126.4 mg·mL^{-1} (sample 26 GL – loose-leaf green tea), expressed as an equivalent of caffeic acid (Table 1, Fig. 1). The values were comparable with those reported by Hilal and Engelhardt [19]. The highest content of polyphenols was found in the green teas (average 100.5 mg·mL^{-1}), just as in the experiments described by McAlpine and Ward, Kiran and Kumar, as well as Shannon *et al*. [20–22]. The white teas were characterized by a slightly lower (mean 97.3 mg·mL^{-1}) content of the active compounds.

On average, the black teas contained 69.2 mg·mL^{-1} of polyphenols, whereas the red tea as well as rooibos and yerba mate exhibited mean values of 47.1 and 50.5 mg·mL^{-1}, respectively. Similar results were reported by a number of researchers [7, 9, 21, 22]. However, Plust *et al*., as well as Hilal and Engelhardt, found higher contents thereof in white teas and lower contents in green teas and, especially, in black teas [3, 19]. In our experiment, higher total contents of polyphenols and flavonoids in green tea, compared to white tea, might be due to slight oxidation of white (nonfermented) tea polyphenols during production. In fact, white teas do not undergo the inactivation of enzymes before withering, so enzymes remain active and white tea polyphenols are oxidized slowly [12].

The antioxidant activity (assessed with FRAP and DPPH) and the polyphenol content demonstrated a significant correlation (r^2 = 0.435–0.732) and a considerable diversity of the results. This correlation between the results of the three tests confirmed the validity of the procedure we used for analysis. However, Almajano *et al*. found no linear correlation between the antiradical activity of the analyzed infusions and the content of polyphenolic compounds [9]. They suggested that antiradical performance was influenced not only by the content of polyphenols but also by their quality and presence of other compounds that might enter the infusions during extraction.

A slightly different relationship was discovered by Rusaczonek *et al*. and Castiglioni *et al*., i.e. a linear correlation between the total polyphenol content, flavonoids content, and antioxidant properties measured with ABST [7, 17]. Similar results were reported by Plust *et al*., Molan *et al*. and Aldiab, who used the FRAP method [3, 11, 23]. We should emphasize that the available literature describes a variety of methods for determination of antioxidant properties and polyphenolic content of tea infusions. Also, the authors employ different methods of extraction (temperature, time, solvent) while preparing solutions for analyses and express their results in different ways. Therefore, the results are sometimes hardly comparable [1, 9, 11].

The antioxidant activity of the teas measured with the FRAP method was in the range of 379.6–2257.2 µmol g d.w.$^{-1}$ The highest antioxidant activity was exhibited by the white and green tea samples, while the lowest activity was found for the red teas (over twice as low). A similar trend was observed by Atalay and Erge as well as Shannon *et al*. [12, 22].

On average, the black teas had 35% weaker antioxidant properties than the green teas and a 43% lower Fe ion reduction ability than the white teas. These results were in line with the findings of Aldiab [23]. The antioxidant activity of this group of teas exhibited a wide range of 461.5–1317.7 µmol g d.w.$^{-1}$, which indicated high variation of tea on the domestic market.

Table 2 Single correlation coefficients between chosen features of teas

	FRAP 10	DPPH 10	FV 10
PL 10	0.732***	0.435**	0.582***
FRAP 10		0.668***	0.548***
DPPH 10			0.666***

PL 10 – polyphenols content after 10 min of brewing; FRAP 10 – FRAP antioxidant activity after 10 min of brewing; DPPH 10 – DPPH antioxidant activity after 10 min of brewing; FV 10 – flavonoids content after 10 min of brewing
***, **, * – significant at $P \leq 0.001$, 0.01 or 0.05

These teas had slightly weaker antioxidant properties than green teas due to the oxidation of catechin derivatives during processing and the presence of less active theaflavins and thearubigins in the infusions [12]. The weaker antioxidant properties and a lower polyphenol content in these tea types were confirmed by other authors [12, 19]. Noteworthily, the loose-leaf white and red teas were characterized by lower activity than the bagged teas, while Plust *et al*. reported an opposite trend for other tea types [3].

Generally, the analyzed red teas had a significantly lower content of polyphenols and exhibited weaker antioxidant activity. This result was probably due to the differences in the manufacturing process, which involves additional drying of tea leaves before they are twisted, leading to slight fermentation of tea. Additional drying may result in an increased loss of active substances. Incomplete fermentation causes polymerization of simple polyphenols, as in black teas, but it does not last long. Another difference is the absence of heat treatment for black tea, which may induce differences in the composition of the pu-erh type teas.

DPPH is a stable free radical that antioxidants can react with, providing it with electrons or hydrogen atoms. As presented in Tables 1 and 2, the value of antioxidant activity determined with the DPPH method was closely related to the results obtained using the FRAP method ($r^2 = 0.668$).

Concurrently, there were substantial differences in the antioxidant activity of the analyzed extracts (from 3.8 to 93.3% radical scavenging). Just as in the study of McAlpine and Ward, the lowest DPPH radical scavenging percentage was found for red tea (20.9–52.2%) and rooibos (20.4–54.9%), while the highest values were reported for white (61.3–81.3%) and green teas (24.4–93.3%) [21]. Similarly, Shannon *et al*. confirmed a higher DPPH radical scavenging capacity of green tea, followed by black tea [20].

The higher antioxidant activity of green tea determined by both methods can be attributed to an epigallocatechin gallate content, showing a greater free radical scavenging capacity than the other catechins [12]. Our study found that the more processed the tea was, the lower its antioxidant capacity. Similar results were reported by Kiran and Kumar, as well as Cleverdon *et al*. [21, 24]. However, they did not agree with the findings of Fik and Zawiślak, possibly due to the application of a different solvent during preparation of infusions and differences in the geographical regions of tea cultivation, harvesting periods, or storage conditions [8].

The most considerable differences between the antioxidant activities of the analyzed extracts were observed for the black teas. The loose-leaf types exhibited higher activity than the bagged teas (Table 1). Additionally, just as in Cleverdon *et al*., true teas had at least a two-fold greater polyphenol content than the herbal varieties [24]. The weaker free radical scavenging capacity of rooibos tea (originating from *Aspalathus linearis* L.) may result from its chemical composition: unlike *Camellia sinensis* L., it does not contain catechins but aspalathin, isoorientin, orientin, and rutin [24, 25].

Similarly, yerba mate (produced from *Ilex paraguariensis* L.) does not contain catechins but substantial amounts of chlorogenic acid [26]. We found that the antioxidant activity of yerba mate was substantially lower than that reported by Boji *et al*. [26]. However, as emphasized by Komes *et al*., yerba mate can exhibit low antioxidant activity compared to white and green teas [27].

It seems that the differences between the polyphenol profiles of "true" and herbal or rooibos teas could be the direct cause of the differences in their antioxidant capacity found in our study. We should take into account that the differences observed in these studies can be related to different sample preparation methodologies and use of different brewing times and tea-to-water ratios. What is more, the comparison of results is sometimes difficult due to the lack of uniformity in the properties of green tea, manufacturing and brewing conditions. Leaf age and size, harvesting season, and manufacturing conditions are all important factors that can affect the results [4].

Thus, according to our study, the teas tested with the FRAP and DPPH methods exhibited antioxidant activity in the following order: white teas > green > black > red > other teas (yerba mate > rooibos).

In all the infusions, the total polyphenol content increased (from 6.9% in red teas to 19.7% in black teas) with the infusion time, with higher values noted for bagged teas (Fig. 1). These findings are in line with other studies conducted on different brands of loosely packed and bagged teas. In particular, Armoskaite *et al*. found that longer periods of extraction of green tea (30 min) led to higher quantities of phenolic compounds [2]. Nikniaz *et al*. reported similar results for black teas [10]. However, storing infusions for 24 h at room temperature had a varied effect on the content of these active substances. Increased polyphenol contents were detected in rooibos, yerba mate, red and white teas.

In our study, we found black and green tea samples with increased polyphenol contents (1 BL, 2 BL, 4 BL, 5 BL, 7 BL, 8 BL, 12 BL, 13 BE, 14 BL, 15 BL and

16 GL, 19 GL, 20 GE, 27 GE, 28 GL), while in the other groups, the content declined from 4 to 42%. Similarly, increased quercetin, flavonoids and total polyphenol contents were recorded along with prolonged tea brewing in Molan *et al*., Castiglioni *et al*., Fernando and Soysa, as well as Palanivel *et al*. [11, 17, 18, 28]. According to Armoskaite *et al*., flavonoids (and catechins, their fraction) are basic phenolic compounds in green tea responsible for antioxidant activity [2].

What is more, Saklar *et al*. reported that green tea catechins may be converted from epi forms to non-epi forms due to epimerization reactions at long brewing times [4]. Jin *et al*. proved that concentrations of epicatechins peaked at 10 min, after which they decreased drastically, while levels of non-epicatechins increased steadily for 5 h [5]. However, the antioxidant activity was more dependent on brewing temperature than brewing time. It is worth emphasizing that tea catechins can act as antioxidants by donating hydrogen atoms or by chelating metals, but epicatechins are known to be stronger than their corresponding non-epi isomers [9].

Further, we assessed the Fe ion reduction ability of the infusions brewed for 10 and 30 min (Fig. 2).

In the 45 teas analyzed, both a decline and an increase in antioxidant activity (particularly in red teas and yerba mate) were detected over the prolonged brewing time. These results were in line with Nikniaz *et al*. who reported higher values for bagged teas [10]. The most substantial differences were found among the black teas. In particular, infusions 1 BL, 2 BL, 3 BE, 4 BL, and 5 BL exhibited a decreased Fe ion reduction ability in a range of 4.2–26.6%, whereas the other infusions were characterized by an increased ferric reducing antioxidant power FRAP (from 1.4 to 44.5%). Increased antioxidant activity was detected in the infusions of the other tea types (except for green tea samples 23 GL, 25 GL, and 26 GL).

Previously published studies reported that the antioxidant capacity and total polyphenols in tea extracts correlated with the extraction time [10, 11, 19]. It is worth underlining that according to Armoskaite *et al*. and Pastoriza *et al*., the time of extraction and antioxidant activity may not always be in direct proportion: longer extraction time results in lower antioxidant activity in some green teas and higher in others [2, 6].

In our study, the FRAP antioxidant activity of the tea infusions stored for 24 h at room temperature varied in a non-uniform manner and the statistical analysis did not confirm the significance of the differences. The magnitude of a decrease or an increase in antioxidant activity was not correlated with the tea type: both were noted in the groups of the same tea types. On average, the black teas exhibited a 14.8% increase in the Fe ion reduction ability. However, some infusions from this group were characterized by a decreased Fe ion reduction ability (samples 10 BE, 13 BE, 14 BL, and 15 BL).

In the other tea groups, we noted a decline in antioxidant activity, particularly in the red and green teas. At the same time, each group comprised some samples with a Fe ion chelating ability that increased throughout storage (samples 16 GL, 17 GL, 18 GE, 19 GL, 20 GE, 23GL of the green teas, samples 31 WL and 32 WL of the white teas, sample 36 RE of the red teas, and samples 41 RoE, 42 RoE, and 43 YML of the teas other than *Camellia sinensis*). According to Komes *et al*., this variation might be due to a great abundance and variability of tea constituents that participate in various reactions during storage in the presence of oxygen, such as polymerization, or even degradation of some tea compounds [30]. Therefore, as indicated by Jayabalan *et al*., the qualitative and quantitative composition of tea infusions undergoes change over time with significant differences detectable only after several days of brewing [29].

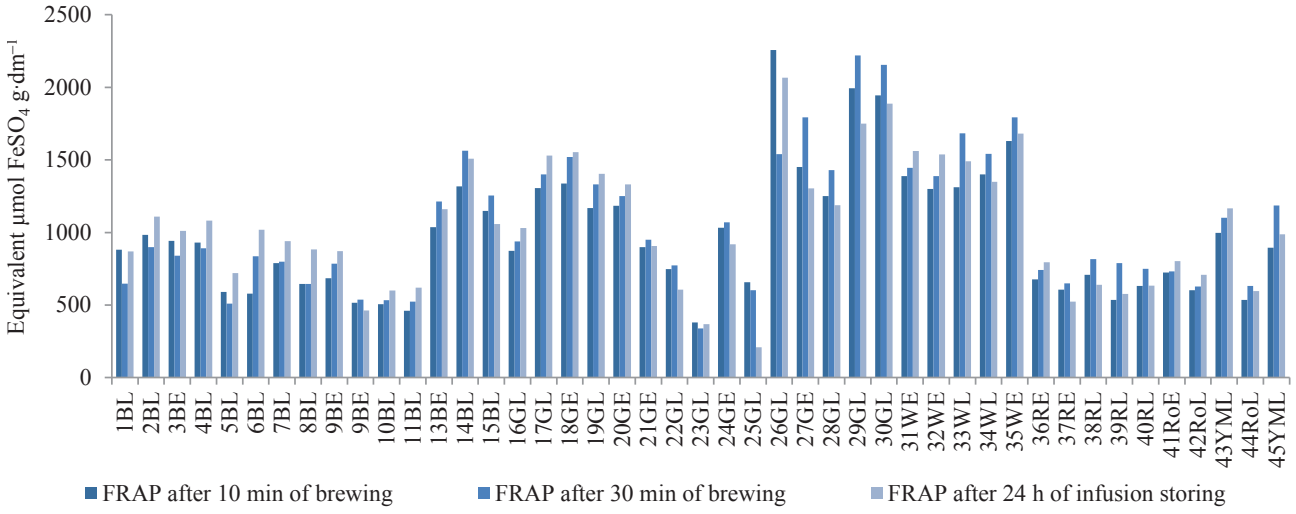

Figure 2 Changes in antioxidant activity of FRAP (Equivalent µmol $FeSO_4$ g·dm^{-1}) in the tested teas

CONCLUSION

Our experiment demonstrated a considerable diversity of teas available on the Polish market, contributing to the variability of active compounds and antioxidant activity of tea infusions. The teas under analysis were characterized by varied free radical scavenging abilities, and the amount of antioxidants depended primarily on the type of tea. The non-fermented teas (white and green) exhibited the highest antioxidant activity (measured with the FRAP and DPPH methods) and total polyphenol content. Weaker antioxidant properties and a lower content of polyphenols were detected in the black teas. The lowest values were found in rooibos and yerba mate, as well as in the red teas.

Our study showed a positive correlation between the polyphenol content and antioxidant activity. In addition, the DPPH antiradical performance of the examined extracts was comparable to their Fe ion chelating ability. The prolonged brewing time had a significant effect on the antioxidant activity of the infusions and polyphenolic compounds contained therein, which was not a linear correlation. Similarly, storage for 24 h at room temperature induced changes in the antioxidant activity of the infusions and altered their total polyphenol content.

The information contained in our study can be useful for tea consumers in their choice of tea, as well as preparation and storage methods that ensure the best pro-health properties. In addition, the substantial differences in the content of active compounds and antioxidant properties of the examined tea types manufactured by various producers suggest a need for establishing appropriate technological parameters, quality requirements, and systematic control of their composition and properties. Given the fact that there are no standards for teas other than black in Europe, the quality of all types of tea traded on the Polish market should be standardized to ensure similar quality of products and fair market competition.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Methodology for identification and quantification of chicken meat in food products

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Abstract:
Introduction. The problem of food adulteration is highly relevant today. Food manufacturers are increasingly replacing expensive raw materials with cheaper poultry. We aimed to develop an effective method for identification and quantification of chicken meat and egg products in multicomponent meat systems using real-time PCR.
Study objects and methods. We studied native animal tissue, namely that of chicken, pork, beef, turkey, quail, duck, horse meat, rabbit, sheep, and goat. Standard samples were taken from pure fresh chicken muscle tissue. We also used raw, boiled, and powdered chicken eggs. For a semiquantitative analysis of chicken mass in the sample, we compared the threshold cycle (C_t) of chicken DNA and the threshold cycles of calibration samples. To ensure the absence of PCR inhibition, we used an internal control sample which went through all the stages of analysis, starting with DNA extraction.
Results and discussion. We developed a methodology to qualitatively determine the content of chicken tissue in the product and distinguish between the presence of egg products and contamination on the production line. The method for chicken DNA identification showed 100% specificity. This genetic material was detected in the range of 0.1% to 0.01% of chicken meat in the sample. The efficiency of the duplex PCR system for chicken DNA detection was more than 95% (3.38 on the Green slope channel and 3.45 on the Yellow slope channel). The analytical sensitivity of the primers was 40 copies/reaction.
Conclusion. Our methodology is suitable for analyzing multicomponent food products, raw materials, feed, and feed additives. It can identify the content of chicken meat at a concentration of up to 1%, as well as distinguish egg impurities from contamination of various origin. PCR allows differentiation between chicken meat and egg products.

Keywords: Multicomponent products, canned food, chicken meat, egg melange, PCR, adulteration, sausages

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INTRODUCTION

The Russian Federation strategy to improve the quality of food products until 2030 prioritizes research in the field of quality management.

Today, the problem of food adulteration is of particular concern. Food manufacturers are increasingly replacing expensive raw materials, such as good quality beef, with cheaper poultry. According to the public report "Consumer Protection in the Russian Federation in 2017", Rospotrebnadzor (Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing) detected 3410 adulterated products out of 310 000 inspected food samples [1]. In 2018, the volumes of rejected meat, poultry, and their products doubled compared to 2017. In particular, Rospotrebnadzor rejected 519 batches of meat and meat products weighing 3509 kg (compared to 459 batches of 1685 kg in 2017) and 168 batches of poultry, eggs, and their products weighing 1951 kg (compared to 159 batches of 975 kg in 2017).

Species identification of meat and meat products is becoming more important due to increased international trade and labeling rules introduced in many countries. Morphological and anatomical characteristics are used to identify fresh and unprocessed meat. However, processed meat loses its characteristic morphological features, which creates favorable conditions for adulteration, namely for replacing one type of meat with another, less valuable type. Poultry – a cheaper raw material compared to pork, beef or other meats – is often used to adulterate products, both semi-finished and finished. Especially difficult is species identification of multicomponent products containing several types of meat, egg impurities, various food additives, enzyme preparations, as well as products subjected to rigorous mechanical or thermal processing, such as canned foods and pastes [2–7]. According to Rospotrebnadzor, most violations of the technical standards in 2018 were detected in canned meat and sausages [1].

At the moment, the Russian Federation has no method for quantifying the content of chicken and/or egg melange in food products and isolating possible contamination on the production line.

Scientific literature reports numerous methods for qualitative identification of meat species [8–11][I]. A group of scientists from Gorbatov's Federal Scientific Center for Food Systems and the National Center for Fishing Products Safety attempted to identify egg melange at the 30th PCR cycle [12, 13]. However, there were no data on the quantitative identification of impurities [14, 15]. Therefore, we need to develop a quantitative method for identifying ingredients in the analyzed products to prevent producers from replacing a specified content of meat with cheaper raw materials and to distinguish between adulteration and inevitable contamination in production [16–20].

The highly sensitive PCR method can reveal even trace amounts of meat ingredients, which are essentially technical impurities. However, in order to distinguish a minor technical impurity from intentional adulteration, we need a methodology for a quantitative or semi-quantitative evaluation of meat, for example, chicken, in food products [20–38].

Therefore, we aimed to develop an effective method for identification and quantification of chicken meat and egg products in multicomponent meat systems using the real-time PCR.

STUDY OBJECTS AND METHODS

Our objects of study included native animal tissue purchased in retail chain stores (chicken, pork, beef, turkey, quail, duck, horse meat, rabbit, sheep, and goat) or obtained at the Russian State Center for Animal Feed and Drug Standardization and Quality, Moscow (mink, cat, and dog). Pure fresh chicken muscle tissue was used as standard samples. The species identity of all the materials was confirmed by the Sanger DNA sequencing method based on the standard CytB gene [3]. In addition, we used raw, boiled, and powdered chicken eggs.

We used only certified equipment, materials, reagents, and utensils.

The tests were conducted using the following methods:

– taking laboratory samples from different product groups (State Standard 31904-2012[II]);

– adsorption DNA extraction based on silicon dioxide (State Standard R 56140-2014[III]);

– guanidine-chloroform-based DNA extraction (State Standard R ISO 21571-2014[IV]). This method can purify DNA from fatty and protein impurities, reduce the inhibition of the reaction, and eliminate the influence of food additives on the final result (it also works well with egg impurities);

– real-time polymerase chain reaction with hybridization-fluorescence detection (State Standard ISO 22119-2013[V]);

– evaluation of metrological characteristics of measurement procedures (RIS 61-2010[VI]);

– certification of measurement procedures (State Standard R 8.563-2009[VII]).

When sampling and preparing test samples, we took measures to prevent the seeding of environmental objects in line with State Standard 8756.0-70[VIII] and State Standard 31719-2012[IX]. The samples were homogenized and 0.05 g weighed, placed in a 1.5 cm Eppendorf type disposable microcentrifuge tube, labeled, and used to isolate DNA.

Three sets of samples were prepared in duplicate. The first set was not subjected to heat treatment. The samples of the second set were mixed with 100 mm^3

[I] MU A 1/022 Sekvenirovanie fragmentov mitokhondrial′nogo genoma zhivotnykh i ryb dlya opredeleniya vidovoy prinadlezhnosti myasa v odnokomponentnoy produktsii [MU A 1/022 Sequencing fragments of the mitochondrial genome of animals and fish to determine meat species in mono-component products].

[II] State Standard 31904-2012. Food products. Methods of sampling for microbiological analyses. Moscow: Standartinform; 2014. 8 p.

[III] State Standard R 56140-2014. Medicine biological remedies for veterinary use. Polymerase chain reaction for the *Mycoplasma* DNA detection. Moscow: Standartinform; 2015. 12 p.

[IV] State Standard R ISO 21571-2014. Foodstuffs. Methods of analysis for the detection of genetically modified organisms and derived products. Nucleic acid extraction. Moscow: Standartinform; 2016. 46 p.

[V] State Standard ISO 22119-2013. Microbiology of food and animal feeding stuffs. Real-time polymerase chain reaction (PCR) for the detection of food-borne pathogens. General requirements and definitions. Moscow: Standartinform; 2014. 15 p.

[VI] RIS 61-2010. State system for ensuring the uniformity of measurements. Accuracy, trueness and precision measures of the procedures for quantitative chemical analysis. Methods of evaluation. Moscow: Standartinform; 2013. 62 p.

[VII] State Standard R 8.563-2009. State system for ensuring the uniformity of measurements. Procedures of measurements. Moscow: Standartinform; 2011. 20 p.

[VIII] State Standard 8756.0-70. Canned food products. Sampling and preparation of samples for test. Moscow: Standartinform; 2010. 8 p.

[IX] State Standard 31719-2012. Foodstuffs and feed. Rapid method of identification of raw composition (molecular). Moscow: Standartinform; 2014. 24 p.

of water and heated at 99°C on a Termite solid-state thermostat (DNA-Technology, Russia) for 30 min. The third set was sampled in quadruplicate and autoclaved at 110°C and 0.5 atm. for 30 min and an hour, respectively. For the purity of the experiment, we used chicken muscle tissue (breast fillet and drumstick), parenchymal and hollow internal organs (kidney, heart, liver), skin and cartilage, as well as minced pork meat containing 1% and 10% chicken.

Since chicken eggs are widely used in the food industry, we had to determine their effect on the PCR results. For this, we analyzed raw, boiled and powdered eggs, as well as pancake flour. In addition, we investigated 20% egg in minced pork, 10% raw egg in water, and 10% egg in minced chicken. A model panel was made from the above samples.

To eliminate the likelihood of PCR inhibition, we used an internal control sample (ICS) which was added to each test sample starting from the DNA extraction stage.

DNA was extracted by the sorbent method recommended by State Standard R 52723-2007[x], using a standard set of DNA-Sorb-S reagents (Central Research Institute of Epidemiology, Russia). A number of experiments performed with the extracted DNA showed that a 100% chicken content (whether fillet, hollow and parenchymal internal organs or connective tissue) produced a threshold cycle (C_t) ≤ 15, whereas 10% and 1% chicken contents in minced meat produced $C_t \leq 18$ and $C_t \leq 21$, respectively. There is a correlation with the ICS detection. When egg is present, the values decrease to $C_t \geq 23$ and the ICS also drops to $C_t \geq 28$ due to inhibition ($C_t \geq 24$ with no inhibitors). DNA is obviously less degraded in a pure product (raw and boiled egg) than in egg powder, but C_t is inversely related: $C_t \geq 27$ and $C_t \geq 20$ for the egg powder sample and the ICS, respectively; $C_t \geq 30$ and $C_t \geq 28$ for the raw and boiled egg sample and the ICS, respectively.

Thus, we can conclude that raw and boiled eggs contain PCR-inhibiting substances. The presence of 10% raw eggs in minced chicken leads to ICS $C_t \geq 27$ versus ICS $C_t \leq 21$ for 100% minced chicken. It is impossible to evaluate the results when the reaction is so strongly inhibited. Therefore, we chose a different DNA extraction method described by Minaev *et al.* [2]. For this, we used a SORB-GMO-B kit (Syntol, Russia) in accordance with the manufacturer's recommendations. The PCR results are shown in Table 1. As we can see, the ICS threshold cycle values indicate insignificant inhibition of the reaction, confirming the right choice of the DNA isolation method.

We selected those primers and probes that fluoresce to the target DNA of chicken and the ICS in the Green and Yellow channels. The solutions of direct and reverse PCR primers and a probe at a known molar concentration were diluted to a working molar concentration of 6 μmol/dm^3 and 3 μmol/dm^3, respectively. For PCR, we used a dNTF solution (Syntol, Russia), a PCR buffer-Flu and TaqF DNA polymerase (Central Research Institute of Epidemiology, Russia).

The DNA extracted from each test sample was analyzed in at least two replicates. For amplification control reactions, we used recombinant plasmids based on the pAL-2 vector (solutions of plasmid DNA at a concentration of 0.01 mg/dm^3) as positive reaction controls. They were a plasmid containing a chicken DNA fragment (pCh) and a plasmid of the internal control sample (pICS).

For real-time PCR, we used Rotor-Gene Q amplifiers (QIAGEN, Germany) and Rotor-Gene 6000 amplifiers (Corbett Research Pty Ltd., Australia). We programmed the device according to the operating instructions and optimized the PCR-RT conditions for the duplex format. The primer annealing temperature was 60°C, with a PCR total temperature profile of 40 cycles.

RESULTS AND DISCUSSION

The PCR results for the model meat systems before and after heat treatment (at various temperatures) are presented in Table 1. The Background Threshold was set at 15% and the Threshold was 0.05. We interpreted the results based on the presence (or absence) of the intersection between the fluorescence curve and a threshold line set at an appropriate level. The conditions for analysis were as follows: for a positive PCR control, the threshold cycle values of $C_t < 26$ were present in the Green and Yellow channels; for a negative extraction control and a negative PCR control, the threshold cycle values were absent in all the channels; the threshold cycle value for the ICS was not lower than $C_t \leq 24$ for qualitative determination, since higher values indicate PCR inhibition.

As we can see in Table 1, all the raw samples containing meat or offal (including extremely low concentrations) were identified at no later than the 19th cycle; egg impurities, no earlier than the 25th cycle; and egg powder and pancake flour, at the 29–30th cycle. Interestingly, pure chicken meat, whether fillet or offal, was identified at no later than the 14th cycle, while connective tissue, no later than the 17th cycle. The chicken contents of 10% and 1% produced $C_t \leq 15$ and $C_t \leq 19$, respectively. These results allowed us to conclude that:

– $C_t < 15$ indicated over 10% chicken in the test sample;

– $C_t < 19$ indicated over 1% chicken or high concentrations of connective tissue in the test sample.

This conclusion makes it impossible to quantify the chicken content at this stage of the study. However, it leaves a possibility of a semi-quantitative analysis, whose result can be expressed as "chicken content at least N%".

[x] State Standard R 52723-2007. Foodstuffs and feeds. Rapid method of identification of raw composition (molecular). Moscow: Standartinform; 2007. 22 p.

Table 1 PCR results for model samples

Product	Weight content, %	Threshold cycle of the model sample (chicken meat content)		
		Not heat-treated	99°C, 30 min	110°C, 0.5 atm., 1 h
Minced breast	100	12.67	13.54	16.26
Minced drumstick	100	12.54	13.39	14.62
Minced liver	100	11.92	13.01	15.52
Minced kidneys	100	12.15	13.83	16.01
Minced heart	100	11.64	13.88	15.04
Cartilage	100	14.97	16.81	19.23
Skin	100	16.04	18.16	22.08
Minced chicken breast and pork	10	14.26	16.44	20.34
Minced chicken breast and pork	1	18.26	20.16	25.72
Liquid egg	100	24.29	26.89	29.05
Liquid egg	10	33.40	32.99	–
Minced pork and egg	20% egg	22.00	22.81	25.91
Minced chicken breast and egg	10% egg	13.29	14.91	16.91
Chicken ovalbumin (egg powder)	100	27.65	29.34	–
Pancake flour	4%*	28.95	30.06	–

* The average amount of egg powder in 12 formulations

The heat-treated samples containing meat or offal (including extremely low concentrations, up to 1%) were identified at no later than the 21st cycle and egg impurities, no earlier than the 21st cycle. A 10% chicken content in minced meat produced $C_t \leq 17$, whereas 1% chicken showed $C_t \leq 21$. From these results, we concluded that $C_t < 21$ indicated more than 1% chicken in the test sample.

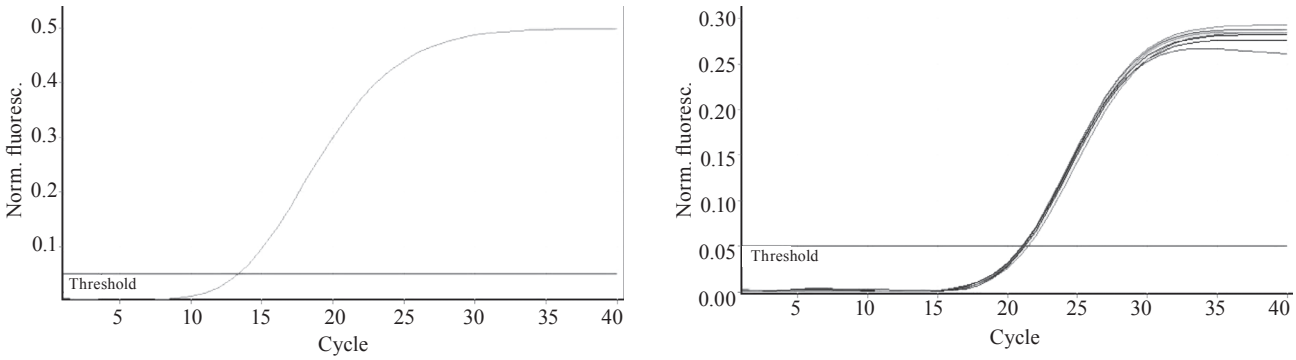
The autoclaved samples containing chicken meat or offal were identified at no later than the 17th cycle, whereas the samples with extremely low concentrations of chicken meat (up to 1%) and egg impurities, no later than the 26th cycle. The chicken contents of 10% and 1% resulted in $C_t \leq 21$ and $C_t \leq 25$, respectively. Thus, the detection of $C_t < 25$ indicated over 1% chicken in the test sample.

Next, we proceeded to the development of a semi-quantitative method for determining chicken meat in food products, since a quantitative method was not possible due to the equality of cycles for the 10% minced chicken samples and the connective tissue samples. As adulterating a product with less than 1% meat (1 g chicken meat per 1 kg of product) seems impractical, we decided that the methodology should allow us to determine the content of chicken in the product in relation to several threshold values of calibration samples, namely:

– "at least 1%" if C_t 10% < sample's $C_t \leq C_t$ 1%;
– "at least 10%" if C_t 50% < sample's $C_t \leq C_t$ 10%;
– "high content" if the sample's $C_t \leq C_t$ 50%;
– "low DNA, possible egg presence" if the sample's $C_t > C_t$ 1%.

Further, we evaluated the following criteria: sensitivity and specificity of the primers, detection limits, and a range of values for calibration samples and internal control samples. Each experiment was performed by two different researchers, at different times, with reagents of different series, on different amplifiers of the same type. Each sample was tested in duplicate.

To assess the specificity of PCR, we created a panel of DNA samples isolated from chicken, pork, beef,



(a) Chicken meat DNA, Yellow

(b) ICS, Green

Figure 1 Specificity assessment of the chicken identification methodology

Table 2 Specificity assessment of the duplex PCR system for chicken identification

Expected amplification result		Actual amplification result, threshold cycle values, C_t ± SD				Name of sample
		Replicate № 1		Replicate № 2		
FAM, ICS	Yellow, chicken	FAM, ICS	Yellow, chicken	FAM, ICS	Yellow, chicken	
+	+	21.66 ± 0.05	12.76 ± 0.18	21.61 ± 0.10	13.46 ± 0.01	chicken
+	–	+	–	+	–	pork
+	–	+	–	+	–	beef
+	–	+	–	+	–	goat
+	–	+	–	+	–	mink
+	–	+	–	+	–	turkey
+	–	+	–	+	–	quail
+	–	+	–	+	–	duck
+	–	+	–	+	–	horse
+	–	+	–	+	–	rabbit
+	–	+	–	+	–	cat
+	–	+	–	+	–	dog
+	–	+	–	+	–	sheep
+	–	+	–	+	–	Ci*
–	–	–	–	–	–	–C**

* Ci – isolation control (shows the absence of inhibition at the stage of DNA isolation)
** –C – negative PCR control (shows the purity of the reaction, mixes, and the laminar, as well as the absence of contamination)

turkey, quail, duck, horse, mink, rabbit, cat, dog, goat, and sheep. The results are shown in Fig. 1 and Table 2.

Within the proposed panel, the chicken DNA identification methodology showed 100% specificity: we observed the ICS amplification only on the Green channel and the target chicken DNA on the Yellow channel.

The assessment of the control panel for validation confirmed a 100% convergence of the results.

To determine the analytical sensitivity of the primers, we isolated DNA from a sample of 100% chicken meat and prepared a series of 10-fold dilutions. The maximum dilution was determined which allowed reproducible (in duplicate) detection of DNA.

In addition, we used plasmid DNA solutions at a specified concentration containing a cloned chicken gene fragment and a ICS fragment. Two series of ten-fold dilutions were prepared in a TE buffer with various concentrations: series № 1 – pICS plasmid DNA solution; series № 2 – pCh plasmid DNA solution. The initial concentration of plasmid DNA in each series was 4 ng/μL, which corresponds to ~ 20 000 genomic copies in PCR (5 μL of a DNA solution for a 25 μL reaction). The results are presented in Table 3.

To determine the absolute limit of detection (LOD) at which the PCR method is able to detect and quantify chicken genetic material, we performed 10 PCRs, with 5, 10, 20, and 40 genomic copies of chicken DNA in each. Our PCR methodology detected chicken even in the strongest dilution, with only five genomic copies in the PCR.

To determine the limit of detection of chicken and egg products in multicomponent raw and heat-treated products, we used a number of model samples prepared in two replicates and containing 10, 1.0, 0.1, 0.01, and 0.001% chicken in minced pork (isolated DNA). The samples were preliminarily cooked at 99°C for 30 min. To determine the LOD of chicken and egg products in canned foods, the model samples were autoclaved at 110°C and 0.5 atm. The minimum chicken content in minced pork was determined, at which chicken DNA was reproducibly (in duplicate) detected. The results are shown in Table 4.

Table 3 Sensitivity of the duplex PCR system (initial concentration of plasmid DNA – 4 ng/μL)

Number of genomic copies in the reaction	C_t ± SD, Yellow (chicken)	C_t ± SD, Green (ICS)
20 000	23.16 ± 0.10	24.41 ± 0.15
2 000	26.87 ± 0.10	28.19 ± 0.05
200	30.58 ± 0.56	32.01 ± 0.18
20	34.00 ± 0.79	35.29 ± 1.07
2	–	–

Table 4 PCR results for LOD determination

Chicken DNA content, %	Threshold cycle C_t ± SD for raw and cooked products		Threshold cycle C_t ± SD	
	Chicken (Yellow)	ICS (Green)	Chicken (Yellow)	ICS (Green)
10	16.06 ± 0.11	23.15 ± 0.03	24.03 ± 0.12	23.15 ± 0.04
1.0	19.16 ± 0.03	23.18 ± 0.20	26.15 ± 0.02	23.18 ± 0.19
0.1	21.84 ± 0.28	23.02 ± 0.01	28.73 ± 0.29	23.02 ± 0.02
0.01	24.56 ± 0.01	23.25 ± 0.03	30.35 ± 0.02	23.25 ± 0.02
0.001	26.56 ± 0.23	22.29 ± 0.03	32.46 ± 0.19	22.29 ± 0.04

Table 5 Constancy of C_t ranges for calibration samples

Series	Calibration sample's C_t											
	Cooked for 30 min at 99°C						Autoclaved for 60 min at 0.5 atm					
	1%		10%		50%		1%		10%		50%	
1	18.97	18.68	16.08	16.21	13.85	13.91	27.92	27.89	24.05	23.99	19.74	19.85
2	19.63	19.70	15.84	16.02	11.78	12.13	25.34	25.40	24.63	24.52	20.04	20.21
3	19.34	19.55	17.04	17.15	13.81	13.89	27.83	27.95	22.16	22.03	19.10	19.03
4	19.22	19.43	17.54	17.66	13.77	13.98	26.45	26.53	22.25	22.15	20.93	20.84
5	19.93	20.15	14.50	14.37	12.88	13.00	27.27	27.17	23.69	23.45	21.52	21.68
6	18.29	18.52	16.32	16.45	12.70	12.96	25.77	25.89	24.67	24.77	19.66	19.76
7	18.04	18.19	15.52	15.63	13.84	13.79	25.05	25.30	22.16	22.26	19.76	19.82
8	19.71	19.59	16.96	16.67	13.55	13.75	25.58	25.41	22.25	22.05	19.34	19.11
9	19.94	20.17	14.43	14.87	12.67	12.56	26.90	26.99	23.70	23.84	20.81	20.94
10	20.40	20.62	14.03	14.25	13.02	13.17	27.60	27.52	24.71	24.66	21.70	21.64
11	18.01	18.14	16.47	16.44	11.99	12.21	25.53	25.64	22.86	23.02	21.45	21.15
12	20.78	20.84	15.33	15.66	12.40	12.23	26.15	26.45	24.35	24.23	20.93	21.03
13	20.96	20.86	17.83	17.64	12.56	12.71	25.74	26.03	22.88	23.09	21.63	21.72
14	20.85	20.91	17.98	17.83	12.83	13.01	27.14	27.33	24.64	24.98	21.12	21.23
15	20.21	20.16	15.25	15.44	13.34	13.43	26.59	26.84	24.10	24.28	21.84	21.92
Maximum, C_t	20.96	20.91	17.98	17.83	13.85	13.98	27.92	27.89	24.71	24.98	21.84	21.92
Minimum, C_t	18.01	18.14	14.03	14.25	11.78	12.13	25.05	25.30	22.16	22.03	19.10	19.03
SD	0.94	0.93	1.20	1.10	0.65	0.63	0.92	0.88	0.98	1.02	0.91	0.93
RSD	4.81	4.70	7.49	6.82	5.02	4.84	3.47	3.33	4.16	4.34	4.40	4.50

* SD – standard deviation, RSD – relative standard deviation

The limit of detection for chicken DNA ranged from 0.1 to 0.001% of the chicken content in the sample.

The methodology should allow us to assess the content of chicken and egg products in food products relative to several selected threshold values of calibration samples. To prepare calibration samples of various compositions for the semi-quantitative analysis of raw and cooked products, we mixed 100% minced chicken meat with 100% minced pork (1%, 10%, and 50% chicken) and heated at 99°C for 30 min.

We decided to evaluate both cooked and raw products in relation to the values of heat-treated calibrators, since fresh chicken meat was used to prepare model samples of raw products, which cannot be guaranteed by product manufacturers. Moreover, samples for analysis do not always get delivered to the laboratory directly, bypassing the stages of storage or freezing, which increases the likelihood of DNA degradation. The calibration samples for canned products were autoclaved at 110°C and 0.5 atm. The uniformity coefficient of the calibrators was 0.99 (99%).

To confirm the constancy of the calibrators' C_t ranges, we performed a series of tests. In particular, we studied 15 series of calibration samples prepared on different days, by different people, each in two replicates. For each series, we determined the minimum and maximum values of the threshold cycle on the Yellow-chicken channel, a standard deviation, and a relative standard deviation. The results are presented in Table 5.

As a result, we selected the following threshold cycle values on the "Yellow-Chicken DNA" channel for the calibrators of:

– raw products and those subjected to light heat treatment: $18 \le C_t$ 1% < 21; $14 \le C_t$ 10% < 18; C_t 50% < 14;

– autoclaved products (canned food): $25 \le C_t$ 1% < 28; $22 \le C_t$ 10% < 25; C_t 50% < 22.

Also, a threshold cycle value of at least $C_t \le 24$ was chosen as acceptable on the "Green-ICS" channel for the calibrators (C_{tics} 1%, C_{tics} 10%, C_{tics} 50%) and the negative control sample.

CONCLUSION

We developed a method (certified methodology) for a semi-quantitative assessment of chicken content in multicomponent food systems of varying degrees of heat and mechanical treatment: raw, heat-treated, canned, finely ground, and homogenized. Having tested various DNA extraction methods, we concluded that the guanidine-chloroform method reduces the content of PCR-inhibiting substances compared to the sorption method.

Our methodology was tested on model samples, as well as product samples from retail stores, to exclude the possibility of PCR inhibition by food additives, stabilizers, emulsifiers, etc. With PCR, we can distinguish between chicken meat and egg products in raw and cooked products (over 21 cycles), as well as canned foods (over 28 cycles). Our results suggest that this methodology is suitable for analyzing

multicomponent food products, raw materials, feeds, and feed additives. In addition, it can identify the content of chicken meat at a concentration of up to 1%, as well as detect egg impurities and contamination of various origins.

Taking into account the current need for distinguishing adulteration from the inevitable contamination on the production line, as well as preventing adulteration of expensive raw materials with chicken meat by introducing egg products, we believe that our methodology could make a significant contribution to the production of high-quality foods.

CONTRIBUTION

Each of the authors was directly involved in the development, testing, and validation of the above methodology, as well as in writing this article.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Volatile N-nitrosamine, residual nitrite, and ascorbic acid levels in sausages during storage

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Abstract:

Introduction. The increasing global consumption of processed meat products has led to certain concerns. For instance, processed meat products are known to contain carcinogen precursor compounds, thus creating the risk of chronic diseases. The present study was performed to estimate the food safety status of processed meat products available in Iran and evaluate the related effective factors.

Study objects and methods. 140 samples of seven most popular commercial types of cooked sausages were obtained from four major meat factories (A, B, C and D) in 140 samples were collected from seven most popular commercial types of cooked sausages as follows: beef salami 90%, chicken salami 90%, dry cured sausage 70%, dry cured salami 60%, beef sausages 55%, chicken sausages 55% and Frankfurt sausage 40% (n = 5) from four major meat factories (A, B, C and D) in Tehran. The samples were screened for residual nitrite, ascorbic acid, and nitrosamine contents on days 0, 7, 14, 21, and 28. The results indicated that products from meat factory B had lower residual nitrite content in the samples with high content of meat. Beef salami (90% of meat) and Frankfurt sausage (40% of meat) contained the lowest and highest amounts of residual nitrite on day 0 – 73.99 and 177.42 mg of nitrite per 1 kg of meat, respectively.

Results and discussion. Beef salami contained 90% of meat, chicken salami – 90%, dry cured sausage –70%, dry cured salami – 60%, beef sausages – 55%, chicken sausages – 55%, and Frankfurt sausage – 40% (n = 5). Nitrite reduction rates in sausages with a smaller diameter, e.g. Frankfurt sausage, were significantly lower ($P < 0.05$), compared to salami samples. The difference can be explained by the shorter cooking time. Nitrosamine formation increased during refrigerated storage; however, it was not significant in all samples. During refrigerated storage, nitrosamine formation depended on the level of added nitrite, the amount of residual nitrite, ascorbic acid, pH, and cooking temperature. Ascorbic acid content decreased significantly ($P < 0.05$) during refrigerated storage.

Conclusion. The findings demonstrate significant correlation between the meat content, cooking time, nitrite content, and nitrosamine formation.

Keywords: Meat industry, processed meat, meat products safety, carcinogenic agents, preservation

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INTRODUCTION

To reduce the adverse effects of red meat and artificial additives on human health, one can reduce the consumption of red meat or processed meat products. However, studying possible ways to reduce the harmful effects might be as effective as limiting consumption levels. Nitrite/nitrate salts are usually added to meat products to guarantee their safety, since these salts inhibit the growth of *Clostridium botulinum* and prevent heat-resistant spores from producing toxins. Moreover, nitrite/nitrate salts improve the color, flavor, and aroma of the finished product and postpone lipid oxidation processes [1].

In spite of the numerous advantages, an excessive intake of nitrite can produce adverse effects on human health. Nitrite can be transformed to a nitrosating agent (NO+), which reacts with biogenic amines and creates carcinogenic N-nitrosamines. Bacterial and meat

enzymes cause decarboxylation of free amino acids, which leads to the formation of biogenic amines. Several intrinsic and extrinsic factors, e.g. salt content, can also affect the biogenic amine formation level [2–4].

