Volume 3, Issue 3, 2020

ПИЩЕВЫЕ СИСТЕМЫ
FOOD SYSTEMS

An international science journal

ISSN 2618-9771 (Print)
ISSN 2618-7272 (On line)
http://www.fsjour.com

Федеральное агентство научных организаций

Федеральное государственное бюджетное научное учреждение «Федеральный научный центр пищевых систем им. В.М. Горбатова» РАН

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Учредитель и издатель
Федеральное государственное бюджетное научное учреждение «Федеральный научный центр пищевых систем им. В.М. Горбатова» РАН

АДРЕС РЕДАКЦИИ И ТИПОГРАФИИ:
109316, Россия, Москва, Талалихина, 26,
Федеральный научный центр пищевых систем им. В.М. Горбатова РАН.
www.fsjour.com

Журнал зарегистрирован в Роскомнадзоре
Регистрационные данные:
ПИ № ФС77–71610 от 13.11.2017
ЭЛ № ФС 77–72022 от 26.12.2017
Периодичность — 4 номера в год.
Издается с 2018 года.
Подписано в печать 30.09.2020.
Тираж 300 экз. Заказ № 330.
Типография ФНЦПС.

ISSN 2618-9771 (Print)
ISSN 2618-7272 (Online)

Federal Agency of Scientific Organizations (FANO of Russia)

V.M. Gorbatov Federal Research Center for Food Systems of Russian Academy of Sciences (Gorbatov Research Center for Food Systems)

ПИЩЕВЫЕ СИСТЕМЫ FOOD SYSTEMS

Founder and publisher:
V.M. Gorbatov Federal Research Center for Food Systems of Russian Academy of Sciences

PRINTING OFFICE:
109316, Talalikhina str. 26, Moscow, Russia, Gorbatov Research Center for Food Systems.
www.fsjour.com

The journal is registered in ROSKOMNADZOR.
Registration data:
ПИ № ФС77–71610 from 13.11.2017
ЭЛ № ФС 77–72022 from 26.12.2017
Frequency — 4 issues a year.
Published in 2018.
Signed print 30.09.2020.
Circulation — 300 copies. Order № 330.
Printing house — FNCPS.

ISSN 2618-9771 (Print)
ISSN 2618-7272 (Online)

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DOI: https://doi.org/10.21323/2618-9771-2020-3-3-4-7

Available online at https://www.fsjour.com/jour
Original scientific paper

RESULTS DESIGNING AND ANALYSIS WHEN INTRODUCING NEW BEVERAGE IDENTIFICATION CRITERIA

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KEY WORDS:
experiment designing, beverage identification criteria, evolutionary operation

ABSTRACT
Building a digital profile of food product with use of modern mathematical apparatus of basic matrices is a solution to the problem of designing innovative beverage recipes. In this regard, for the effective use of the food resource base, modeling and production of high-quality food products, there is an acute problem of developing a methodology for identifying food products using the full range of the currently available analytical base. The article discusses an algorithm for constructing a flexible experimental design for the new identification criteria development, taking into account the laboratory research peculiarities in the beverage industry. The application of software in experiment designing is considered and a practical example of integrated designing based on the construction of an identification criterion for wine materials is presented.

FUNDING: The article was published as part of the research topic No. 0585–2019–0011 of the state assignment of the V. M. Gorbatov Federal Research Center for Food Systems of RAS

1. Introduction

One of the priorities in Russia over the past decade has been to provide the population with high-quality and safe food. The development of technologies in the agro-industrial area makes it possible to produce food products with various consumer characteristics. In this regard, a steady trend has been established to introduce personalized nutrition elements.

The variety of food assortment and, to a greater extent, the dishonesty of some manufacturers do not allow the construction of robust evaluative quality criteria based on one or a narrow group of specialized methods. In this regard, for the effective use of the food resource base, modeling and production of high-quality food products, there is an acute problem of food products identifying methodology developing using the full range of the currently available analytical base.