In meat and meat products, carcinogenic N-nitrosamines are usually formed under acidic conditions caused by the electrophilic reaction between a nitrosating agent, e.g. nitrite or nitrous acid, and secondary/tertiary amines that result from protein and lipid degradation [5]. The mechanism of nitrosamine formation in meat products has been thoroughly studied. Nitrosamines with generic chemical structure of $R_2NN = O$ are produced under certain conditions of low pH, high temperature, and presence of some reducing agents associated with processing and composition of a particular meat product [6]. There are several hurdle technologies that can reduce the concentration of nitrite required to inhibit bacterial growth, e.g. ascorbic/erythorbic (isoascorbic) acid and essential oils, certain processing conditions, or various non-thermal methods [1, 7]. Exposure level of N-nitrosodimethylamine (NDMA) compound via consumption of food and beverages was estimated to be 0.09 and 0.1 μg/day in the Netherlands and Germany, respectively [8, 9]. The level of these compounds in nitrite-preserved meat products varies significantly. It depends on the ingoing volume of nitrite, meat quality, and fat content, as well as on processing, ripening, and storing conditions. There have been many reports on nitrosamines detected in processed meat products [10, 11].

The research objective was to examine the safety of emulsion-type cooked sausages available on the Iran markets by assessing the contents of residual nitrite, ascorbic acid, and nitrosamine. In addition, we also studied the effects of processing conditions and related factors on these compounds to provide information for health professionals and food manufacturers.

STUDY OBJECTS AND METHODS

Samples. The samples were collected from four major meat factories in Tehran (A, B, C, and D) out of the total of 189 meat factories in Iran. Seven most popular commercial types of cooked sausages were randomly purchased. Seven most popular commercial types of cooked sausages were randomly purchased as follows: beef salami 90%, chicken salami 90%, dry cured sausage 70%, dry cured salami 60%, beef sausages 55%, chicken sausages 55% and Frankfurt sausage 40%.

Five samples (2 kg) from each type were examined on days 0, 7, 14, 21, and 28 of storage. The samples contained beef (15% fat) orchicken (10% fat), water, oil, sodium caseinate, sodium polyphosphate, garlic, salt, wheat flour starch, spices, gluten, natural flavorings (paprika, curcumin, ginger, and cinnamon), ascorbic acid, and sodium nitrite. The samples were immediately transferred to the laboratory and kept refrigerated until tested.

Residual nitrite determination. The nitrite content was evaluated during 28 days on days 0, 7, 14, 21, and 28 using slightly modified calorimetric method of AOAC method no. 973.31 [12]. The samples were cut into pieces and homogenized. An aliquot of about 2.5 g of minced sausage was added to 5 mL of saturated borax and 25 mL of deionized water (> 70°C) in Falcon tubes. After stirring and cooling in room temperature, 1 mL of each Carrez solution (I and II) was added to each sample and adjusted to the volume of 50 mL. Carrez solution I was prepared by dissolving 10.6 g of ferrocyanide in distilled water (100 mL) according to the method introduced by Ramezani *et al*. [13]. Carrez solution II was also made by mixing 21.9 g of zinc acetate with acetic acid (3 mL) and adjusted to the volume of 100 mL using distilled water. After centrifugation at 4000 rpm for 5 min (HeltichRotorfix 32A), 25 mL of supernatant was transferred to a 100 mL tube. Ten ml of sulfanilamide reagent and 6 mL of dilute HCl were added to the supernatants and kept in the dark for 5 min. Then, 2 mL of naphtyl-ethylenadiamine solution was added to obtain high intensity azo dyes. The samples were kept in the dark for 10 min to achieve complete reactions. Absorbance was measured at 538 nm.

Ascorbic acid determination. To determine the ascorbic acid content, 2 g of minced sausage was mixed with 10 mL of meta-phosphoric acid solution (3%), tertiary butylhydroquinone (TBHQ) (0.1%), and acetic acid (8%). After that, they were centrifuged at 4000 rpm for 10 min. After filtration, 20 μL of supernatant solution was injected to a Cecil CE-4900 high-performance liquid chromatograph coupled to a UV-vis detector (HPLC-UV/VIS, Cambridge, England). The analytical HPLC was equipped with two CE-4100 pumps, vacuum degasser, six port valves (Rheodyne, USA), mixing chamber, multiple solvent delivery unit, and an ODS column (250 mm·4 I.D., 5 μm). The mobile phase consisted of acetonitrile and sodium phosphate buffer solution (50:50). The flow rate was 1.2 $mL \cdot min^{-1}$ at room temperature [14].

N-nitrosamine determination. The experiment evaluated seven volatile nitrosamines in the popular cooked sausages from four major Iran meat factories. The nitrosamines included N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitroso-morpholine (NMOR), N-nitrosopyrrolidine (NPYR), N-nitrosopiperidine (NPIP), N-nitrosodi-n-butylamine (NDBA), and N-nitrosodiphenylamine (NDPheA). The presence of nitrosamine was determined and quantified according to the method described in our previous study [13]. The method presupposed using microwave-assisted extraction coupled with dispersive liquid–liquid micro extraction (DLLME) followed by gas chromatography–mass spectrometry (GC-MS).

Statistical analysis. All experiments were carried out in triplicate. One-way ANOVA was performed to determine significant differences. Duncan's multiple

range test was used to define the differences of mean value. Data analysis was performed using SPSS version 21 (SPSS Inc., Chicago, IL, USA); $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The present research featured samples from seven most popular types of sausages in Iran. The selected sausages differed in the amount of nitrite, ascorbic acid, and nitrosamine. This difference is associated with the variations in concentration of added nitrate and nitrite salts (sodium and potassium), storage conditions, and different pH values [15, 16]. Following the EU legislations, 2006/52/EC directive limited the usage of nitrite to 150 mg per 1 kg of meat. However, the national laws in Iran are more restricting. Iranian provisions allow for maximum 120 mg of nitrite per 1 kg of meat (ppm) in sausages [17]. Table 1 displays the residual nitrite, ascorbic acid, and nitrosamine contents in the meat samples under study during storage.

Figure 1 illustrates the results of nitrite content changes (mg/kg meat) in the meat samples obtained from factory B as representative of all the four meat factories.

As it was expected, the amount of nitrite residue in meat products of all four meat factories decreased during 28 days of refrigerated storage. The increase in meat content increased the reduction rate of sodium nitrite in the products. In other words, lower residual nitrite content was detected in high content meat samples. The highest reduction rate of nitrite content was observed in chicken and beef salami (90% of meat). On the first day of refrigerated storage, the Frankfurt sausage (40%) and both beef and chicken sausages (55%) contained 80 μg/kg of nitrite.

The detected amount exceeded the level permitted by Iranian laws as defined by the Institute of Standard and Industrial Research of Iran (ISIRI). The maximum permissible burden of nitrite is 66 and 63 μg/kg for processed meat products with 40% and 55% of meat content, respectively[1]. The concentration of nitrite was higher than the expected levels even after 28 days of storage. However, the residual nitrite content was not exactly equal to the initial added nitrite. First, it was partly degraded by heating process. Second, it decreased when ascorbic acid was applied to the meat product during the heating process to accelerate conversion of nitrite to nitric oxide.

The nitrite content varied due to interaction with heme-containing components, non-heme proteins, and fat tissues, conversion to nitrate, production of such gases as N_2, CO_2, and NO, and nitrosamines [18]. Therefore, residual nitrite was associated with meat content due to different contents of myoglobin [19]. As a reactive agent, nitrite converts to nitrite oxide and forms

[1] ISIRI 932. Test method for determination of nitrite in meat and meat products (reference method). Iran: Iran Institute of Standards and Industrial Research; 2014.

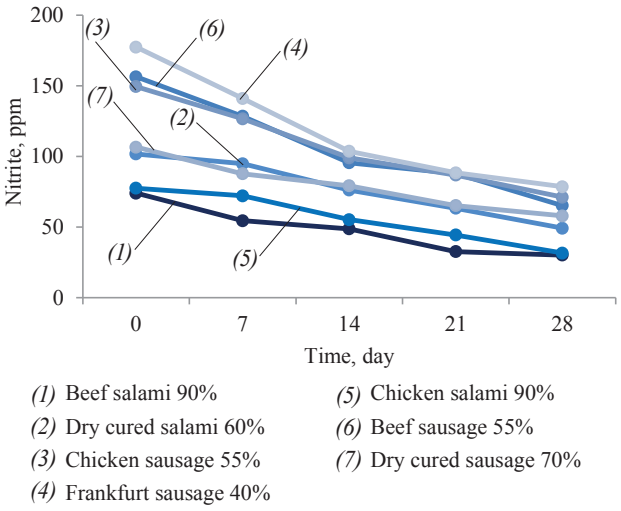


Figure 1 Residual nitrite content (mg/kg meat) in sausages with different meat content from factory B on days 0, 7, 14, 21, and 28

nitrite-heme-nitrosomyoglobin complex with myoglobin, thus producing nitrosomyochromogen. The latter is responsible for the characteristic bright pink color of cured meat products [20].

Hence, higher content of myoglobin results in lower nitrite content in the final meat product. The results obtained by statistical analysis indicate that residual nitrite in Frankfurt sausage (40%) was significantly higher ($P < 0.05$), whereas in beef salami (90%) it was at its lowest. No significant differences were observed in other sausage and salami products ($P > 0.05$).

Ascorbic acid, or ascorbate, is applied to meat products as an additive with high water solubility for three major reasons. First, the nitrosomyoglobin-forming reduction of nitrite to nitric oxide produces the required color. Second, the antioxidative activity of ascorbic acid slows down oxidation of pigments and lipids, which results in color and flavor stability. Third, residual nitrite decreases due to binding to nitrite in heated samples [21].

Ascorbate proved effective in nitrosamine inhibition. This quality is associated with rapid reactions of ascorbate with nitric oxide compared to nitrosating agents, e.g. amines. Therefore, nitrosamines are formed when the reaction rate constant of ascorbate is not much larger than amines [16]. Figure 2 and Table 1 demonstrate that ascorbic acid content of samples decreased significantly ($P < 0.05$) during refrigerated storage.

Meat products with higher meat content exhibited lower ascorbic acid reduction rate, which was due to lower residual nitrite content. Ascorbic acid degradation in meat products could be related to oxygen content, temperature, light, water activity, presence of metal ions, e.g. copper and ferric iron, and storage time [22–24]. Degradation of ascorbic acid is increased in acidic conditions (pH = 3.3–5.5). In the current study,

Table 1. Residual nitrite, ascorbic acid and nitrosamine contents in sausages produced by four major meat factories during storage

Factory A		Day 0	Day 7	Day 14	Day 21	Day 28
Beef salami (90% of meat)	Nitrite, mg/kg	67.91 ± 0.605^{a}	59.95 ± 0.360^{b}	46.06 ± 0.326^{c}	27.75 ± 0.119^{d}	16.07 ± 0.294^{e}
	Nitrosamine, ng/g	5.43 ± 0.004^{a}	6.71 ± 2.887^{b}	13.44 ± 0.033^{c}	17.25 ± 0.563^{d}	22.01 ± 0.038^{e}
	Ascorbic acid, ng/g	214.08 ± 0.262^{a}	216.81 ± 0.026^{a}	194.98 ± 0.018^{b}	171.95 ± 0.017^{c}	167.82 ± 0.026^{c}
Chicken salami (90% of meat)	Nitrite, mg/kg	73.20 ± 0.376^{a}	62.90 ± 0.134^{b}	51.72 ± 0.226^{c}	33.00 ± 0.156^{d}	17.75 ± 0.356^{e}
	Nitrosamine, ng/g	6.30 ± 0.135^{a}	10.91 ± 0.173^{b}	16.47 ± 0.610^{c}	18.68 ± 0.856^{c}	22.44 ± 0.616^{d}
	Ascorbic acid, ng/g	175.39 ± 0.039^{a}	168.53 ± 0.003^{a}	159.21 ± 0.027^{b}	140.84 ± 0.039^{c}	134.40 ± 0.021^{d}
Dry cured salami (60% of meat)	Nitrite, mg/kg	90.93 ± 1.036^{a}	85.00 ± 0.457^{b}	72.64 ± 0.678^{c}	61.36 ± 0.719^{d}	36.73 ± 0.381^{e}
	Nitrosamine, ng/g	6.93 ± 0.0561^{a}	14.77 ± 0.037^{b}	18.03 ± 0.060^{c}	21.82 ± 0.518^{d}	24.67 ± 0.091^{e}
	Ascorbic acid, ng/g	185.86 ± 0.021^{a}	180.79 ± 0.003^{b}	172.05 ± 0.062^{c}	168.86 ± 0.683^{d}	162.35 ± 0.706^{d}
Beef sausage (55% of meat)	Nitrite, mg/kg	181.80 ± 0.274^{a}	137.28 ± 0.760^{b}	108.10 ± 0.716^{c}	83.97 ± 0.080^{d}	70.78 ± 0.387^{e}
	Nitrosamine, ng/g	14.47 ± 0.399^{a}	24.80 ± 1.06^{b}	32.31 ± 0.389^{c}	33.60 ± 0.566^{c}	42.52 ± 1.077^{d}
	Ascorbic acid, ng/g	198.22 ± 0.149^{a}	203.91 ± 0.057^{a}	196.78 ± 0.031^{a}	182.49 ± 0.033^{b}	175.88 ± 0.018^{c}
Chicken sausage (55% of meat)	Nitrite, mg/kg	187.42 ± 0.315^{a}	145.40 ± 0.447^{b}	114.00 ± 0.151^{c}	91.21 ± 1.279^{d}	76.29 ± 0.390^{e}
	Nitrosamine, ng/g	7.71 ± 0.075^{a}	25.96 ± 0.1063^{b}	33.32 ± 0.053^{c}	34.29 ± 0.067^{c}	41.91 ± 0.0167^{d}
	Ascorbic acid, ng/g	165.27 ± 0.009^{a}	163.34 ± 0.009^{a}	161.99 ± 0.024^{a}	158.26 ± 0.072^{b}	149.46 ± 0.031^{c}
Dry cured sausage (70% of meat)	Nitrite, mg/kg	82.11 ± 0.757^{a}	69.78 ± 0.758^{b}	54.94 ± 0.115^{c}	46.35 ± 0.314^{d}	39.79 ± 1.206^{e}
	Nitrosamine, ng/g	14.32 ± 0.052^{a}	15.36 ± 0.012^{a}	17.86 ± 0.083^{ab}	20.81 ± 0.765^{b}	21.55 ± 0.045^{b}
	Ascorbic acid, ng/g	129.67 ± 0.416^{a}	120.55 ± 0.007^{ab}	117.99 ± 0.033^{b}	106.79 ± 0.039^{c}	105.82 ± 0.705^{c}
Factory B		Day 0	Day 7	Day 14	Day 21	Day 28
Beef salami (90% of meat)	Nitrite, mg/kg	73.99 ± 0.011^{a}	54.53 ± 0.130^{b}	48.76 ± 0.649^{c}	32.60 ± 0.626^{d}	30.20 ± 0.598^{d}
	Nitrosamine, ng/g	3.06 ± 0.058^{a}	5.13 ± 0.204^{ab}	7.05 ± 0.284^{b}	13.40 ± 0.122^{c}	14.18 ± 0.1054^{c}
	Ascorbic acid, ng/g	194.99 ± 0.016^{a}	192.30 ± 0.021^{a}	165.00 ± 0.699^{b}	144.62 ± 0.004^{c}	121.60 ± 0.060^{d}
Chicken salami (90% of meat)	Nitrite, mg/kg	77.55 ± 0.556^{a}	72.12 ± 0.505^{a}	55.24 ± 0.247^{b}	44.41 ± 0.830^{c}	31.62 ± 0.347^{d}
	Nitrosamine, ng/g	4.85 ± 0.045^{a}	4.83 ± 0.018^{a}	6.43 ± 0.09^{a}	11.43 ± 0.065^{b}	14.81 ± 0.031^{b}
	Ascorbic acid, ng/g	220.79 ± 0.022^{a}	192.84 ± 0.554^{b}	168.95 ± 0.134^{c}	164.89 ± 0.025^{c}	134.07 ± 0.492^{d}
Dry cured salami (60% of meat)	Nitrite, mg/kg	101.79 ± 0.290^{a}	94.85 ± 0.362^{a}	76.18 ± 0.359^{b}	63.30 ± 0.244^{c}	49.24 ± 0.396^{d}
	Nitrosamine, ng/g	11.93 ± 0.081^{a}	15.20 ± 0.256^{ab}	15.32 ± 0.298^{ab}	21.68 ± 0.307^{b}	28.38 ± 0.575^{c}
	Ascorbic acid, ng/g	201.24 ± 0.127^{a}	188.65 ± 0.038^{b}	172.34 ± 0.401^{c}	168.01 ± 0.002^{d}	145.69 ± 0.018^{e}
Beef sausage (55% of meat)	Nitrite, mg/kg	156.45 ± 0.774^{a}	128.46 ± 0.637^{b}	95.54 ± 0.323^{c}	87.82 ± 0.296^{d}	65.26 ± 0.971^{e}
	Nitrosamine, ng/g	13.21 ± 0.057^{a}	14.94 ± 0.04^{a}	23.64 ± 0.075^{b}	29.54 ± 0.94^{c}	37.73 ± 0.059$_{d}$
	Ascorbic acid, ng/g	200.90 ± 0.132^{a}	193.71 ± 0.015^{ab}	187.60 ± 0.345^{b}	183.60 ± 0.031^{b}	174.74 ± 0.297^{c}
Chicken sausage (55% of meat)	Nitrite, mg/kg	149.47 ± 0.197^{a}	126.70 ± 0.516^{b}	98.98 ± 0.708^{c}	86.83 ± 0.681^{d}	71.21 ± 0.192^{e}
	Nitrosamine, ng/g	7.85 ± 0.118^{a}	14.70 ± 0.077^{b}	24.73 ± 0.053^{c}	29.77 ± 2.93^{d}	37.99 ± 0.133^{e}
	Ascorbic acid, ng/g	181.71 ± 0.014^{a}	160.27 ± 0.020^{b}	138.63 ± 0.013^{c}	121.13 ± 0.003^{d}	104.42 ± 0.005^{e}
Dry cured sausage (70% of meat)	Nitrite, mg/kg	106.62 ± 0.277^{a}	87.71 ± 0.850^{b}	79.26 ± 0.211^{c}	65.21 ± 0.306^{d}	57.90 ± 0.226^{e}
	Nitrosamine, ng/g	8.18 ± 0.202^{a}	9.71 ± 0.469^{a}	15.22 ± 0.085^{b}	25.88 ± 0.1589^{c}	33.16 ± 0.622^{d}
	Ascorbic acid, ng/g	197.86 ± 0.215^{a}	156.10 ± 0.021^{b}	131.45 ± 0.073^{c}	127.63 ± 0.015^{d}	126.30 ± 0.007^{d}
Frankfurt sausage (40% of meat)	Nitrite, mg/kg	177.42 ± 0.677^{a}	141.07 ± 0.075^{b}	103.64 ± 0.280^{c}	88.45 ± 0.662^{d}	78.61 ± 0.710^{e}
	Nitrosamine, ng/g	14.34 ± 0.05^{a}	16.12 ± 0.6746^{b}	27.20 ± 0.116^{c}	34.97 ± 0.202^{c}	45.06 ± 0.229^{d}
	Ascorbic acid, ng/g	192.57 ± 0.062^{a}	187.08 ± 0.032^{b}	169.47 ± 0.057^{c}	155.29 ± 0.008^{d}	137.55 ± 0.006^{e}
Factory C		Day 0	Day 7	Day 14	Day 21	Day 28
Beef salami (90% of meat)	Nitrite, mg/kg	59.08 ± 0.199^{a}	35.41 ± 0.347^{b}	25.26 ± 0.952^{c}	18.25 ± 0.297^{d}	8.42 ± 0.088^{e}
	Nitrosamine, ng/g	2.65 ± 0.0123^{a}	8.37 ± 0.058^{b}	15.51 ± 0.021^{c}	19.27 ± 0.057^{c}	33.83 ± 0.123^{d}
	Ascorbic acid, ng/g	232.27 ± 0.0321^{a}	187.13 ± 0.060^{b}	135.76 ± 0.025^{c}	122.71 ± 0.031^{cd}	108.52 ± 0.026^{d}
Chicken salami (90% of meat)	Nitrite, mg/kg	62.90 ± 0.220^{a}	50.30 ± 0.589^{b}	36.67 ± 0.347^{c}	19.66 ± 0.714^{d}	9.26 ± 0.076^{d}
	Nitrosamine, ng/g	2.78 ± 0.071^{a}	9.68 ± 0.078^{b}	14.47 ± 0.064^{c}	18.59 ± 0.083^{d}	25.34 ± 0.117^{e}
	Ascorbic acid, ng/g	162.54 ± 0.055^{a}	155.95 ± 0.086^{b}	121.19 ± 0.060^{c}	103.93 ± 0.112^{d}	92.68 ± 0.049^{e}
Dry cured salami (60% of meat)	Nitrite, mg/kg	69.96 ± 0.912^{a}	65.00 ± 0.721^{a}	50.22 ± 0.977^{ab}	36.57 ± 0.386^{b}	19.17 ± 0.448^{c}
	Nitrosamine, ng/g	3.51 ± 0.007^{a}	11.91 ± 0.042^{b}	19.61 ± 0.047^{c}	22.57 ± 0.052^{d}	23.76 ± 0.052^{d}
	Ascorbic acid, ng/g	248.29 ± 0.004^{a}	213.54 ± 0.630^{b}	198.32 ± 0.016^{b}	165.78 ± 0.032^{c}	119.79 ± 0.296^{d}
Beef sausage (55% of meat)	Nitrite, mg/kg	107.41 ± 0.682^{a}	92.93 ± 0.114^{ab}	85.60 ± 1.314^{b}	64.39 ± 0.142^{c}	31.88 ± 0.397^{d}
	Nitrosamine, ng/g	8.61 ± 0.157^{a}	17.01 ± 0.05^{b}	20.51 ± 0.547^{b}	34.51 ± 0.068^{c}	44.20 ± 0.051^{d}
	Ascorbic acid, ng/g	200.69 ± 0.45^{a}	196.17 ± 0.057^{a}	189.30 ± 0.047^{ab}	175.08 ± 0.107^{b}	142.58 ± 0.117^{c}
Chicken sausage (55% of meat)	Nitrite, mg/kg	116.20 ± 0.725^{a}	93.36 ± 0.354^{ab}	81.96 ± 0.279^{b}	55.90 ± 0.374^{c}	47.44 ± 0.658^{d}
	Nitrosamine, ng/g	9.85 ± 0.011^{a}	16.19 ± 0.145^{ab}	20.51 ± 0.547^{b}	35.51 ± 0.068^{c}	44.20 ± 0.051^{d}
	Ascorbic acid, ng/g	199.63 ± 0.588^{a}	184.59 ± 0.068^{b}	165.67 ± 1.013^{c}	102.61 ± 0.173^{d}	79.79 ± 0.033^{e}

Continuation of the table 1

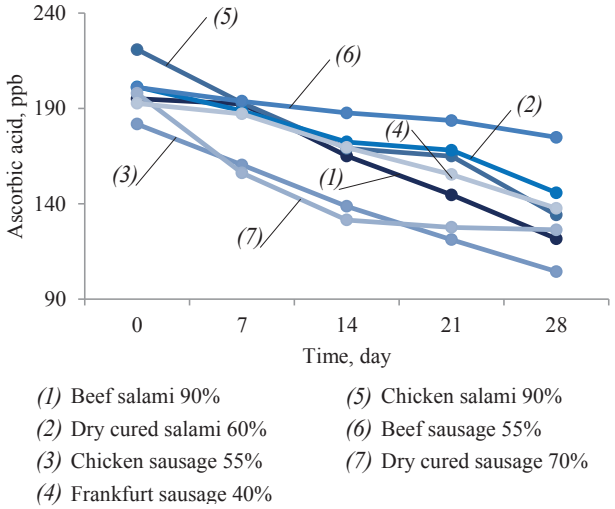
Dry cured sausage (70% of meat)	Nitrite, mg/kg	84.48 ± 0.188^{a}	71.59 ± 0.763^{b}	57.72 ± 0.274^{c}	50.18 ± 1.247^{c}	27.29 ± 0.242^{d}
	Nitrosamine, ng/g	10.05 ± 0.033^{a}	10.35 ± 0.048^{a}	17.68 ± 0.031^{b}	20.81 ± 0.080^{c}	23.44 ± 0.037^{d}
	Ascorbic acid, ng/g	149.83 ± 0.017^{a}	114.28 ± 0.177^{b}	103.91 ± 0.122^{c}	89.73 ± 0.145^{d}	71.32 ± 0.035^{e}
Frankfurt sausage (40% of meat)	Nitrite, mg/kg	135.79 ± 1.314^{a}	98.41 ± 1.405^{b}	82.62 ± 0.592^{c}	80.51 ± 0.754^{c}	52.84 ± 0.489^{d}
	Nitrosamine, ng/g	10.05 ± 0.087^{a}	17.95 ± 0.086^{b}	24.09 ± 0.105^{c}	37.80 ± 0.065^{d}	41.32 ± 0.090^{e}
	Ascorbic acid, ng/g	141.12 ± 0.059^{a}	138.09 ± 0.012^{a}	102.27 ± 0.027^{b}	91.43 ± 0.068^{b}	74.10 ± 0.707^{c}
Factory D		Day 0	Day 7	Day 14	Day 28	
Beef salami (90% of meat)	Nitrite, mg/kg	96.65 ± 0.646^{a}	75.59 ± 0.754^{b}	59.11 ± 1.413^{c}	52.95 ± 1.361^{cd}	47.94 ± 0.628^{d}
	Nitrosamine, ng/g	28.96 ± 0.026^{a}	34.52 ± 0.042^{ab}	39.02 ± 0.054^{b}	44.38 ± 0.048^{bc}	49.52 ± 0.077^{c}
	Ascorbic acid, ng/g	233.43 ± 0.202^{a}	195.42 ± 0.054^{b}	163.34 ± 0.095^{c}	157.90 ± 0.158^{c}	132.90 ± 0.167^{d}
Dry cured salami (60% of meat)	Nitrite, mg/kg	101.88 ± 0.611^{a}	90.53 ± 0.437^{a}	78.70 ± 0.650^{b}	66.24 ± 0.271^{c}	53.22 ± 0.917^{d}
	Nitrosamine, ng/g	31.53 ± 0.017^{a}	35.12 ± 0.051^{ab}	39.81 ± 0.029^{b}	44.46 ± 0.036^{c}	48.46 ± 0.036^{c}
	Ascorbic acid, ng/g	169.07 ± 0.115^{a}	157.47 ± 0.032^{b}	114.25 ± 0.174^{c}	103.32 ± 0.151^{d}	89.92 ± 0.341^{e}
Beef sausage (55% of meat)	Nitrite, mg/kg	114.24 ± 0.862^{a}	99.66 ± 0.920^{ab}	86.06 ± 0.526^{b}	73.10 ± 1.452^{c}	61.39 ± 0.122^{d}
	Nitrosamine, ng/g	38.02 ± 0.031^{a}	74.74 ± 0.136^{b}	94.90 ± 0.021^{c}	124.58 ± 0.033^{d}	131.558 ± 0.063^{d}
	Ascorbic acid, ng/g	198.59 ± 0.035^{a}	104.30 ± 0.240^{b}	54.95 ± 0.079^{c}	35.61 ± 0.162^{d}	29.11 ± 0.452^{d}
Chicken sausage (55% of meat)	Nitrite, mg/kg	143.09 ± 0.787^{a}	127.56 ± 0.488^{b}	98.24 ± 0.196^{c}	72.37 ± 0.808^{d}	55.95 ± 0.069^{e}
	Nitrosamine, ng/g	38.02 ± 0.562^{a}	68.88 ± 0.125^{b}	70.90 ± 0.152^{b}	85.94 ± 0.099^{c}	89.91 ± 0.059^{c}
	Ascorbic acid, ng/g	201.10 ± 0.351^{a}	173.31 ± 0.494^{b}	138.33 ± 0.442^{c}	90.33 ± 0.953^{d}	68.82 ± 0.721^{d}
Frankfurt sausage (40% of meat)	Nitrite, mg/kg	150.45 ± 1.003^{a}	135.95 ± 0.765^{b}	109.52 ± 1.216^{c}	97.55 ± 0.084^{d}	80.41 ± 0.784^{e}
	Nitrosamine, ng/g	12.91 ± 0.085^{a}	38.73 ± 0.039^{b}	100.42 ± 0.093^{c}	153.60 ± 0.253^{d}	178.60 ± 0.293^{e}
	Ascorbic acid, ng/g	282.14 ± 0.310^{a}	181.60 ± 0.025^{b}	170.03 ± 0.169^{c}	117.97 ± 0.037^{d}	75.22 ± 0.897^{e}

Different letters in the same row during storage within the same section (i.e. factories A-D) indicate a significant difference ($P < 0.05$)

pH values were in the range of 5–6 in all samples. Therefore, ascorbic acid degradation might have been due to the relative acidic condition of meat products and heating process.

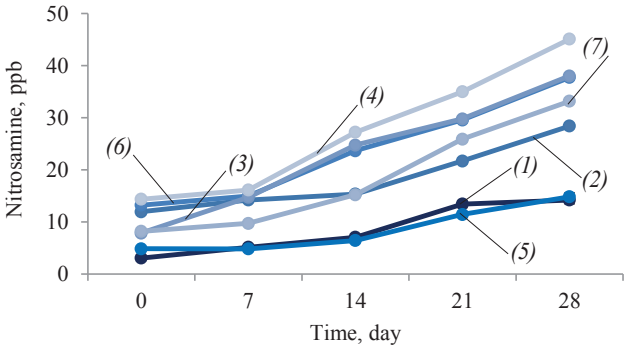
The obtained results showed an increase in nitrosamine content of samples during refrigerated storage. However, it was not significant in all samples (Fig. 3).

The nitrite reduction rates in the sausages with a smaller diameter, e.g. Frankfurt sausage, appeared significantly lower ($P < 0.05$) than in the samples with a bigger diameter, e.g. salami. This difference can be explained by the longer cooking time for salami (5–6 h) compared to Frankfurt sausages (3–4 h), which is associated with a higher reduction rate of residual nitrite. According to the results, the total volatile nitrosamine level of Iranian meat products with lower meat contents was generally higher than that of samples with high meat contents. The nitrosamine content in meat products was associated with the added nitrite and the residual nitrite [25]. The residual nitrite is a reactive agent that can be reduced by heating treatment or exposure to such meat components as proteins, lipids, and pigments [26, 27].



(1) Beef salami 90%
(2) Dry cured salami 60%
(3) Chicken sausage 55%
(4) Frankfurt sausage 40%
(5) Chicken salami 90%
(6) Beef sausage 55%
(7) Dry cured sausage 70%

Figure 2 Ascorbic acid content (ng/kg meat) in sausages with different meat content from factory B on days 0, 7, 14, 21, and 28



(1) Beef salami 90%
(2) Dry cured salami 60%
(3) Chicken sausage 55%
(4) Frankfurt sausage 40%
(5) Chicken salami 90%
(6) Beef sausage 55%
(7) Dry cured sausage 70%

Figure 3 Nitrosamine content (ng/kg of meat) in sausages with different meat content from factory B on days 0, 7, 14, 21, and 28

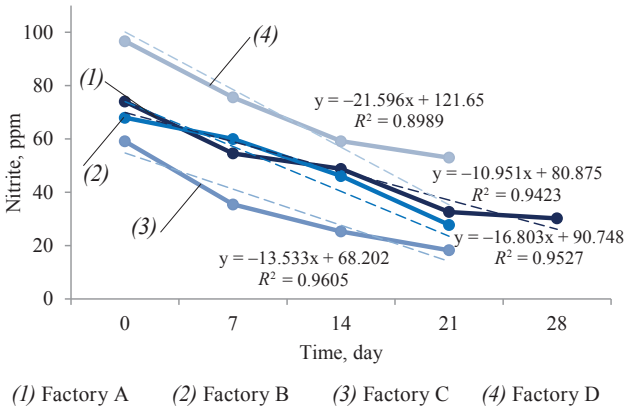


Figure 4 Correlation between residual nitrite content and nitrosamine in beef salami (90% meat) samples

Figure 4 shows that there was a significant correlation between the amounts of added nitrite and the nitrosamine contents in beef salami (90%) samples from all four meat factories.

In this study, the correlation factor was 0.9, which asserts the effects of added nitrite and also residual nitrite contents on nitrosamine formation in the processed meat product. Therefore, the products with lower meat content showed higher residual nitrite and, consequently, a greater nitrosamine formation. In meat products, presence of nitrosating agents increases the concentration of nitrosamine, whether grouped in NO_2 related agents, e.g. N_2O_4, or grouped in nitrous acid derivatives, e.g. N_2O_3 and HNO_2 [28].

There are several suggested strategies to reduce nitrosamine formation in meat products to improve their healthy status and safety. The present research clarified that the levels of nitrosamines in the samples depended on the amount of residual nitrite, ascorbic acid, pH, and cooking temperature. Higher levels of residual nitrite were detected in the samples with a lower amount of meat, compared to those with a higher amount of meat.

CONCLUSION

In the current study, the residual nitrite, ascorbic acid, and nitrosamine contents of seven most popular Iranian processed meat products, namely sausages with different amounts of meat were evaluated to monitor the safety status of the meat industry. The samples contained various concentrations of nitrite and nitrosamine, which were above the permitted standard level. Several factors were found to affect the residual nitrite and nitrosamine contents, the meat content being a significant variable. Nitrite interacted with heme-containing components, non-heme proteins, and fat tissues, thus conversed to nitrate and nitrosamine. Therefore, the contents of residual nitrite and, consequently, nitrosamine in products with lower meat content were significantly higher. A longer cooking time also decreased residual nitrite and nitrosamine concentration.

Further research is needed to identify new substances that could replace nitrites, as well as factors that reduce the required amount of nitrite in meat products.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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Health risk assessment: heavy metals in fish from the southern Black Sea

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Abstract:
Introduction. The coastal contamination of the Black Sea has been an important issue for several decades. Heavy metals are the most harmful contaminants which affect people health. The research objective of the present study was to determine the amounts of Cd, Hg, Pb, Cu, and Zn found in the whiting (*M. merlangus* L.) and the red mullet (*M. barbatus* L.). These Black Sea bottom fish species have the highest commercial value. The obtained data were used to assess the risk which the fish represents for human consumers.
Study objects and methods. The elements were detected using an inductively coupled plasma mass spectrometer (ICP-MS). The amounts of the metals arranged in the following order: Zn > Cu > Pb > Hg > Cd.
Results and discussion. The mean values of Cd, Hg, Pb, Cu, and Zn in the edible tissues were 0.013, 0.024, 0.07, 0.195, and 9.05 mg/kg wet wt. for whiting and 0.017, 0.036, 0.05, 0.29, and 6.4 mg/kg wet wt. for red mullet, respectively. These levels proved lower than the permitted values set by the Ministry of Agriculture, Forestry, and Fisheries of the UK (MAFF), Turkish Food Codex (TFC), and EU Commission Regulation. The target hazard quotient (THQ) for all the elements via consumption of whiting and red mullet were also low.
Conclusion. Hazard index (HI) was < 1, which means that the fish caused no health problems in people who consumed whiting and red mullet caught in the southern Black Sea during the fishing seasons of 2017–2018. The carcinogenic risk index (CRI) for whiting and red mullet was also considered insignificant.

Keywords: Heavy metals, Black Sea, fish, risk assessment, target hazard quotient, carcinogenic risk index

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INTRODUCTION

Fish is usually located at the top of the food chain in the marine ecosystem. It accumulates contaminants from water, food, bottom sediment, and suspended particles in the water column. Even though available and accessible literature shows that heavy metals accumulated in the Black Sea commercial fish have no detrimental effect on human health [1], this issue remains a matter of public concern. However, the present research confirmed the fact that Black Sea fish is unaffected by environmental situation and is safe to eat.

A review conducted by Bat *et al*. showed some concern about the increase of unregulated settlements and anthropogenic activities along the marine coastal area of the Black Sea [2]. The growing urbanization and industrialization, as well as the fast development of agriculture, tourism, and fishery, increase the concentration of heavy metals discharged by major rivers into the coastal waters of the Black Sea. The resulting increase in heavy metals adversely affects the coastal ecosystem.

The contaminants eventually accumulate in marine biota, particularly in fish [3, 4]. Subsequently, metals pass on to people that consume contaminated fish, thus threatening their health [5]. As a result, the environmental issues related to heavy metal contamination of the Black Sea are relevant to all countries along the Black Sea coast. After Romania and Bulgaria entered the European Union, the problem affected the whole of Europe.

The Marine Environment Policy of the Marine Strategy Framework Directive (MSFD) concerns the matters of monitoring chemical elements in edible tissues of seafood and avoiding heavy metal transfer from sea biota to human body via food chain [6]. The MSFD targets the

subject of sea contamination in Descriptor 8 "Concentrations of contaminants are at levels not giving rise to pollution effect" and Descriptor 9 "Contaminants in fish and other seafood for human consumption do not exceed levels established by Community legislation or other relevant standards" [6]. The objective of the MSFD with concern to Descriptors 8 and 9 is to ensure that contaminants are represented in foods in safe amounts.

According to the main guideline of the European Union, European seas are to obtain the Good Environmental Status (GES) by 2020. The abovementioned facts make studies of chemical elements in commercial fish extremely relevant.

The current study featured two commercial demersal fish species and assessed the heavy metal contamination, as well as the risk that the detected heavy metals represent for human health. The current study concentrated on the effect of Cd, Hg, Pb, Cu, and Zn on consumers' health. The concentrations of the metals were measured in the muscle tissues of whiting and red mullet caught along the Sinop coast of the southern Black Sea and sold on fish markets. The research also included a thorough analysis of scientific literature on the amounts of Cd, Hg, Pb, Cu, and Zn in Black Sea whiting and red mullet. The obtained results could help in achieving the goals set by MSFD 2008/56/EC [6].

STUDY OBJECTS AND METHODS

Sample Collection. Twenty specimens of whiting and red mullet were purchased on fish markets. The sampling was conducted during the fishing seasons of 2017 and 2018 on the Sinop coast of the Black Sea (Fig. 1). The fish samples were processed according to the method depicted by Bernhard and UNEP [6–8]. The edible tissues of *M. merlangus* L. and *M. barbatus* L. were dissolved with Suprapur® HNO_3 (nitric acid) using a microwave digestion system. The elemental concentrations (Cd, Hg, Pb, Cu, and Zn) of the digested edible samples of whiting and red mullet were studied using the methods recognized by the Environmental Food Analysis Lab Industry and Trade Inc.

Fish tissues were prepared using an inductively coupled plasma mass spectrometer (ICP-MS), based on m-AOAC 999.10 (Association of Official Analytical Chemists with TS EN ISO IEC 17025 AB-0364-T references number) and CSN EN 15763 European Standards. The presence and quantity of the metals were detected according to the instrumental reaction of the equipment. The results were given as $mg \cdot kg^{-1}$ wet weight (wt.).

Health risk assessment. The risk assessment for infants, children, and adults was performed to estimate the possible hazard associated with the consumption of heavy metals contained in the Black Sea fish. The risk exposure demands taking the mean daily intake of the heavy metals (mg/kg/day). The estimated daily intake (EDI) is subjected to the element levels and the amount of ingestion of fish. The EDI of heavy metals was calculated according to the equation below:

$$EDI = \frac{C_{metal} \times W_{fish}}{BW} \quad (1)$$

where C_{metal} is the amounts of elements in edible tissues;

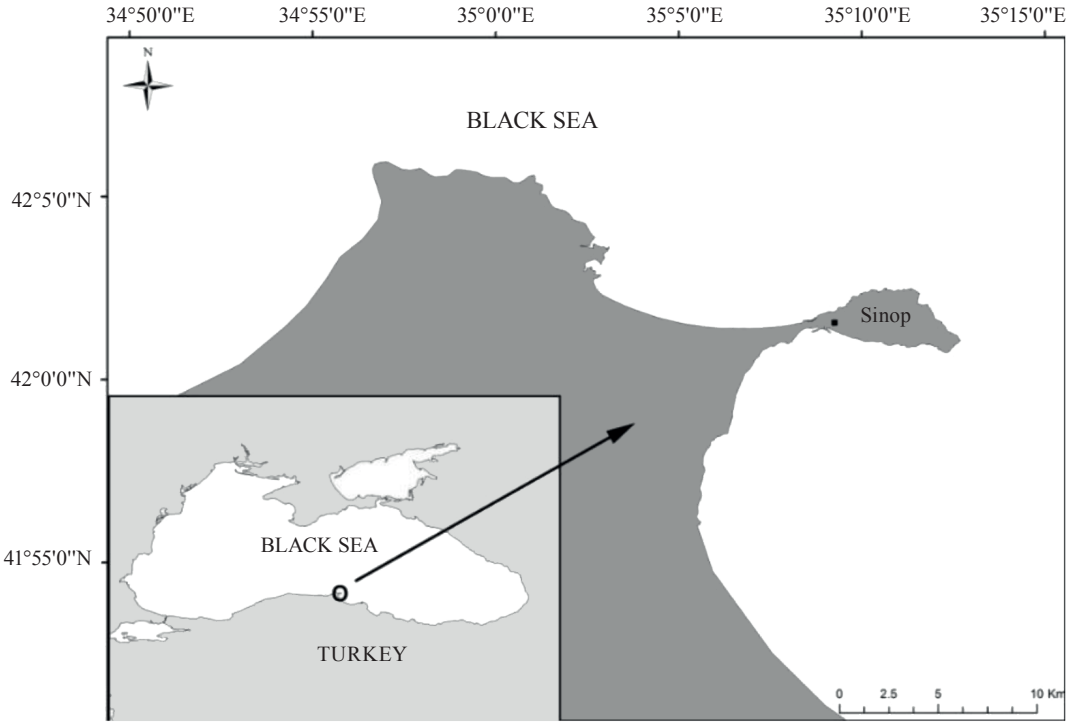

Figure 1 Fishing area

W_{fish} represents the daily mean ingestion of fish given as 0.013, 0.027, and 0.041 kg/day for infants, children, and adults, respectively [10]; BW is the body weight of 10 kg for infants, 30 kg for children, and 70 kg for adults.

The target hazard quotient (THQ) has been used in many studies to analyze the potential non-carcinogenic effect of the metals in the edible tissues of fish. The EDI (mg/kg of body wt. per day) of each heavy metal was related with the reference dose (Rf. D, mg/kg/day) as described in the equation below [11–13]:

$$THQ = \frac{EDI}{Rf.D.} \quad (2)$$

Rf. D. is the oral reference dose for Zn, Cu, Pb, Hg, and Cd as suggested by the US Environmental Protection Agency, i.e. 0.3, 0.04, 0.004, 0.0005, and 0.001 mg/kg/day, respectively [14, 15]. However, in the Risk Assessment Information System (RAIS), the mercury inorganic salts Rf. D. value is 0.0003, and there is no Rf. D. value for lead and compounds [15]. In contrast, oral slope factor is given only for lead and compounds as 0.0085 mg/kg/day [16]. The hazard index (HI) was defined as the sum of the THQs as described in the equation below:

$$HI = THQ(Zn) + THQ(Cu) + THQ(Pb) + THQ(Hg) + THQ(Cd) \quad (3)$$

The HI was used in this study to describe the cumulative non-carcinogenic effect. If HI > 1.0, then the EDI of a specific element exceeds the Rf. D, showing that there is a potential risk associated with that element.

The risk index (RI) represents the probability of developing any type of cancer over a lifetime. It is calculated by integrating the EDI with the respective oral slope factors (SF) for heavy metals. Slope factors (SF) are used to reckon the risk of cancer along with exposure to a carcinogenic or probably carcinogenic matter [17]. The description is presented in the equation below:

$$RI = EDI \times SF \quad (4)$$

The RI was considered insignificant if the RI was $< 10^{-6}$; the RI was considered allowable or tolerable if RI was $10^{-6} < RI < 10^{-4}$; the RI was considered significant if the RI was $> 10^{-4}$.

RESULTS AND DISCUSSION

The average amounts of the heavy metals in Black Sea whiting and red mullet are given in Fig. 2. The amounts of heavy metals in both *M. merlangus* L. and *M. barbatus* L. decreased in the following order: Zn > Cu > Pb > Hg > Cd. The essential metals Zn and Cu were represented in higher amounts due to their biological functions, whereas the toxic metals Pb, Hg, and Cd have no biological functions, and their amounts in fish tissues were considerably lower.

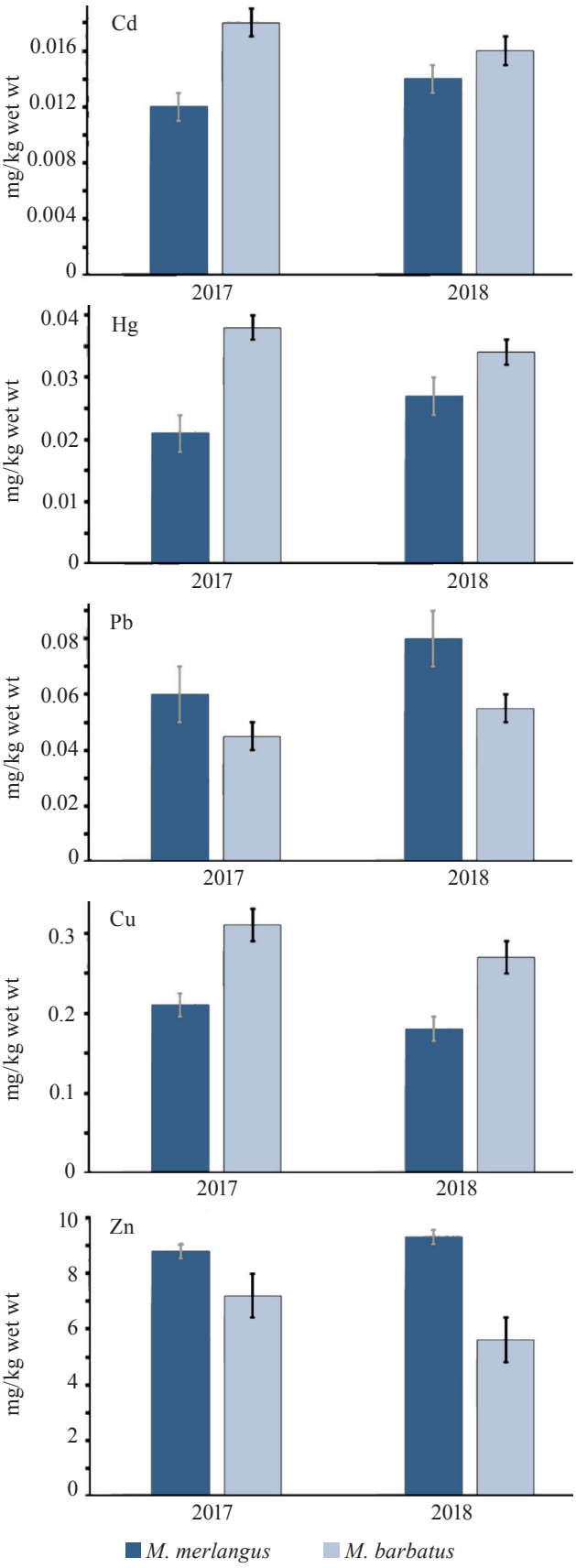

Figure 2 Heavy metal amounts with standard deviation for Cd, Hg, Pb, Cu, and Zn in the edible tissues of *M. merlangus* L. and *M. barbatus* L. from the Black Sea coasts caught in 2017 and 2018

Table 1 Estimated daily intakes (EDI) of elements in the edible tissues of *Merlangius merlangus* L. from the southern Black Sea

Heavy metals	EDI (2017), mg/day/kg body wt.			EDI (2018), mg/day/kg body wt.		
	Infants	Children	Adults	Infants	Children	Adults
Cd	0.0000156	0.0000108	0.0000070	0.0000182	0.0000126	0.0000082
Hg	0.0000273	0.0000189	0.0000123	0.0000351	0.0000243	0.0000158
Pb	0.0000780	0.0000540	0.0000351	0.0001040	0.0000720	0.0000468
Cu	0.0002730	0.0001890	0.0001230	0.0002340	0.0001620	0.0001054
Zn	0.0114400	0.0079200	0.00515428	0.0120900	0.0083700	0.0054471

Table 2 Estimated daily intakes (EDI) of elements in edible tissues of *Mullus barbatus* L. from the southern Black Sea

Heavy metals	EDI (2017), mg/day/kg body wt.			EDI (2018), mg/day/kg body wt.		
	Infants	Children	Adults	Infants	Children	Adults
Cd	0.0000234	0.0000162	0.0000105	0.0000208	0.0000144	0.00000937
Hg	0.0000494	0.0000342	0.0000222	0.0000442	0.0000306	0.0000199
Pb	0.0000585	0.0000405	0.0000263	0.0000715	0.0000495	0.0000322
Cu	0.0004030	0.0002790	0.0001815	0.0003510	0.0002430	0.00015814
Zn	0.0093600	0.0064800	0.0042171	0.0072800	0.0050400	0.0032800

Table 3 Target hazard quotients (THQ) and hazard index (HI) of elements consumed with *Merlangius merlangus* L. caught near the southern coast of the Black Sea in 2017 and 2018

Heavy metals	THQ (2017)			THQ (2018)		
	Infants	Children	Adults	Infants	Children	Adults
Cd	0.0156000	0.0108000	0.00702857	0.0182000	0.0126000	0.0082000
Hg	0.0546000	0.0378000	0.0246000	0.0702000	0.0486000	0.03162857
Pb	0.0195000	0.0135000	0.008785714	0.0260000	0.0180000	0.011714286
Cu	0.0068250	0.0047250	0.0030750	0.0058500	0.0040500	0.00263571
Zn	0.0381330	0.0264000	0.01718095	0.0403000	0.0279000	0.01815714
HI	0.1346580	0.0932250	0.060670238	0.1605500	0.1111500	0.072335714

Table 4 Target hazard quotients (THQ) and hazard index (HI) of elements consumed with *Mullus barbatus* L. caught near the southern coast of the Black Sea in 2017 and 2018

Heavy metals	THQ (2017)			THQ (2018)		
	Infants	Children	Adults	Infants	Children	Adults
Cd	0.0234000	0.0162000	0.010542857	0.0208000	0.0144000	0.009371429
Hg	0.0988000	0.0684000	0.044514286	0.0884000	0.0612000	0.039828571
Pb	0.0146250	0.0101250	0.006589286	0.0178750	0.0123750	0.008053571
Cu	0.0100750	0.0069750	0.004539286	0.0087750	0.0060750	0.003953571
Zn	0.0312000	0.0216000	0.014057143	0.0242666	0.0168000	0.010933333
HI	0.1781000	0.1233000	0.080242857	0.160116667	0.1108500	0.072140476

In this study, the heavy metal amounts in edible tissues varied according to the species. Cd, Hg, and Cu were high in *M. barbatus*, whereas *M. merlangus* proved rich in Pb and Zn. These differences may be related to habitat and feeding habits. The red mullet is demersal fish found near sand, gravel, and mud bottoms of the continental shelf. It feeds on small benthic mollusks, crustaceans, and worms. The whiting is benthopelagic fish found mostly near gravel and mud bottoms. Less frequently, it can be found on rock and sand. The whiting feeds on crabs, shrimps, mollusks, polychaetes, and small fish [17].

Cu and Zn are relatively safe for living biota. Therefore, the permissible values of such essential heavy metals as Cu and Zn are not available in the current European Union and TFC regulations. However, they can be harmful if consumed in large amounts. According to the Ministry of Agriculture, Forestry, and Fisheries of the UK (MAFF), the maximal tolerable limits of Cu and Zn are 20 and 50 mg/kg wet wt., respectively [18]. In this study, the amount of heavy metal detected in whiting and red mullet was found to be significantly lower than these values.

Similarly, the present study revealed that toxic metal values (Cd, Hg and Pb) in edible tissues of whiting and red mullet were below the permissible values (0.05, 0.5, and 0.3 mg/kg wet wt.) set by European Union Commission Regulation and Turkish Food Codex [19, 20]. The Global Agriculture Information Network (GAIN) Report of the Russian Federation defined the

Table 5 Carcinogenic concentration of consumed fish (CDI), hazard quotient (HQ), risk index (RI), and hazard risk (HI) of elements in *Merlangius merlangus* L. caught near the southern coast of the Black Sea in 2017 and 2018

Heavy metals	2017			2018		
	CDI, mg/kg/day	HQ	RI	CDI, mg/kg/day	HQ	RI
Cd	0.0000026	0.0070290		0.0000030	0.0082000	
Hg	0.0000045	0.0246000		0.0000058	0.0316290	
Pb	0.0000130	–	0.00000011	0.0000170	–	0.00000014
Cu	0.0000450	0.0030750		0.0000390	0.0026360	
Zn	0.0019144	0.0171810		0.0020232	0.0181570	
HI		0.0518850	0.00000011		0.0606220	0.00000014

Table 6 Carcinogenic concentration of consumed fish (CDI), hazard quotient (HQ), risk index (RI), and hazard risk (HI) of elements in *Mullus barbatus* L. caught near the southern coast of the Black Sea in 2017 and 2018

Heavy metals	2017			2018		
	CDI, mg/kg/day	HQ	RI	CDI, mg/kg/day	HQ	RI
Cd	0.0000039	0.0105430		0.0000034	0.0093710	
Hg	0.0000082	0.0445140		0.0000082	0.0398290	
Pb	0.0000097	–	0.000000083	0.0000119	–	0.0000001
Cu	0.0000670	0.0045390		0.0000580	0.0039540	
Zn	0.0015663	0.0140570		0.0012182	0.0109330	
HI		0.0736530	0.000000083		0.0640870	0.0000001

permissible amounts of Cd, Hg, and Pb as 0.2, 0.5, and 1 mg/kg wet wt., respectively [21].

Tables 1 and 2 present the EDI values for whiting and red mullet caught near the Sinop coast of the Black Sea in 2017 and 2018 for infants, children, and adults. Tables 3 and 4 feature the THQ and HI values.

The EDI levels of Cd, Hg, Pb, Cu, and Zn were very low for both whiting and red mullet. These values were observed to be lower than their Rf. D. values. Likewise, THQ levels of these elements were very low. The HI values for infants were observed to be higher than those for children and adults. This result suggests that, at a relatively high level of exposure, infants will be more likely at risk than children and adults. Obviously, infants weigh much less than children and adults. However, the total non-carcinogenic indices (HI), which is the sum of THQ values for all the heavy metals studied for each sampling year, were lower than the threshold value of 1.0. Therefore, there were no health risks for infants, children, and adults who consumed whiting and red mullet caught near the southern coast of the Black Sea during the fishing seasons of 2017 and 2018.

In the Risk Assessment Information System, the SP value is given for Pb and its compounds only. The lifetime of a person is stated to be 70 years on average, while the exposure duration is assumed to be 26 years [16]. Tables 5 and 6 show carcinogenic concentration of consumed fish (CDI), hazard quotient (HQ), risk index (RI), and hazard risk (HI) of heavy metals in *M. merlangus* and *M. barbatus* caught near the southern coast of the Black Sea. The carcinogenic risk for whiting and red mullet was lower than 10^{-6} and is considered insignificant. The lowest RI was found in red mullet in 2017.

The results of this study were compared with the studies that featured *Merlangius merlangus* and *Mullus barbatus* from the Black Sea. They are presented in Tables 7 and 8, respectively.

In general, the amount of heavy metal found in both *Merlangius merlangus* and *Mullus barbatus* proved to be lower than that in other studies. Likewise, Zn is the heaviest metal found in both species. It is followed by Cu, Pb, Cd, and Hg. When compared, Zn, Cu, and Pb were found in high amounts in the whiting collected near the Amasra coasts of the southern Black Sea [31]. Hg was the highest in the whiting caught near the shores of Istanbul in the Black Sea [30]. Cd was detected in both fish species caught near the Trabzon shores. The highest Hg level species was obtained from *M. barbatus* caught near the shores of Istanbul and Kocaeli in the Black Sea [37]. The highest Pb value was found in the red mullet fished near the Kastamonu shores of the Black Sea [45].

The differences in the amounts of heavy metals found in these fish species may be due to the fact that they were caught during different fishing seasons and in different areas of the Black Sea. Metabolism, physiology, and feeding habits of the fish are different in different seasons. The pollution load also varies in different areas of the Black Sea coast [2]. Similarly, one should not dismiss different applications in heavy metal measurements, equipment accuracy, and human error. Although there are some exceptions, the amounts of heavy metals in these fish species proved to be low. Therefore, they posed no threat to human health.

CONCLUSION

The research featured the effect of Cd, Hg, Pb, Cu, and Zn on the health of infants, children, and adults who

Table 7 Comparison of the amounts (ppm) of heavy metals in the edible tissues of *Merlangius merlangus* L. caught near various areas of the Black Sea coast

Location	dw/ww	Metals					Ref.
		Zn	Cu	Pb	Cd	Hg	
Black Sea	d.w.	48.6 ± 3.9	1.25 ± 0.10	0.93 ± 0.07	0.55 ± 0.04	–	[22]
Black Sea	d.w.	8.86–163.28	0.91–8.95	–	–	–	[23]
Trabzon	w.w.	8.62 ± 0.54	0.88 ± 0.12	0.25 ± 0.07	0.01 ± 0.00	–	[24]
Sinop		12.9 ± 4.14	2.90 ± 0.78	0.46 ± 0.08	0.04 ± 0.01	–	
Bartın		5.73 ± 0.37	0.77 ± 0.07	0.18 ± 0.04	0.02 ± 0.00	–	
İstanbul	d.w.	6.03 ± 0.55		0.50 ± 0.10	0.19 ± 0.02	–	[25]
Black Sea	w.w.	65.4 ± 4.2	1.32 ± 0.11	0.53 ± 004	0.21 ± 0.02	84 ± 5 $\mu g \cdot kg^{-1}$	[26]
Sinop	d.w.	–	–	< 0.05	< 0.02	< 0.05	[27]
Samsun		–	–	< 0.05	< 0.02	< 0.05	
Samsun, Ordu, Trabzon, Rize	d.w	20.6 ± 2.1	1.8 ± 0.2	0.46 ± 0.05	0.18 ± 0.02	–	[28]
Samsun, Sinop, Terme, Fatsa Ordu	d.w.	31.34 ± 1.61	3.72 ± 0.59	0.58 ± 0.03	0.002 ± 0.000	not detect	[29]
İstanbul	w.w.	4.248–30.842	0.001–4.915	0.004–1.581	0.001–0.151	0.003–0.491	[30]
Amasra-West Black Sea	w.w.	77.99 ± 46.91	8.53 ± 2.14	6.80 ± 5.88	0.40 ± 0.29	–	[31]
Samsun- Turkey	d.w.	58 ± 3.5 28.3 ± 1	2.3 ± 0.7 2.7 ± 0.7	0.9 ± 0.2 not detect	0.2 ± 0.03 not detect	–	[32]
Terkos	d.w.	–	–	15	0.35	0.07	[33]
Sakarya		–	–	12	0.24	< 0.01	
Bafra		–	–	15	0.07	0.09	
Ordu		–	–	13	0.22	0.5	
Trabzon-Turkey	d.w.	22.76 ± 2.01	1.02 ± 0.05	0.08 ± 0.03	0.04 ± 0.01	0.05 ± 0.01	[34]
Black Sea	d.w.	8.49	0.51	0.01	–	–	[35]
Sinop	d.w.	22.82–34.33	2.85–5.26	0.02	0.08–0.18	–	[36]
Black Sea	d.w.	18 ± 1.4	2.5 ± 0.06	0.05 ± 0.01	0.03 ± 0.01	0.33 ± 0.02	[37]
Eastern Black Sea, Turkey Ordu-Samsun	d.w.	21.5	1.56	0.024	0.031	–	[38]
West Black Sea	w.w.	18.1 ± 0.3	1.28 ± 0.07	–	–	–	[39]
Sinop	d.w.	16.34 ± 3.83	1.20 ± 0.31	0.69 ± 0.34	0.027 ± 0.012	–	[40]
Trabzon-Turkey	w.w.	–	–	0.02 ± 0.00	4.05 ± 0.14	–	[41]
Sinop	w.w.	3.4	< 0.5	< 0.05	< 0.02	< 0.05	[42]
Sinop	w.w.	43 ± 6	0.41 ± 0.02	0.88 ± 0.006	0.075 ± 0.006	not detect	[43]
Samsun	w.w.	5.04 ± 0.58	1.28 ± 0.09	1.41 ± 0.23	0.06 ± 0.02	–	[44]
Sinop		3.47 ± 0.27	0.92 ± 0.08	0.63 ± 0.06	0.05 ± 0.003	–	
Kocaeli		3.99 ± 0.5	1.46 ± 0.18	0.69 ± 0.12	0.06 ± 0.01	–	
Kastamonu	w.w.	5.45 ± 1.12	4.52 ± 0.70	6.12 ± 1.45	0.24 ± 0.02	–	[45]
Giresun	w.w.	3.77 ± 0.22	2.40 ± 0.25	0.05 ± 0.00	0.66 ± 0.08	–	[46]
Trabzon		5.65 ± 0.58	1.62 ± 0.25	1.30 ± 0.31	0.12 ± 0.03	–	
Rize		4.08 ± 0.36	1.65 ± 0.26	1.29 ± 0.21	0.08 ± 0.02	–	
Southwestern Black Sea	w.w.	23.54 ± 6.77	2.44 ± 0.54	0.36 ± 0.42	0.02 ± 0.01	0.01 ± 0.01	[47]
Black Sea	w.w.	–	–	0.099	0.013	0.081	[48]
Sinop	w.w.	7.11–17.88	0.18–0.33	0.03–0.09	0.007–0.0085	0.01–0.017	[49]
Sinop	w.w.	9.70 ± 1.9	2.90 ± 0.99	1.17 ± 1.01	0.02 ± 0.01	–	[50]
Kastamonu		6.74 ± 1.63	2.35 ± 0.36	1.18 ± 0.45	0.03 ±0.01	–	
Zonguldak		6.24 ± 0.8	2.25 ± 0.25	0.86 ± 0.34	0.03 ± 0.01	–	
Sinop	w.w.	12.63 ± 0.22 18.52 ± 0.60	0.59 ± 0.06 2.10 ± 0.67	0.19 ± 0.02 0.90 ± 0.28	0.03 ± 0.00 0.22 ± 0.03	0.13 ± 0.01 0.23 ± 0.00	[51]
Western Black Sea	w.w.	–	–	–	–	0.01 ± 0.01	[52]

d.w.= dry wt.; w.w. = wet wt.

Table 8 Comparison of the amounts (ppm) of heavy metals in the edible tissues of *Mullus barbatus* L. caught near various areas of the Black Sea coast

Location	dw/ ww	Metals					Ref.
		Zn	Cu	Pb	Cd	Hg	
Black Sea	d.w.	106 ± 9.1	0.98 ± 0.07	0.84 ± 0.07	0.45 ± 0.04	–	[22]
Black Sea	d.w.	1.424–63.290	0.380–2.714	–	–	–	[23]
Trabzon	w.w.	8.26 ± 0.77	1.30 ± 0.13	0.22 ± 0.08	0.02 ± 0.00	–	[24]
Sinop		10.5 ± 2.03	0.87 ± 0.09	0.39 ± 0.03	0.03 ± 0.00	–	
İstanbul	d.w.	7.573 ± 0.389	–	0.727 ± 0.141	0.208 ± 0.017	–	[25]
Black Sea	w.w.	75.5 ± 5.3	0.96 ± 0.08	0.36 ± 0.03	0.17 ± 0.02	36 ± 2 μg·kg^{-1}	[26]
Sinop	d.w.	–	–	0.0525	< 0.02	< 0.05	[27]
Samsun		–	–	0.0815	< 0.02	< 0.05	
Samsun, Ordu, Trabzon, Rize	d.w.	17.8 ± 1.8	1.4 ± 0.1	0.40 ± 0.04	0.23 ± 0.02	–	[28]
Samsun, Sinop, Terme, Fatsa Ordu	d.w.	23.71 ± 0.71	3.14 ± 0.31	0.92 ± 0.12	0.020 ± 0.002	–	[29]
Amasra	w.w.	16.03 ± 14.05 (3.48–40.72)	4.08 ± 2.79 (1.23–9.21)	1.11 ± 1.60 (0.09–7.00)	0.11 ± 0.13 (0.02–0.55)	–	[31]
Trabzon-Turkey	d.w.	27.36	1.12	0.10	0.02	0.11	[32]
Sinop	d.w.	6.95–18.43	4.93–7.74	0.09–0.31	0.02	–	[53]
Black Sea (İstanbul and Kocaeli)	d.w.	14.6 ± 1.3	1 ± 0.18	0.02 ± 0.01	0.02 ± 0.01	0.47 ± 0.02	[37]
Eastern Black Sea, Turkey Ordu-Samsun	d.w.	19.7	1.36	0.020	0.018	–	[38]
West Black Sea	d.w.	36.4 ± 3.2	2.28 ± 0.03	–	–	–	[39]
Sinop	d.w.	17.15 ± 3.78	0.95 ± 0.41	0.82 ± 0.34	0.035 ± 0.018	–	[40]
Trabzon-Turkey	w.w.	–	–	< LOD	3.38 ± 0.06	–	[41]
Sinop	d.w.	3.2	< 0.5	< 0.05	< 0.02	< 0.05	[42]
Sinop	d.w.	10.64–19.53	2.79–5.45	0.11–0.45	0.03–0.19	–	[54]
Samsun	w.w.	4.95 ± 0.6	1.27 ± 0.19	1.76 ± 0.40	0.20 ± 0.11	–	[44]
Sinop		9.49 ± 0.38	2.38 ± 0.12	2.94 ± 0.81	0.07 ± 0.02	–	
Kocaeli		5.71 ± 0.88	1.4 ± 0.12	0.88 ± 0.12	0.06 ± 0.005	–	
Kastamonu	w.w.	6.14 ± 1.46	2.35 ± 0.38	7.21 ± 1.56	0.28 ± 0.03	–	[45]
Giresun	w.w.	6.02 ± 0.45	1.99 ± 0.18	0.45 ± 0.05	0.04 ± 0.00	–	[46]
Trabzon		7.15 ± 0.64	1.74 ± 0.13	1.03 ± 0.10	0.12 ± 0.03	–	
Rize		5 ± 0.31	1.81 ± 0.15	1.30 ± 0.16	0.09 ± 0.02	–	
Southwestern Black Sea	w.w.	20.80–34.94	1.36–11.85	0.03–1.70	0.02–0.05	0.01–0.03	[47]
Ordu	d.w.	44.85 ± 7.11 83.13 ± 8.4	1.64 ± 0.37 3.95 ± 0.74	0.81 ± 0.04 1.54 ± 0.36	0.8 ± 0.02 0.91 ± 0.02	–	[55]
Black Sea	w.w.	–	–	0.165	0.016	0.032	[48]
Sinop	w.w.	5.61–11.8	0.27–0.49	0.025–0.06	0.007–0.011	0.015–0.021	[49]
Romania	w.w.	–	3.486 ± 2.45	0.32 ± 0.25	0.026 ± 0.001	–	[56]
Romania	w.w.	–	–	–	–	0.035 ± 0.01 (0.021–0.072)	[57]
West Black Sea	w.w.	–	–	–	–	0.03 ± 0.02	[52]

d.w.= dry wt.; w.w. = wet wt.

consumed whiting (*M. merlangus* L.) and red mullet *(M. barbatus* L.) caught near the southern coast of the Black Sea in 2017 and 2018. For all age groups, the EDI values for each heavy metal decreased in the following order: Zn > Cu > Pb > Hg > Cd. The mean values of Cd, Hg, Pb, Cu, and Zn in the edible tissues were 0.013, 0.024, 0.07, 0.195, and 9.05 mg/kg wet wt. for whiting and 0.017, 0.036, 0.05, 0.29, and 6.4 mg/kg wet wt. for red mullet, respectively. The differences might have been caused by the fact that the samples were caught during different fishing seasons and in different areas of the Black Sea.

In all cases, HI values for each metal were < 1, suggesting no health risk. The concentrations also met the standards set up by regulatory bodies of Turkey and the European Union. The RI values for whiting and red mullet did not exceed the insignificant limit (10^{-6}). In addition, these two commercial species caught near the Sinop coast showed no carcinogenic potential.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Comparative assessment of sorbic and benzoic acid via express biotest

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Abstract: Negative physiological and biochemical effects of chronic and subchronic doses of benzoates and sorbates may pose a certain risk to human health. Identifying new biomarkers responsible for the body's response to these compounds could provide significant details in determining the mechanism of their toxicity. To assess comparatively physiological, cytological, cytogenetic, and biochemical parameters in onion roots cells we used an Allium test. The roots were previously treated with sorbic and benzoic acids. The study recorded the dose-dependent toxic effect of these preservatives on the root mass growth. The EC_{50} values obtained for benzoic and sorbic acids (10 mg/L and 110 mg/L respectively) were significantly lower than the regulated concentrations prescribed by the standards for their content in certain types of food products. With an increase in concentrations of these acids, the mitotic index of meristematic cells decreased in experimental groups compared to control groups. The data obtained confirmed the necessity of estimating the mitotic index when choosing onion for the Allium test. The necessity resulted from the fact that low proliferative activity could cause false positive results. Sorbic and benzoic acids in concentrations below the corresponding EC_{50} increased the frequency of chromosomal aberrations in apical meristematic cells of the roots compared to control. Thus, benzoic and sorbic acids had reliable mitodepressive and genotoxic effects on the dividing cells of onion roots. The study explored the dynamics of lipid oxidation biomarker accumulation (malon dialdehyde, MDA) after exposure to benzoic and sorbic acids. The toxic effect of benzoic acid appeared not to be associated with oxidative damage to root cell lipids, whereas sorbic acid in concentrations from 20 to 200 mg/L resulted in a multiple increase in MDA concentration in the test samples compared to control. At the same time, lipid peroxidation showed a higher level of sensitivity compared to other indicators of this test. Further, the data obtained on the toxic influence of sorbic and benzoic acids can be used in express methods to assess food and ecological security of these acids.

Keywords: Food preservatives, *Allium cepa*, biotesting, lipid peroxidation toxicity, cytogenetic analysis, biomarkers

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INTRODUCTION

Food preservation has remained a problem throughout the human history. It is caused by the activity of environmental microorganisms and enzymatic reactions in the products during their production and storage [1, 2]. About a third of the population in the developed countries are estimated to suffer from diseases transmitted through food especially falsified [3]. Food safety is directly related to the development of chemicals that prevent or slow down the spoilage of these products.

Sorbic and benzoic acids, as well as their salts, are known to be widely used as food preservatives. Their production is steadily increasing. These acids are contained in some fruits, berries, dairy products. Sorbic acid is an unsaturated fatty acid and is used only as a preservative in food, animal feed, tobacco, cosmetics and pharmaceuticals. It is metabolized like normal fatty acids, so this acid was assumed to have no side effects. Benzoic acid is a synthetic additive, used as a preservative and antioxidant. It is excreted by the human body through the kidneys.

There are numerous data on the health safety of these compounds in regulated food products. Recently, however, there are more discussions on the necessity to develop scientific approaches to studying mechanisms of their toxicity [4, 5]. The interest in this problem is due to by the detected adverse effects of the chronic and subchronic benzoate and sorbate intake by both animals and humans. Thus, adding benzoic acid to pig feed increased the liver enzymes activity and changed the blood formula negatively, eventually damaging the liver and spleen, respectively [6].

In vitro studies of human erythrocytes demonstrated that sodium benzoate reduced the level of key metabolic enzymes of amino acids (aspartate and alanine aminotransferase) and alkaline phosphatase significantly [7]. There is strong evidence that attention deficit and hyperactivity syndrome in children and anxiety conditions in rats could be associated with high doses of sodium benzoate [8–9]. Other researchers showed that sodium benzoate caused numerous negative physiological and biochemical changes in mice and rats. The changes included reducing the mass of reproductive organs and embryos and the level of sex hormones in mice [10]. As for human blood cell culture, sorbic acid demonstrated the inhibitory effect on biochemical reactions in the activated immune response [11].

However, the mechanism of toxicity for these preservatives is still unclear. In addition, creating a new algorithm for assessing food safety is debated a lot. The algorithm especially concerns foods containing several food additives because of their potential additive and synergetic effect of toxicity [12]. It is yet to be found out if the food preservatives may exert increased activity in people with specific diseases or genetic defects.

To rise up to the challenge, it is necessary to go beyond standard toxicity tests to identify molecular biological protection mechanisms and to identify biomarkers responsible for the body reaction to the effects of chemical compounds. All the more so, as modern methodology and instrumentation system are able to tackle these complex problems. New approaches should not only monitor and evaluate toxic effects, but also result in the adequate test systems for modelling detoxification and metabolism of food preservatives in the human body. It is important to develop new model systems. They should be simple to execute, cheap, and able to simulate the reactions of the human body, both on the physiological and molecular levels, with the maximum available accuracy.

In this aspect, the special interest is given to the work on a comprehensive assessment of biomarkers of neurotoxicity and antioxidant enzymes activity in daphnia under the influence of food sweetener sucralose. It is due to the evidence that *Gammarus zadachi* and *Daphnia magna* crustaceans exposed to this sweetener altered their swimming behavior [13]. The tests were carried out on these organisms to compare the activity of acetylcholinesterase (AChE), lipid peroxidation enzymes, and the ability to absorb oxygen radicals (ORAC assay) in them. The authors observed the stimulating effect of sucralose on the activity of AChE and lipid peroxidation, but not on the antioxidant capacity (ORAC). In humans, an increased AChE activity was also associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and restless legs syndrome. It is important to note that the data obtained in this work are consistent with those in other experimental studies on human cell cultures and vertebrates. However, plant test systems are also of interest, in particular *Allium cepa* L. onion roots (Allium test).

Traditionally, the Allium test has been used as a bioindicator in numerous studies on toxicity, cytotoxicity, and genotoxicity of various chemical compounds. It is recommended by WHO experts as a standard for the cytogenetic monitoring of the environment. Recently, it has been increasingly used to assess the genotoxic potential of medicinal plants, food additives, and even ionizing radiation [14–17]. The Allium test was an excellent eukaryot model *in vivo*. It was one of the few direct methods for measuring damages in biological systems after exposure to various toxicants and mutagenes. Its main advantages include the following characteristics. First, the apical meristematic root cells can show constant mitotic division. Second, the roots may incubate directly with the object being tested. Third, these cells have large chromosomes, which allows a comprehensive analysis of DNA damage. In addition, the test indicators were shown to be more sensitive than the models on microorganisms, cell cultures, and even animals [15, 18].

Allium cepa was also presented as an effective test object in studying the reaction of plant cell biomarkers to chemical toxicants of different nature. It is known that chemical pollutants can induce the formation of active forms of oxygen. In its turn, oxygen can activate enzymes of peroxidation and result in damaging various biological molecules, including lipids. Thus, it was found that herbicide glyphosate and copper salts significantly increased lipid peroxidation in plant cells [19, 20]. In our opinion, the Allium test can help significantly expand our knowledge of the mechanisms of damage to biological systems of eukaryotes, including the damage after exposure to sorbic and benzoic acids. Moreover, no information was found on an effect of these preservatives on physiological and biochemical parameters in the meristematic cells of onion roots. The aim of the research was to compare changes in the mass growth, activity of lipid peroxidation enzymes, cytological and cytogenetic parameters of *Allium cepa* roots after treatment with sorbic and benzoic acids.

STUDY OBJECTS AND METHODS

In the research we used such preservatives as sorbic acid (Alfa Aesar by Thermo Fisher Scientific) and benzoic acid (Alfa Aesar by Thermo Fisher Scientific).

Allium cepa onion bulbs (Stuttgarter sort) of the same size (2.5–3 cm in diameter) and mass (5–7 g) were selected as a test organism. Dry scales were removed from the bulbs before incubation. Preliminarily, the germination was conducted in 15 mL test tubes with bottled water for 2 days in the dark at 25°C.

The bulbs with roots over 1 cm long were selected for further studies. Before treatment with benzoic and sorbic acid solutions, the average mass of the roots was determined in a separate group of the control bulbs. Then the bulbs were transferred to the solutions of these acids in the bottled water and incubated for 2 or 3 days depending on the purpose of the experiment. After the incubation, the roots were cut off, dried with filter paper, and weighed [21]. The EC_{50} value was determined by the concentration of the preservative, which retarded the root mass growth by 50% compared to control, considering the average mass of the roots before treatment with acids. For cytogenetic analysis, the apical meristematic cells of the roots were stained with acetoorcein (1 g of orcein dye was diluted in 50 mL of 45% CH_3COOH). The roots were placed in a 70% solution of ethyl alcohol for the long-term storage. Next, instant squash preparations were obtained, the analysis of which was carried out with the help of a light microscope Axioskop 40, Zeiss.

The lipid oxidation level was determined by the concentration of malon dialdehyde (MDA) in the onion roots [22]. The sample weight of approximately 0.25 g to the fourth decimal place was measured in a 15 mL test tube. Then 1 mL of trichloroacetic acid solution (Merck, Germany), concentration of 200 g/dm^3, was added. The mixture was thoroughly stirred with a glass stick. Then the stick was washed with 3 mL of the same solution of trichloroacetic acid. The tubes were tightly corked and centrifuged at 1000 g and 4°C for 15 min. One milliliter of supernatant was transferred to a clean 15 mL test tube. Four milliliters of thiobarbituric acid solution (0.5 g of thiobarbituric acid (Diaem, Russia)) was added to 100 mL of trichloroacetic acid solution (200 g/dm^3). The test tubes were closed and placed in a water bath at 95°C for 30 min. Then the test tubes were pulled out and cooled in an ice bath. The cooled solutions were centrifuged at 1000 g and 20°C for 10 min.

The spectrophotometric detection was performed with the obtained solutions at 600 and 532 nm. The MDA content was calculated according to the formula:

$$x = \frac{(ABS_{532} - ABS_{600}) \times K}{K_e \times l \times m_{wt}}$$

where ABS_{532} is the absorption value at 532 nm;

ABS_{600} is the absorption value at 600 nm;

K is the dilution factor;

K_e is the molar coefficient of extinction;

l is a beam path length, cm;

m_{wt} is the weight of the sample, g.

The statistical processing of the results was carried out in Microsoft Excel and Statistica programs (v. 12). In the paper, the analysis of average values by Student's criterion with Fisher's angular transformation was used for comparative estimation of percentages.

RESULTS AND DISCUSSION

The macroscopic parameters were studied and comparatively evaluated, particularly, for the levels of mass growth in the onion roots after treatment with benzoic and sorbic acid solutions. According to the literature review, the macroscopic parameters appeared more sensitive in comparison with the cytological and cytogenic parameters [23]. This conclusion seemed logical because these parameters reflected the final effect of all disorders in the plant cells. In this work, when calculating the growth of root mass, the average weight of roots was subtracted both in control and experimental samples before their treatment with preservatives solutions. Thus, the EC_{50} overstatement error was eliminated in these samples. In the preliminary experiments the solutions of preservatives were used with the concentrations not exceeding the permissible levels for some food products, namely, 1 g/L and 2 g/L. Death of the roots was observed after 2 days of incubation. Therefore, we reduced the range of acid concentrations significantly. As a result, the root growth and dose-dependent toxic effects were observed during the same incubation period (Figs. 1 and 2). The roots in the samples remained white and unchanged in shape throughout the incubation. However, there were statistically significant differences between the control and test samples, namely, when treated with benzoic acid at concentrations of 0.01 ($P < 0.1$); 0.05 ($P < 0.05$); 0.1 ($P < 0.05$) and 0.2 g/L ($P < 0.05$) and with sorbic acid at concentrations of 0.02 ($P < 0.1$); 0.1 ($P < 0.05$); 0.2 ($P < 0.05$) and 0.3 g/L ($P < 0.05$). EC_{50} was 10 mg/L for benzoic acid and 110 mg/L for sorbic acid. Thus, these values differed significantly from the domestic regulatory norms on the content of these food additives in certain types of food.

As far as we know, this is the first study in which EC_{50} values were identified for these preservatives in the Allium test. At the same time, cyanobacteria with

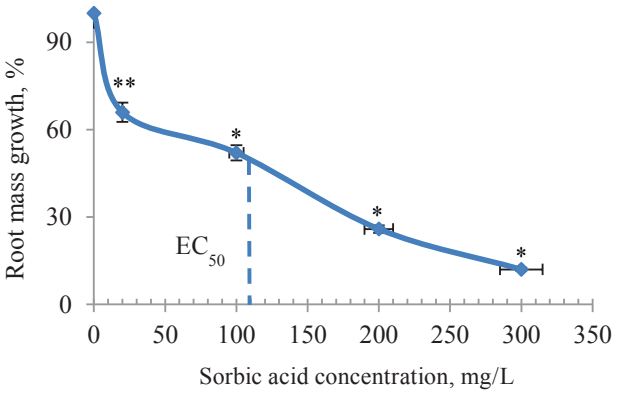

Figure 1 Root mass growth inhibition after treatment with sorbic acid (n = 10). * $P < 0.05$, ** $P < 0.1$

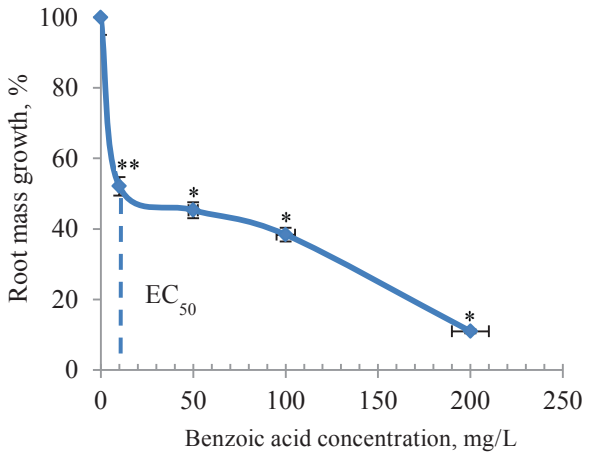

Figure 2 Root mass growth inhibition after treatment with benzoic acid (n = 10). * $P < 0.05$, ** $P < 0.1$

EC_{50} from 9 mg/L were the most sensitive to benzoic acid in similar studies using different organisms living in water when treated for 14 days. In molluscs, fish and amphibians, EC_{50}/LC_{50} values were determined within 100–1291 mg/L for 24–96 h [24]. The results of these studies confirmed the high sensitivity of the macroscopic parameters in the Allium test.

However, the studies on the toxicity of benzoic and sorbic acids using the Allium test focused mainly on the microscopic indicators reflecting the peculiarities of cell division and chromosomal aberrations occurring in its process, and EC_{50} was not determined. At the same time, the tested concentrations of preservatives were usually much higher than the EC_{50} values we found. So the exposure of the roots to the preservative solutions in these studies usually did not exceed several hours [25, 26]. We believe that such experimental conditions are suitable only for acute toxicity testing. They are totally unacceptable for the study of subchronic and chronic consequences of negative effects, especially at the biochemical level. The last aspect should be considered the most interesting in the case of food preservatives. Therefore we believe that the Allium test scheme previously proposed for environmental monitoring did not lose its relevance for studying the toxic effects of these preservatives. The Allium test included a comparative analysis of macro- and microindicators at concentrations of toxicants within their EC_{50} [23].

The mitotic index is one of the microindicators in the Allium test which is used as an indicator of the level of cell proliferation. It is known that the dose-dependent deviation of the mitotic index in the experimental samples compared to the control values, both increasing and decreasing, indicates cytotoxicity of the tested chemical. In our previous study, the mitotic index of cells of their meristem was decreasing significantly with an increase in concentrations of preservatives (Table 1). In testing highly toxic doses of sorbic acid (from 1 to 2 g/L), the mitotic index decreased only slightly when the concentration of this acid increased [26]. This data confirmed the previous assumption that there may be difficulties in interpreting the research results due to the high concentration of preservative.

The cytogenetic analysis was carried out on the squash preparations of the apical meristematic cells of onion roots obtained in the previous study. The analysis determined the accumulation dynamics of chromosomal aberrations when the concentrations of sorbic and benzoic acids were increased. According to Table 1, when acid concentrations increased, the proportion of mitosis pathologies also increased, peaked, and then decreased. It is noteworthy that the highest percentage of chromosomal aberrations coincided with acid concentrations close to the corresponding EC_{50} of these preservatives. The drop in chromosomal aberrations at high concentrations of acids is probably associated with a significant decrease in the number of divisible cells in the meristematic cells of roots.

The types of major chromosomal aberrations detected in the experiment are shown in Fig. 3. The analysis of the data allows us to conclude that stickness of chromosomes in metaphase and chromosomes with laggard in anaphase make the main contribution to the spectrum of chromosomal aberrations. These anomalies account respectively for aberrations ranging from 23.8% to 70% (for sticky metaphase) and from 13.6% to 45.2% (for chromosome with laggard). Also, there were the following aberrations of the mitosis process detected in micropreparations: C-mitosis, multiple fragmentation of chromosomes, change in the spatial orientation of chromosomes at the metaphase stage in cells. The least observed anomalies included bridges and fragments (about 2%, depending on the concentration of the tested substances).

It seems remarkable to consider the whole spectrum of aberrations. The most numerous anomalies found while analyzing biomaterial can be due to the effects

Table 1 Mitotic index and chromosomal aberrations in meristematic cells of onion roots after exposure to benzoic and sorbic acids (n = 10)

Acid concentration, g/L	Mitotic index, %	Chromosomal aberrations based on the total number of cells, %
	Benzoic acid	
0.01	12.02 ± 0.48^{c}	0.88 ± 0.14^{a}
0.02	10.20 ± 0.49^{a}	1.00 ± 0.16^{a}
0.1	6.89 ± 0.33^{a}	0.50 ± 0.09^{b}
0.2	0.75 ± 0.42^{a}	0.05 ± 0.04^{a}
	Sorbic acid	
0.02	9.63 ± 0.42^{a}	0.95 ± 0.14^{a}
0.1	6.81 ± 0.38^{a}	0.79 ± 0.12^{a}
0.2	1.52 ± 0.16^{a}	0.10 ± 0.04^{a}
0.3	0.00 ± 0.00^{a}*	0.00 ± 0.00^{a}*
Control	12.97 ± 0.48	0.30 ± 0.08

[a] $P < 0.05$, [b] $P < 0.1$, [c] $P < 0.15$, * 6503 cells were observed

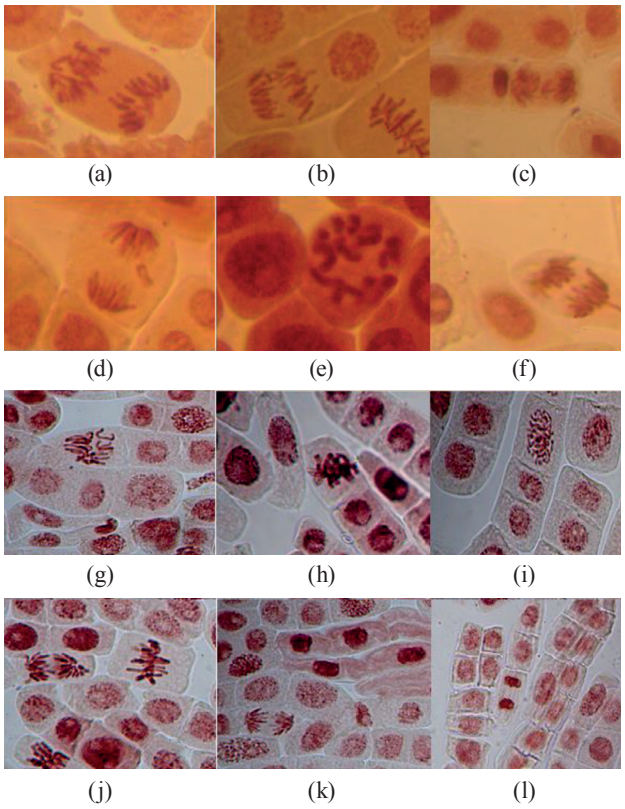

Figure 3 Stained preparations of meristemic cells of oninon roots: (a, b) fragmentation in anaphase; (c, d) fragments of chromosomes in anaphase; (e) C-mitosis; (f) anaphase with laggards; (g) fragmentation in metaphase; (h) sticky metaphase, without pathologies: (i) prophase; (j) metaphase; (k) anaphase; (l) telophase

of mitotic spindle disorder and changes in the surface of chromosomes. The aberrations occur in this group probably due to the influence of the tested substance on the proteins regulating the work of the mitotic spindle in the cell [27]. On the other hand, bridges, fragments, and micronuclei are associated with clastogenic aberrations (arising from the fracture of the chromosome and anomalies of the further molecular genetic processes, unequal translocation or inversion of the chromosome segments). In the study [28], the analysis of genotoxicity of sodium benzoate (in concentrations from 20 to 100 mg/kg) discovered the prevalence of aberrations related to mitotic spindle disorders and changes in the surface of chromosomes. These are the main types of agglutination and C mitosis disorders. The clastogenic effect of the factor was not recorded at all for this indicator.

On the other hand, studies with high concentrations of sodium benzoate exposed a much wider spectrum of chromosomal aberrations. The aberrations included agglutination and fragmentation of chromosomes, their reduction, the formation of binuclear cells, chromosomal bridges and other disorders [29]. According to data [30], treating cells with sorbic acid resulted in the

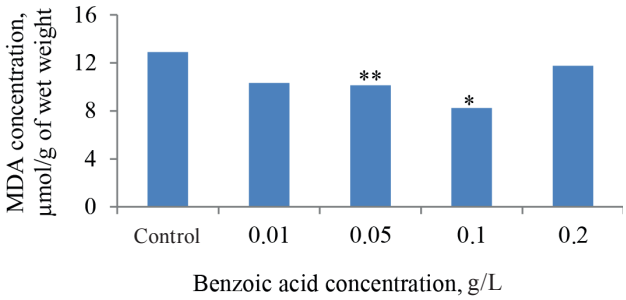

Figure 4 Effects of different doses of benzoic acid on MDA level in roots after 2 days of incubation. * $P < 0.05$, ** $P < 0.1$

chromosomal aberrations associated with mitotic spindle disorder. Clastogenic aberrations were not detected. Similar data were obtained in the study of the effects of sorbic acid on the formation of micronuclei in cells [31].

This study recorded reliable mitodepressive and genotoxic effects at very low concentrations of preservatives (10 and 20 mg/L for benzoic and sorbic acids, respectively). It is important to note that the data obtained were consistent with the results on genotoxicity of these acids and their salts for human and animal cell culture. The results were published in a number of papers, describing the exposure to both low and high doses of these preservatives. Thus benzoic acid caused sister chromatid exchange, chromosomal aberrations, and micronuclei formation in human lymphocyte cells [32]. Other researchers demonstrated the genotoxic effect of sodium sorbate on Chinese hamster cells, as well as clastogenic, mutagenic and cytotoxic effects of sodium benzoate on the cell culture of human lymphocytes [29, 33].

MDA concentration is commonly used as an indicator of lipid peroxidation when the tissues are exposed to chemical toxicants. MDA was measured in the onion roots of the control and experimental groups obtained in our previous study. In the experimental groups of onion roots this biomarker analysis showed

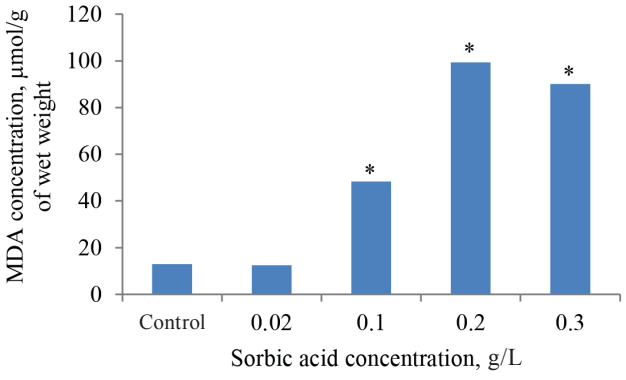

Figure 5 Effects of different doses of sorbic acid on MDA level in roots after 2 days of incubation (n = 10). * $P < 0.05$

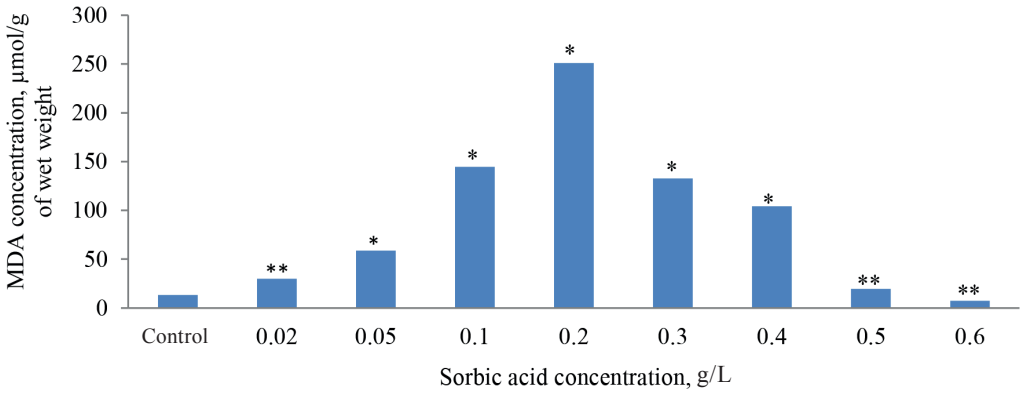

Figure 6 Effect of different doses of sorbic acid on MDA level in roots after 3 days of incubation (n = 10). * $P < 0.05$, ** $P < 0.1$

a significant dose-dependent increase in the MDA levels (by 760%) compared to the control samples. At the same time benzoic acid did not have a significant effect on the process (Figs. 4 and 5). Since sorbic acid can be subjected to partial hydrolysis, MDA was measured in a solution of sorbic acid (0.2 g/L) after 4 days of incubation, but its content exceeded only by 57% compared to the values in the control samples of onion roots.

Since the biomarkers of oxidative stress usually showed a two-phase response rather than a linear response, we expanded the range of sorbic acid dilutions and increased the period of incubation with acids up to 3 days to identify the dynamics of MDA biosynthesis. Indeed, this pattern of the two-phase response was confirmed again [13]. With an increase in sorbic acid concentration, the level of this biomarker increased evenly at first, reached its peak, and then dropped dose-dependent until it reached MDA value in the control samples (Fig. 6). Like in the previous study, the maximum concentration of MDA was recorded for sorbic acid at 200 mg/L. The concentration was above that in the control group by almost 2000%, i.e. lipid oxidation level also increased with exposure time.

As far as we know, these are the first experiments to study the dynamics of lipid oxidation biomarker generation in the Allium test after exposure to benzoic and sorbic acids. Both acids reduced root growth and the mitotic index of apical meristematic cells. However, these negative phenomena were accompanied by the simultaneous increase in MDA only in the case of sorbic acid. These results are consistent with the data on the treatment of wheat seeds with benzoic acid [34]. This study did not detect any change in lipid peroxidation activity different from control when treated with low concentrations of this preservative.

In the case of benzoic acid, its toxic effects were probably not associated with oxidative damage to lipids. In addition, the study showed the protective reaction in the plant cell to benzoic acid in concentrations of 1 to 10 mM. The reaction was accompanied by an increased activity of glutamate and malate dehydrogenase, enzymes activating catabolic and metabolic processes [35]. In the current study, both the MDA level and root mass growth increased with an increase in the concentration of sorbic acid from 20 to 200 mg/L (Figs. 5 and 6). Thus, there was a clear correlation between the physiological index and MDA, the latter being even more sensitive.

According to the literature, the meristematic cell mitotic index in the control samples when using the Allium test is both close to our result (12.97 ± 0.48) and well below it [20, 36, 37]. This indicator could change depending on the quality of the batch of onions, its variety, and storage conditions. However, the question remained whether there was a dependence between the

Table 2 Inhibition of the root mass growth and the mitotic index after treatment with sorbic acid (n = 9)

Sorbic acid concentration, g/L	Root mass growth, g	Mitotic index, %	Chromosomal aberrations based on the total number of cells, %
0.02	0.19 ± 0.07	6.84 ± 0.40[a]	0.29 ± 0.09
0.2	0.05 ± 0.02[a]	0.00 ± 0.00[a]*	0.00 ± 0.00[a]*
Control	0.27 ± 0.04	7.97 ± 0.89	0.15 ± 0.06

[a] $P < 0.05$, *1748 cells were observed

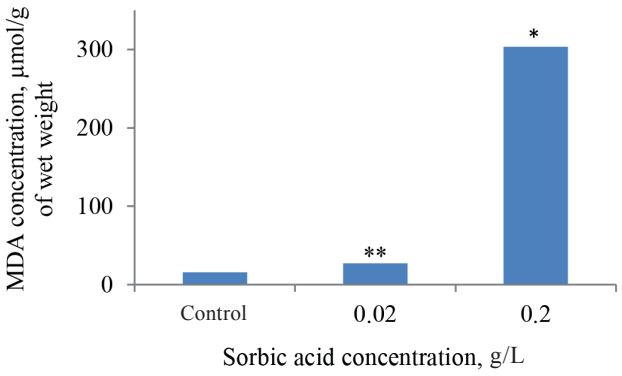

Figure 7 Effect of different doses of sorbic acid on MDA level in roots after 2 days of incubation (n = 9). * $P < 0.05$, ** $P < 0.1$

initial level of the mitotic index and the ability of root cells to fully respond to the effects of toxicants.

To this end, another study was conducted to examine the toxic effect of sorbic acid solutions at low and high concentration on onions with a small part of meristematic dividing cells in control. In this study, the miotic index in the control samples (Table 2) was 40% lower than that obtained in the previous study (Table 1). The conditions of the Allium test in the previous and the current study did not differ. The comparative analysis showed the following negative trends in the results. First, the roots in the experimental groups became soft and acquired a yellowish hue after 2 days of incubation with the acid. Second, there was no gain in the root mass compared to control (Table 2) when treated with a high-concentration acid solution (0.2 g/L), whereas in the previous study the gain was 25%, and roots did not change the color (Fig. 1). Similar negative changes were recorded at the biochemical level in MDA measurement (Fig. 7). The lipid oxidation activity in these samples compared to the previous study was significantly higher both in the test and control samples.

According to the obtained results, it seems advisable to select batches of bulbs before the Allium test. This selection is necessary as the low values of the mitotic index may result in false positive results, both in terms of EC_{50} estimates and biochemical indicators.

CONCLUSION

The results of this study showed that sorbic and benzoic acids caused toxic effects in the roots of *Allium cepa*. These preservatives affected the physiological, biochemical, cytological, and genetic characteristics of the plant system. Treating onion roots with these acids in concentrations of 1 and 2 g/L, which are acceptable for some food products, was so highly toxic as to lead to their death. When concentrations of these acids decreased, EC_{50} limits for benzoic and sorbic acids were shown to be 10–20 and 20–100 mg/L, respectively. These concentrations of preservative solutions induced a 50% retardation in root growth, a significant decrease in the mitotic index, especially in the case of sorbic acid, and almost a triple increase in chromosomal disorders.

Thus, these preservatives at very low concentrations gave a chronic and subchronic toxic effect. Based on the conducted studies, it is necessary to use the concentration of food preservatives within their detected EC_{50} values to assess these toxicity indicators in the Allium test. If these conditions are met, it is possible to simulate the processes of detoxification and metabolism for these compounds, both at the cellular level and the whole organism.

Therefore, it can help gain a better understanding of the biological actions of these agents. Indeed, the negative effects found under these conditions for sorbic acid, but not benzoic acid, were correlated with the lipid oxidation biomarker. In this regard, we believe that the study of this biomarker can provide valuable information for monitoring and predicting early effects of sorbic acid on animal and human cells. Yet, it is probably necessary to study the role of catabolic processes to determine the molecular mechanisms of activation of enzymes with benzoic acid [35].

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Microbial indices of industrial and traditional medicinal herbs in Ahvaz, Iran

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Abstract:
Introduction. Medicinal herbs are susceptible to microbial contamination which can have profound effects on the consumer's health. Our study aimed to evaluate microbial contamination of common medicinal herbs in Ahvaz.
Study objects and methods. We collected 80 samples of traditional and industrial medicinal plants from the supply market, namely valeriana, fennel, licorice, and shirazi thyme. The reference method was used to determine microbial indices such as the total count of microorganisms, yeast and mold, *Bacillus cereus*, coliforms, and *Escherichia coli*.
Results and discussion. We found that the total microbial count, yeast and mold, *B. cereus*, and coliform contamination accounted for 45, 77, 55, and 55% of the total samples, respectively, exceeding the allowed limits. There was a significant difference between the industrial and traditional samples in fungal and coliform contamination, with the traditional samples being more highly contaminated. However, no significant difference was observed between them in total count and *B. cereus* contamination. *E. coli* contamination was detected in 31.2% of the samples, mostly in traditional. Total microbial count and yeast and mold contamination were highest among valeriana plants. Fennel showed the highest *B. cereus* and coliform contamination. The lowest contamination was observed in licorice.
Conclusion. The results showed that a considerable percentage of the medicinal herbs under study were contaminated at levels exceeding the standard limits. Plants could be contaminated during harvesting, processing or storage. Finally, different species of plants have different antimicrobial activities that affect their microbial contamination.

Keywords: Microbiology, microbial contamination, quality control, medicinal herbs, total microbial count

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INTRODUCTION

Medicinal plants become contaminated by a variety of sources such as heavy metals, insect larvae and seed, different bacteria, and fungi [1]. Heat and humidity of the environment, long-time drying, irrigation with contaminated water, and lack of farmer training may result in considerable microbial contaminations and reduce the quality of plants. Moreover, microbial contamination of plants may take place during unhealthy collection, cleaning, storage, transportation, and packaging. Contact of herbal products with external factors such as plastic, glass, and other materials may lead to cross contamination.

Medicinal plants can be contaminated by a wide range of microorganisms, such as fungi, yeasts, protozoa, and viruses, most of which are transferred from soil [2, 3]. Total microbial count is an important factor in determining the health status or probable detection of a contamination source [4]. Yeast and mold are the most common contaminants of medicinal herbs. Various species of molds and yeasts that proliferate on food stuff secrete metabolic toxic materials such as mycotoxins, which are harmful for humans and animals [5]. The WHO (World Health Organization) has a large amount of data in this direction [6].

Coliforms (*Escherichia*, *Enterobacter*, and *Klebsiella*) from the *Enterobacteriaceae* family inhabit human and animal intestines. Most of them are not pathogenic, although some *E. coli* strains could be highly pathogenic and cause food poisoning [7]. According to Iran's national standard, coliform contamination in most dried vegetables should not exceed the maximum level of 1000 CFU/g, while the presence of *E. coli* is not allowed[I]. *Bacillus cereus* is widely distributed in the environment and some its strains are harmful for human health and can cause food poisoning. This bacterium secretes enterotoxin, hemolysin, and lecithinase C which are responsible for disease [9]. Dried vegetables contamination by *Bacillus cereus* should not exceed the maximum level of 100 CFU/g.

Valeriana (*Nardostachys jatamansi* L.) from the *Valerianceae* family is known for its anticonvulsant, sedative, anti-asthmatic, and cardiotonic properties [9]. Fennel (*Foeniculum vulgare* L.) from the Umbelliferae family has culinary and medicinal properties (anti-inflammatory, anti-spasmodic, diuretic, laxative, analgesic, antioxidant, and wound-healing) [10]. Licorice (*Glycyrrhiza glabra* L.) growing in Mediterranean countries, Central Asia, and Europe has a wide range of pharmacological effects such as antioxidant activity, liver protection, and regulation of the immune system [11]. Shirazi thyme (*Zataria multiflora Boiss* L.) is used in the south of Asia as tea or spice and in traditional medicine as a gastrointestinal disinfectant, diuretic, or an anti-inflammatory remedy [12].

Contaminants such as microorganisms, heavy metals, and pesticides affect the quality and the efficacy of herbal products. Since it is impossible to remove all contaminants, precautionary measures should be taken to prevent or limit contamination [2, 3]. Therefore, our study aimed to show the effect of these contaminations on consumer's health.

STUDY OBJECTS AND METHODS

Collection and preparation of samples. For this study, samples were randomly collected from medicinal herb retailers and drugstores of Ahvaz (Iran) from December 2017 for 6 months. A total of 80 samples were used: 40 traditional (10 samples for each traditional herb) and 40 industrial herbs from different companies (19 shirazi thyme, 6 fennel, 8 valeriana, and 7 licorice samples). The amounts of industrial samples were not equal due to their insufficient availability.

Total microbial count. Total microbial count was performed as described by Standard No. 5272, Iran[II]. Different dilutions of medicinal herbs were prepared and cultured on Plate count agar (PCA, Merck, Germany). Triplicate plates for each dilution were cultured and incubated for 72 h at 30°C. Then, the average of counted colonies was measured taking into account the dilution coefficient.

Mold and yeast count. Fungal count was performed according to Standard No. 10899, Iran[III]. Different dilutions of medicinal herbs were inoculated on Sabouraud dextrose agar (SDA, Merck, Germany) in triplicates and incubated at 25°C for 5 days. Then, the average number of molds and yeasts per gram of herb was estimated.

***Bacillus cereus* detection.** To detect and count *B. cereus* (Standard No. 2324, Iran), dilutions of medicinal herbs were prepared and cultured in triplicate on Mannitol-egg yolk-polymyxin (MYP) agar (Merck, Germany) at 30°C for 48 h. The agar contained an egg yolk emulsion and polymyxin B sulfate (Shijiazhuang Pharma, China)[IV]. Large and pink colonies (lack of manitol fermentation) with a sedimentary halo (lecithinase producer) were counted as probable *B. cereus*. To confirm the suspected colonies, a hemolysis test was performed on Blood agar (Merck, Germany).

Coliform detection and enumeration. Coliform detection and enumeration were performed according to Standard No. 9263, Iran[V]. Different dilutions of medicinal herbs were inoculated (pour plate and two-layer culture) in triplicate on Crystal violet neutral red bile lactose (VRBL, Merck, Germany) agar and incubated at 37°C for 24 h. Typical red purple colonies were confirmed on Brilliant green bile lactose (BGBL) broth (Merck, Germany) contained in Durham tubes at two temperatures (37 and 44°C) for 24 h.

***Escherichia coli* detection.** Following coliform detection, positive BGBL tubes (gas production) were inoculated into peptone water and incubated at 44°C for 48 h. Gas production in BGBL and production of indole in peptone water were recorded for presence of *E. coli*[VI].

Statistical analysis. Analysis of data was performed using SPSS statistical software. The significance of the results was evaluated by McNemar nonparametric test with significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Total microbial contamination. According to the results of total microbial count (Fig. 1), 45% of the total

[I] Standard No. 5939, Microbiology of dehydrated vegetables-specifications. Institute of Standards and Industrial Research of Iran; 2008.

[II] Standard No. 5272, Microbiology of food and animal feeding stuffs-horizontal method for the enumeration of microorganisms-colony count technique at 30°C. Institute of Standards and Industrial Research of Iran; 2007.

[III] Standard No. 10899, Microbiology of food and animal feeding stuffs-horizontal method for the enumeration of yeasts and molds. Institute of Standards and Industrial Research of Iran; 2008.

[IV] Standard No. 2324, Microbiology of food and animal feeding stuffs-horizontal method for the enumeration of presumptive *Bacillus cereus*-colony count technique at 30°C. Test method. Institute of Standards and Industrial Research of Iran; 2006.

[V] Standard No. 9263, Microbiology of food and animal feeding stuffs-horizontal method for the enumeration of coliforms-colony count technique. Institute of Standards and Industrial Research of Iran; 2007.

[VI] Standard No. 2946, Microbiology of food and animal feeding stuffs-detection and enumeration of presumptive *Escherichia coli*. Most probable number technique. Institute of Standards and Industrial Research of Iran; 2005.

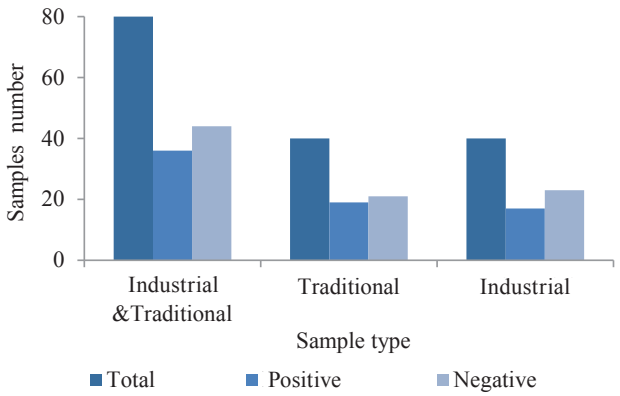

Figure 1 Total microbial contamination in medicinal herbs. Total is number of samples. Positive – contamination over the limit (10^5 CFU/g). Negative – contamination below the limit

herbs (36 samples out of 80) showed contamination over the limit (10^5 CFU/g). Of them, 19 (48%) and 17 (43%) were traditional and industrial, respectively. The microbial load in the samples with contamination over the limit varied from 5.03 ± 0.03 to 6.25 ± 0.03 log CFU/g. This ranged from 5.14 ± 0.01 to 6.25 ± 0.03 in the traditional samples and from 5.03 ± 0.3 to 6 ± 0.05 log CFU/g in the industrial samples. However, there was no significant difference between the total microbial contamination in the traditional and industrial samples ($P > 0.05$). Among the studied herbs, valeriana (*Nardostachys jatamansi* L.) and licorice (*Glycyrrhiza glabra* L.) showed the highest and the lowest contamination – 56 and 18%, respectively. Also, the total microbial contamination of shirazi thyme (*Zataria multiflora Boiss* L.) and fennel (*Foeniculum vulgare* L.) was over the limit in 55% and 44% of the samples, respectively.

Fungal contamination. The results of fungal contamination are presented in Fig. 2. As we can see, 61 (77.5%) out of 80 samples had mold and yeast contamination over the limit (10^3 CFU/g). They comprised 34 (85%) traditional and 27 (67.5%) industrial samples. The fungal load in the samples with contamination over the limit varied from 3.02 ± 0.00 to 4.78 ± 0.06 log CFU/g. This reached from 3.02 ± 0.00 to 4.78 ± 0.06 in the traditional samples and from 3.04 ± 0.01 to 4.60 ± 0.01 log CFU/g in the industrial samples. It should be mentioned that the traditional samples were significantly contaminated with mold and yeast ($P < 0.05$). Meanwhile, valeriana (100%) and licorice (47%) showed the highest and the lowest contamination, respectively. Also, 79% and 75% of shirazi thyme and fennel, respectively, showed over the limit fungal contamination.

***Bacillus cereus* contamination.** *B. cereus* contamination in 44 samples (55%) of the total herbs exceeded the limit (10^3 CFU/g) (Fig. 3), including 23 (57.5%) traditional and 21 (52.5%) industrial herbs. Over the limit *B. cereus* contamination varied from 2.03 ± 0.03 to 3.84 ± 0.06 log CFU/g. In the traditional samples, it ranged from 2.03 ± 0.03 to 3 ± 0.06 log CFU/g and in the industrial samples, from 2.03 ± 0.03 to 3.84 ± 0.06 log CFU/g. Our results showed that there was no significant difference between the traditional and industrial samples in *B. cereus* contamination ($P > 0.05$). The contamination in fennel (94%) and valeriana (66%) was significantly higher ($P < 0.05$). Shirazi thyme (41%) and licorice (29%) showed a lower level of *B. cereus* contamination.

Coliform contamination. Coliform contamination was found over the limit (10^3 CFU/g) in 44 samples (55%) of the total herbs. Among these samples, 29 (72.5%) and 15 (37.5%) were from traditional and industrial herbs, respectively (Fig. 4). Over the limit coliform contamination ranged from 3.01 to 4.16 ± 0.03 log CFU/g, namely from 3.01 to 4.16 ± 0.03 in the traditional samples and from 3.03 ± 0.02 to 4.15 ± 0.03 log CFU/g in the industrial samples. According to the results, the traditional samples showed a significantly higher coliform contamination than the industrial samples ($P < 0.05$). Fennel (81%) and valeriana (66%)

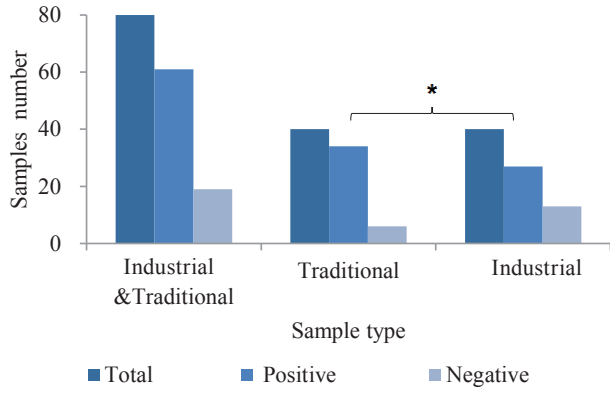

Figure 2 Mold and yeast contamination in medicinal herbs. Total is number of samples. Positive – contamination over the limit (10^3 CFU/g). Negative – contamination below the limit. * $P < 0.05$

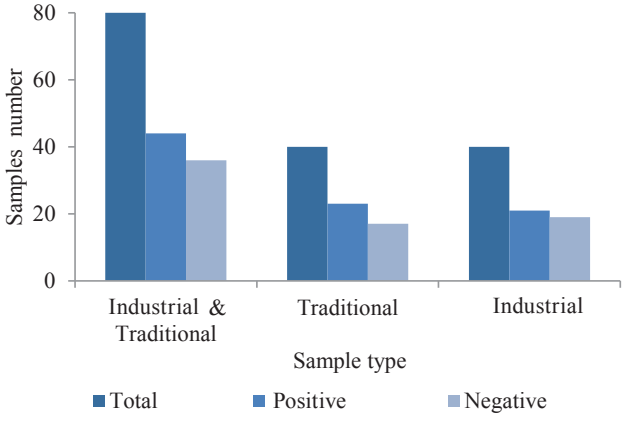

Figure 3 *Bacillus cereus* contamination in medicinal herbs. Positive – contamination over the limit (10^2 CFU/g). Negative – contamination below the limit. * $P < 0.05$

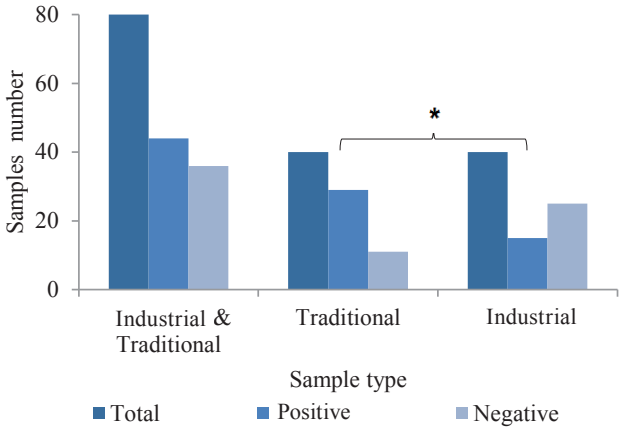

Figure 4 Coliform contamination in medicinal herbs. Positive – contamination over the limit (10^3 CFU/g). Negative – contamination below the limit. * $P < 0.05$

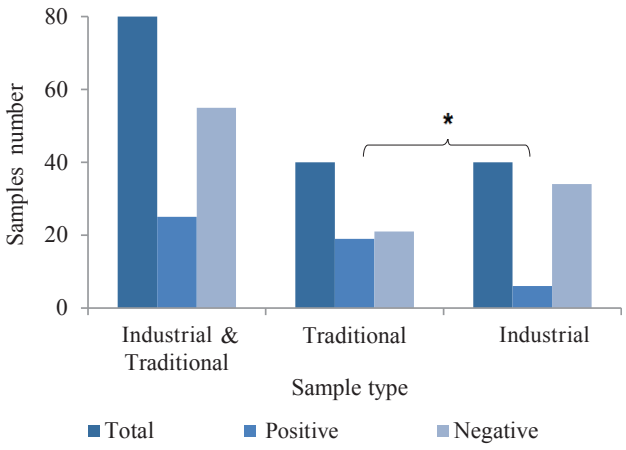

Figure 5 *Escherichia coli* contamination in herbs. Total is number of samples. Positive – presence of *E. coli*. Negative – absence of *E. coli*. * $P < 0.05$

revealed a very high level of contamination ($P < 0.01$), while the lowest level was recorded in licorice (41%) and shirazi thyme (41%).

***Escherichia coli* contamination.** There should be no *E. coli* contamination in dried vegetables [8]. According to our results, 25 samples (31.2%) of the total herbs showed *E. coli* contamination. Of them, 19 (47.5%) and 6 (15%) were from traditional and industrial herbs, respectively (Fig. 5). *E. coli* contamination in the traditional samples was significantly higher than in the industrial samples ($P < 0.05$). The contamination in fennel (43.7%) and valeriana (33.3%) was significantly higher ($P < 0.05$) than that in licorice (23.5%) and shirazi thyme (27.5%).

One of the important aspects is contamination of medicinal herbs by different types of harmful factors such as microbes, heavy metals, as well as radioactive and chemical materials [2]. Our results, in many cases, indicated high contamination of the herbs under study with different microbial agents. These contaminations could occur during different stages of cultivation, extraction, drying, packing or distribution [2, 3, 13]. In our study, the microbial contamination level in the traditional samples was higher than that in the industrial samples. This result could be due to different production and packaging conditions.

Researchers have reported that the differences in technological level and preparation, supply and production of medicinal herbs could affect their contamination level [13–15]. Worldwide, a high level of contamination has been reported in a variety of medicinal herbs. For example, Banerjee *et al*., in a study of 154 dried plants collected from shops in India, showed that the total microbial count was over the limit in 51% of the samples, and 97% of them had mold contamination [16].

Moreover, Abba *et al*., in a study of powdered medicinal plants in Nigeria, reported that 87% of them had high microbial contamination [17]. Their contamination level was significantly higher than that in our study (45%), which could be due to environmental factors, soil or inappropriate packing conditions. Some studies in different locations showed that many of the investigated medicinal herbs were contaminated with various fungi [5, 16, 18, 19]. Alwakeel, in a study on 32 samples of various medicinal plants in Saudi Arabia, showed *Bacillus cereus* as the most common microbial contaminant [20]. Martins *et al*. found the same result in more than 90% of the studied medicinal plants in Portugal [21].

In our study, fennel and valeriana showed the highest and licorice showed the lowest levels of contamination with *B. cereus* and coliform. In a study of the antimicrobial activity of Turkish spices, fennel showed a lower antibacterial effect on *B. cereus* [22]. Moreover, Lang *et al*. in Austria reported that fennel had a lower inhibitory effect on coliform than licorice [23]. It seems that the antimicrobial properties of medicinal herbs could also explain the differences in their contamination levels. In the previous studies, high microbial contaminations were reported in valeriana [19, 24]. We found that valeriana had the highest contamination level. This result can be due to the fact that most of its active medicinal ingredients are in the root of the plant, which is in direct contact with soil, so more microorganisms can be transmitted to it [19]. In our study, licorice showed a considerably lower contamination level than other plants. It could be due to differences in plant production, its active constituents, and distribution processes [3, 25]. In addition, most studies have shown that licorice had higher antimicrobial and antifungal activities than other plants, especially against *B. cereus* and *E. coli* [22, 35–38].

CONCLUSION

In our study, we tested 80 samples of traditional and industrial herbs, such as valeriana, fennel, licorice, and shirazi thyme, for microbial contamination. The

results of the experiment showed that microbial indices in considerable percentage of the samples exceeded the standard limit. Valeriana had the highest total microbial count and yeast/mold contamination, fennel – *B. cereus* and coliforms, while licorice was not massively contaminated.

We also revealed that fungal, coliform, and *E. coli* contamination in traditional herbs was considerably higher than that in industrial samples. However, there was no significant difference between them in total microbial count and *B. cereus* contamination.

Thus, our results demonstrated the importance of monitoring medicinal plants contamination to control the quality of herbal products.

CONTRIBUTION

Maryam Ekhtelat developed the original idea and the protocol. Abdolghani Ameri, Maryam Ekhtelat and Sara Shamsaei developed the protocol, analyzed the data, and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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The extended methylene blue reduction test and milk quality

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Abstract:
Introduction. This study aimed to evaluate the quality of milk produced by six cattlemen's associations in small, isolated farming communities of Carchi, Ecuador. It involved a herd of 814 cows and lasted eight consecutive months. Another aim was to propose a suitable methodology for milk quality evaluation.
Study objects and methods. All milk samples were analyzed for total solids, protein, fat, acidity, density, total bacterial count (TBC) and somatic cell count (SCC). Each sample was subjected to an extended qualitative methylene blue reduction test (MBRTe) for which 10 mL of milk, with 0.5 mL of methylene blue, was incubated at 37°C for 24 h.
Results and discussion. As a result, we obtained the following types of clots: MBRTe-I (homogeneous solid/liquid clot), MBRTe-II (lumpy clot), MBRTe-III (gaseous clot) and MBRTe-IV (lumpy + gaseous clot). The study showed significant differences in the quality of milk between different associations, suggesting that some of them did not comply with good practices of milking, handling and storage of fresh milk. The quality of milk was classified as good in one association, as regular in another association, and as low in four associations. The MBRTe classified 37% of the samples as MBRTe-I, 18% as MBRTe-II, 14% as MBRTe-III and 12% as MBRTe-IV. Of the MBRTe-I samples, 95% showed the TBC and SCC values of first quality milk. The MBRTe-II had the TBC values of first quality milk, but exceeded the SCC, while the MBRTe-III had good SCC values, but exceeded the TBC. Finally, the MBRTe-IV samples exceeded the permissible levels of both TBC and SCC.
Conclusion. It was proved that the MBRTe can help milk producers evaluate the quality of milk and alert them to the possible presence of mastitis in the herd. The MBRTe is a reliable and cheap method that is quick and easy to perform.

Keywords: Dairy industry, raw milk, dairy cattle, microorganisms, somatic cells

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INTRODUCTION

Ecuador produces between 5.5 to 5.8 million liters of milk on a daily basis and this production has been steadily growing in recent years [1]. About 75% of milk is produced in the Andean region, mostly by small associations of farmers, far from large urban centers [2].

The quality of milk determines the quality of dairy products. It refers to the content of microorganisms (pathogenic or not) and somatic cells, as well as the presence of antibiotics and medicines [3]. Milk quality is guaranteed by the health of the herd, as well as good management and milking practices (GMMP). To check the microbiological quality of raw milk, dairy producers commonly use the counts of total bacteria (TBC), somatic cell counts (SCC), and the methylene blue reduction test (MBRT) [4].

The presence of somatic cells in milk has been mainly related to the increase of white cells (leukocytes) as a result of an immune system's response to mastitis. It is a livestock disease caused by the inflammation of the udder due to the action of pathogenic microorganisms such as *Staphilococcus aureaus*, *Streptococcus*

Table 1 Types, characteristics, and possible causes of clots obtained from the extended methylene blue reduction test (MBRTe)

Type	Classification	Characteristics	Causes	Image
MBRTe-I	Solid or liquid homogenous clot*	Homogeneous clot, with an acidic odor and taste, without cracks or fissures, white in color, without or with few bubbles	Presence of *Lactobacillus spp*. or antibiotics in milk	Figure 1a
MBRTe-II	Clumped heterogeneous clot	Heterogeneous clot with lumps, with a whitish, yellowish serum, or other abnormal colors	Produced by germs with bitter tastes and unpleasant odors; mastitic milk at the end of lactation; or milk cooled for a long time	Figure 1b
MBRTe-III	Gaseous heterogeneous clot	Heterogeneous clot with bluish shades and numerous bubbles and gaseous grooves	Coliform bacteria; milk obtained and preserved in poor sanitary conditions or refrigerated for a long time	Figure 1c
MBRTe-IV	Clumped + gaseous heterogeneous clot	Heterogeneous clot with lumps and numerous bubbles or gaseous furrows	Combined action of coliform bacteria and somatic cells; milking and conservation of milk without complying with the GMMP; mastic milk or milk refrigerated for a long time	Figure 1d

* If it is liquid, check the presence of antibiotics or substances that can inhibit microbial growth (such as detergents, pesticides, etc.)

dysgalactiae and *Streptococcus agalactiae*. Mastitis alters the chemical composition of milk and decreases its yield [5, 6].

However, the TBC and SCC are usually carried out in accredited laboratories located in urban areas, far from small farmers and their associations. They are expensive for milk producers and, moreover, the latter have to wait quite long for the results before they can improve the microbiological quality of their milk.

The MBRT, on the other hand, is an old but effective method which has been correlated, with some success, with the total microbial load and, therefore, with the microbiological quality of milk [7–9][I]. It is a simple and fast method, although sometimes it lacks the expected precision.

The goals of this work were to evaluate the quality of milk produced by a group of cattlemen's associations in the province of Carchi, Ecuador, and to suggest a cheap, fast, and reliable alternative methodology that would allow the associations to evaluate the quality of their milk *in situ*.

STUDY OBJECTS AND METHODS

Herd size and geographic location. The study involved six cattlemen's associations (A–F) located in the Andean province of Carchi in Ecuador. There were 11 small farmers in Association A, 27 in Association B, 16 in Association C, 20 in Association D, 15 in Association E and 19 in Association F. Their milk was sampled for eight months, from October 2016 to May 2017. As a result, 709 samples were taken from a herd of 814 milking cows (34 from Association A, 235 from Association B, 50 from Association C, 120 from Association D, 230 from Association E, and 145 from Association F). All the samples were analyzed for total solids, total protein, fat, acidity and density. The total bacteria and somatic cells were also counted.

Physicochemical and microbiological properties of the samples. The determinations of total solids, total protein, fat, acidity and density, as well as somatic cells (SC) and total bacteria counts (TBC) were performed in an accredited laboratory of the Phyto- and Zoo-Sanitary Regulation and Control Agency of Ecuador (AgroCalidad) (www.agrocalidad.gob.ec) located in Tumbaco (Quito, Pichincha, Ecuador) [10][II,III,IV,V]. The somatic cell count (SCC, SC/mL) was performed in a Foosmatic™7 (Foss, Hilleroed DK-3400, Denmark) according to the standard procedure[VI]. The total bacterial count (TBC, CFU/mL) was performed in a BactoScan™FC+ (Foss, Hilleroed DK-3400, Denmark), obtaining values equivalent to those that would be obtained from a standard plate count (SPC)[VII].

Standard and extended methylene blue reduction test. The standard methylene blue reduction test (MBRT) and the 24 h extended methylene blue reduction

[I] ISO 4833-2:2013. Microbiology of the food chain – Horizontal method for the enumeration of microorganisms – Part 2: Colony count at 30°C by the surface plating technique. Geneva: International Organization for Standarization; 2013.

[II] ISO 6731:2010 [IDF 21:2010]. Milk, cream and evaporated milk: determination of total solids content (reference method). Geneva: International Organization for Standarization; 2010. 5 p.

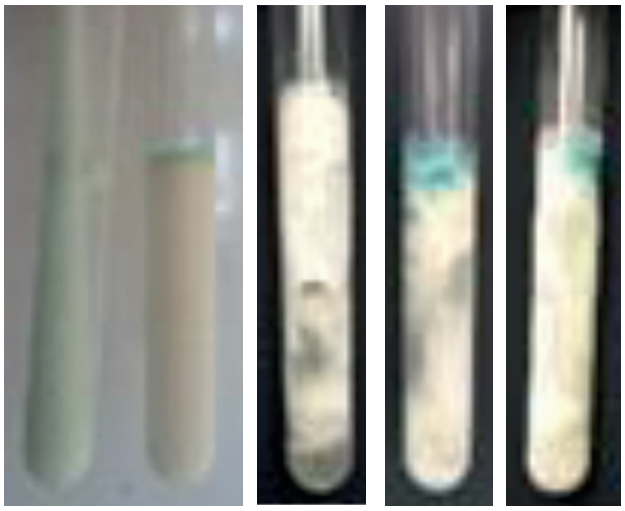
[III] ISO 8968-1:2014 [IDF 20-1:2014]. Milk and milk products – Determination of nitrogen content – Part 1: Kjeldahl principle and crude protein calculation. Geneva: International Organization for Standarization; 2014. 18 p.

[IV] ISO 1211:2010 [IDF 1:2010]. Milk – Determination of fat content – Gravimetric method (Reference method). Geneva: International Organization for Standarization; 2010. 18 p.

[V] ISO/TS 11869:2012 [IDF/RM 150:2012]. Fermented milks – Determination of titratable acidity – Potentiometric method. Geneva: International Organization for Standarization; 2012. 7 p.

[VI] ISO 13366-2:2006 [IDF 148-2:2006]. Milk – Enumeration of somatic cells – Part 2: Guidance on the operation of fluoro-opto-electronic counters. Geneva: International Organization for Standarization; 2006. 13 p.

[VII] ISO 4833-1:2013. Microbiology of the food chain – Horizontal method for the enumeration of the microorganisms – Part 1: Colony count at 30°C by the pour plate technique. Geneva: International Organization for Standarization; 2013. 9 p.



(a) (b) (c) (d)

Figure 1 Four types of clots. (a) MBRTe-I (homogeneous solid/liquid clot), (b) MBRTe-II (lumpy clot), (c) MBRTe-III (gaseous clot), and (d) MBRTe-IV (lumpy + gaseous clot)

test (MBRTe) were carried out in the associations' own laboratories. For the quantitative MBRT, 10 mL of a sample was incubated at 37°C with 0.5 mL of methylene blue and the time (in hours) for the blue coloration to disappear was measured according to the technique described in ISO 4833-2:2013[I]. We used the methylene blue reagent produced by Merck KGaA (Darmstadt, Germany). The samples were incubated at 37°C in a conventional water bath (Thermo Scientific™ TSGP10, Waltham, Massachusetts, USA).

The procedure of the qualitative MBRTe was similar to that of the MBRT, but the samples were incubated for 24 h. As a result, we obtained clots of the following four types (Table 1, Fig. 1): a homogeneous solid or liquid clot (MBRTe-I); a heterogeneous lumpy clot (MBRTe-II); a heterogeneous gaseous clot (MBRTe-III), and a heterogeneous lumpy + gaseous clot (MBRTe-IV).

The MBRT is, therefore, a quantitative test (measured in hours), while the MBRTe is a qualitative test (one of the four possible sample types after 24 h incubation with methylene blue).

Statistical Analysis. The statistical analysis was applied using the free statistical package R version 3.6.1 (2019-07-05).

RESULTS AND DISCUSSION

We analyzed 709 samples for eight continuous variables (SCC, TBC, MBRT, fat, protein, total solids, density and acidity) and three categorical variables:
(1) eight dates (Oct-16, Nov-16, Dec-16, Jan-17, Feb-17, Mar-17, Apr-17, and May-17);
(2) six associations (A, B, C, D, E, and F); and
(3) four MBRTe clots (MBRTe-I, MBRTe-II, MBRTe-III, and MBRTe-IV).

The Lilliefors test (a normality test based on the Kolmogorov-Smirnov test) [11–13] was used to explore the continuous variables, and none of them showed a normal distribution of the samples ($P < 0.05$).

Recently, a similar finding has been reported in a study conducted to determine the quality of milk (total bacterial and somatic cell counts) among small livestock producers where the values obtained did not follow a normal distribution [14]. This is probably due to the non-homogeneity of the samples, the differences between the producers with respect to compliance with good practices, as well as uncontrolled factors that fall outside the framework of the studies.

The Kruskal-Wallis rank-sum test was performed to establish the influence of categorical variables over continuous variables [15, 16]. A pairwise comparison with the Wilcoxon nonparametric rank-sum test was used to determine which of the associations or MBRTe types differed from each other ($P < 0.05$) for each specific continuous variable [17]. Different letters near each of the magnitude values showed significant differences ($P < 0.05$).

In this study, the values of acidity, total solids, MBRT, SCC and TBC differed significantly ($P < 0.05$) between the associations, while density, protein and fat concentrations were not different ($P > 0.05$) (Fig. 2).

As we can see in Fig. 2, only Association A, which fully implemented the GMMP, showed a better quality of fresh milk during the whole period. Association B, which began to implement the GMMP during the study period, achieved high quality in the final months of the study. Associations C, E and F are still in the process of organizing their quality assurance system, and their results oscillate between regular and low quality. Finally, Association D always had contamination problems and showed poor quality milk, so all of their work protocols need revising.

The values of SCC, TBC, MBRT, fat, total solids, and acidity were significantly influenced by the type of MBRTe ($P < 0.05$), whereas there were no differences ($P > 0.05$) for the protein content and density (Fig. 3).

The significant difference ($P < 0.05$) observed in the fat content between the MBRTe-II and MBRTe-III samples (Fig. 3c) could be due to a high concentration of somatic cells and a low concentration of total bacteria in the MBRTe-II sample group. In fact, the method of fat determination presupposes the addition of sulfuric acid which causes the breakdown of somatic cells incorporated into milk fat. It was also possible that exogenous bacteria species that contaminated milk, which were present in the MBRTe-III samples, exerted a greater lipolytic effect on the fat and lowered its concentration in milk, compared to the rest of the MBRTe samples.

In Fig. 3c, we can observe an increase in acidity and a decrease in total solids when moving from MBRTe-I

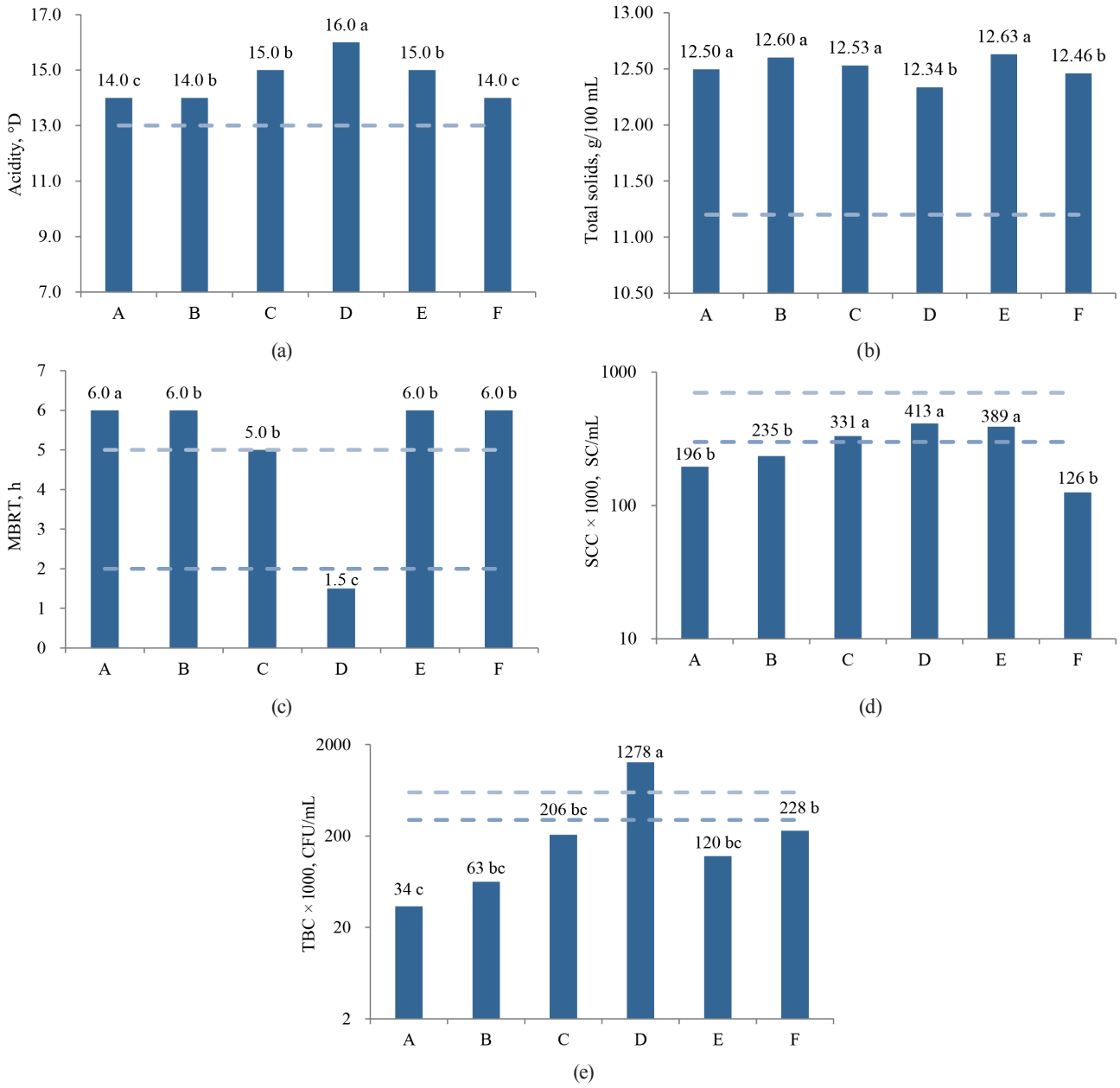


Figure 2 Average values of (a) acidity (°D), (b) total solids (g/100 mL), (c) MBRT (h), (d) SCC (SC/mL), and (e) TBC (CFU/mL) for each association during eight months of the study. The dashed red lines represent the values that delimit the thresholds of good quality, regular quality and poor quality of milk or the minimum acceptable values by the Ecuadorian standards. Different letters mean statistically significant differences ($P < 0.05$)

to MBRTe-IV. This trend seems to be associated with a combined increase in the microbial load and somatic cells in these groups, enhancing the presence of organic acids and therefore leading to higher acidity, and a decrease in carbon sources, such as lactose, leading to lower total solids.

When comparing the magnitudes of the qualitative MBRTe and the quantitative MBRT with the SCC and TBC values in a Kruskal-Wallis rank-sum test, we can see that unlike the MBRT, which is only significantly influenced ($P < 0.05$) by TBC, but not SCC ($P > 0.05$), the qualitative variable of MBRTe correlates significantly ($P < 0.05$) with both the TBC and SCC values.

In the proposed MBRTe test, some of the samples incubated with and without the presence of methylene blue had a similar behavior and formed the same type of clot after 24 h. This finding suggests that the presence of the methylene blue dye does not play the same role as it does in the MBRT test. However, there is a need for more detailed experiments to corroborate the influence or necessity of this dye in the MBRTe test. They need to use the same samples, incubate them under the same conditions for 24 h and then observe the type of clot forming after that time.

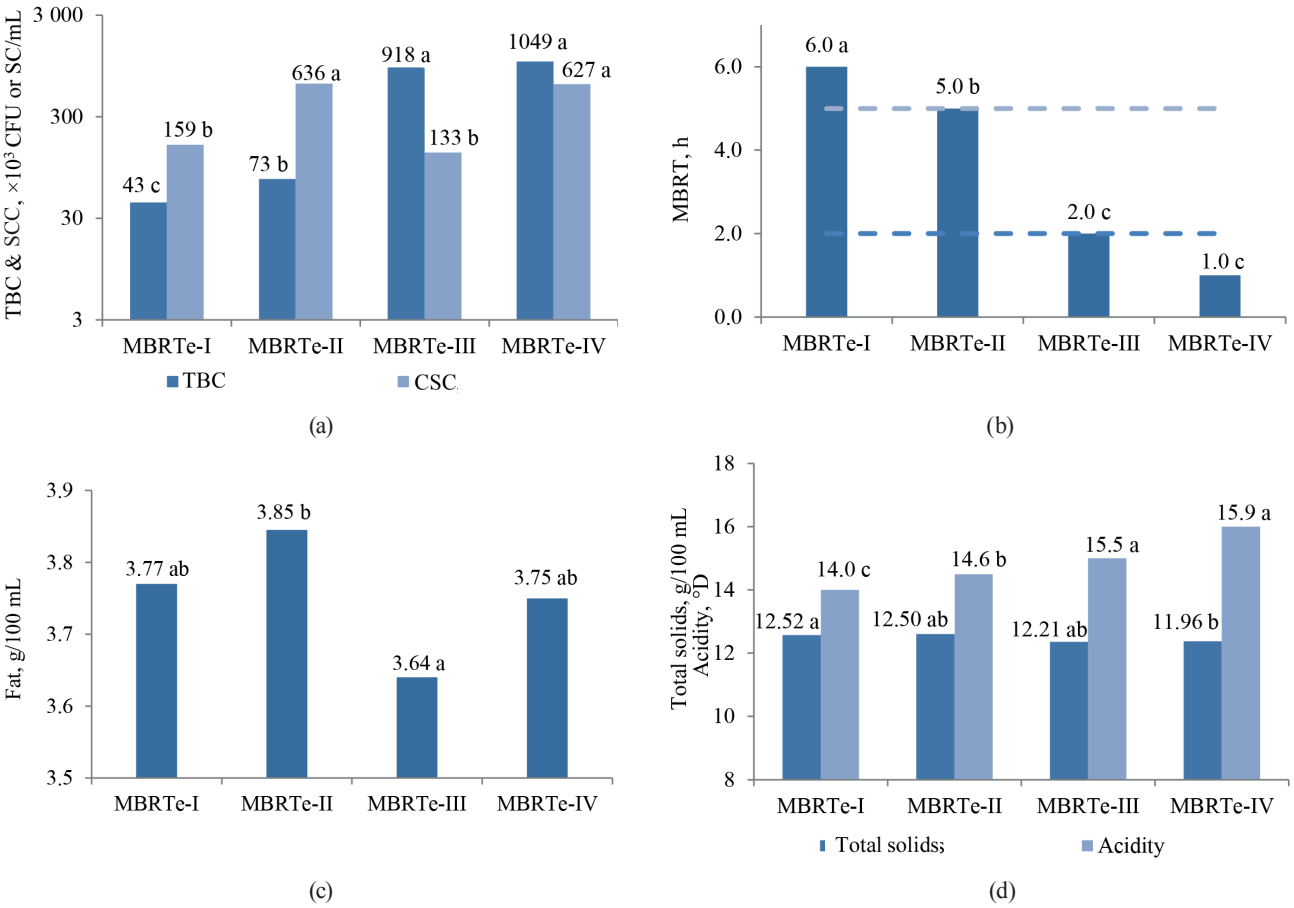


Figure 3 Relationship between average values of (a) TBC + SCC, (b) MBRT, (c) Fat (g/100 mL), and (d) Total solids (g/100 mL) + Acidity (°D) and the MBRTe-types. Equal letters mean no significant differences ($P < 0.05$) according to the Wilcoxon nonparametric rank-sum test [17]

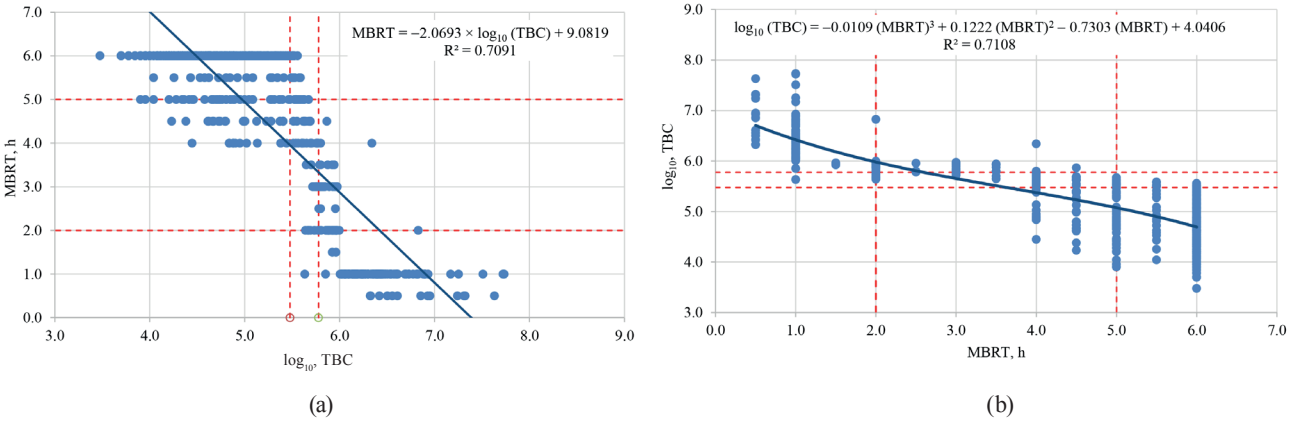


Figure 4 Correlation between (a) MBRT vs $\log_{10}$ (TBC) and (b) $\log_{10}$ (TBC) vs MBRT. The dashed red lines represent the values that delimit milk quality thresholds

When correlating the MBRT values with the TBC, or vice versa, we can observe similar correlations to those previously reported by other authors [8, 9], although with somewhat lower correlation coefficients R^2 (Fig. 4).

Thus, the qualitative MBRTe not only would allow us to assess the microbiological quality of milk samples through TBC values, but it could also detect a healthy dairy herd (< 310 000 SC/mL) or the presence of mastitis in its preclinical (310 000 ≤ SCC ≤ 700 000 SC/mL) or clinical (> 700 000 SC/mL) stages, which is impossible to do with the standard MBRT test.

Fig. 5 shows the distribution of the MBRTe samples

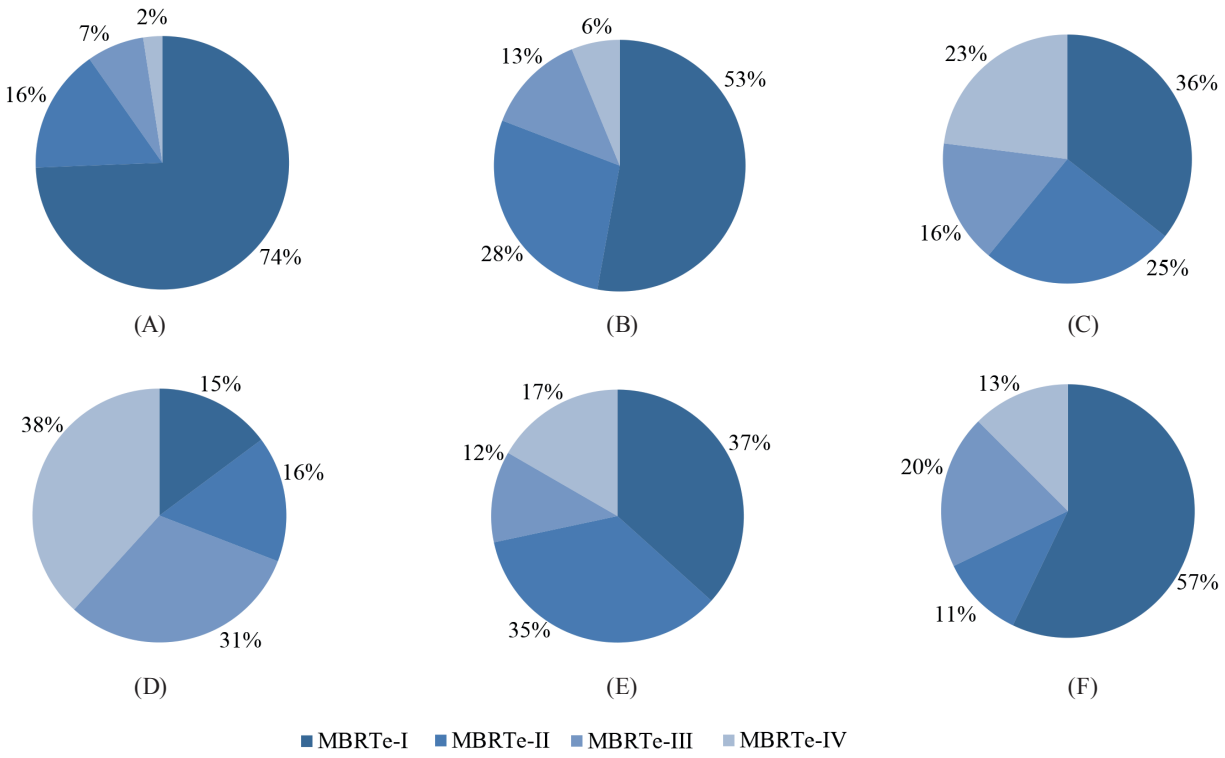


Figure 5 Distribution of MBRTe samples in each association (A–F) showing the herd health and compliance with good practices

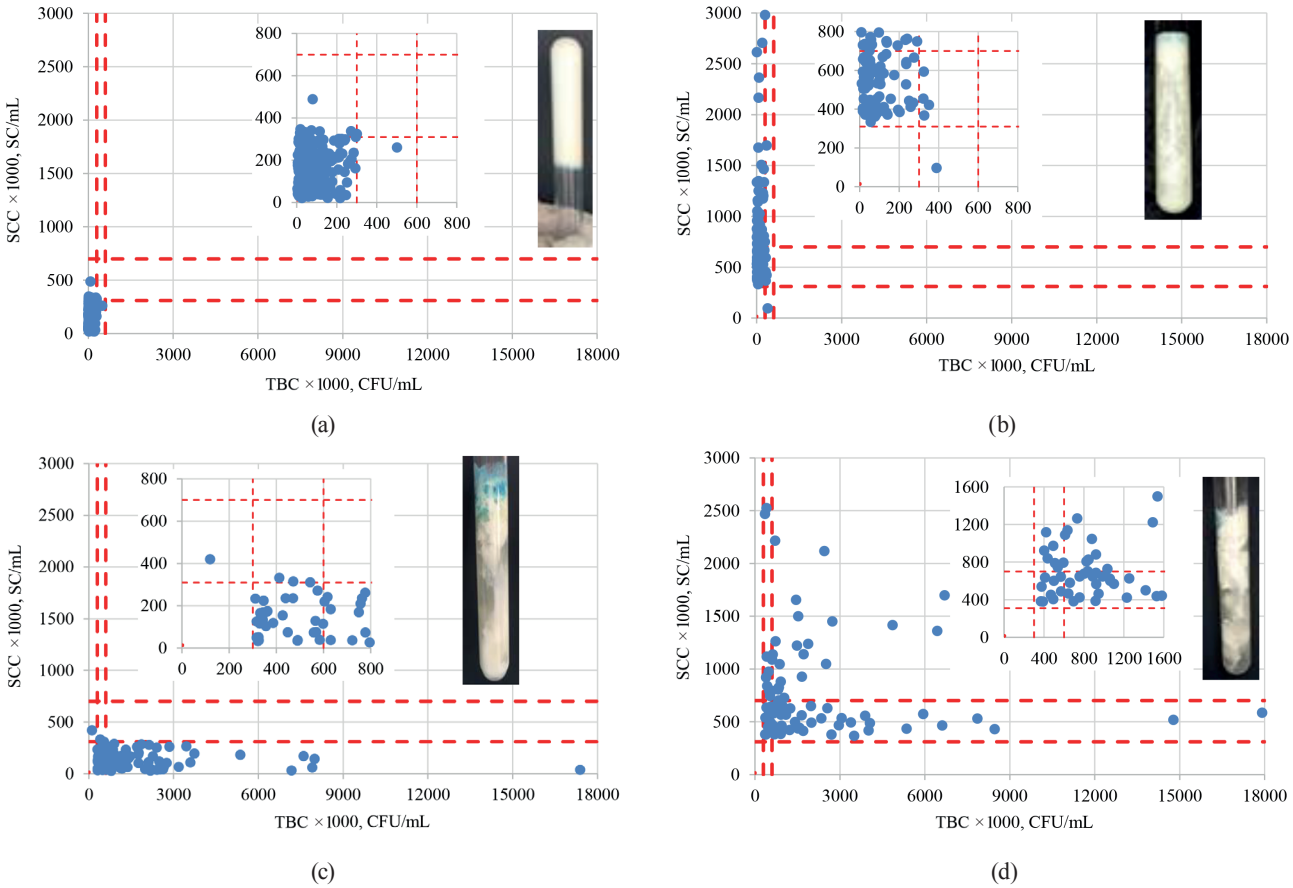


Figure 6 Correlation between the MBRTe and TBC + SCC (CFU or SC/mL, respectively). (a) MBRTe-I: n = 265 (37% of the total); (b) MBRTe-II: n = 130 (18% of the total); (c) MBRTe-III: n = 99 (14% of the total); (d) MBRTe-IV: n = 84 (12% of the total)

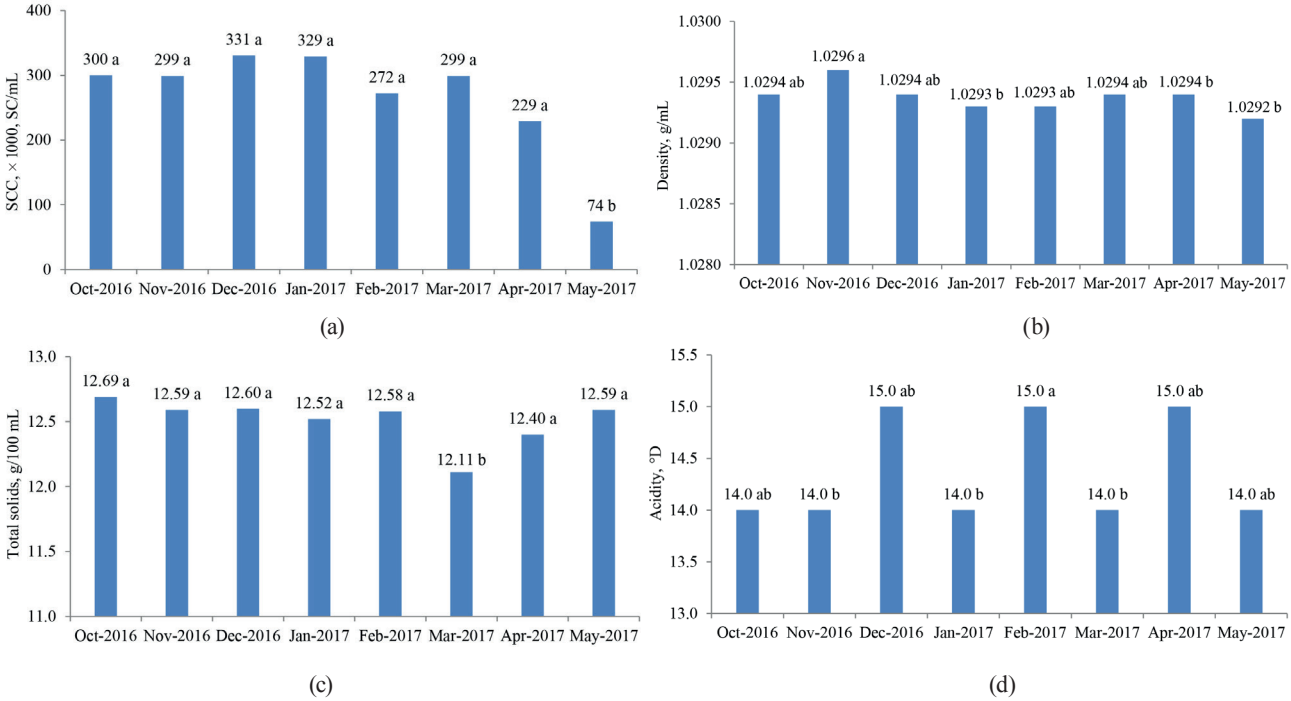


Figure 7 Dependence of average values of (a) SCC (SC/mL), (b) density (g/mL), (c) total solids (g/100 mL), and (d) acidity (°D) on the month of sampling. Equal letters mean no significant justified ($P < 0.05$)

in each association. As we can see, the associations with the highest proportions of MBRTe-I, compared to the other types, had the best quality milk.

As we can see in Fig. 5, Association A provided better milk quality than the rest of the associations. The lowest-quality milk was produced by Association D. This means that the health of its herd and the procedures for milking, handling, and storage of fresh milk should be reviewed.

When correlating the TBC and SCC values with the qualitative MBRTe, we observed that the MBRTe could adequately predict not only the samples with a high bacterial load, but also those with a significant presence of somatic cells. The latter might indicate preclinical or clinical mastitis in the herd (Fig. 6).

As we can see in Fig. 6a, more than 94% of the MBRTe-I samples had SC values below 310 000 SC/mL and TBC values below 300 000 CFU/mL. Fig. 6b shows that more than 94% of the MBRTe-II samples had TBC values below 300 000 CFU/mL, while 57% of them had SC values between 310 000 and 700 000 SC/mL, which could indicate a preclinical condition of mastitis. Moreover, 42% of the MBRTe-II samples had SC values of over 700 000 SC/mL, which suggests the presence of mastitis in at least part of the dairy herd. Of the MBRTe-III samples, 97% had SC values below 310 000 SC/mL, which indicates a healthy dairy herd, without mastitis problems.

However, as we can see in Fig. 6c, 32% of those samples showed moderate values of microbial contamination (between 300 000 and 600 000 CFU/mL) and 67% of them had high values (> 600 000 CFU/mL). These data suggest that the samples came from a healthy dairy herd, but the GMMP were not followed properly. Finally, all the MBRTe-IV samples (Fig. 6d) showed moderate to high values of both TBC and SCC, suggesting a dairy herd with mastitis problems and bad management and milking practices. Such a product cannot be recommended for direct consumption – it has to be carefully pasteurized before being used in the manufacture of dairy products.

Likewise, we analyzed a possible relationship between the physicochemical and hygienic-sanitary properties of the samples and the month in which these samples were taken (from October 2016 to May 2017). For this, a Kruskal-Wallis rank sum test was applied to each of the measurements made to each sample and the month of sampling. We found that the TBC, MBRT, protein and fat contents did not depend on the months in which the samples were taken. However, the determinations of density, total solids and SCC, in at least a couple of months, were influenced by the month of sampling. To determine the significance ($P < 0.05$) of these differences, we performed a pairwise comparison using the Wilcoxon nonparametric rank-sum test and the Bonferroni method (Fig. 7), as we did with the previous categorical variables (type of association and MBRTe) [17].

The differences associated with the month in which the samples were analyzed could be explained by some uncontrolled factors in the experiments. These include variations in the periods of rain, which could influence the type and abundance of the grass consumed by the dairy herd, and changes in the management of the herd, as well as milking and storage of fresh milk. Also,

Table 2 MBRTe types and the quality of fresh milk

Type	Quality of milk	Remark
MBRTe-I	Good	Suitable for consumption and all uses.
MBRTe-II	Intermediate to good	Suitable, after pasteurization, for producing dairy products.
MBRTe-III	Poor to intermediate	
MBRTe-IV	Poor	Its consumption is not recommended.

possible measures taken by the associations to deal with mastitis problems may have been reflected in the SC values, as well as the time they were taken.

To sum up, we can say that the MBRTe correctly pre-qualified fresh milk and, therefore, allowed us to suggest possible industrial uses for it and set fair market prices (Table 2).

We all know of difficulties that small cattlemen's associations have with assessing the microbiological quality of milk and detecting sub-clinical mastitis in real time to continuously improve the quality of milk delivered to the industry and consumers. Accredited laboratories that perform somatic cells and total bacterial counts, as well as the methylene blue reduction test (MBRT), are located in provincial cities or capitals, far away from the rural areas where most of the small farmers' associations are, at least in Ecuador [16][VIII]. This means that the farmers' associations usually have to wait a few days (an average of 3 days) for the test results. Thus, they cannot quickly identify individual producers that affect the milk quality of the whole association to take prompt corrective measures.

Moreover, the cost of such analysis in Ecuador, including transportation (for a distance of ~ 50 km), is approximately $9.56 per sample. In contrast to that, the qualitative MBRTe takes only one day and costs approximately $0.46 per sample. In addition, it is easy to perform and its interpretation is straightforward and simple: fresh milk is pre-qualified as good (MBRTe-I), intermediate to good (MBRTe-II), poor to intermediate (MBRTe-III), and poor (MBRTe-IV).

The qualitative MBRTe would allow us not only to know if the association follows good practices of milking, handling and storage of milk, but also to examine the health of the dairy herd, as far as mastitis is concerned. In addition, it is a cheap test since it requires only a conventional thermostatic bath, the blue methylene reagent, and a set of common glass tubes. It is significantly cheaper than modern equipment for the detection and counting of somatic cells.

The above makes the MBRTe suitable for small associations of livestock farmers that are isolated from cities and towns where accredited laboratories are generally located.

[VIII] NTE INEN 9:2012. Norma Técnica Ecuatoriana. Servicio Ecuatoriano de normalización. Leche cruda. Requisitos [Ecuadorian Technical Standard. Ecuadorian Normalization Service. Raw milk. Requirements]. Quito: INEN; 2012. 7 p.

Additionally, the MBRTe can be applied not only to raw fresh milk collected from all the farmers in the association, but also from individual farmers who are its members. This last feature could help identify individual cattlemen who own dairy herds with preclinical or clinical mastitis or those who do not comply with good practices of milking and handling of fresh milk. By doing so, the association can make a corrective plan to improve the microbiological quality of raw fresh milk in the near future and establish better market prices for its producers.

CONCLUSION

In this work, we evaluated the quality of milk produced by six dairy associations of small farmers in the province of Carchi for eight consecutive months. We determined the hygienic and sanitary status of milk and dairy herd, respectively. The study found an adequate correlation between the quality of milk and the farmer's compliance with good practices of milking, handling and storage of fresh milk. Thus, it served to encourage some of the associations to comply with these good practices.

We demonstrated a relationship between the qualitative MBRTe and somatic cells and total bacteria counts. As a result, we proposed the MBRTe to the cattlemen's associations in the Ecuadorian highlands to pre-qualify milk collected from both the entire association and individual farmers. Also, the proposed methodology can be useful for isolated ranchers, away from accredited labs, to check the quality of their milk by themselves. This test can identify the presence of sub-clinical or clinical mastitis and inadequate management of milking, handling, storage and transportation of fresh milk. The results can be used to make appropriate improvement plans to correct these deficiencies and enhance the quality of milk.

CONTRIBUTION

Mayra Pérez-Lomas collected the data. Milton Cuaran-Guerrero, Lucía Yépez-Vásquez, Holger Pineda-Flores, Jimmy Núñez-Pérez and Rosario Espin-Valladares contributed the data and analysis tools. José Pais-Chanfrau conceived and designed the analysis. Edmundo Recalde-Posso performed the analysis. Luis E. Trujillo-Toledo wrote the paper in cooperation with José M. Pais-Chanfrau.

CONFLICT OF INTEREST

The authors declare that there were no conflicts of interest during the elaboration of this work or later, during the preparation of the manuscript.

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Plastics: physical-and-mechanical properties and biodegradable potential

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Abstract:
Introduction. Processing agricultural waste into plant biodegradable plastics is a promising way for its recycling. This work featured the main physical-and-mechanical properties of plant plastics without adhesive substances obtained from millet husk and wheat husk and wood plastic obtained from sawdust, as well as their biodegradation potential.
Study objects and methods. Objects of the study were plastics without adhesives based on wood sawdust, millet husk, and wheat husk.
Results and discussion. We analyzed of the physical-and-mechanical parameters of the plant plastic based on millet husk, wheat husk, as well as wood plastic based on sawdust. The analysis showed that, in general, the strength characteristics of the wood plastics were higher than those of the plastics based on millet husk, especially flexural strength. Thus, the average value of the density of the wood plastic exceeded that of the plant plastic from millet husk by 10%, hardness by 40%, compression elasticity modulus by 50%, and flexural modulus by 3.9 times. It was found that wood and plant plastics obtained from sawdust, millet husk, and wheat husk without adhesives had a high biodegradation potential.
Conclusion. The plastics obtained can be used as an insulating, building, and decorative material in the steppe regions experiencing a shortage of wood and wood powder.

Keywords: Plastic, agricultural waste, grain, husk, biodegradation

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INTRODUCTION

The concept of organic agriculture, which first appeared in European countries, has gained its popularity in Russia in the last few years. According to the concept, agricultural industry should ensure the environmental and biological safety of technologies, raw materials, and products [1–3]. In Russia, a new federal law on organic products comes into force in 2020 that regulates the activity of agricultural enterprises. It prohibits the use of packaging and transport package which damages to the environment and encourages the application of methods and technologies aimed at ensuring a favorable state of the environment, strengthening human health, as well as at maintaining soil fertility.

Besides, one of the problems is the utilization of agricultural wastes, which are currently mainly stored or disposed. Only a small part of them is used to produce coarse low-value feed and bedding for animals, fertilizers, or fuel [4]. These include straw, flax shive, coffee grounds, nutshells, plant waste from cereals and flour manufacture, as well as from fruit and berries processing. In Russia, efficient agricultural enterprises generate several million tons of such waste annually. In the Altai Territory, for example, the amount of grain husks obtained at elevators is on average 2–3 million tons per year [5].

One of the alternatives of plant waste recycling is the production of bioplastics and paper [6–9]. In the world practice, plant fillers from cereal husks

and agricultural plant fibers are widely used in the production of biocomposites and reinforced bioplastics. They can be produced both from plant materials only and with the addition of classical petrochemical products [10–12]. The improved technologies and performance characteristics of bioplastics as well as production cost reduction make it possible to fill new niches in the market [13]. Bioplastics without adhesive substances based on plant materials are environmentally friendly. Plant materials can be wood powder, sawdust, cereal husk, as well as flax and hemp fibers [6, 13]. Such plastics are biodegradable materials, and their degradation is due to microbial enzymes [14–16].

The use of plant biodegradable plastics, including plastics without adhesives, to produce containers with a short life cycle, building and packaging materials can be a promising way for agricultural waste recycling, which corresponds to the concept of organic agriculture [7, 17, 18].

Taking into consideration the obvious advantages of biodegradable plastics, the study of the main physical-and-mechanical properties of plant plastics without adhesives obtained from millet husk and wheat husk and analysis of their biodegradation potential are relevant.

STUDY OBJECTS AND METHODS

The objects of the study were plastic samples without an adhesive substance based on wood and plant materials. The samples were made at the Ural State Forestry University. Wood-based plastics made from industrial sawdust (State Standard 18320-78[I]) were used as control samples. Experimental samples were plant plastics obtained from millet husk and wheat husk, cereal production wastes.

The plant fillers of 18.0 and 30.0 g in mass were subjected to pressing to obtain disks of 2.0 and 4.0 mm in thickness and 90 mm in diameter (moisture content of the molding material was 12%). The conditions of pressing were as follows: molding material mass, 10.0 g; pressing pressure, 124.0 MPa; pressing time, 10 min; and cooling time under pressure, 10 min. The physical-and-mechanical characteristics of the samples were analyzed both before and after biostability and biodegradation tests. We determined water absorption (State Standard 4650-80[II]) and strength parameters such as density, flexural strength, hardness, elasticity number, compression elasticity modulus, elastic modulus in flexure, breaking stress, yield strength (State Standard 4648-71[III], State Standard 4670-77[IV], State Standard 10634-88[V]).

To study biodegradation potential, the test samples were kept in soil for 21 days, then the main visual morphological characteristics of their biodegradation were evaluated. Soil was prepared in accordance with State Standard 9.060-75[VI]. At the beginning of the test, pH of the soil extract was 7.0 and biological activity coefficient was 0.8. The soil microbiocenosis was formed by native field strains of microorganisms of the initial components of the soil.

We observed such biodegradation signs as splitting, swelling, loosening, macro- and microcavities formation, changes in the shape and size of the main plant component particles, fibrillation and fragmentation of particles, the local discoloration of the sample, the presence of colonies of microorganisms, hyphae, fungal fruit inside or on the sample surface, as well as its mucilagination. The samples that did not display biodegradation signs were tested for strength and water absorption.

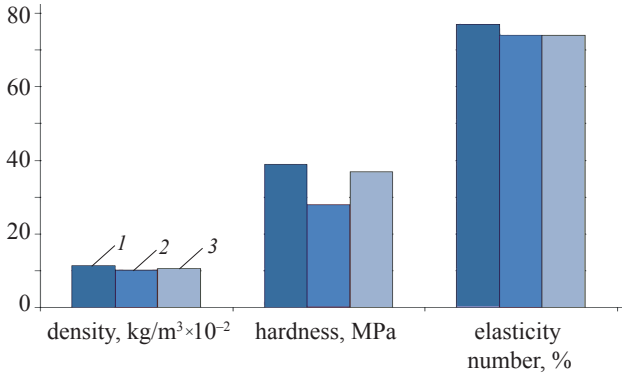
In addition, the test with the germination of oat and clover seeds on a substrate containing the samples under study was carried out. For this, the substrate was prepared that included two layers of multi-purpose soil (60%) alternating with two layers of the samples (40%). Multi-purpose soil was used as control sample. Oat and clover seeds were sown in the substrate, germinated for 21 days, after that growth rate, as well as stem and leaves formation were evaluated in the experimental and control samples. The root system of the plants was determined in visible light and in ultraviolet light.

RESULTS AND DISCUSSION

We analyzed the physical-and-mechanical parameters of plant plastics based on husks of millet and wheat and wood plastic based on sawdust. The samples did not include adhesives. The analysis showed that the strength characteristics of the wood-based samples were higher than those of the plastics based on millet husk, especially flexural strength. Thus, the average density of wood plastics exceeded that of plant plastics from millet husk by 10%, hardness by 40%, compression elasticity modulus by 50%, and flexural modulus by 3.9 times (Figs. 1 and 2).

A comparative analysis of the physical-and-mechanical properties of the wood plastic samples with the samples of plant plastics from wheat husk showed similar results. Thus, the average value of the density of the wood-based plastics exceeded that of the plant samples from wheat husk by 15%, hardness by 10%, compression elasticity modulus by 10%, and flexural modulus by 2.6 times.

[I] State Standard 18320-78. Technological wooden sawdust for hydrolysis. Specifications. Moscow: Izdatel′stvo standartov; 1986. 7 p.
[II] State Standard 4650-80. Plastics. Methods for the determination of water absorption. Moscow: Izdatel′stvo standartov; 2008. 7 p.
[III] State Standard 4648-71. Plastics. Method of static bending test. Moscow: Izdatel′stvo standartov; 1992. 11 p.
[IV] State Standard 4670-77. Plastics and ebonites. Method for determination of hardness by ball indentation under a given load. Moscow: Izdatel′stvo standartov; 1992. 6 p.
[V] State Standard 10634-88. Wood particle boards. Methods for determination of physical properties. Moscow: Izdatel′stvo standartov; 1991. 10 p.
[VI] State Standard 9.060-75. Unified system of corrosion and ageing protection. Fabrics. Method of laboratory tests for microbiological destruction stability. Moscow: Izdatel′stvo standartov; 1994. 9 p.



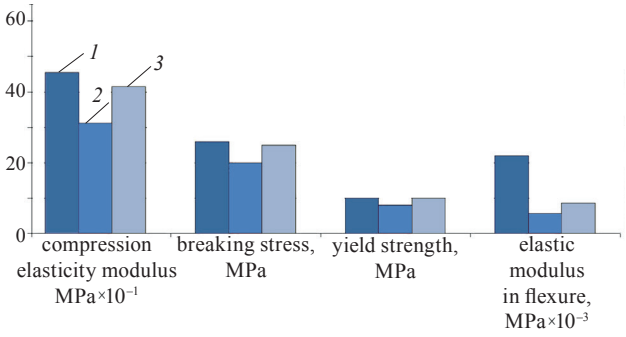
(1) wood plastic *(2)* plant plastics from millet husk
(3) plant plastics without from wheat husk

Figure 1 Average values of density, hardness, and elasticity number of plastics without adhesives

According to the results of the comparative analysis of the physical-and-mechanical properties of the two plant plastic samples, the average values of hardness, compression elasticity modulus, and elastic modulus in flexure of the plastic from millet husk were higher than those of the samples based on wheat husk by 1.3, 1.3, and 1.5 times, respectively.

An increased pressing temperature (from 180 to 170°C) led to an improvement in the physical-and-mechanical properties of the plant plastics compared to the wood-based samples. Thus, the values of elasticity number, the elasticity moduli, breaking stress, and yield strength increased. Presumably, this is due to the difference in the dynamics of lignin polymerization reactions in wood and plant plastics. This factor should be taken into consideration when selecting pressing conditions.

The study of the biodegradation potential of plastics without an adhesive component based on wood sawdust, millet husk, and wheat husk showed that all the samples



(1) wood plastic *(2)* plant plastics from millet husk
(3) plant plastics without from wheat husk

Figure 2 Average values of compression elasticity modulus, breaking stress, yield strength, and elastic modulus in flexure of plastics without adhesives

Figure 3 Plant plastic from millet husk, plant plastic from wheat husk, and wood plastic kept in active soil for 21 days (all plastics are made without adhesives)

studied had a relatively equal high biodegradation potential.

We analyzed the morphological signs of biodegradation of the materials kept in active soil for 21 days. The analysis showed that all the samples had surface mucilagination, edge swelling, and local discoloration of the surface (Fig. 3).

60% of the samples based on millet husk, 58% of the samples from wheat husk, and 47% of the wood plastics based on sawdust displayed longitudinal and transverse splitting, loosening, and macrocavities formation (Fig. 4). The splitting and loosening sites ranged from 1.5 to 5.5 mm in size.

Microscopy was used to assess signs of the sample destruction. The analysis revealed marginal fibrous structure; the fragmentation and destruction of individual particles of the plant component; focal darkening of particles; and microcavities formation between the particles of plant material. Moreover, all the samples under study showed bacterial contamination. 74% of the samples with millet husk, 85% of the samples from wheat husk, and 62% of the wood samples had

Figure 4 Wood plastic fragment with signs of splitting, swelling, marginal and longitudinal fragmentation

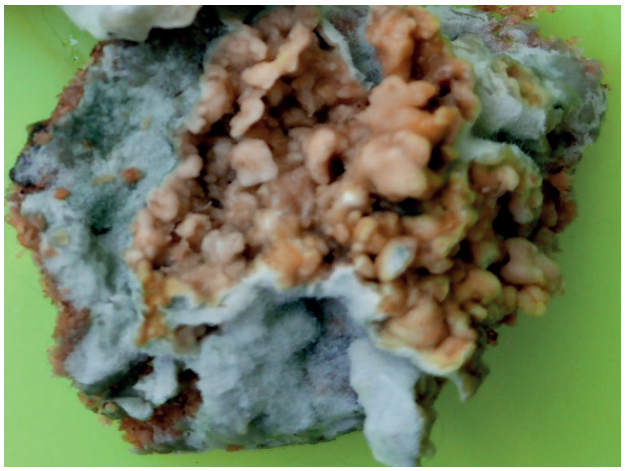


Figure 5 Fragment of plant plastics from wheat husk (without adhesives) with signs of mold growth

multiple large colonies of mold fungi of different growth phases (Fig. 5).

The plant plastics demonstrated a more pronounced biological destruction compared to the wood samples. Thus, they had changes throughout the sample, while the wood plastics were characterized by edge and surface changes.

Further, we evaluated the growth rate and organs formation of oat and clover grown on control and experimental substrates. Multi-purpose soil was used as the control substrate, while the experimental substrate contained multi-purpose soil and the samples under study. According to the results of the experiment, morphological signs of retardation and deviation in the oat and clover development were not detected. The root system of the plants did not also have significant differences. The roots penetrated into the plant and wood samples, fragmenting them. The soil – root conglomerate was analyzed in ultraviolet light. The result was typical of these plant species; no dependences on the presence of the samples under study in the substrate were found (Fig. 6).

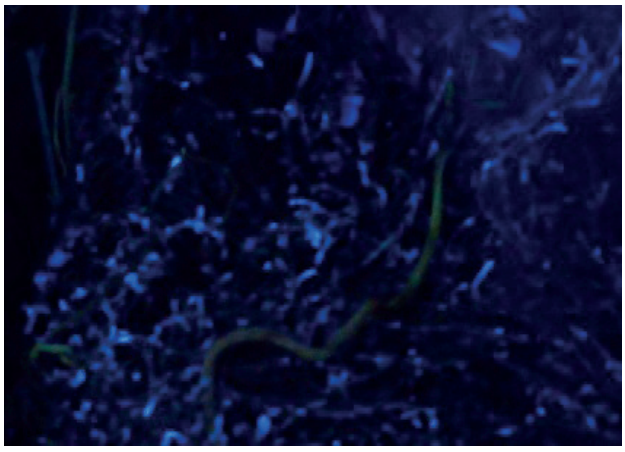


Figure 6 Root system of oat on a substrate with plant plastic from wheat husk without adhesives in ultraviolet light with luminescence foci

Based on the sings found, the plastics with wheat husk had the highest degree of biodegradation among all the samples under study.

The keeping of the plant and wood samples in active soil for three weeks led to thier physical-and-mechanical properties deterioration. Then, hardness decreased by 66, 70, and 62%, elasticity number by 43, 47 and 46%, and compression elasticity modulus by 76, 80, and 73% for the wood plastics, plant plastics from millet husk, and plant plastics from wheat husk, respectively. Breaking stress and yield strength values decreased by 64% and 63% for the wood-based plastics and by 60% and 68% for the plant-based samples, respectively.

A comparative analysis of flexural strength values of the plastics under study without adhesives showed that the highest average value of this indicator was for the wood plastic samples (4 MPa), and the lowest for the plant plastic based on wheat husk (1 MPa). Water-absorbing capacity was 96, 85, and 94% for the samples with wheat husk, with wheat husk, and with sawdust, respectively.

CONCLUSION

According to the results of the study, plant plastic obtained under the same pressing conditions which are used for wood plastic production had lower strength characteristics. A decrease in the pressing temperature by 10°C improved such strength characteristics of the husk-based samples as elasticity number, moduli of elasticity in compression and flexure, as well as breaking stress. Also, the wood and plant plastics without adhesives based on sawdust, millet husk, and wheat husk were found to have a high biodegradation potential. Therefore, it makes it possible to utilize such materials naturally, i.e. without composting, in contrast with biodegradable wood-polymer composites.

On the other hand, a high biodegradation potential also indicates a low biostability of materials. Special conditions should be applied to use of products from plant plastics based on husk of millet and wheat, as well as from wood with sawdust. The conditions include a low humidity, no contact with water and soil, or with using antiseptics and waterproofing agents. The advantages of plant plastics without adhesives based on agricultural waste – millet husk and wheat husk – include their ecological safety, relative ease of production, low cost, raw materials availability, a high biodegradation potential, as well as performance characteristics comparable to those of wood plastics. Plant plastics without adhesives obtained from agricultural waste can be relevant in steppe regions as an insulating, building, and decorative material.

CONTRIBUTION

The authors were equally involved in developing the

research concept, obtaining and analyzing data, as well as in writing the manuscript.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Microorganisms during cocoa fermentation: systematic review

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Abstract:
Introduction. Cocoa (*Theobroma cacao* L.) originates from Ecuador. It is one of the oldest foods in the world. The fact that cocoa is the main component in chocolate industry makes it one of the most quoted raw materials today. The chemical, physical, microbiological, and sensory properties of cocoa determine its quality and, as a result, economic and nutritional value. The research objective was to conduct a detailed analysis of cocoa fermentation process and to study the transformations this raw material is subjected to during processing.
Study objects and methods. The present article introduces a substantial bibliographic review based on three databases: Science Direct, Scopus, and Medline. The scientific publications were selected according to several factors. First, they had to be relevant in terms of cocoa fermentation. Second, they were written in English or Spanish. Third, the papers were indexed in high-impact journals. The initial selection included 350 articles, while the final list of relevant publications featured only 50 works that met all the requirements specified above.
Results and discussion. The main characteristics of yeasts, lactic bacteria, and acetic bacteria were analyzed together with their main parameters to describe their activities during different stages of alcoholic, lactic, and acetic fermentation. A thorough analysis of the main enzyme-related processes that occur during fermentation makes it possible to optimize the use of substrates, temperature, time, pH, acidity, and nutrients. As a result, the finished product contains an optimal concentration of volatile compounds that are formed in the beans during fermentation. The study featured the main strains of fermentation-related microorganisms, their activities, main reactions, and products.
Conclusion. This study makes it possible to improve the process of fermentation to obtain beans with a better chemical composition.

Keywords: Biochemistry, fermentation, yeasts, acetic acid

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INTRODUCTION

The Latin name for cocoa, *Theobroma Cocao*, translates as "food for the gods". The plant has its origin in the upper Amazon, where archeologists discovered theobromine alkaloid from the oldest organic cocoa matter in history at the Santa Ana archaeological site in La Florida, in the province of Zamora Chinchipe, Ecuador. From this place, cocoa beans spread throughout the rest of the continent. On the Yucatan Peninsula, fragments of once-vast cocoa plantations have been found on the territories that were occupied by the Mayan civilization. The traces of cultivated cocoa were also discovered in Central America on the territory of the modern Mexico. Currently, cocoa is cultivated in many tropical countries of the world. It grows in the area between 20 degrees latitude north and south of the equator [1, 2].

The cocoa beans come from the *Theobroma cacao* tree. They grow in a pod that contains 30–40 beans

wrapped in a jelly-like mucilaginous substance. Raw cocoa has an unpleasant astringent taste, which means that its volatile compounds have to be generated artificially. During treatment, microorganisms modify their state and components through various processes, e.g. fermentation. After fermentation, beans are dried and exposed to the sun. Only then do the typical sensory properties develop, and the beans acquire the pleasant characteristics we associate with chocolate [3, 4].

Fermentation is considered the most critical step in the processing of cocoa, since the beans are the main raw material in chocolate industry. It is at the fermentation stage that they develop their sensory properties. These properties come from aroma precursors generated during chemical changes in the phenolic content [5]. Fermentation occurs in the pulp of the pod. The pulp is a white carbohydrate-rich mucilaginous mass that surrounds and protects the beans. The process lasts several days and depends on several groups of microorganisms [6].

The microbial activity during cocoa fermentation has complex biochemical implications. In fact, cocoa is one of few foods where so many changes and processes occur during the same process. It includes the successive growth of several species of yeasts, lactic acid bacteria, acetic acid bacteria, and, to greater or lesser extent, species of *Bacillus* and filamentous fungi [7]. Yeasts are active at the earliest stage of fermentation. The most common strains include *Saccharomyces spp*, *Candidda spp*, and *Pichia spp*. The yeast stage is followed by lactic acid bacteria, which are represented by *Lactobacillus plantarum* and *Lactobacillus fermentum*. Acid bacteria acetics belong to the genus of *Acetobacter*, *Acetobacter pasteurianus* being the most common.

What exactly determines the quality of the cocoa beans still remains a mystery: groups of microorganisms or individual species? Most likely, all the strains are essential for the fermentation process, since new genera and new species are constantly being discovered [8, 9]. Therefore, the first objective of this review was to specify the characteristics and reactions of microorganisms during fermentation. The second objective was to formulate some recommendations on improving fermentation conditions or maintaining the optimal ones to achieve the best transformation in the chemical compounds.

STUDY OBJECTS AND METHODS

The bibliographic review was conducted according to three databases, namely Science Direct, Scopus, and Medline. The descriptors included the following key words: cocoa fermentation, microbiology of cocoa fermentation, and phenolic compounds in cocoa fermentation. The articles were in English or Spanish and indexed in high impact journals. They were selected according to their relevance in terms of cocoa fermentation. Of 350 initially selected articles, 149 were excluded as irrelevant, and 101 did not fit the language criterion. Out of 100 English and Spanish articles, only 50 were selected as corresponding with all the specified requirements.

RESULTS AND DISCUSSION

Cocoa fermentation. Cocoa fermentation is a post-harvest process which includes several stages. The first step after cultivation is to open the pods and remove the beans. They are covered with white pulp, or mucilage, which is mainly sugars and water [10]. The initial pH of the pulp is 3.6. It is a nutrient-rich medium that encourages microbial growth. The pulp has the following composition: about 85% of water, 10–15% of sugars (the concentration of glucose, fructose, and sucrose depending on the age and maturation), 2–3% of pentoses, 1–3% of citric acid, and 1.5% of pectin, proteins, amino acids, vitamins, and minerals. Vitamin C and potassium are the most common representatives of vitamins and minerals. They are minor but very important components [10–12].

The microbiological changes during fermentation are obvious. First, yeasts ferment pulp carbohydrates and transform them into ethanol and carbon dioxide. The secretions of their pectinolytic enzymes generate anaerobic environment. The yeast stage takes approximately 36 h. The next stage involves lactic acid bacteria that appear between 16 and 48 h of fermentation. They generate lactic and citric acid, increase the acidity of the medium, and change the composition of the pulp.

As fermentation continues, oxygen begins to come in. As a result, the temperature rises above 37°C, which boosts the growth of acetic acid bacteria. Their population reaches its peak in 88 h. Between 48 and 112 h of fermentation, one can even feel the smell emanating from acetic acid. After alcohol and lactic acid turned to acetic acid, the temperature rises up to 50°C. The heat finally inhibits the microorganisms that have a life span of 120 h. After fermentation, several filamentous fungi have been registered in the surface areas and in the excess fermentation mass [13–16].

Basically, the fermentation of cocoa beans begins with the initial acidity of the mucilage and the low levels of oxygen, which are the optimal conditions for yeasts. As these factors decrease, the lactic acid bacteria reach their maximum growth point. As their amount gradually decreases, it is replaced by acetic bacteria, which prefer ethanol, good aeration, and heat. Aerobic spore-forming bacteria and filamentous fungi often appear at the final stage of fermentation. They are responsible for unpleasant flavors of fermented cocoa beans [17, 18]. On the other hand, prolonged fermentation leads to an increase in bacilli and filamentous fungi, which can also cause unpleasant flavors. The physiological functions of the predominant microorganisms have been the subject of countless studies, which established the crucial

role of microbial succession in the development of the characteristic cocoa aroma [16].

The sensory properties of cocoa beans can be developed by basic conditions or by external factors, especially those connected with fermentation. The cocoa flavor increases as the fermentation time elapses, which means a negative correlation with astringency. In other words, the astringency of the beans decreases during fermentation [19].

Stages and changes of cocoa beans. Fermentation of raw cocoa beans occurs in two stages, which, in turn, are divided into four steps. The first stage involves microbial reactions that take place in the pulp and on the surface of the beans. The second phase involves several hydrolytic reactions that occur within cotyledons [20].

The system formed to ferment the mucilage that covers the cocoa beans is metabolized by a succession of microorganisms. When cocoa beans are harvested and extracted from the pod, they are exposed to natural biodiverse microflora that comes from the contact with environment, crop handling personnel, transport containers, tools, pod surfaces, etc. [21, 22].

Reactions initiated during fermentation continue at the drying and roasting stage. Thus, oxidation reactions reduce acidity and the amount of phenolic compounds responsible for bitterness and astringency. Cocoa beans can be dried in the sun or in special dryers. However, the latter method often implies extra high temperatures, which can harden the cotyledons and decrease the quality of the finished product. The quality of cocoa beans directly depends on the genotype, harvest, fermentation, drying, and roasting. For instance, beans of different cocoa genotypes should not be fermented together, as it can spoil their sensory properties [23, 24].

Microorganisms present in cocoa fermentation. Traditionally, cocoa fermentation is an uncontrolled process initiated by microorganisms that naturally appear in fermentation sites. These fermenting organisms use pulp as the main substrate. At the onset of fermentation, pulp reduces the diffusion of oxygen within the mass of the fermented bean, thus creating anaerobic conditions [25]. As it was already mentioned, there are five main groups of microorganisms that participate in cocoa fermentation: yeasts, lactic acid bacteria, acetic acid bacteria, and various species of bacilli and fungi. Unlike other fermented raw materials, endogenous enzymes play a crucial role in the development of the flavor of cocoa beans: without fermentation, cocoa beans have no flavor. During fermentation, microorganisms eliminate pulp and produce indispensable metabolites [26, 27].

Yeasts. Yeasts are eukaryotic microorganisms with a high biotechnological potential for food industry. Their properties are completely different from prokaryotic bacteria. Yeasts are resistant to antibiotics, sulfa drugs, and other antibacterial agents. This resistance is genetic, i.e. natural: it cannot be modified or transmitted to other microorganisms [28]. Yeast particles are 5×10 μm in size, which is significantly bigger than the size of bacteria (0.5×5 μm) [29].

The yeast species that have been identified as the main colonizers during cocoa fermentation are the *Saccharomyces cerevisiae*, *Candida pelliculosa*, *Candida tropicalis*, *Candida zeylanoides*, *Torulopsis candida*, *Torulopsis castelli*, *Torulopsis holmii*, *Kloeckera apiculata*, *Kloeckera apis*, *Schizosaccharomyces*, *Kluyveromyces marxianus*, *Pichia membranifaciens*, *Pichia kudriavzevii*, and *Pichia membranaefaciens*. The *Saccharomyces cerevisiae* is the most common strain reported in all cocoa plantations. The exact reason why a certain strain of yeast enters fermentation process still remains unknown. However, the *Kloeckera apiculata* does not survive 24 h of fermentation as it is inhibited by the concentration of ethanol produced in the medium. As for the *Kluyveromyces marxianus*, it grows slowly and degrades gradually. Yeasts are active for approximately 48 h and reach the peak of their activity in 24 h. By that time, their activity has changed conditions of the medium, and other microorganisms join in [16, 30, 31].

Yeasts play an important role in the pulp degradation process. Cocoa pulp can be fermented to produce an alcoholic beverage. Yeasts demonstrate pectinolytic activity. The secondary products of yeast metabolism involve organic acids, aldehydes, ketones, higher alcohols, and esters. The production of glycosidases enzymes is important and affects the quality of beans and, subsequently, that of chocolate [32].

Lactic acid bacteria. Lactic acid bacteria comprise a group of microorganisms linked by the formation of lactic acid as the main metabolite. They are a product of carbohydrate fermentation. Depending on the amount of this product, they can be homo- or heterofermentative. They share similar morphological, physiological, and metabolic characteristics. They are Gram-positive, catalase and oxidase negative, not mobile, and they do not form spores. They can be anaerobic, microaerophilic, and airborne [33].

Lactic acid bacteria can appear at the onset of fermentation. However, they increase their number and become active only when the pulp with its sugars begins to hydrolyze and leave the fermentative system, which is boosts yeast metabolism. The main species that have been isolated so far include *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus cellobiosus*, *Leuconostoc mesenteroides*, *Lactococcus (Streptococcus) lactis*, *Pediococcus spp*, and various species of *Bacillus*.

As for heat-resistant flora, *Lactobacillus curieae*, *Enterococcus faecium*, *Fructobacillus pseudoficulneus*, *Lactobacillus casei*, *Weissella paramesenteroides* and *Weissella cibaria* have also been registered, but to a lesser extent. They exist during the first 72 h of fermentation and reach their peak in 36 h.

Their maximum growth period is 16–48 h. The prevailing species include *Lactobacillus plantarum* and *Lactobacillus fermentum*. Lactic acid bacteria mostly produce lactic acid, but they also generate small amounts of alcohol and acetic acid from fructose and glucose. In addition, they can use citric acid to produce acetaldehyde, diacetyl, mannitol, acetic acid, and lactic acid [34–36].

Acetic acid bacteria. Acetic bacteria are Gram-negative and belong to the *Acetobacteraceae* family. They are strict aerobics, non-spore-forming, ellipsoidal or bacillus-shaped. They may occur in pairs or in chains. Members of the *Acetobacter* genus are so common due to their ability to grow in ethanol environment. Acetic bacteria are known to partially oxidize a variety of carbohydrates and to release various metabolites, e.g. aldehydes, ketones, and organic acids, in different media. For a long time, they have been used to perform specific oxidation reactions via processes called "oxidative fermentations" [37]. The first step in the production of acetic acid is the conversion of ethanol from a carbohydrate by yeasts. The second step is the oxidation of ethanol to acetic acid by acetic acid bacteria [38].

During cocoa fermentation, the population of yeasts and lactic acid bacteria decays, thus creating an aerobic environment favorable for the growth of acetic bacteria. The temperature reaches approximately 37°C. The increase in temperature triggers protein hydrolysis and acidification of the beans. As a result, ethanol dissolves into acetic acid, carbon dioxide, and water. Some strains appear at 24 h and reach their growth peak at 88 h. After 120 h, they can no longer be detected. They begin to disappear when the mass reaches 50°C. Part of the generated acid volatilizes, while the rest enters the bean and is responsible for killing the germ [39]. These bacteria play a fundamental role in the generation of volatile compounds that affect the quality of chocolate. The *Acetobacter* and *Gluconobacter* geni are usually observed during fermentation, the most common being *Acetobacter aceti* and *Acetobacter pasteurianus*, as well as the recently discovered *Acetobacter ascendens*, *A. rancens*, *A. xylinum*, *A. lovaniensis*, *A. xylinum*, *A. peroxydans*, and *Gluconobacter oxydans* [16, 40, 41].

Fermentation stages.

Stage I. During the first stage of fermentation, the volume of the pulp that surrounds the beans reduces the diffusion of oxygen within the medium. This is where the beans will be fermented in anaerobic conditions. During this stage, first yeasts and then lactic bacteria consume sugars and organic acids from the pulp, thus producing ethanol, lactic acid, etc. [25].

The yeast population starts with 10^7 CFU/g pulp and reaches a maximum of 10^8 CFU/g pulp. After that, it starts to decline until it reaches the bottom level of 10 cells per gram of pulp. Yeasts are prevailing microorganisms, and their depectinization activity causes liquefaction of the pulp with its subsequent drainage, or "sweating". The pulp loses its viscosity and lets in air [42]. As a result, the simple sugars of the mucilage, namely sucrose, fructose, and glucose, turn into ethanol. The pectin degrades, causing the texture of the bean to change, and eliminates citric acid. The yeasts which are generally responsible for metabolizing this acid are *Candida* spp. and *Pichia* spp., which generate an alkaline pH. This parameter, together with alcohol and oxygen, coincidentally inhibits the yeasts and their activity, but contributes to the development of lactic bacteria. Yeasts also form such organic acids as acetic, oxalic, phosphoric, and malic acids. They help reduce pH fluctuations [43, 44].

Yeasts have become focus of numerous cocoa bean fermentation studies since they release pulp degradation enzymes. Moreover, they are also the main producers of esters and higher alcohols, which can contribute to the complex mix of aromatic volatile compounds that make up the cocoa aroma. The main yeasts that generate these volatile compounds are *Candida sp.*, *Kluyveromyces marxianus*, *Kloeckera apiculata*, *S. cerevisiae*, and *S. cerevisiae var. chevalieri* [45].

The second phase of this stage involves several hydrolytic reactions that occur within the cotyledons. As the fermentation continues and the pulp drains, more oxygen enters the system, thus creating the optimal conditions for the growth of lactic bacteria [42]. They colonize the cocoa mass, degrade the glucose of the pulp into lactic acid, and assimilate the remaining citric acid.

Several studies on microbial fermentation indicate that two most prevalent species in this process are *Lactobacillus plantarum* and *Lactobacillus fermentum*. They also produce acetate esters from acetic acid, which give different tones to cocoa-based products [31]. Lactic bacteria can reach a population of 6.4×10^7 CFU/g pulp. At first, they increase the acidity by producing citric acid, but then they lower the pH by releasing products that are not acidic. Lactic acid bacteria are able to metabolize malic acid. These bacteria have no major proteolytic activity and can only ferment two types of amino acids: serine and arginine. After all these reactions, the environment is totally aerobic, which allows for the growth of acetic bacteria [7, 34, 46].

Stage II. During the second stage, the environment is oxygenated, and the pH has decreased due to the removal of some components and variability of the remaining compounds. At last, acetic acid bacteria can convert the previously obtained ethanol into acetic acid via the oxidation of alcohol. The optimal temperature of the acetic fermentation process is between 28°C and 30°C, and the optimum pH is 4.5. The oxidation of ethanol is carried out in two stages. First, ethanol is oxidized into acetaldehyde. Second, the acetaldehyde becomes acetic acid. Other products include ethyl acetate, butanol, isopropanol, intermediate acetaldehyde compounds, and organic acids [47].

The formation of acetic acid is very important at this stage of the process. It occurs due to the activity of acetic

bacteria. The exothermic reactions of the bacteria raise the temperature of the mass. The population reaches its peak at 1.2×10^7 CFU/g pulp and falls down after three days of activity precisely because of the high temperature it generates. In some cases, the population can reach 3.5×10^3 CFU/g pulp [16, 48].

As the volume of oxygen increases, the pH reaches 3.5–5.0, and the temperature becomes 45–50°C. Under these conditions, several aerobic spores of *Bacillus* bacteria may appear in the fermentation. After the pile of beans has been stirred, one can detect the presence of *Bacillus licheniformis*, *B. megaterium*, *B. pumilus*, *B. pumilus. B. coagulans*, *B. circulans. B. subtilis. B. cereus*, and *B. megaterium*. Most of them are heat-tolerant and can survive during drying and roasting. They are capable of producing numerous enzymes, both proteolytic and lipolytic, which catalyze reactions. However, they give cocoa unpleasant taste and smell since they degrade proteins and fats by producing chemical substances that can distort the flavor [49, 50].

CONCLUSION

The fermentation stage is considered the most important process in the transformation of cocoa to chocolate. The changes that occur in its volatile and aromatic compounds trigger structural changes in the composition. The changes are due to the activity of various microorganisms. Their main objective is to kill the germ and thus stop the metabolism of the bean. The resulting alcohol is broken down into acetic acid and other acids, which produce desirable sensory properties that will be accentuated during drying and roasting.

Yeasts, lactic bacteria, and acetic bacteria play a fundamental role in the fermentation process. By knowing the main strains, their action parameters, main reactions, and products, food scientists can improve this process to obtain cocoa beans with a better chemical composition.

Each strain of microorganisms requires a separate research with regard to the variety of cocoa beans. The species vary from zone to zone, and plantations in different parts of the world are unlikely to have similar characteristics.

CONTRIBUTION

Roberto Ordoñez-Araque and Julio Urresto-Villegas compiled the manuscript. Edgar Landines-Vera and Carla Caicedo-Jaramillo collected the data, checked the structure, and performed the final review.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to the publication of this article.

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The present research is our homage to the indigenous peoples of Ecuador, who started growing cocoa 5.000 years ago and introduced this delicious treat to the world.

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Extracts of *Rhodiola rosea* L. and *Scutellaria galericulata* L. in functional dairy products

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Abstract:
Introduction. Modern scientific research into the biochemical composition and medicinal value of plants makes it possible to use them as functional ingredients in food technology. The research objective was to test rose root (*Rhodiola rosea* L.) and scullcap (*Scutellaria galericulata* L.) for biologically active substances and their potential use in functional dairy products.
Study objects and methods. The research featured biologically active substances (BAS) obtained from rose root and scullcap that grow in mountain areas or on rock outcrops along Siberian rivers. The BAS content was determined using high performance liquid chromatography (HPLC). The biologically active substances were screened and identified using HPLC, thin-layer chromatography (TLC), and infra-red identification (IR). The new functional products were based on whey and cottage cheese made from processed whole milk.
Results and discussion. The analysis of *Rhodiola rosea* rhizomes and roots showed the following BAS content (mg/g): rosavin – 16.9, salidroside – 14.3, rosin – 5.04, rosarin – 2.01, and methyl gallate – 6.8. The roots of *Scutellaria galericulata* had the following BAS content (mg/g): scutellarein – 22.27, baicalin – 34.37, baicalein – 16.30, apigenin – 18.80, chrysin – 6.50, luteolin – 5.40, and vogonin – 3.60. Whey served as a basis for a new functional whey drink fortified with BAS isolated from *Rhodiola rosea* 100 mL of the drink included 50 mL of whey, 20 mL of apple juice, 0.1 mL of rose root concentrate, 3 g of sugar, 0.5 g of apple pectin, 04 g of citric acid, and 30 mL of ionized water. The content of phytochemical elements ranged from 0.11 ± 0.001 to 0.49 ± 0.08 mg/100 g. Cottage cheese served as a basis for another dairy product fortified with BAS obtained from *Scutellaria galericulata*. The formulation included 81 g of cottage cheese, 10 mL of cherry jam, 9 g of sugar, and 0.025 mL of scullcap concentrate. The content of biologically active substances in the finished product varied from 0.09 ± 0.02 for luteolin to 0.48 ± 0.11 for baicalin. The whey drink fortified with the BAS extracted from *Rhodiola rosea* and the cottage cheese product fortified with the BAS isolated from *Scutellaria galericulata* satisfied 40–45% and 55–60% of the reference daily intake for phenolic compounds, respectively. The obtained data made it possible to recommend the new functional foods for commercial production.
Conclusion. A set of experiments was performed to isolate biologically active substances from *Rhodiola rosea* and *Scutellaria galericulata*. The research developed and tested formulations of two new functional products based on whey and cottage cheese.

Keywords: Medicinal plants, Functional food, biologically active substances, whey, cottage cheese

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The experiments involving high-performance liquid chromatography were conducted at the premises of the Co-working Research Center, Kemerovo State University.

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INTRODUCTION

Public nutrition attracts attention of medical science and affects the development of biotechnology in food industry [1]. As a result, most solutions lie in the sphere of functional products designed for particular groups of population [2]. Miners, geologists, polar explorers, astronauts, submariners, athletes, and programmers are prone to various diseases as a result of adverse working conditions. Unsocial working hours make them vulnerable to diseases of digestive system, liver, thyroid gland, cardiovascular system, and bones. Low-income families still experience the consequences of unhealthy diet that lacks natural meat, dairy products, and fresh vegetables. As a result, a lot of people suffer from deficiency of proteins, vitamins, and other biologically active substances. Functional products can compensate for the missing elements as they are fortified with biologically active substances of plant origin, e.g. minerals, macro- and microelements, bioactive peptides, enzymes, etc. [3, 4, 20].

As a rule, food habits are as old as the nations or states they belong to. However, they were shaped not only by the local flora, fauna, climate, soil fertility, water availability, national traditions, and culture, but also by the genetic ability of the people to digest certain types of food [14–16]. Some researchers recommend introducing ancient Eastern traditions to the achievements of Western medicine. In fact, European diet includes less than 2–3% of edible plants while in the East people enjoy a variety of 1000 different edible species [17]. The Japanese, whose life expectancy is one of the longest in the world, consume equal amounts of meat and vegetables [24].

European scientists believe that saturated fats and cholesterol in meat can be reduced by introducing safe fibers into processed foods [5, 6]. Nitrites and polycyclic aromatic hydrocarbons (PAH) are often found in processed meat products and can have a disastrous effect on human health [7]. Functional ingredients extracted from medicinal plants can significantly improve meat, fish, and dairy products [8, 9, 16]. Russian food science has achieved great success in developing new functional dairy products based on whey, cottage cheese, and buttermilk [14–16].

The relevance of the present research lies in the fact that a few plant species are actually used in functional products, including the rose root (*Rhodiola rosea* L.). According to scientific sources, it is usually used in herbal tea mixes, water tinctures, or wine products. As for the scullcup (*Scutellaria galericulata* L.), this plant is protected by law, and this is the first time it has become focus of the attention of food science. Its properties and prospects for functional food industry remain understudied. Thus, the research objective was to identify the biologically active substances that can be extracted from these plants and study their potential for the production of new functional foods based on whey and cottage cheese.

STUDY OBJECTS AND METHODS

The present research featured biologically active substances (BAS) extracted from two plants: the rose root (*Rhodiola rosea*) and the skullcap (*Scutellaria galericulata*).

The rose root can be found all over Russia, from its European part to the Far East. It is especially abundant on the fragmental soil of the Altai-Sayan mountain systems. The plant proliferates on the variety of local minerals and macro- and microelements. They add unique medicinal properties to the phytochemical composition of the plant organs [21, 22, 24]. In fact, the rose root has nearly become extinct due to uncontrolled herborization. As a result, it is now listed in the regional endangered-species lists and in the Red Book of Russia.

The scullcap is endemic to Eastern Siberia: it grows in the Tomsk and Kemerovo regions, in the Republic of Tuva, in Khakassia, and in the Mongolian areas of the Altai Mountains [13]. The plant prefers moist forest woodlands, steep river banks, and sandy terraces. The scullcap is a popular medicinal plant with unique adaptogenic, antioxidant, apoptotic, and antiviral properties. It is also known for its ability to inhibit the development of free radicals in cells [23–26]. The research featured aerial parts, rhizomes, and roots.

The content of BAS was determined using Shimadzu LC-20 Prominense chromatography unit. The device was equipped with a Shimadzu SPD20MA diode array detector and a RID refractometric detector with a Kromasasil C-18 250 × 4.6 mm column.

The TLC chromatography was performed using Sorbfil PTCX-AF-A plates with subsequent densitometry on a TLC Sorbfil plate. The experiment involved a densitometer with a Sony photofixation system (Handycam HDR-CX-405) purchased from IMID LLC, Russia. Sulfuric acid and 25% ethanolic solution of phosphoric-tungsten acid were used for targeted derivatization. After that, photofixation was performed at wavelengths of 254 and 365 nm in the visible range. Elution was conducted in mobile phase systems: chloroform – methanol – water (62:32:6) and ethyl acetate – formic acid – glacial acetic acid – water (100:11:11:26).

During the preparative stage, the chromatographic zones were excised and subjected to further analysis. The targeted BAS were screened and identified using HPLC, TLC, and IR. The obtained statistical data were processed using the Microsoft® Excel program. The tables show the arithmetic mean values. All experiments were performed in triplicates. The quantitative content of the BAS was determined using calibration curves constructed in the concentration range of 0.05–200 μg/mL.

The new functional products were based on whole milk whey and cottage cheese.

RESULTS AND DISCUSSION

In the industrially developed regions of Siberia,

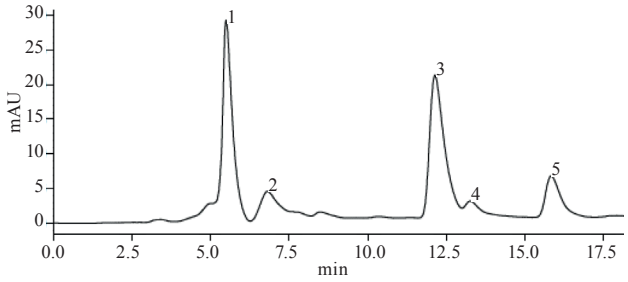

Figure 1 Chromatogram of ethanol extract from rhizomes and roots of *Rhodiola rosea* L.

public health is especially vulnerable. Its maintenance requires an active use of biological resources in food biotechnology [22, 23]. The present research featured the content of BAS in the rhizomes and roots of the rose root (*Rhodiola rosea*) harvested in the subalpine zone of the Kuznetsk Alatau mountains (Figs. 1 and 2). The BAS were isolated using chromatographic methods (Figs. 1 and 2). Rosavin (peak 1) and salidroside (peak 3) appeared to be the most abundant substances. Methyl gallate (peak 5), rosin (peak 2), and rosarin (peak 4) also proved significant. Rosavin, rosarian, and rosin belong to phenylpropanoids.

These compounds possess a lot of beneficial properties. First of all, they have scientifically proven adaptogenic and antioxidant properties [17]. Phenylpropanoids (rosavin, rosin, rosarin) are known to have tonic, antiviral, and immunomodulatory properties. Salidroside is regarded as one of the most promising substances for solving gerontology problems. This fact confirms the hypothesis that BAS extracted from rose root can be used in functional food industry. The actual value of rosavin was 16.9 mg/g, which exceeded other BAS by 15.4–88.2%.

Baicalin has good antioxidant properties. It also neutralizes oxidation processes and prevents the formation of free radicals. Scutellarein and vogonin exhibit mutual synergism and have anticonvulsant and antitoxic properties. Luteolin and vogonin have apoptotic, anti-inflammatory, and other useful properties. The BAS complex obtained from the *Scutellaria* genus is actively used for the treatment and recovery of cancer patients.

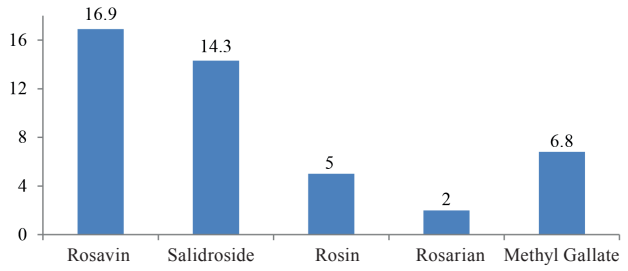

Figure 2 Content of biologically active substances in *Rhodiola rosea* L., mg/g

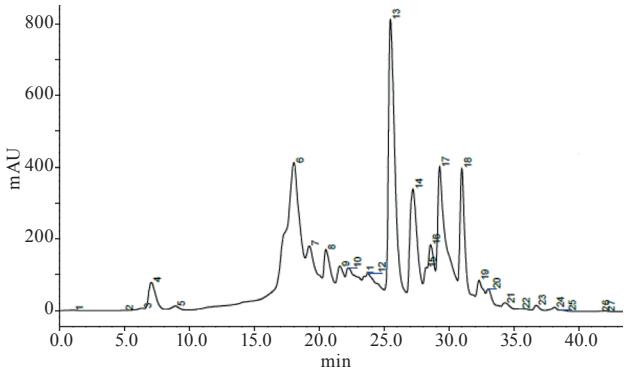

Figure 3 Chromatogram of ethanol extract from roots of *Scutellaria galericulata* L.

The analysis of scullcap roots showed high concentrations of the following BAS: baicalin (peak 13), scutellarein (peak 6), baicalein (peak 17), apeginin (peak 18), chrysin (peak 14), luteolin (peak 16), and vogonin (peak 7) (Figs. 3 and 4).

As for quantification, the content of BAS within this group varied from 5.4 to 34.4 mg/g. Baikalin had the biggest share compared with other BAS: 34.37 mg/g. Its advantage over other components was 35.2–84.3%. The

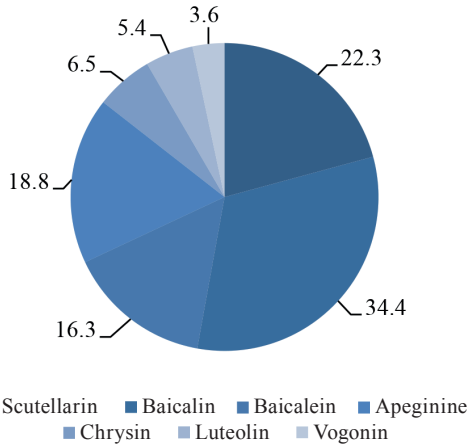

Figure 4 Content of biologically active substances in *Scutellaria galericulata* L., mg/g

Table 1 Formulation of the whey drink fortified with *Rhodiola rosea* L. concentrate

Component	Amount					
	1	2	3	4	5	6
Whey, mL	70.0	60.0	50.0	70.0	60.0	50.0
Apple juice, mL	30.0	40.0	50.0	20.0	20.0	20.0
Sugar, g	3.0	3.0	3.0	3.0	3.0	3.0
Apple pecin, g	0.5	0.5	0.5	0.5	0.5	0.5
Concentrate of *Rhodiola rosea*, mL	0.1	0.1	0.1	0.1	0.1	0.1
Lemon acid, g	0.04	0.04	0.04	0.04	0.04	0.04
Drinking water, mL	–	–	–	10.0	20.0	30.0

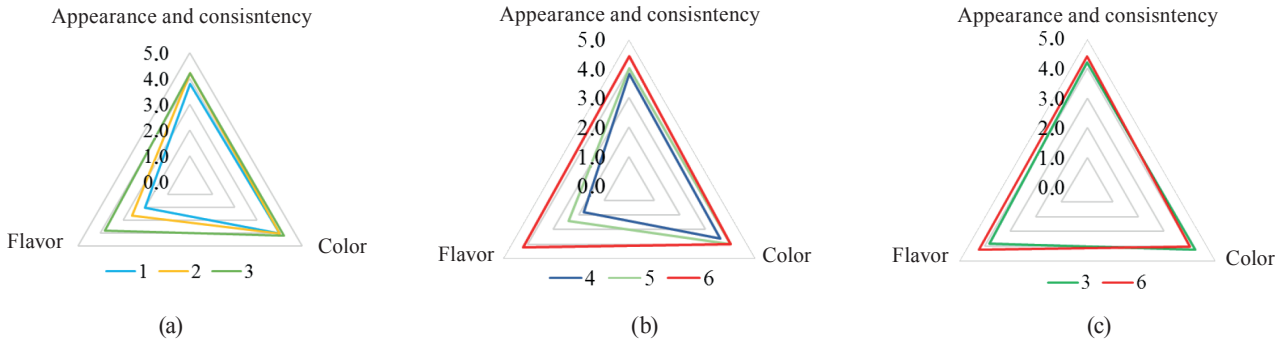

Figure 5 Sensory evaluation of the whey drink: (a) formulations 1–3; (b) formulations 4–6; (c) formulations 3 and 6, which proved optimal

obtained data prove that both plants have good prospects for functional food industry.

Whey and cottage cheese are high-protein dairy products and are beneficial for human health. They served as bases for formulations of two new functional products: a whey drink fortified with BAS extracted from rose root concentrate and cottage cheese fortified with BAS extracted from scullcap roots.

The formulation of the whey drink included cottage cheese whey, apple juice, sugar, rose root concentrate, and drinking water. Citric acid served as a regulator of acidity, while apple pectin was used as a stabilizer (Table 1). We tested six formulations of the new product. The first three samples had a different amount of apple juice. The remaining three samples differed in the amount of water, while the volume of apple juice remained the same. Water affects sensory properties and regulates the acidity of the finished product.

In order to determine the optimal formulation, the drink underwent a sensory evaluation for appearance, consistency, flavor, and color on a five-point scale (Fig. 5).

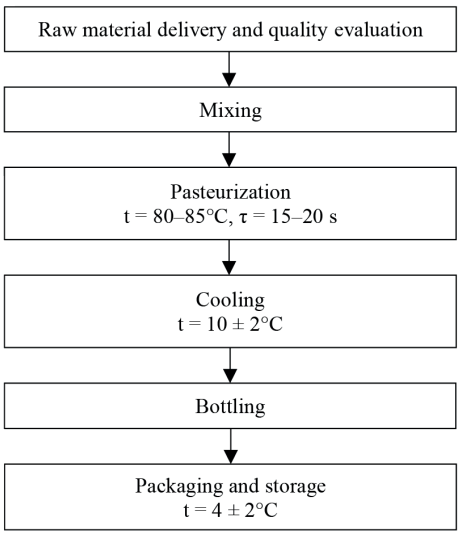

Figure 6 Flow chart for the whey drink fortified with biologically active substances extracted from *Rhodiola rosea* L.

Variants 3 and 6 received the highest score. When they were compared with each other, preference was given to variant 6. It had the highest sensory evaluation both in terms of flavor and color. Therefore, variant 6 was selected for the production of the functional product.

The technological process for the whey drink fortified with rose root concentrate included the following stages: raw material delivery and sensory evaluation, mixing the components, pasteurization, cooling, bottling, packaging, and storage (Fig. 1).

At the first stage, the raw material was evaluated according to the main quality indicators. Raw materials that met the requirements of regulatory and technical documentation passed on to the next stage. The initial mix was made up of the main ingredients, i.e. whey and drinking water, which entered the tank through a pipeline. Apple juice and rose root concentrate were introduced manually. The rose root concentrate was a dense, homogeneous dark brown mass. The dry ingredients, i.e. sugar, pectin, and citric acid, were gradually added to the resulting solution. A continuously working stirrer prevented lump formation. To suppress the development of vegetative microorganisms, the mix was pasteurized at 80–85°C for 15–20 s. The resulting drink was cooled to 10°C, bottled, and capped in uniform vessels.

Tables 2 and 3 show the content of BAS in the finished product and the results of sensory, physico-chemical, and microbiological evaluation. All the BAS introduced into the formulation of the functional drink were represented in quantities that were found sufficient for practical use. State-issued Recommended

Table 2 Biologically active substances in the functional whey drink fortified with *Rhodiola rosea* concentrate L.

Component	Content in the concentrate, mg/g	Content in the finished drink, mg/100 g
Rosavin	16.89 ± 2.11	0.31 ± 0.077
Salidroside	14.35 ± 2.52	0.49 ± 0.08
Rosin	5.04 ± 0.93	0.11 ± 0.001
Rosarin	2.01 ± 0.37	0.17 ± 0.012
Methyl gallate	6.8 ± 1.05	0.12 ± 0.032

Table 3 Sensory, physico-chemical, and microbiological indicators of the functional whey drink fortified with *Rhodiola rosea* L. concentrate

Index	Property
Appearance and texture	Opaque liquid with slight phase layering
Color	Intrinsic, uniform
Taste and smell	Characteristic, no extraneous flavors and odors; tastes a little sour
Mass fraction of solids, %	9.7 ± 0.3
Mass fraction of fat, %	0.02 ± 0.03
Acidity, °T	47.5 ± 0.8
Release temperature, °C	4 ± 2
Coliform bacteria, per 0,01 cm^3	Not detected
Yeast and mold, CFU/cm^3	≤ 1,0×10–1
Pathogens, including salmonella	Not detected

Practice MP 2.3.1.1915-04 highlights the level of BAS consumption. According to the data provided in the document, the new functional drink satisfied 40–45% of the reference daily intake for phenolic compounds and phenylpropanoids. The performed evaluation of sensory, physico-chemical, and microbiological properties of the drink showed that it corresponded to another state-issued standard – Technical Requirements 10.51.55-001-02068309-2019.

The unique properties of skullcap, or *Scutellaria galericulata*, have never become an object of food technology. However, it is rich in flavonoids, and a functional product fortified with its BAS will have a beneficial effect on various systems of human body. Using the above techniques, we obtained another functional dairy product – cottage cheese fortified with skullcap concentrate. The experiment involved five variants: a control sample, two samples with cranberry jam, and two samples with cherry jam.

Table 4 demonstrates the formulation, while Fig. 7 shows the flow chart for the producing of cottage cheese enriched with skullcap concentrate.

Table 4 Formulation of the cottage cheese fortified with *Scutellaria galericulata* L. concentrate

Component	Amount				
	1	2	3	4	5
Cottage cheese, g	91.0	86.0	81.0	86.0	81.0
Cherry jam, mL	–	5.0	10.0	–	–
Cranberry jam, mL	–	–	–	5.0	10.0
Sugar, g	9.0	9.0	9.0	9.0	9.0
Concentrate of *Scutellaria galericulata*, mL	0.025	0.025	0.025	0.025	0.025

The sensory analysis of the cherry jam samples revealed good monogenicity, consistency, and appearance in both variants. Variant 3 was given the best scores for flavor (Fig. 7a). This sample contained 81 mL of cherry jam, 9 g of sugar, and 0.025 mL of scull-cap concentrate per 81 g of cottage cheese. The samples with cranberry jam showed no significant differences. After a comparative analysis of all the options, variant 3 was announced best according to taste properties.

The technology for the new cottage cheese product included the following stages: preparation of the raw material, mixing, heating, homogenization, cooling, packaging, and storage (Fig. 8). Raw materials were evaluated according to the main quality indicators and regulatory documentation. To prepare the mix, cottage cheese was put into the kneading machine. Jam, sugar, and scull-cap concentrate were added manually. The obtained mix underwent a thermal treatment at 62°C for 15–20 s to suppress the development of vegetative microorganisms. To obtain a homogeneous texture, the cottage cheese was homogenized at 62°C. After that, the finished cottage cheese was cooled to 20°C and packaged. The product was stored at 4 ± 2°C.

A biochemical analysis of the finished product revealed sufficient quantities of BAS (Table. 5). Table 6 shows sensory, physico-chemical, and microbiological indicators of the fortified cottage cheese.

Figure 7 shows the results of the sensory evaluation of appearance, consistency, flavor, and color on a five-point scale.

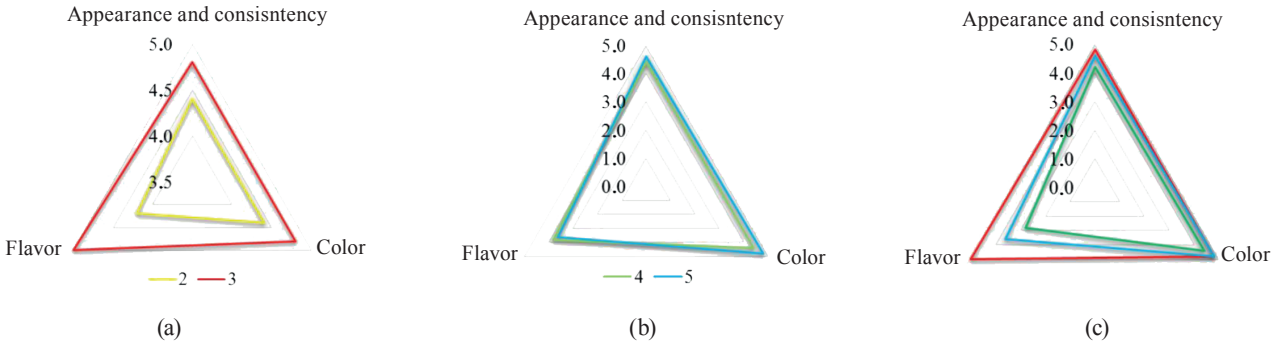

Figure 7 Sensory evaluation of the cottage cheese: (a) formulations 2 and 3; (b) formulations 4 and 5; (c) control formulation 1 and formulations 3 and 5, which proved optimal

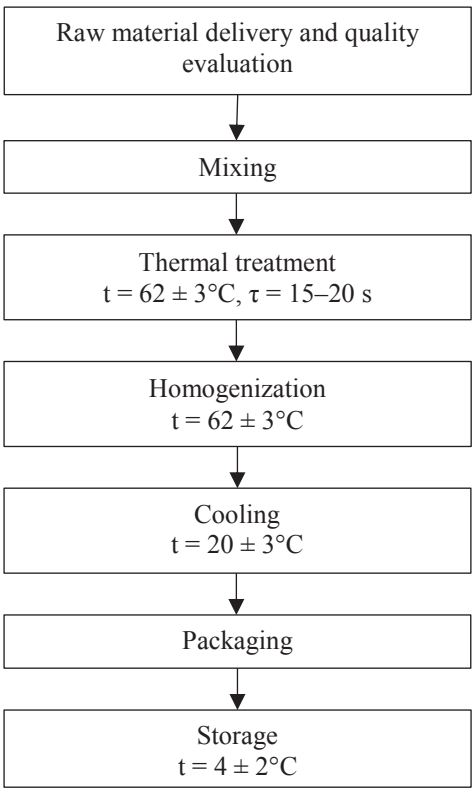

Figure 8 Flow chart for the cottage cheese fortified with biologically active substances extracted from *Scutellaria galericulata* L.

Table 5 Main flavonoids in the cottage cheese fortified with biologically active substances extracted from *Scutellaria galericulata* L.

Component	Content in the concentrate, mg/g	Content in the finished cottage cheese, mg/100 g
Scutellarein	22.27 ± 2.23	0.26 ± 0.019
Baicalin	34.37 ± 3.47	0.48 ± 0.11
Baicalein	16.3 ± 2.19	0.26 ± 0.019
Apigenin	18.80 ± 1.98	0.23 ± 0.019
Chrysin	6.50 ± 1.13	0.14 ± 0.012
Luteolin	5.40 ± 1.00	0.09 ± 0.02
Vogonin	3.60 ± 0.90	0.12 ± 0.014

According to the Recommended Practice MP 2.3.1.1915-04, the new functional cottage cheese product satisfied 55–60% of the reference daily intake for phenolic compounds. The performed sensory, physico-chemical, and microbiological evaluation of the cottage cheese showed that it corresponded to Technical Requirements 10.51.55-001-02068309-2019.

Table 6 Sensory, physico-chemical, and microbiological indicators of the cottage cheese fortified with biologically active substances extracted from *Scutellaria galericulata* L.

Index	Property
Appearance and consistency	Homogeneous, pasty, soft
Color	White, with the hue characteristic of the introduced components
Flavor	Pure, sour-milk, sweet, with a touch of added ingredients
Moisture content, %	59.3 ± 3.9
Mass fraction of protein, %	12.4 ± 0.7
Mass fraction of fat, %	3.8 ± 0.7
Acidity, °T	149.3 ± 10.9
Release temperature, °C	4 ± 2
Lactic acid microorganisms, CFU/cm^3	1×10^7
Coliform bacteria, per 0,01 cm^3	Not detected
Yeast and mold, CFU/cm^3	Not detected
Pathogens, including salmonella	Not detected

CONCLUSION

The present research established the content of biologically active substances obtained from two medicinal plants of the Kemerovo Region. It featured the rhizomes and roots of *Rhodiola rosea* harvested in the subalpine belt of the Kuznetsk Alatau mountains and the roots of the *Scutellaria galericulata* harvested on the rocky outcrops along the Tom' River.

The biomass was tested for biologically active substances and revealed good pharmacological prospects, i.e. high antioxidant, anti-inflammatory, antibacterial, antiviral, and apoptotic properties.

A set of experiments resulted in two formulations of new functional dairy products: a whey drink fortified with biologically active substances extracted from *Rhodiola rosea* concentrate and cottage cheese fortified with biologically active substances extracted from *Scutellaria galericulata*.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to the publication of this article.

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Intensification of cooling fluid process

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Abstract: A number of sectors in the food industry practice cooling substances of biological origin. This contributes to the maintenance of their biological properties, as well as prevents microflora growth in the product. One of the ways to intensify production processes and maintain the quality of raw materials and finished products is their accelerated cooling with the help of low-energy cooling equipment. The use of physical bodies cooled to low temperatures is a promising way to accelerate liquid cooling. We used balls with frozen eutectic solution. In our research, the problem of cooling a liquid system is formulated and solved within the framework of classical linear boundary value problem for the equation of a stationary convective heat transfer. In the area of the actual values of the process parameters on the study object, the solution obtained is used as the basis for numerical experiment on the modelling of the cooling liquid flow with the cooling agent system, namely balls filled with eutectic solution. By calculation, the efficiency of the proposed method for cooling liquid was justified based on such factors as temperature, the number of balls in a two-phase liquid system, and the duration of low-temperature treatment. The presented results of the numerical experiment complied with real heat transfer processes during liquid cooling.

Keywords: Low-temperature treatment, cooling, liquid system, heat transfer, eutectic solution

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INTRODUCTION

An important issue of the intensification of production processes and rational use of raw materials is the possible rapid decrease in the temperature of the liquid system. For example, in processing industries and other sectors of the economy, the cooling of biological origin substances contributes to the maintenance of their biological properties, as well as prevents microflora growth in the product [1, 2].

Currently, the cooling of liquids by using frozen solids is one of the methods for lowering the temperature in a liquid medium [3–7, 11, 14, 15]. This technology is useful and in refrigeration engineering, where frozen physical bodies are balls filled with eutectic solution [8–10, 12]. This is confirmed by results of theoretical and experimental studies conducted by reserchers of Moscow State University of Food Production and Razumovsky Moscow State University of technology and management. The results confirm the advantages of cooling water with frozen balls over other methods, providing a high intensity of the process and reduced energy consumption.

To accelerate the heat transfer process based on water treatment using the technology of enrichment of the working volume of cooling liquid with frozen bodies (cooling agent), it is advisable to carry out this process in the mode of flow of the liquid through a container with frozen balls. At the same time, it should be noted that there are no theoretically based calculation methods for predicting and controlling the heat transfer process, including in the flow, when the cooling process develops in a heterogeneous liquid “water-frozen balls” system.

Bases on the law of conservation of mass and energy, we presented the results of the analytical and numerical studies on the of cooling a liquid flow moving through a heat exchanger filled with balls with a frozen eutectic coolant to justify the intensification of the heat transfer process between a coolant and liquid.

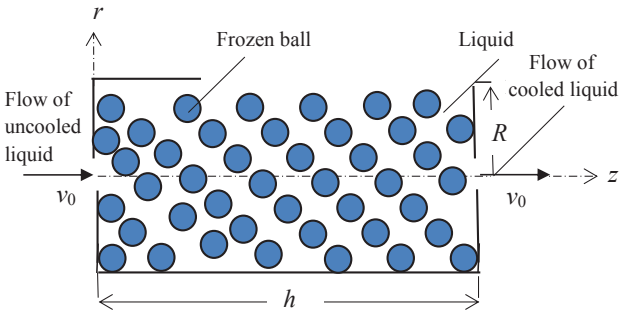

Figure 1 Scheme of liquid cooling with frozen balls

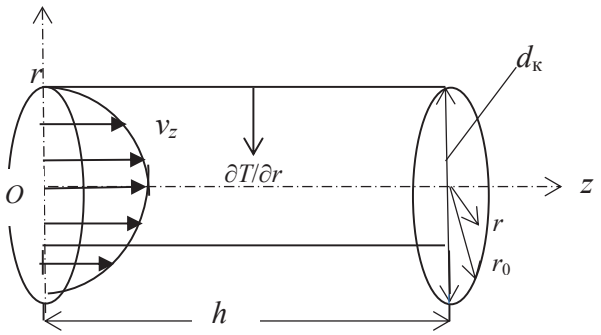

Figure 2 Scheme to calculate the cooling process of liquid with frozen balls

The setting of the problem. Let a liquid flow (for example, water) be supplied to a certain container filled with balls with frozen eutectic solution in a stationary mode, with a small productivity Q and a low flow rate v_0 (Fig. 1). We will consider the selected motion model of a two-phase liquid system as the filtration flow of a fluid through a porous medium formed by balls (coolant) [6].

It is assumed that the balls are statistically uniformly distributed inside a cylinder of length h and radius R containing a liquid (water) and solid (balls) phases.

To simplify the process for quantitative analysis of the heat removal from the liquid to the balls, we replaced the pore volume occupied by the liquid (preserving the values of porosity) with a set of cylindrical tubes (conditionally, capillaries). Each of them had an axis parallel to the axis of the vessel, length h and reduced radius r_0 (Fig. 2).

For the selected geometric model of the liquid volume, we introduce the following notations: d_b is the ball diameter and m is the surface porosity of the system ($m < 1$), numerically equal to the ratio of the volume of liquid filled pores to the volume of the system [8].

Since the volume and surface porosities of the working volume of the capacitance are quantitatively the same [8], the approximate ratio, based on the porosity definition can be written as follows:

$$\pi r_0^2/m = \pi d_b^2/[4(1-m)]$$

which yields the relationship:

$$r_0 = 0{,}5d[m/(1-m)]^{1/2} \quad (1)$$

henceforward, $r_0 = r_0(d,m)$, $d = d_b$ is the diameter of the ball.

Thus, as can be seen, the problem of calculation reduces to the quantitative analysis of temperature in the isolated capillary.

The solution of the problem. Due to the possible axisymmetric nature of heat transfer from the walls of the channel to the liquid, the quantitative modeling of this process will be carried out in a cylindrical coordinate system, in the meridional section of the channel rOz (Fig. 2).

For the selected simulation geometric model, we use the equation of stationary convective thermal conductivity related to cylindrical coordinates as a basic differential equation describing the heat transfer phenomenon in the flow [9]:

$$\frac{\partial(Tv_z)}{\partial z} = a\left(\frac{\partial^2 T}{\partial r^2} + \frac{1}{r}\frac{\partial T}{\partial r}\right) \quad (2)$$

where r, z – radial and axial coordinate, respectively; T – the temperature of water; v_z – axial component of fluid flow rate in the capillary; $a = \lambda/(c\rho)$, a – is the coefficient of thermal diffusivity of water, λ – thermal conductivity coefficient, c – specific conductivity coefficient, and ρ – is the density of water.

In practice, fluid rate v_z can be replaced with its averaged value over the cross section of the channel, with a small error. Then a simplified form of Eq. (2) can be written as follows:

$$\frac{\partial T}{\partial z} = \beta\left(\frac{\partial^2 T}{\partial r^2} + \frac{1}{r}\frac{\partial T}{\partial r}\right) \quad (3)$$

where

$$\beta = a/v_z \quad (4)$$

$v_z = v_0/m$, $v_0 = Q/S$ – volume rate of fluid flow in the capacitance (filtration rate [8]), $S = \pi R^2$ – cross-sectional area of the capacitance; β is the specific coefficient of thermal diffusivity calculated taking into consideration axial velocity v_z.

For simplicity, assume inlet temperature to be constant:

$$T(r,z) = T_0 \text{ at } 0 \le r \le r_0,\ z = 0 \quad (5)$$

The condition of symmetry of the temperature distribution along the channel diameter corresponds to the condition of the maximum temperature in the middle the capillary walls.

$$\partial T/\partial r \text{ at } r = 0,\ 0 < z \le h \quad (6)$$

Taking into account the fact that heat energy develops from the liquid to the capillary wall, the boundary condition on the surface of this channel is:

$$H[T(r_0,z) - T_k] - \partial T(r_0,z)/\partial r = 0\ (0 < z \le h) \quad (7)$$

where $H = \alpha/\lambda$, α is heat transfer coefficient from the liquid to the capillary wall, $T_к$ is the eutectic temperature of frozen balls (Fig. 1).

According to (5)–(7), in the framework of the terminology adopted in the theory of heat transfer, we have a problem with boundary conditions of the third kind for differential equation (3).

From the point of view of quantitative analysis of the thermal regime in a steady stream of fluid inside the capillary, the considered problem is formally equivalent (provided that the diameter of the capillary $d_к$ is much less than the capacitance height h) to the problem of the temperature distribution over time in an unsteady mode in an unlimited cylinder. What is more, in Eqs. (3)–(7) the duration of the heat transfer process is displayed on the axial coordinate z.

Thus, the solution to the problem with an unsteady mode of heat transfer in an unbounded cylinder can be adapted to the boundary value problem (3)–(7) of the temperature distribution in the convective fluid stream in the capillary and is formulated as a dependence:

$$\theta = 1 - \sum_{i=1}^{n} A_i J_0(\nu_i r/r_0) \times \exp(-\nu_i^2 \times Fo^*),\ n \to \infty \quad (8)$$

where

$$\theta = \theta(d,m,r,z) = (T - T_0)/(T_к - T_0) > 0 \quad (9)$$

is specific value reflecting the differential temperature of the ball $T_к$ and the initial temperature of the liquid T_0, as well as the current differential temperature of the liquid $T(d,m,r,z)$ and the coolant temperature T_0.

$$A_i = 2J_1(\nu_i)/\{\nu_i[J_0(\nu_i)^2 + J_1(\nu_i)^2]\} \quad (10)$$

where J_0, J_1 – Bessel function of the first kind of zero and first order respectively; positive roots of transcendental equations.

$$J_0(\nu)/J_1(\nu) = \nu/\mathrm{Bi} \quad (11)$$

$$\mathrm{Bi} = \mathrm{Bi}(\alpha,d,m) = \alpha r_0/\lambda \text{ – the Biot number} \quad (12)$$

$$Fo^* = Fo^*(z,d,m) = \beta z/r_0^2 \text{ – the modified Fourier number} \quad (13)$$

RESULTS AND DISCUSSION

Quantitative modeling of the heat transfer process was carried out based on relations (8)–(13) using the Mathcad medium.

We used the following process parameters: the length of the capacitance h = 0.5 m; the diameter of the capacitance D = 0.1 m; equipment productivity (by water) $Q = 2\times10^{-4}$ m^3/s; kinematic viscosity coefficient $v = 10^{-6}$ m^2/s; ball eutectic temperature $T_к$ = –10°C; thermal conductivity coefficient λ = 0.58 W/(m·K); thermal diffusivity coefficient $a = 13.8\times10^{-8}$ m^2/s; and ball diameters d = 0.0375, 0.04, and 0.0425 m. The calculation of the current temperature of the liquid was carried out according to two variants of porosity: m = 0.5 and m = 0.35.

In accordance with the selected parameter values, the volume rate of flow (filtration rate) for all calculation options was $v_0 = 4Q/(\pi D^2) = 4\times2\times10^{-4}/(3.14\times0.1^2) = 0.0254$ m/s.

As a calculated value of the temperature θ given over the radius r inside the capillary, we used its value θ_{av}, averaged over the channel cross-sectional area:

$$\theta_{av}(d,m,z) = \frac{2}{r_0^2}\int_0^{r_0} \theta(d,m,r,z) r dr \quad (14)$$

The following dependence was used as a calculated dependence for the liquid temperature based on the operating parameters of the axis coordinate z and the time of the process τ:

$$T(z) = T_0 + (T_к - T_0)\theta, \quad (15)$$

To calculate the number of balls N in the capacitance, we used the formula:

$$N(m,d) = 1.5D^2h\times(1 - m)/d^3 \quad (16)$$

where m is the porosity if the liquid system, d is the diameter of the ball, D is the diameter of the capacitance, and h is the length of the capacitance.

Thus, according to the geometrical parameters, the number of balls of diameter d = 0.04 m was 58 for m = 0.5 and 76 for m = 0.35.

Previously, to assess the convergence of series (8), we performed test calculations using formula (14). Temperature T_0 was 36°C and heat transfer coefficient α was 440 W/(m^2·K) (Fig. 3). Based on $\mathrm{Bi} = \alpha r_0/\lambda = 440\times0.02/0.58 \approx 15$, we found partial sums of this series, from the first to the sixth sum inclusively.

Since determination of the roots of transcendental equation (11) when varying the parameters of the Bi criterion involves laborious calculations, we used tabular data. As in all calculations a slight difference in the value of partial sums was noted only starting from the sixth sum (Fig. 4), the sum of six members of this series was used in the calculations (8).

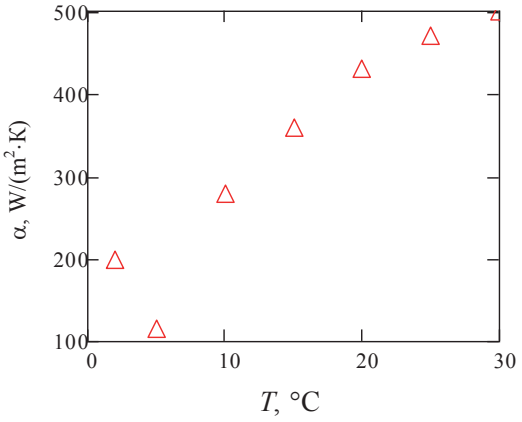

Figure 3 Heat-transfer coefficient as a function of the temperature of water for the "water-ice" system

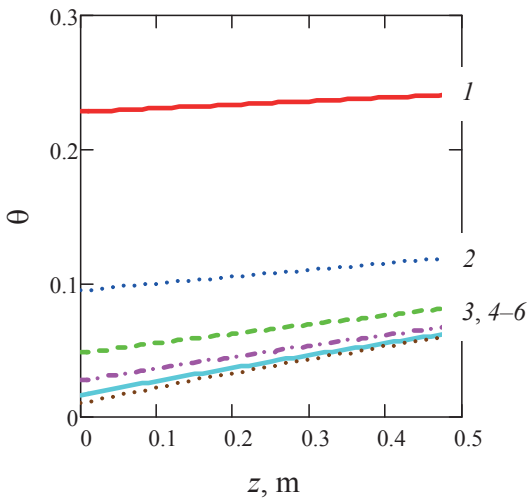

Figure 4 Relation between the radius mean specific temperature of liquid and the axial coordinate z for partial sums of the series (8). d = 0.04 m; m = 0.5; *1*, *2*, and *3* are numbers of summands from 1 to 3; and *4–6* from 4 to 6

In addition, to find the dependence of temperatures in the capillary on the diameter of the balls for each of the options, we considered the situation when balls with a diameter of 0.0375 or 0.0425 m (closed to the diameter of the test ball d = 0.04 m) acted as a coolant. This made it possible, with some approximation, to use tabular data [13] on the roots of equation (11) based on $\mathrm{Bi} = \alpha r_0/\lambda = 15$ which corresponded to d = 0.04 m.

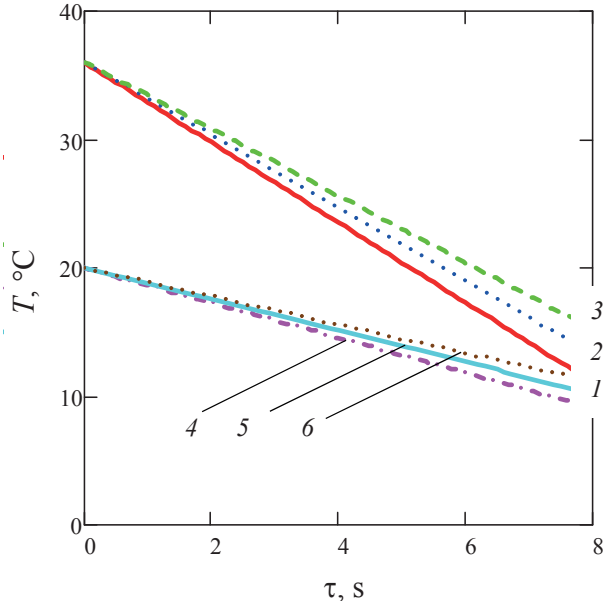

Figure 5 Relation between the outlet temperature of liquid T and time τ at different values of parameters (z = 0.5 m; m = 0.5; T_0 = 36°C; α = 440 W/(m²·K); Bi = 15: *1* – d = 0.0375 m, *2* – d = 0.04 m, *3* – d = 0.0425 m; T_0 = 20°C; α = 230 W/(m²·K); Bi = 8: *4* – d = 0.0375 m, *5* – d = 0.04 m, *6* – d = 0.0425 m)

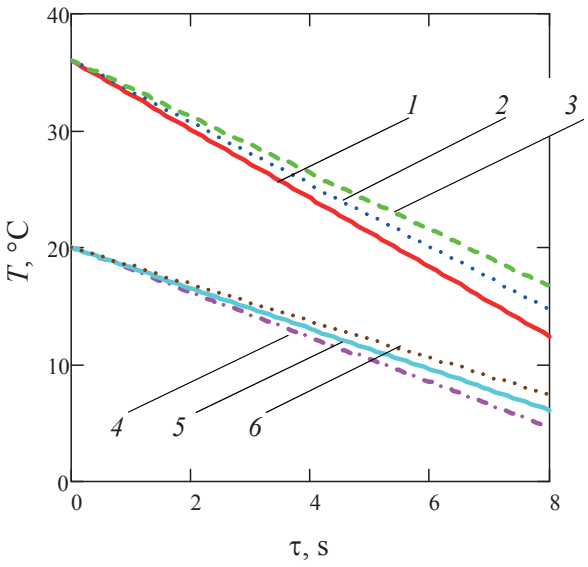

Figure 6 Relation between the outlet temperature of liquid T and time τ at different values of parameters (z = 0.5 m; m = 0.35; T_0 = 36°C; α = 440 W/(m²·K); Bi = 15: *1* – d = 0.0375 m, *2* – d = 0.04 m, *3* – d = 0.0425 m; T_0 = 20°C; α = 230 W/(m²·K); Bi = 8: *4* – d = 0.0375 m, *5* – d = 0.04 m, *6* – d = 0.0425 m)

From relations (8)–(13) and (15), in the range of variation of the parameters of water cooled by frozen balls, 7 calculation results were obtained and graphs were plotted (Figs. 5–7). Based on the analysis of the graphs, the dependence of the variables of the considered problem on the operating parameters was revealed.

Since the calculated temperature of the coolant reduced along the channel (Fig. 4), the specific temperature of the liquid θ increased in the same direction.

In turn, the rate of change in the temperature of the liquid decreased with time (Figs. 5–7). This is because of the reduction of the specific surface area of the ball, which is the ratio of the surface area of the ball to its volume. This resulted in decreasing heat exchange on the border between solid and liquid phases in the liquid system (for example, in Fig. 5 graph *3* corresponding to the diameter of the ball d = 0.0425 m is above graph *2* for the ball diameter d = 0.04 m).

In addition, as it can be seen from Fig. 7, in the case of water productivity Q fixed for all variants, the increase in the rate of flow of the coolant along with the decrease in the porosity of the liquid system naturally leads to a decrease in the rate of cooling of the liquid. Thus, for example, in Fig. 7 graph *4* corresponding to porosity m = 0.35 and the diameter of the ball d = 0.04 m is above graph *1* for porosity m = 0.35 and the ball with the same diameter.

However, it should be noted that, despite the qualitative consistency of the calculated results with

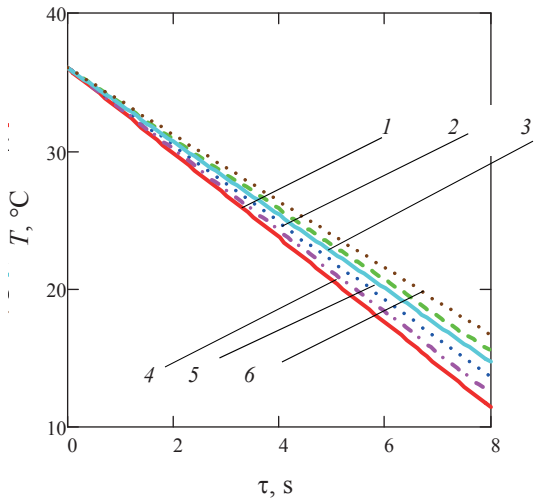

Figure 7 Dependency temperature T cooled fluid from a feed from time τ with different values of *m* porosity, coefficient α transfer heat, criterion Bi, diameter *d* ball (*z* = 0,5 m; *m* = 0,5; T_0 = 36°C; α = 440 W/(m²·K); Bi = 15: *1* – *d* = 0,0375 m, *2* – *d* = 0,04 m, *3* – *d* = 0,0425 m; *m* = 0,35; T_0 = 36°C; α = 440 W/(m²·K); Bi = 10: *4* – *d* = 0,0375 m, *5* – *d* = 0,04 m, *6* – *d* = 0,0425 m)

the physical meaning of the process under study, the quantitative assessment that characterizes the course of this process needs additional comments.

First of all, this relates to the question of formalizing the boundary condition at the interface, which is typical for many works on the theory of heat conductivity, where, when setting the problem, the temperature is assumed unchanged, while for the heat exchange between phases (for example, between liquid and coolant) phase temperature tends to level off. This leads to a decrease in the intensity of heat transfer from the liquid to the ball as naturally as in the studied problem.

Therefore, the results of the the cooling rate of water presented in Figs. 5–7 on, in fact, are overestimated compared to real data.

At the same time, despite the simplifications based on the theory of heat conduction and used in the formulation of the problem, physical and mathematical modeling of processes makes it possible to predict and control their development. To the same extent, this is also applied to the complex problem of justifying the rate of liquid cooling due to the accumulation of frozen balls with a developed heat exchange surface that is analyzed in this paper.

CONCLUSION

To justify heat transfer from the fluid flow to balls with eutectic frozen solution, we applied an analytical tool forecasting the course of this process in the innovative technology for cooling this liquid.

In the quantitative analysis of this problem kinetic aspects of filtration fluid motion was used, namely, when the working volume occupied by the liquid between the balls was simulated by equivalent plurality of ordered cylindrical capillary channels. This allowed us, from the point of view of analytical and numerical analysis of the thermal regime in a steady fluid flow inside the working volume of the capacitance, to adapt the solution of this problem to the study of this regime in an isolated capillary.

The accepted conditions, namely the size of the capacitance and balls and the volume fraction of balls in the capacitance created the preconditions for conducting quantitative modeling of the process under study based on the calculated dependences of the temperature distribution in an unlimited cylinder under an unsteady regime.

To assess the efficiency of the cooling process of fluid flow in a heat exchanger with a frozen solid phase and a developed heat exchange surface in the field of the real values of the process parameters, the obtained temperature dependences were used to carry out a numerical modeling of the cooling process of this medium.

Based on the results of the analytical and numerical study of the problem, an acceptable region for varying the mechanical and thermotechnical parameters of these processes was determined. This region is of importance for engineering calculations of the low-temperature processing of raw materials and finished products of biological origin.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to the publication of this article.

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FTIR spectroscopy for quality evaluation of sports supplements on the Polish market

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Abstract:
Introduction. Our study aimed to apply medium infrared (MIR/FTIR) spectroscopy to evaluate the quality of various sports supplements available in the Polish shops and gyms.
Study objects and methods. The study objects included forty-eight sports supplements: whey (15 samples), branched-chain amino acids (12 samples), creatine (3 samples), mass gainers (6 samples), and pre-workouts (12 samples). First, we determined the protein quantity in individual whey supplements by the Kjeldahl method and then correlated the results with the measured FTIR spectra by chemometric methods. The principal component analysis (PCA) was used to distinguish the samples based on the measured spectra. The samples were grouped according to their chemical composition. Further, we correlated the spectra with the protein contents using the partial least squares (PLS) regression method and mathematic transformations of the FTIR spectral data.
Results and discussion. The analysis of the regression models confirmed that we could use FTIR spectra to estimate the content of proteins in protein supplements. The best result was obtained in a spectrum region between 1160 and 2205 cm^{-1} and after the standard normal variate normalization. R^2 for the calibration and validation models reached 0.85 and 0.76, respectively, meaning that the models had a good capability to predict protein content in whey supplements. The RMSE for the calibration and validation models was low (2.7% and 3.7%, respectively).
Conclusion. Finally, we proved that the FTIR spectra applied together with the chemometric analysis could be used to quickly evaluate the studied products.

Keywords: Spectroscopy, FTIR, medium-infrared, chemometric, PCA, PLS, sports supplements, whey, creatine, BCAA, gainers, pre-workouts

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INTRODUCTION

Food supplements are concentrated sources of nutrients (i.e. minerals and vitamins) or other substances with a nutritional or physiological effect that are marketed in "dose" form (e.g. pills, tablets, capsules, or liquids in measured doses) [1]. In the EU, food supplements are regulated as foods. Therefore, it is the responsibility of the manufacturer, importer, supplier or distributor to ensure the safety of food supplements placed on the market.

The use of dietary supplements is mainly widespread in sport. People are continually searching for supplements to help them lose weight, boost energy, and build muscles. There are some supplements which are commonly used to achieve these goals [2].

One of them is a whey protein supplement, the most important nutrient to boost athletic performance. Whey protein is popular among athletes, bodybuilders, fitness models, as well as people seeking to improve their performance in the gym. Numerous studies show that it can help increase strength, gain muscle, and lose significant amounts of body fat [3, 4]. Some specific types of protein are made for certain scenarios, such as casein protein for a slow-release protein and whey protein for a faster release. The main types of whey protein are concentrates (WPC), isolates (WPI), and hydrolysates (WPH).

The branched-chain amino acids (BCAAs) – leucine, iso-leucine, and valine – are among the nine essential amino acids for humans that account for 35% of essential amino acids in muscle proteins. They are unique as the only amino acids used directly by muscles as energy during exercise [5].

The next commonly used supplement in sport is creatine which increases lean body mass, skeletal muscle strength, as well as muscle power and endurance [6]. Creatine supplementation appears to raise the creatine level in muscle cells and cause weight gain through an increase in lean body mass with no effect on fat mass [7, 8].

Pre-workout supplements are multi-ingredient dietary formulas designed to boost energy and athletic performance. While some pre-workout supplements have carbohydrates, most are carbohydrate- and calorie-free. Others contain caffeine, beet juice, or amino acids, such as arginine, citrulline, and ornithine, to increase blood flow to the muscles.

Mass gainers are products mostly directed for men who find it difficult to build lean muscle mass. They contain high amounts of calories, as well as carbohydrates and protein, making them a perfect meal replacement for people with quick metabolism.

Due to increased consumption of sports supplements and EU regulations, there is a need for a quick and precise method to evaluate their quality. The defects of traditional measurements create a possibility of adulteration. For example, the commonly used the Kjeldahl method, which determines protein content in samples, is time-consuming and unable to distinguish the protein nitrogen from the non-protein nitrogen [9]. Nowadays, adding inexpensive amino acids and amino acid derivatives to protein supplements to modify their content has become a common adulteration method which is hard to detect [9]. Moreover, dishonest producers provide incorrect information on the packaging regarding the amounts of ingredients.

Some methods have been proposed to ensure the quality of sports supplements. Jiao *et al.* used the Raman spectroscopy combined with multivariate analysis for rapid detection of adulterants in whey protein [8]. High values of R^2 and low errors of prediction for partial least squares (PLS) analysis prove that it could be used to detect adulterants in WPC. Champagne and Emmel demonstrated the Fourier transform infrared (FTIR) with attenuated total reflectance (ATR) as a tool for detecting adulteration in raw materials of dietary supplements [10]. The researchers proved that vibrational spectroscopy could be used to identify the presence of known adulterants intentionally spiked into dietary ingredients, including erectile dysfunction drugs, steroids, weight loss drugs, and Melamine.

Pereira *et al.* proposed using fluorescence spectroscopy to detect and characterize adulterated whey protein supplements [11]. The adulteration was performed by adding creatine, caffeine, and lactose to WPC samples at different levels (10%, 20%, and 30% w/w). The time-resolved fluorescence analysis showed increased mean intensity lifetime in all adulterated samples, compared to pure WPC. This study proved that fluorescence spectroscopy was able to evince adulteration in WPC powders.

Another use of the fluorescence technique was reported by Pulgarin *et al.* [12]. The authors used the emission spectroscopy to characterize several whey samples subjected to different treatments and conditions. Their results indicated that the fluorescent amino acids, tyrosine and tryptophan, were responsible for the intrinsic fluorescence of whey. Martin *et al.* predicted the protein content in single wheat kernels using hyperspectral imaging, while Ingle *at al.* applied NIR spectroscopy to determine the protein content in powder mix products [13, 14].

High-performance liquid chromatography (HPLC) is one of the most common techniques used to determine the concentration of ingredients. The HPLC technique was applied by several authors to measure the concentration of taurine, caffeine or vitamins in energy drinks [15–17]. These studies exemplify a growing demand for new, more efficient techniques to assess the quality of food products and their ingredients. Compared to conventional techniques or chromatography analysis, infrared spectroscopy allows measuring the sample's eco-friendliness – without sample preparation or the use of chemical reagents. In addition, FTIR spectroscopy can be successfully used in the analysis of amino acid profiles, as confirmed by [18, 19].

In this study, we applied FTIR spectroscopy coupled with chemometrics to evaluate the quality of sports supplements. This method is very efficient as the spectral profile in one measurement can provide various information about the product that could not be given by any conventional technique in common use.

Our main objectives were to create a regression model using PLS analysis to determine the total amount of protein in the product and to distinguish various ingredients by the FTIR spectra and principal component analysis (PCA).

STUDY OBJECTS AND METHODS

Samples. Our study objects included forty-eight samples of sports supplements from different producers: whey (15 samples), BCAAs (12 samples), creatine (3 samples), mass gainers (6 samples), and pre-workouts (12 samples). The samples were in the form of powders or liquids.

Protein determination. The protein content in whey protein samples was assessed by the Kjeldahl method, using a conversion factor of total nitrogen to protein (6.38 for milk products, 6.25 for meat products, and 5.70 for vegetables) [20]. Three parallel trials were performed for each sample. The percentage of protein in a sample (X) was calculated according to the formula [20]:

$$X = (a \cdot n \cdot 1.4 \cdot f)/m$$

where a is the amount of the standard solution of hydrochloric acid used for titration of ammonia in a specific sample, cm^3; n is the molar concentration of hydrochloric acid used for titration; m is the sample

Table 1 Protein content in whey protein samples measured by the Kjeldahl method [22]

Producer	Type of protein	Declaration, g	Determined, g	Standard deviation, g
Producer 1	WPC+WPH+WPI	72.72	72.72	1.98
Producer 2 (vegetarian)	–	56.7	54.72	0.29
Producer 3	WPI	85	86.30	4.39
Producer 4	WPI+WPC	78.5	81.49	0.28
Producer 5	WPC+WPI+WPH	71	74.18	1.25
Producer 6	WPC+WPI	71	73.04	1.08
Producer 7	WPC+WPI+WPH	63	65.31	1.67
Producer 8	WPC+WPI	82	75.92	2.48
Producer 9	WPI	84.7	73.87	0.91
Producer 10	WPI+WPC	79.2	78.02	1.45
Producer 11	WPC+WPI	71	68.69	0.31
Producer 12	WPC	70	71.08	0.36
Producer 13	WPC+WPI	80	78.21	0.52
Producer 14	WPI	88	85.32	0.81
Producer 15	WPI	85	76.73	0.87

WPI – whey protein isolate, WPC – whey protein concentrate, WPH – whey protein hydrolysate

mass, g; *f* is the conversion factor of total nitrogen to protein (6.38 for milk products, 6.25 for meat products, and 5.70 for vegetables); 1.4 is the amount of nitrogen corresponding to 1 cm^3 of 0.1 molar solution of hydrochloric acid, mg.

FTIR measurements. Medium infrared spectra were performed on a 4700 FTIR spectrometer (Jasco, Japan). Single beam spectra of the sample were collected and rationed against the background of air. For each sample, MIR spectra were recorded from 4000 to 600 cm^{-1} by co-adding 16 interferograms at a resolution of 4 cm^{-1}. The measurements were performed in triplicate.

Data analysis. Principal component analysis (PCA). Principal component analysis was performed on the FTIR spectra of whey protein supplements to distinguish the samples. PCA is a multivariate technique that linearly transforms an original set of variables into a substantially smaller set of uncorrelated variables that represents most of the information in the original data set. Data for PCA are arranged in a two-way matrix, in which column vectors represent variables and row vectors represent the "objects" whose variables are measured [21]. The PCA analysis was carried out using Unscrambler X (CAMO, Oslo, Norway) software.

Partial least squares (PLS). The partial least squares (PLS) regression method was used to determine the relation between the samples' spectra and the content of protein in whey supplements. We selected regions of spectra and data preprocessing options to optimize the model. In total, 45 spectra were measured (15 samples in triplicate). The set of independent variables *X* was the FTIR spectra and the set of dependent variables *Y* was the protein content. Full cross-validation was applied to the regression model.

The regression models were evaluated using the adjusted R^2 and the root mean-square error of cross-validation (RMSECV), as the term indicating the prediction error of the model. The quality models were evaluated by the ratio of the standard deviation of reference data for the validation samples to the RMSEP (RPD). The predicted values were compared to the reference values. The PLS analysis was carried out using Unscrambler X (CAMO, Oslo, Norway) software.

RESULTS AND DISCUSSION

Protein determination. The protein contents in whey protein samples (measured by the Kjeldahl method) are given in Table 1.

The results show that the producers declared similar values to those marked. For most producers, the differences from the declared values did not exceed 5 g/100 g of protein, which is considered as acceptable. The highest difference between the value declared and that determined by the Kjeldahl method was observed for three samples. They were from Producer 9 (84.7 g/100 g vs. 73.87 g/100 g), Producer 8 (82 g/100 g vs. 75.92 g/100 g), and Producer 15 (85 g/100 g vs. 76.73 g/100 g). However, most producers declared the correct protein value on the package of their products.

Sports supplements spectra in medium infrared range. The medium infrared absorption spectra of the sports supplements measured against air are presented in Fig. 1.

According to the data reported in [23], the absorption spectra of whey products had two prominent features, Amide I (about 1650 cm^{-1}) and Amide II (about 1540 cm^{-1}) bands. The former arose primarily from the C=O stretching vibration and the latter was attributed to the N-H bending and C-N stretching vibrations of the peptide backbone. The band with the maximum absorption at about 3268 cm^{-1} was assigned to Amide A. The band at 3000–2825 cm^{-1} corresponded to the C-H stretching vibration, while the low intensity bands at

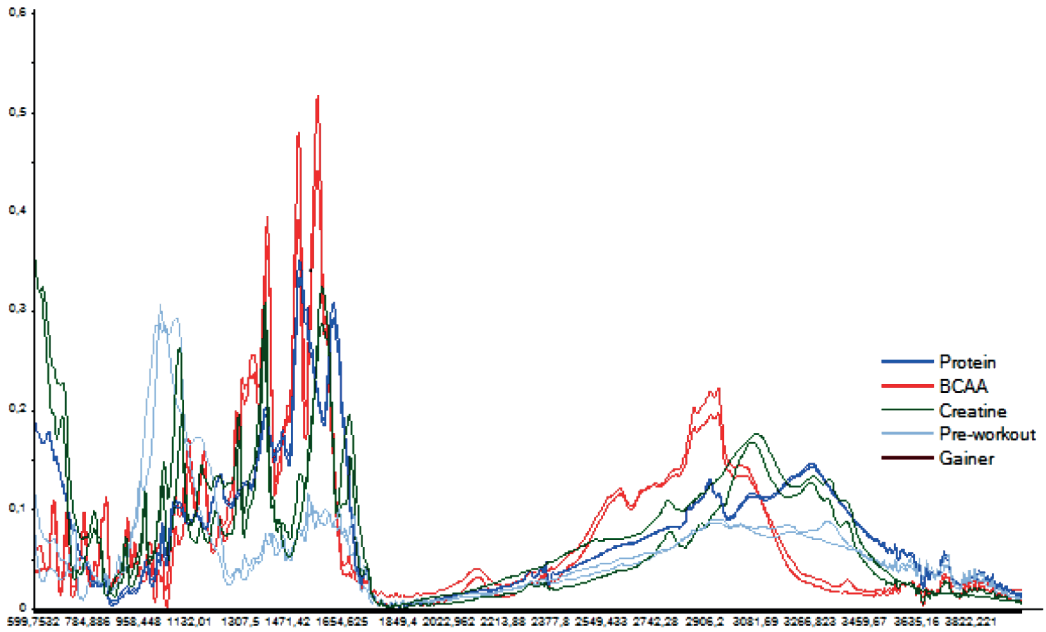

Figure 1 Absorption spectra of sports supplements in medium infrared region (4000–600 cm^{-1}) [22]

about 1241 cm^{-1} and 1100 cm^{-1} were assigned to the P-O stretching vibrations [23].

Zhu *et al.* [24] found that the absorption spectra of branched chain amino acids (BCAA) showed the concentration of effective wavelengths of amino acids (e.g. valine, leucine, isoleucine, and glycine) mainly in the fingerprint region (500–1700 cm^{-1}). Based on the literature, we can describe the main bands in these products. The band with two maximum absorption peaks at about 1575 cm^{-1} and 1509 cm^{-1} could be assigned to isoleucine [24]. The band with the maximum at about 1400 cm^{-1} corresponded to glycine that does not contain asymmetric carbon atoms [24]. The valine bands were also observed in the fingerprint region. The band at 665 cm^{-1} was assigned as a bending mode of CO_2. The bands at about 753 cm^{-1} and 776 cm^{-1} were assigned as wagging and bending of CO_2 group, while vibrations between 900 and 965 cm^{-1} as mainly due to the C–C stretching vibration. The medium intensive band at 2817–3000 cm^{-1} was assigned to the C-H hydroxyl group [24].

The creatine MIR absorption spectra have not been widely reported in literature. Based on the chemical composition of creatine (which is also an organic acid), we can infer that the creatine spectrum should be similar to the BCAA spectrum. The differences in the intensity and shape of some bands are probably due to a high concentration of aminoacetic acid and guanidine in the creatine sample.

The pre-workout absorption spectra have not been widely described in literature either. According to the studies of caffeine determination in Singh *et al.* or Abdalla, pre-workouts containing caffeine have some typical bands for that component [25, 26]. Thus, it could be used to confirm the presence of this ingredient in the product.

Principal component analysis (PCA). The PCA was used to distinguish the medium infrared spectra obtained from different types of sports supplements. The PCA data were plotted on a graph of first principal component (PC1) vs. second principal component (PC2), as shown in Fig. 2. The PCA was conducted for all the products and for groups of products. The results were diversified into (1) all supplements, (2) protein supplements, (3) creatine supplements, (4) BCAAs, (5) mass gainers, and (6) pre-workout supplements (Fig. 2).

Sports supplements are products to which producers add various mixes of ingredients depending on market needs and prevailing trends. These ingredients may include vitamins, minerals, herbs, and amino acids. In our study, we applied the PCA analysis to the spectra acquired from forty-eight samples which were measured in triplicate and then averaged. For the whole spectrum (4000–400 cm^{-1}), the first and second principal components (PC1 and PC2) described 78% of total variation (61% and 17%, respectively), as shown in Fig. 2a.

Based on the data in Fig. 2a, we identified three main groups of products. The first one included products characterized by positive values of PC1 and negative values of PC2 (BCAAs and pre-workouts). These products differed from the others in their physical state (they were liquids). The second group was products which primarily contained proteins and amino acids. They included mostly proteins, mass gainers, and creatine. The third group (mostly with a positive PC1) was composed of BCAAs and pre-workouts. The main ingredient in these products was branched amino acids.

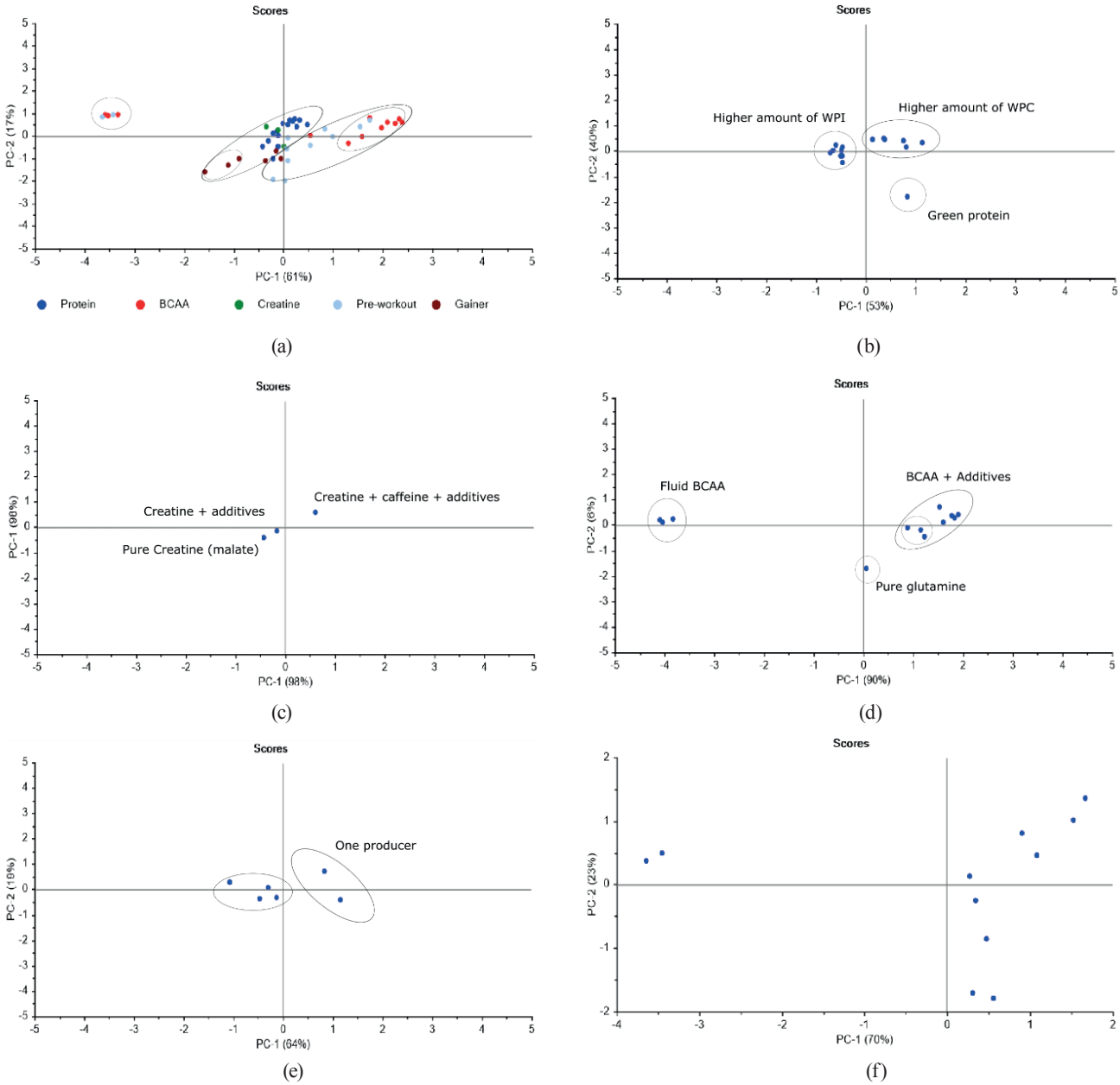

Figure 2 PCA results for medium infrared spectra of sports supplements. Scores plot for two significant principal components: PC1 vs PC2. (a) all samples and full spectra at 4000–600 cm^{-1}; (b) proteins; (c) creatines; (d) BCAA; (e) gainers; (f) pre-workouts [22]

The PCA results for protein supplements are presented in Fig. 2b. For the whole spectrum, the first and second principal components described 93% of total variation (53% and 40%, respectively). Based on the distribution of the samples, we distinguished three groups of protein products. The first group included supplements with high amounts of whey protein isolate (WPI) and negative values of PC1. The second group contained supplements made from whey protein concentrate and characterized by positive PC1 and PC2. Finally, the third group included products based on green protein (plant proteins for vegans) with positive PC1 and negative PC2.

Fig. 2c presents the PCA results for creatine samples. According to the data, creatine with the addition of caffeine was characterized by positive values of PC1 and PC2. Pure creatine and creatine with additives were in the opposite sites (negative values of PC1) and close together.

The PCA results for BCAA supplements are shown in Fig. 2d. The first and second principal components described 96% of total variation (90% and 6%, respectively). Based on the distribution of samples, we identified three main groups of BCAA products. Fluid BCAAs were characterized by negative values of PC1 and positive values of PC2. Solid samples had a positive

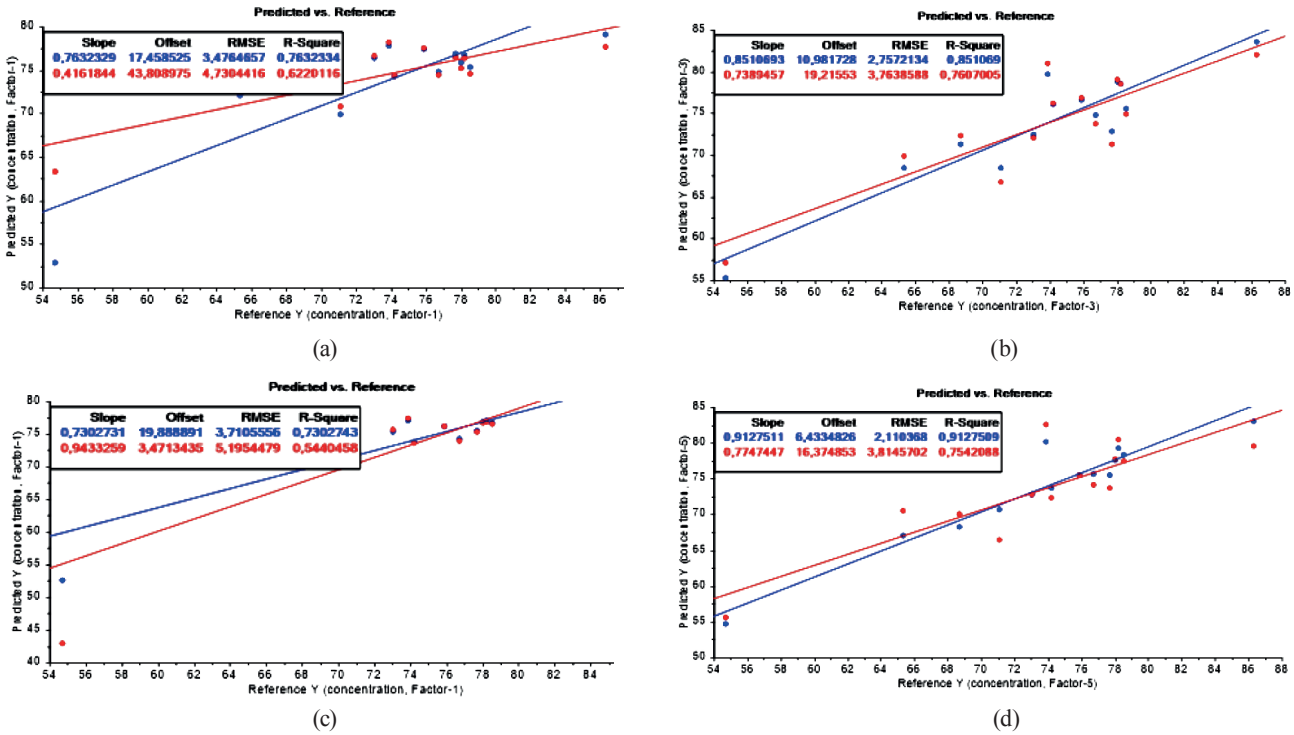

Figure 3 Predicted versus actual concentration of protein in whey supplements obtained by PLS calibration. (a) full FTIR spectrum; (b) sub-region: 1467–1600 cm^{-1}; (c) full spectra after SNV normalization; (d) sub-region: 1160–2205 cm^{-1} after SNV normalization [22]

PC1. Pure glutamine was in the quarter which had a positive PC1 and a negative PC2. We also found some BCAA samples with additives in that quarter. According to the information on the packaging, these samples contained glutamine. The rest of the BCAA supplements (with positive values of PC1 and PC2) were samples without glutamine.

Fig. 2d features the PCA results for mass gainer supplements. We found that the first and second principal components described 83% of total variation (64% and 19%, respectively). Based on the data, we distinguished two groups of gainers. The first group was characterized by negative values of PC1 while the second, by positive values of PC1. Due to insufficient information on the packaging, it was hard to determine the differences between them. It is worth emphasizing that group A contained supplements from various producers, while group B had only two products of the same producer. In addition, the products in group B could be found on a low-price shelf on the market.

The last group of sports supplements exposed to PCA included pre-workout products (Fig. 2e). The first and second principal components described 93% of total variation (70% and 23%, respectively). We identified two groups of pre-workout supplements. The first group included liquid samples with negative values of PC1, while the second contained solids with positive values of PC1. The samples in the second group differed from each other in the amount of caffeine. Those with lower amounts of caffeine had negative values of PC2, while those with higher amounts of caffeine had positive values of PC2.

Partial least squares regression (PLS). PLS was used to quantitatively evaluate the concentration of protein in whey protein supplements based on their spectral characteristics. Different types of mathematical pre-processing were applied to the spectra before building the model. First, we analyzed complete spectra in all the spectral regions. Next, we chose specific sub-regions, relying on the regression coefficients for the complete spectra and the chemical information in the specific sub-regions (Fig. 3).

The PLS regression results for the full spectrum (4000–400 cm^{-1}) without any pretreatment revealed a correlation between the spectra and the protein composition. R^2 for the calibration and validation models amounted to 0.76 and 0.62, respectively. It meant that the models had a medium-good capability to predict the protein content in whey supplements. The RMSE for the calibration and validation models was also low (3.5% and 4.7 %, respectively), confirming their medium-good quality (Fig. 3a). The regression results were improved when specific spectral regions were used instead of the complete spectra. R^2 for the calibration and validation models reached 0.85 and 0.76, respectively. It meant that the models had a good capability to predict the protein content in whey supplements. The RMSE for the calibration and validation models was also low (2.7%

Table 2 Protein concentration by FTIR method versus Kjeldahl method

Predicted values by PLS, g*	Determined values by Kjeldahl, g	Difference between FTIR and Kjeldahl, g
54.70	54.72	0.02
75.62	76.73	1.11
79.31	78.21	1.10
70.68	71.08	0.40
75.57	75.92	0.35
80.20	73.87	6.33
73.62	74.18	0.56
67.11	65.31	1.80
72.75	73.04	0.29
75.45	77.72	2.27
75.85	76.02	0.17
77.54	78.02	0.48
68.30	68.69	0.39
78.39	78.53	0.14
83.08	86.30	3.22

*Results for sub-region: 1160–2205 cm^{-1} after SNV normalization (Fig. 3d)

and 3.7%, respectively), confirming the good quality of the models (Fig. 3b).

Next, we performed the mathematical pre-processing of the spectra (using SNV normalization). R^2 for the calibration (full spectrum) and validation models equaled 0.73 and 0.54, respectively. The RMSE was also low (3.7% and 5.2%, respectively), which confirmed that the quality of the models was medium-good (Fig. 3c). The regression results were improved when specific spectral regions were used instead of the complete spectra. R^2 for the calibration and validation models amounted to 0.91 and 0.75, respectively. This suggested a good capability of the models to predict the protein content in whey supplements. The RMSE for the calibration and validation models was also low (2.1% and 3.8%, respectively), which confirmed their good quality (Fig. 3d).

Based on the results, we found that the rapid FTIR method had an accuracy comparable to the Kjeldahl method. The difference between the values determined by the Kjeldahl method and those predicted by FTIR was about 1.2 g (Table 2). In addition, our complementary method offered several advantages: it is simple, fast (less than a minute) and requires no chemicals or reagents, compared to traditional methods.

CONCLUSION

Our study aimed to investigate the potential of medium infrared (FTIR) radiation in combination with a multiway analysis in monitoring the quality of sports supplements. The spectra of selected sports supplements had a different shape and intensity, depending on the chemical composition. Based on the characteristic spectra, the FTIR could be used to confirm the presence or absence of a given ingredient in the sample.

The results of the PCA analysis (sample distribution) showed that the FTIR spectra coupled with PCA offered a promising tool for distinguishing sports supplements based on their ingredients.

The regression analysis (PLS) indicated that FTIR spectroscopy could replace the time-consuming Kjeldahl method as a much faster technique to predict the concentration of protein in whey supplements that does not require any reagents.

Thus, we found FTIR spectroscopy to be a promising approach to quality evaluation of sports supplements.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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Antioxidant activity of aqueous and alcohol extracts of *Salvia leriifolia* L. and *Linum usitalissmum* L. subjected to a pulsed electric field

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Abstract:
Introduction. More attention has been paid in recent decades to extracts and essential oils from various plants as natural antioxidant sources due to their positive effects on food oxidation reactions. Our study aimed to compare the antioxidant activity of aqueous and alcoholic extracts from *Salvia leriifolia* L. and *Linum usitalissmum* L.The extracts were subjected to a pulsed electric field with intensities of zero (without pretreatment), 3 and 6 kV·cm^{-1}, and a constant pulse number of 30. For this purpose, parameters such as total phenolic compounds and antioxidant activity were investigated by DPPH and TEAC methods.
Results and discussion. Our results showed that a higher intensity of a pulsed electric field pretreatment and the use of an alcoholic solvent significantly raised total phenolic compounds in the extracts and their antioxidant activity at a 95% confidence level. We found significant effects of the plant source (*Linum usitalissmum* and *Salvia leriifolia*), pretreatment (pulse electric field at intensities of 0.3 and 6 kV·cm^{-1}), and a solvent (aqueous and alcohol) on the extracts' antioxidant activity ($P < 0.05$). In addition, there was a significant correlation between the results of the DPPH and the TEAC antioxidant activities ($P < 0.01$ and $r = 0.932$).
Conclusion. The total antioxidant activity (based on both TEAC and DPPH methods) and total phenolic compounds extracted from *Salvia leriifolia* were higher than those from *Linum usitalissmum* ($P < 0.05$). Based on the results, the extract obtained from *Salvia leriifolia* with an alcoholic solvent and a pulsed electric field pretreatment (at 6 kV·cm^{-1} and 30 pulses) was selected as possessing desired antioxidant properties.

Keywords: Antioxidant, extraction, pulsed electric field, *Linum usitalissmum*, *Salvia leriifolia*

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INTRODUCTION

Lipid oxidation is one of the major chemical changes that occur during food processing, storage, and preparation. Lipid molecules are rapidly oxidized in the presence of oxygen, especially in the case of unsaturated fatty acids [1]. Antioxidants are widely used today to reduce the rate of oxidation reaction of fats in foods.

Antioxidants are molecules or compounds that act against free radicals which damage to molecules, resulting in the loss of their function. Antioxidants provide a primary defense against such oxidative degradations [2]. In industrial processes, synthetic antioxidants – such as butyl hydroxy toluene and butyl hydroxy anisol – are mainly used to increase the food's shelf life. In this regard, nutritionists have found that these compounds can have adverse effects on the body [3].

Therefore, it is necessary to use strong antioxidants with lower toxicity and greater efficacy. In recent decades, natural antioxidants have drawn the attention of food researchers due to their safety in food formulation. These are extracts and essential oils of various plants that produce positive effects on nutrient oxidation reactions.

Pre-extraction seed treatment is one of the most essential steps to ensure high quality extraction. One of the treatment methods is the use of a pulsed electric field. It is an important non-thermal method of treating foodstuffs by placing them in a chamber between two

electrodes and subjecting to high-voltage pulses for a short time. A pulsed electric field focuses mainly on the microscopic scale so that pores are created in the cell membrane, accelerating the exit of intercellular compounds. This process preserves qualitative, nutritional, and energy consumption properties, as well as increases productivity in food production [4].

Most importantly, a pulsed electric field destroys the cell wall and its membrane and increases the mass transfer rate. Indeed, when a living cell is affected by such a field, the cell wall and its membrane are naturally damaged. The inside material is easily removed and the surrounding material enters the cell, resulting in its destruction. With increased permeability of plant and animal cells, their intracellular material is extracted more easily and quickly. Therefore, this treatment can be used as a pre-processing step in the extraction of valuable cellular materials [5, 6].

Salvia leriifolia L. is one of the plants that contain antioxidant compounds. It is a native species of *Lamiaceae* family to Khorasan and Semnan provinces, Iran [7]. It grows in cold and semi-arid or arid regions at altitudes between 900 and 1650 meters, with an average rainfall of 80 mm. A special shape of its leathery leaves, especially white villi on both sides, and a wide growth on the surface of the soil make this plant resistant to harsh winter winds or severe heat [8].

Various studies have reported therapeutic properties of *Salvia leriifolia*. For example, its aqueous and alcoholic root extracts have neuroprotective properties against topical anemia in the rat brain [9]. The analgesic and sedative activity of *Salvia leriifolia* leaf extract in the amount of 500 mg/kg is comparable to that of diazepam in the amount of 5 mg/kg [8]. In treating chronic inflammation, the plant's extract is similar to diclofenac [10]. Its aqueous and alcoholic leaf extracts were found to prevent gastric ulcers in rats similarly to Sucralfate [11].

In addition, the plant's root and leaf extracts showed considerable antimicrobial activity [9]. They also have strong antioxidant properties that prevent the oxidation of oils. This property is competitive with that of antioxidants commonly used in the food industry, such as butylated hydroxy toluene and alpha-tocopherol. It is due to the presence of a secondary metabolite of chalcones, called butin, in this plant. Finally, *Salvia leriifolia* is of industrial importance. In this regard, researchers have found that its seeds contain 26% yellow oil, with a very low peroxide index and a high antioxidant index, which increases its shelf life compared to other oils [12].

Another plant with antioxidant properties is *Linum usitalissmum* L. It is a one-year-old plant of *Linaceae* family that grows in bushes. This plant has over 200 species but only *Linum usitalissmum* has economic importance. In addition, its seeds have several powerful antioxidants, including lignans. 100 g of *Linum usitalissmum* contains about 9.2 mg of vitamin E, mainly in the form of gamatocopherol [13].

The most common method for extracting compounds from plant tissues uses aqueous and ethanol solvents. Therefore, we aimed to evaluate effects of an electrical pulse pre-treatment and to compare the aqueous and alcoholic extracts of *Linum usitalissmum* and *Salvia leriifolia* seeds.

STUDY OBJECTS AND METHODS

Preparation of raw materials. For this study, *Linum usitalissmum* L. seeds and *Salvia leriifolia* L. aerial limbs, leaves, and stems were obtained from a certified apothecary. We also used chemicals produced by Merck (Germany).

Extraction of aqueous and alcohol extracts from *Linum usitalissmum* and *Salvia leriifolia* seeds pretreated with a pulsed electric field. Initially, *Linum usitalissmum* and *Salvia leriifolia* seeds were cleaned and the external materials and impurities were separated and dried in an oven at 45°C. The samples were powdered in a household mill (Fama Model Cs, Germany) and passed through a 40-mesh sieve. Finally, they were packed in air- and water-proof packages and kept in a freezer at –18°C until further experiments to preserve the extract's antioxidant and functional properties.

The aqueous and alcoholic extracts were made using the Kabiri and Seyyedlangi method [14, 15]. For this, the prepared powders were mixed with a water solvent (aqueous extract) or 80% methanol (alcoholic extract) at the ratio of 50:1.

Subsequently, to apply a pulsed electric field pretreatment, each of the extracts was subjected to an alternating electric field with zero (without pretreatment), 3, and 6 $kV \cdot cm^{-1}$ intensity and a constant pulse number of 30 (Table 1). The linear electric current in this device is transmitted to a series of capacitors and the energy stored in the capacitors is discharged to the chamber containing two electrodes with a pulse switch. The discharge chamber is made of Plexiglass 1 and the distance between the two electrodes is 4 cm. These waves were applied to facilitate the extraction.

Evaluation of antioxidant properties of *Linum usitalissmum* and *Salvia leriifolia* aqueous and alcoholic extracts. Total phenolic compounds. The amount of total phenolic compounds was measured by the Folin-Ciocalteu method according to Oardoz *et al.* [16]. For this purpose, 10 g of extracts was first extracted with 200 mL of methanol for 24 h at room temperature using a magnetic stirrer. The extract was filtered with Whatman Paper No. 1 and the sediment was extracted again under the same conditions. The solvent was then removed by a vacuum evaporator at less than 40°C and concentrated as far as possible. Then, 0.5 mL of the extract was mixed with 2.5 mL of 0.2N Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate solution.

Table 1 Treatments investigated in the study

Treatment code	Intensity of pulsed electric field pre-treatment, kV·cm^{-1}	Herbal source	Extraction method
1	0	*Linum usitatissimum*	aqueous
2			alcoholic
3	3		aqueous
4			alcoholic
5	6		aqueous
6			alcoholic
7	0	*Salvia leriifolia*	aqueous
8			alcoholic
9	3		aqueous
10			alcoholic
11	6		aqueous
12			alcoholic

The mixture was kept at room temperature for 120 min. The absorbance rate of the solution was then read by a spectrophotometer at 760 nm. The total content of phenolic compounds was expressed in mg/g of extract using the line equation drawn on the basis of gallic acid. The calibration curve was plotted as follows.

Different concentrations of gallic acid were first prepared and 0.5 mL of each was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent (v/v) and 2 mL of 7.5% sodium carbonate for half to 8 min (w/v). The samples were stored at room temperature for 30 minutes and then absorbed at 760 nm [17]. Distilled water was used as a control.

Antioxidant activity by DPPH method. To extract antioxidant compounds, 10 g of aqueous and alcoholic extracts with 100 mL methanol was stirred with a magnetic stirrer at a speed of 100 rpm at 25°C for 24 h and finally filtered with Whatman filter paper. The solution was then transferred to a freezing dryer for methanol removal and, finally, the dried extract was stored at –20°C [18]. The antioxidant activity of the samples was further evaluated by the method of Brand Williams *et al.* [19]. 3.9 mL of DPPH stock was poured into the cell and read by a spectrophotometer at 515 nm. Then, 0.1 mL of each extract was added to the DPPH stock solution and after 90 minutes of incubation, the absorbance of the samples was read at 515 nm. The inhibition percentage of DPPH radical was calculated using Eqs. (1) and (2).

$$I(\%)= 100 \times (A_0 - A_s)/A_0 \quad (1)$$

where A_0 is control absorption and A_s is sample absorption.

The results were then expressed as IC_{50} (the amount of antioxidant required to reach 50% of the initial DPPH concentration). To draw a standard curve, we used a Trolox solution with a concentration of 1000–100 µmol. First, the percentage of radical neutralization activity was obtained for each sample. Then, we calculated the antioxidant activity of the samples using a standard curve in µmol of Trolox per gram dry weight (µmol/g).

Antioxidant activity by TEAC[I] method. To extract antioxidant compounds, 10 g of the milled sample with 100 mL of methanol was mixed with a magnetic stirrer at 100 rpm and 25°C for 24 h and then filtered with a Whatman filter. Then, the methanol was transferred to a freezing dryer and, finally, the dried extract was stored at –20°C [18]. The antioxidant activity of the samples was further evaluated by the method of Yu *et al.* [20].

First, we made an aqueous solution of ABTS[II] at a concentration of 1 mM to prepare the radical ABTS. Potassium persulfate was then added to this solution to reach a final concentration of 2.45 mM. The resulting solution was incubated at room temperature and darkness for 2 h. During this time, the ABTS molecule produced the ABTS•$^+$ cation radical. Then, 4 µL of the samples was taken with a Peptide and mixed with 4 mL of the ABTS•$^+$ solution in the cell. Its absorption at a 734 nm wavelength was verified at 6 min after mixing (for 30 s). A standard curve was plotted, corresponding to the reaction of 40 µL of Trolox (at concentrations of 50, 100, 250, 500, 750, and 1000 µM) to 4 mL of the ABTS•$^+$ solution. The inhibition percentage of ABTS•$^+$ of the samples was calculated according to Eq. (2). Also, the ABTS•$^+$ radical inhibition activity was expressed based on the standard Trolox curve as the Trolox solution equivalent antioxidant capacity (mM TEAC).

Statistical design and analysis of results. The results of our study were evaluated with SPSS 16 software.

To extract the essential oil, we used a completely randomized design with a three-factor arrangement. In particular, the three factors were a plant source (*Salvia leriifolia* and *Linum usitalissmum*), a type of pretreatment (pulsed electric field at the intensity of zero (no pretreatment), 3 and 6 kV·cm^{-1}), and a type of solvent (aqueous and alcoholic).

The samples were obtained in three replications and the means were compared by the Duncan test at a significant level of 5% ($P < 0.05$). Finally, Excel software was used to plot the diagrams.

$$AA\%= [A_{blank} - A_{sample}/A_{blank}] \times 100 \quad (2)$$

where A_{blank} is the absorption of a control sample without the active compound and A_{sample} is the absorption of a sample containing a distilled extract).

RESULTS AND DISCUSSION

Total phenolic compounds. Fig. 1 presents independent effects of the factors, Fig. 2 shows their binary effect, while Table 3 indicates the interaction between the three factors in their effect on the content of phenolic compounds in the extracts. We found that a higher intensity of a pulsed electric field and the use of an alcoholic solvent significantly increased total

[I] Trolox equivalent antioxidant capacity
[II] 2,2'-Azino-Bis(3-ethylbenzothiazoline-6-Sulphonic Acid)

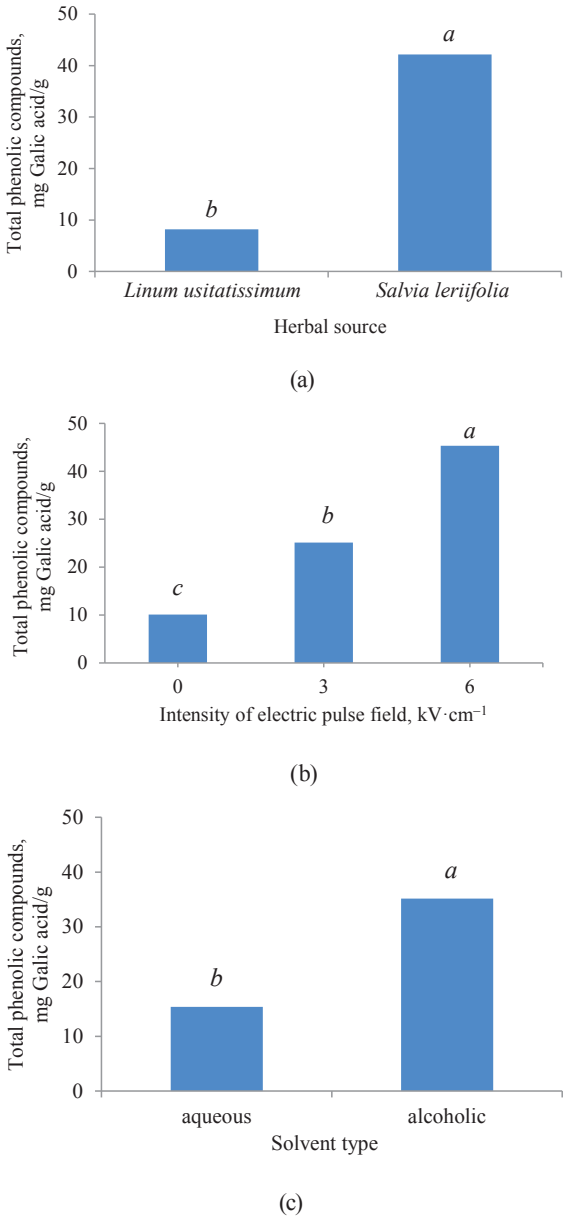

Figure 1 Independent effects of herbal source (a), pre-treatment (b), and solvent type (c) on total phenolic compounds ($P < 0.05$)

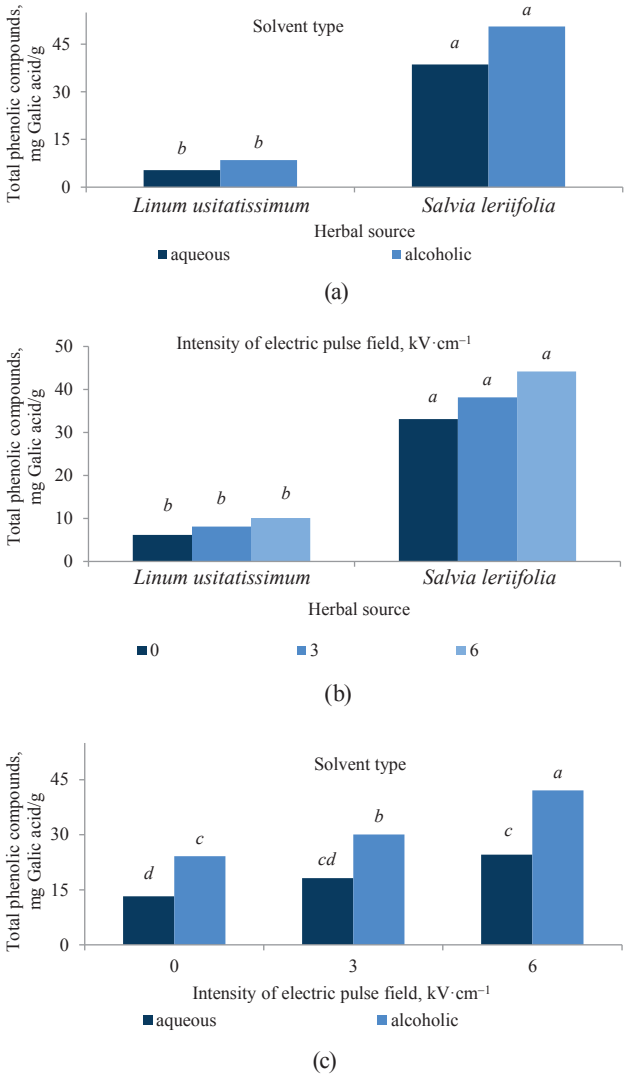

Figure 2 Binary effects of herbal source and pre-treatment (a), herbal source and solvent (b), and pre-treatment and solvent (c) on total phenolic compounds ($P < 0.05$)

phenolic compounds at a 95% confidence level. On the other hand, the content of total phenolic compounds was higher in the *Salvia leriifolia* L. extract, compared to *Linum usitalissmum* L. ($P < 0.05$).

Various factors, such as plant variety, harvest area, and harvest time, appear to affect the content of phenolic compounds. Different studies have found different amounts of total phenolic compounds in the *Salvia leriifolia* plant. For example, Hamrouni-Sellami *et al.*, Ahmadi *et al.*, Najafi *et al.*, Abadi *et al.*, and Bahadori *et al.* reported total phenolic compounds of 0.399–2.37, 40.47–61.32, 11.28–23.88, 12.68–83.85, and 17.3–294.9 mg of gallic acid per gram of extract, respectively [21–25]. In our study, this value reached 33.24–63.98 mg of gallic acid per gram of extract, depending on

Table 2 Interaction between herbal source, pre-treatment, and solvent type in their effects on total phenolic compounds

Herbal source	Type of solvent	Intensity of pulsed electric field pre-treatment, kV·cm^{-1}	Total phenolic compounds (mg of galic acid/g)
Linum usitatis-simum	aqueous	0	4.28 ± 0.21^{c}
	alcoholic		8.11 ± 0.54^{c}
	aqueous	3	5.33 ± 0.37^{c}
	alcoholic		8.49 ± 0.181^{c}
	aqueous	6	5.73 ± 0.33^{c}
	alcoholic		10.37 ± 0.81^{c}
Salvia leriifolia	aqueous	0	33.24 ± 0.37^{b}
	alcoholic		51.75 ± 1.63^{ab}
	aqueous	3	38.63 ± 0.76^{b}
	alcoholic		54.81 ± 1.30^{ab}
	aqueous	6	44.19 ± 0.63^{b}
	alcoholic		63.98 ± 1.11^{a}

$P < 0.05$

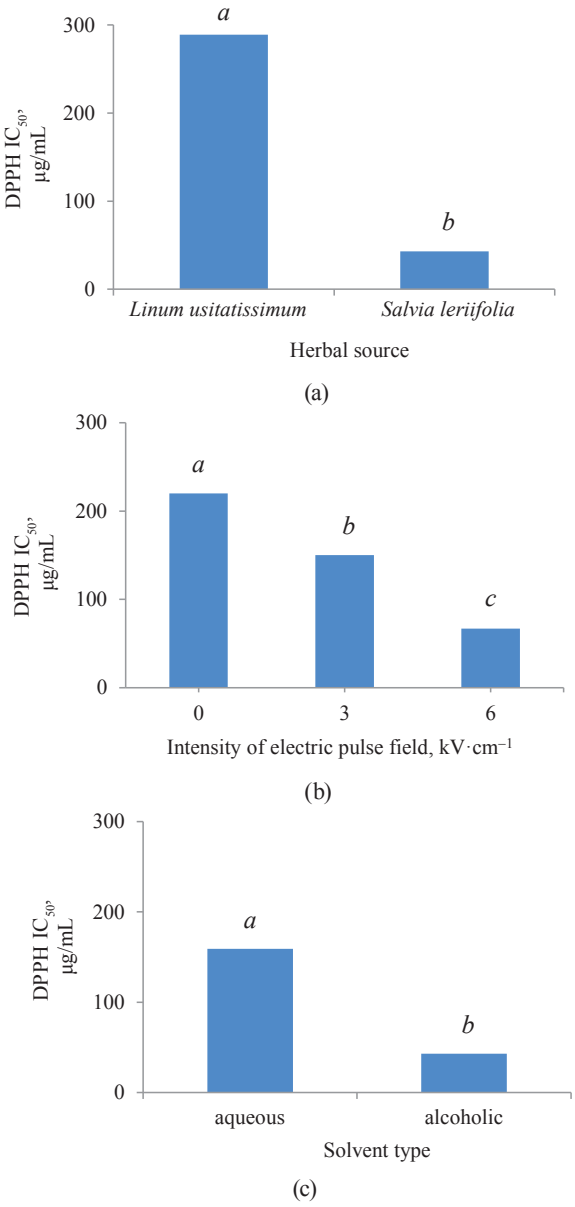

Figure 3 Independent effects of herbal source (a), pre-treatment (b), and solvent type (c) on antioxidant activity by DPPH method ($P < 0.05$)

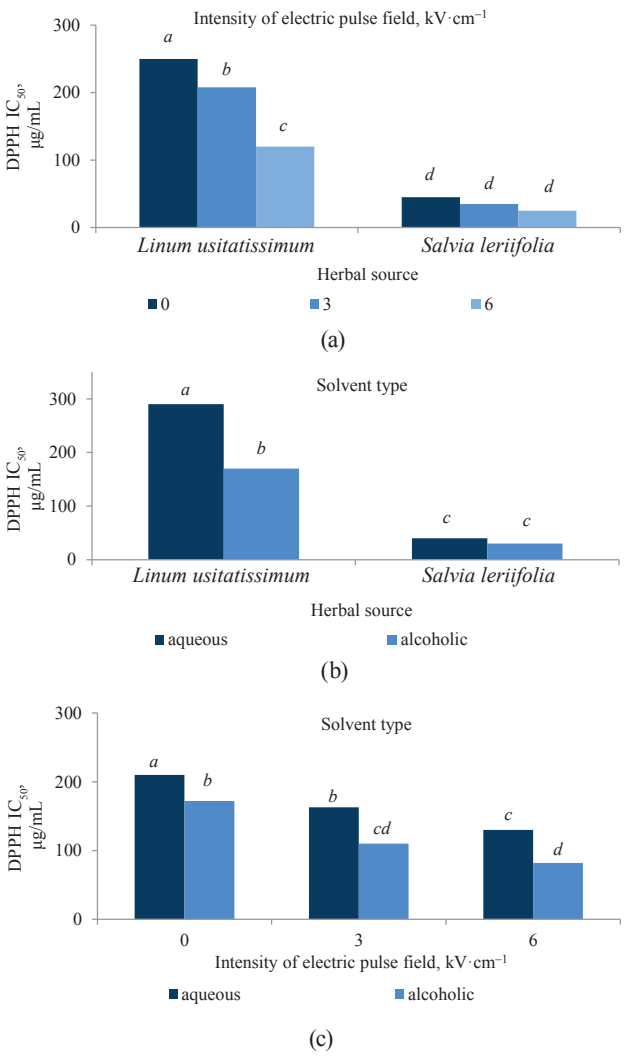

Figure 4 Binary effects of herbal source and pre-treatment (a), herbal source and solvent type (b), and pre-treatment and solvent type (c) on antioxidant activity by DPPH method ($P < 0.05$)

the solvent type and the use of a pulsed electric field pretreatment.

Total phenolic compounds in *Linum usitalissmum* oilseed have been reported by Oomah *et al.*, Brodowska *et al.*, and Russo and Reggiani at 8–10 mg of caffeic acid per gram of extract), 0.988 mg of catechin per gram of extract, and 4.64–9.40 mg caffeic acid per gram of extract, respectively [26–28]. In our study, their amount ranged from 4.28 to 10.37 mg gallic acid per gram of extract, depending on the solvent type and the use of a pulsed electric field pretreatment.

The studies showed that the amount of extracted phenolic compounds increased with a higher intensity of a pulsed electric field, reaching their maximum at a 6 kV·cm^{-1} pre-treatment. Schroeder *et al.* attributed this to the electrical degradation of cells and their increased permeability due to the use of a pulsed electric field [29]. In this regard, Bozinou *et al.* investigated the extraction of phenolic compounds and antioxidant activity of dried oak leaves with a 7 kV·cm^{-1} pulse electric field pretreatment [30]. They stated that the highest amount of phenolic compounds was obtained with a pulse time of 20 ms, a pulsing duration of 40 min, and a pulse interval of 100 ms.

Liu *et al.* examined the enhancement of extracted phenolic compounds in onion pre-treated with a pulsed electric field [31]. They stated that the optimum conditions for this purpose were a pulsed electric field of 2.5 kV, 90 pulses, and a temperature of 45°C. In these conditions, the amounts of extracted phenolic and flavonoid compounds were 86.82 mg of gallic acid per 100 g and 37.58 mg of quencherine per 100 g, respectively. These values were 2.2 times and 2.7 times as high as those in the control samples, respectively.

Table 3 Interaction between herbal source, pre-treatment, and solvent type in their effects on antioxidant activity by DPPH method

Herbal source	Solvent type	Intensity of pulsed electric field pre-treatment, kV·cm^{-1}	DPPH IC_{50}, μg/mL
Linum usitatis-simum	aqueous	0	312.51 ± 12.10^{a}
	alcoholic		220.22 ± 8.06^{b}
	aqueous	3	304.31 ± 10.11^{a}
	alcoholic		207.63 ± 12.10^{b}
	aqueous	6	267.81 ± 5.81^{ab}
	alcoholic		157.32 ± 4.16^{bc}
Salvia leriifolia	aqueous	0	41.38 ± 3.17^{c}
	alcoholic		33.54 ± 2.87^{c}
	aqueous	3	40.14 ± 1.36^{c}
	alcoholic		31.67 ± 2.23^{c}
	aqueous	6	36.91 ± 5.17^{c}
	alcoholic		25.28 ± 1.10^{c}

$P < 0.05$

Antioxidant activity by DPPH method. Fig. 3 presents independent effects of the agents, Fig. 4 shows their binary effects, and Table 3 indicates their interaction in relation to the antioxidant activity of the extracts derived with the DPPH method. As we can see, their antioxidant activity significantly increased, at a 95% confidence level, with a higher intensity of a pulsed electric field and the use of an alcoholic solvent. At the same time, we found that the antioxidant activity of *Salvia leriifolia* extracts was higher than that of *Linum usitalissmum* ($P < 0.05$).

The content of phenolic compounds is not an accurate measure of antioxidant activity. Since the Folin-Ciocalteu reagent nonspecifically reacts with phenolic and other compounds, such as organic acids, sugars are also able to reduce this reagent. Therefore, it is also necessary to measure antioxidant activity in other ways, for example, by the DPPH method, which we used in our study [32].

Phenolic compounds donated hydrogen or electron to the groups exposed to oxidation [33]. Thus, the content of phenolic compounds can be used as an important indicator of antioxidant activity. As noted above, various factors, such as plant variety, harvest area, and harvest time, appear to affect the amount of phenolic compounds and, subsequently, antioxidant activity.

In our study, the antioxidant activity of extracts (IC_{50}) from *Salvia leriifolia* plant extracted by the DPPH method ranged between 25.28 and 41.38 μg/mL, depending on the type of solvent and the intensity of a pulsed electric field.

We also investigated various sources of antioxidant activity (IC_{50}) in *Linum usitalissmum*. This value was reported by Brodowska *et al.* and Alachaher *et al.* to reach 299.00 and 220.05 μg/mL of extract, respectively [27, 34]. In our study, the amount of total phenolic compounds extracted from *Linum usitalissmum* varied

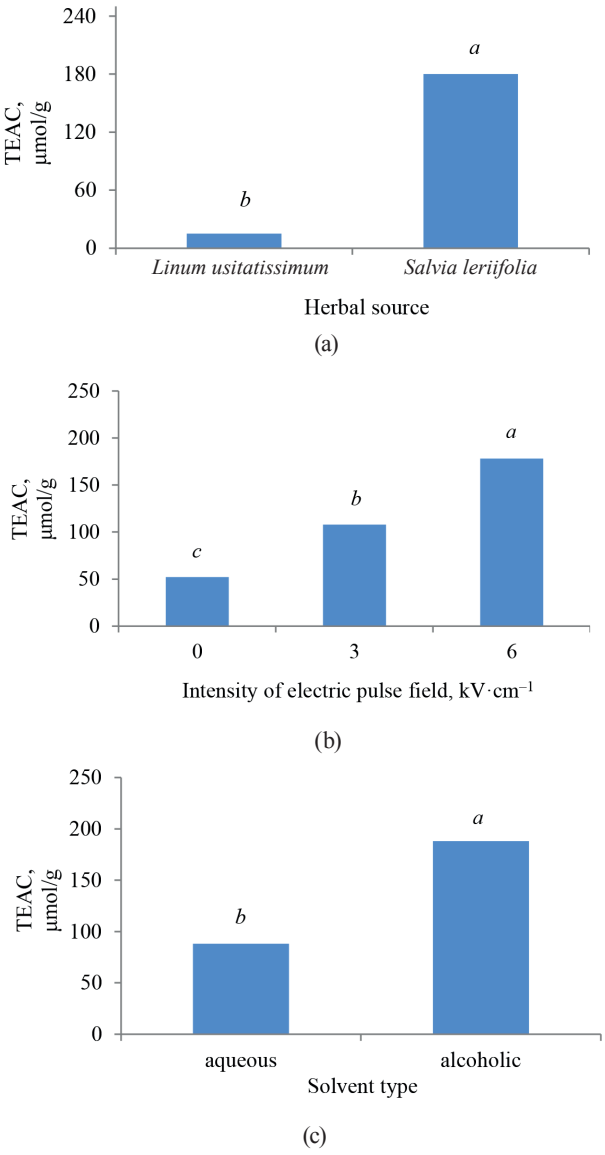

Figure 5 Independent effects of herbal source (a), pre-treatment (b), and solvent type (c) on antioxidant activity by TEAC method ($P < 0.05$)

from 157.37 to 312.51 μg/mL, depending on the solvent type and the use of a pulsed electric field pretreatment.

On the other hand, we found that the extracts' antioxidant activity increased with a higher pulsed electric field intensity. The highest values were observed in the samples with a 6 kV·cm^{-1} pretreatment. This was quite predictable from the measurement of total phenolic compounds, whose content also increased with a higher intensity of the applied electric field. In this regard, Bozinou *et al.* investigated the extraction of phenolic compounds and antioxidant activity of dried oak leaves with a 7 kV·cm^{-1} pulse electric field pretreatment [30]. They stated that the antioxidant activity was proportional to the content of total phenolic compounds: the higher the amount of phenolic compounds, the higher the antioxidant activity. In their study, phenolic compounds were highest with a pulse time of 20 ms,

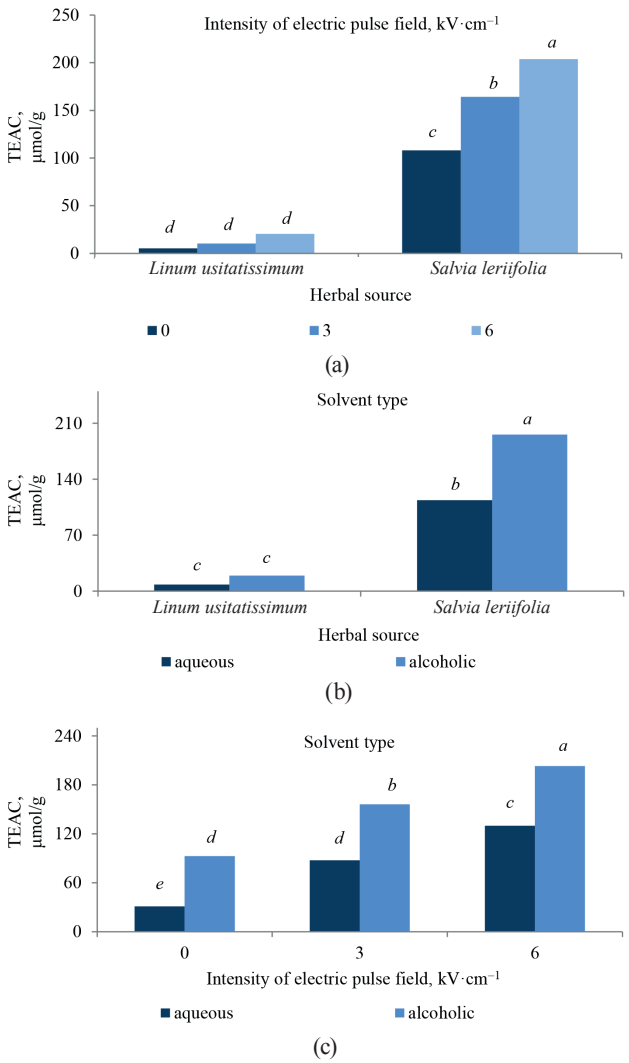

Figure 6 Binary effects of herbal source and pre-treatment (a), herbal source and solvent type (b), and pre-treatment and solvent type (c) on antioxidant activity by TEAC method ($P < 0.05$)

a pulsing duration of 40 min, and a pulse interval of 100 ms. Under these conditions, the sample's antioxidant activity was maximum.

Liu *et al.* studied the enhancement of extracted phenolic compounds in onion subjected to a pulsed electric field [31]. They stated that the optimum conditions for this purpose were a pulsed electric field of 2.5 kV, 90 pulses, and a temperature of 45°C. The researchers also found that the extract's antioxidant activity increased with a higher pulse electric field intensity and a larger number of pulses applied. Their finding also proved the correlation between the antioxidant activity and the amount of phenolic compounds.

Lopez Giral *et al.* also investigated a pulsed electric field pretreatment to improve the extraction of phenolic compounds from three different grape varieties (Graciano, Tempranillo, and Grenache) during two

Table 4 Interaction between herbal source, pre-treatment, and solvent type in their effects on antioxidant activity by TEAC method

Herbal source	Solvent type	Intensity of pulsed electric field pre-treatment, kV·cm^{-1}	TEAC (micro-mole Trolox per g dry herb weight)
Linum usitatis-simum	aqueous	0	0.89 ± 0.18^{c}
	alcoholic		12.44 ± 0.21^{c}
	aqueous	3	1.32 ± 0.01^{c}
	alcoholic		13.96 ± 1.45^{c}
	aqueous	6	6.92 ± 0.07^{c}
	alcoholic		21.48 ± 1.23^{c}
Salvia leriifolia	aqueous	0	108.54 ± 2.82^{bc}
	alcoholic		188.59 ± 2.37^{ab}
	aqueous	3	132.43 ± 1.06^{b}
	alcoholic		196.93 ± 2.24^{ab}
	aqueous	6	156.76 ± 1.14^{b}
	alcoholic		235.87 ± 2.87^{a}

$P < 0.05$

production periods [35]. The pretreatment conditions included a pulsed electric field of 7.4 kV·cm^{-1}, a pulse width of 20 ms, and a frequency of 400 Hz. They stated that using a pulsed electric field increased the color intensity, total phenol index, anthocyanin index, and total antioxidant power. These researchers therefore introduced a pulsed electric field pretreatment as a suitable technology for extracting phenolic compounds. However, they acknowledged that the ability of the method depended on the type of grape and the initial amount of phenolic compounds.

Similarly, Minussi *et al.* demonstrated a positive relationship between antioxidant power and the content of total polyphenolic compounds in grape juice, particularly compounds such as gallic acid, catechin, and epi-catechin [36].

Antioxidant activity by TEAC method. Independent, binary, and combined effects of the agents on the antioxidant activity of the extracts extracted with the Trolox method are presented in Fig. 5, Fig. 6, and Table 4. As observed, a higher intensity of a pulsed electric field pretreatment and the use of an alcoholic solvent significantly increased the TEAC number of the extracts at a 95% confidence level. On the other hand, the number of TEAC extracts of *Salvia leriifolia* was higher than that of *Linum usitalissmum* ($P < 0.05$).

As noted earlier, the amount of phenolic compounds alone is not a precise measure for antioxidant activity. Since the Folin-Ciocalteu reagent nonspecifically reacts with phenolic and other compounds such as organic acids, sugars also can reduce this reagent. Therefore, it is also necessary to measure antioxidant activity with other methods [32]. Therefore, we used the Trolox Equivalent Antioxidant Capacity (TEAC) method to measure antioxidant activity.

There was a significant correlation between the values of antioxidant activity measured by the DPPH

method and the TEAC method ($P < 0.01$ and $r = 0.932$). The Trolox equivalent antioxidant capacity test and diphenyl picryl hydrazyl are both synthetic free radicals with similar application. However, the Trolox equivalent antioxidant potential can be used to measure antioxidant activity of polar and nonpolar compounds [37].

The ABTS•$^+$ cation radical is more active than the DPPH radical and is therefore widely used in the measurement of antioxidant activity. In this test, ABTS oxidation first occurred following the reaction with potassium persulfate. The ABTS•$^+$ cation radical subsequently reacted with antioxidants or other hydrogen donating radicals and transformed in a reduced form [37]. Consequently, the antioxidant inhibition percentage can be measured by determining the absorption reduction rate. The radical inhibition activity in this test was reported based on the Trolox equivalent antioxidant capacity.

As noted above, various factors (plant variety, harvest area and time) appear to affect the amount of phenolic compounds and, subsequently, antioxidant activity.

We found that the antioxidant activity of *Salvia leriifolia* extracts measured with the TEAC method ranged between 108.54 and 235.87 µmol of Trolox per g dry plant weight, depending on the type of solvent and the intensity of pulsed electric field pretreatment.

Some studies evaluated the antioxidant activity of *Linum usitalissmum* with the TEAC method. Russo and Ragiani and Deng *et al.* reported the value of 560–860 (for oilseed *Linum usitalissmum*) and 22 000 µmol Trolox/g dry weight, respectively [28, 28]. In our study, the amount of total phenolic compounds extracted from *Linum usitalissmum* ranged from 0.89 to 21.48 µmol Trolox/g dry weight, depending on the solvent type and the intensity of pulsed electric field pretreatment.

On the other hand, the antioxidant activity of the extracts increased with a higher pulsed electric field intensity. A pre-treatment of 6 kV·cm^{-1} provided the highest amount of compounds. This result was predictable from the measurement of total phenolic compounds, which also increased with a higher intensity of the applied electric field. In this regard, Bozinou *et al.* investigated the extraction of phenolic compounds and antioxidant activity of dried oak leaves by using a pulsed electric field pretreatment at 7 kV·cm^{-1} [30]. They stated that the level of antioxidant activity was proportional to the amount of total phenolic compounds, so a higher content of phenolic compounds increased the antioxidant activity. In their study, the highest amount of phenolic compounds was associated with a pulse time of 20 ms, pulse duration of 40 min, and pulse interval of 100 ms. Under these conditions, the level of antioxidant activity was also maximum.

CONCLUSION

Our study aimed to compare the antioxidant activity of aqueous and alcoholic extracts derived from *Salvia leriifolia* L. and *Linum usitalissmum* L. subjected to a pulsed electric field at the intensities of zero (without pre-treatment), 3 and 6 kV·cm^{-1} with a constant pulse of 30. We investigated such parameters as total phenolic compounds and antioxidant activity. According to our results, the *Salvia leriifolia* extract had more phenolic compounds and higher antioxidant activity than the *Linum usitalissmum* extract under the same conditions.

On the other hand, a pulsed electric field pretreatment and the use of an alcoholic solvent (methanol) for extraction increased the content of phenolic compounds and the extract's antioxidant activity. In fact, the solubility of phenolic compounds depended on the type of solvent and their interaction. Finally, the extract derived from *Salvia leriifolia* with an alcoholic solvent and a pulsed electric field pretreatment (at 6 kV·cm^{-1} with 30 pulses) was selected as possessing desirable antioxidant properties.

CONTRIBUTION

The authors equally participated in the research and preparation of manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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