The main document, regulating the relations of all counterparties of the food industry — manufacturers, processors, distributors, retail chains, consumers and regulatory authorities — is Federal Law No. 29-FZ "On the quality and safety of food products." Within the framework of this document, the identification of food products is defined as "the activity to establish the compliance of certain food products with the requirements of regulatory, technical documents and information on food products contained in documents attached to them and on labels" [1].

In GOST R51293–99 "Product identification. General provisions" [2] the process of product identification is described as "establishing the conformity of a specific product to a sample and/or its description", which can also be traced in other regulatory documents [3,4]. Despite the fact that this GOST comprehensively describes the identification process and the requirements for its initiation, and also contains recommendations on the hierarchical structure of the identification methods application, in this work we will move away from the commodity study view of the identification process and will use more theorized definition, which in further will call clustering or discriminating, which postulates identification as a methods set for establishing differences between food groups [5,6].

Since the organoleptic and many biochemical indicators of beverages are not linear, moreover, they are mathematically unstable characteristics, sensitive to minimal recipe changes, the most common linear regression identification method gives acceptable results only in a limited area of the factor space. The clustering methodology based on experiment designing is promising and uses the whole range of analytical methods when constructing discriminatory criteria. The methodology does not use aggregating information on laboratory analyzes, but dynamically operates with unmodified data of the entire multidimensional factor space, which will minimize the error percentage and accurately predict qualitative relationships in beverage technology [7].

2. Materials and methods

The starting point for the identification methods construction and the development of new beverage formulations is the design of new laboratory studies to find new discriminating factors. For this, a full-factorial design of the experiment or its analogs is constructed [8,9,10].

In particular, when determining the factors significance, a design with a verification point is used. To build an experimental design, you need to calculate the number of units in a row, equal to the limiting factors number that can be tested for significance. If the number of factors is less than the number of characters in a row, the extra factors are called fictitious and are simply not taken into account. Hence follows a certain information surplus, which makes it possible to put all design tests in one replication, while insignificant losses occur in the description accuracy. If there are no fictitious factors, then the optimal solution is to use a verification test (verification point). This test, by default, is the center point, that is, the row in which all factors are at the average (in coded form — zero) level.

When constructing an experiment design, the most important stage is the choice of the variation interval. On the one hand, the variation interval should not be too small, otherwise, due to the small difference in responses, a significant factor

FOR CITATION: **Semipyatny V. K., Ageikina I. I., Lazareva E. G., Mikhailova I. Yu.** Results designing and analysis when introducing new beverage identification criteria. Food systems. 2020; 3(3): 4–7. https://doi.org/10.21323/2618-9771-2020-3-3-4-7

may be mistaken for insignificant. On the other hand, the interval of variation should not be too large, since there is always a risk of an extremum "missing". The variation interval is mainly determined by the experiment tasks, the creative part is the discretization of the selected interval for conducting point tests.

Nevertheless, there are so-called "saturated" and "oversaturated" designs, for which the number of degrees of adequacy freedom is equal to zero or even less. In the theory of experiment designing, such designs are widely known [10]. In practice, these designs represent for insignificant factors "elimination" (Table 1).

Table 1

Standard columns for constructing Plackett-Berman designs with different number of factors

Number of factors	7	11	15	19	23
Factor values	1	1	1	1	1
	1	1	1	1	1
	1	−1	1	−1	1
	−1	1	1	−1	1
	1	1	−1	1	1
	−1	1	1	1	−1
	−1	−1	−1	1	1
		−1	1	1	−1
		−1	−1	−1	1
		1	1	1	1
		−1	1	−1	−1
			−1	1	−1
			1	−1	1
			−1	−1	1
			−1	−1	−1
			−1	−1	−1
				1	1
				1	−1
				−1	1
					−1
					−1
					−1
					−1

When constructing regression models, it should be noted that the description error, other things being equal, is determined by the main diagonal of the correlation-covariance matrix $(X*X)^{-1}$, where X is the experimental design matrix. In the case of using an irregular design, the errors of the coefficients can greatly increase due to the growth of numbers that make up the main diagonal of the correlation-covariance matrix.

It is possible to develop various designs if we take a B_2 design as a basis, that is, a full-factor design. The rest of the designs differ from him in the size of the "star" shoulder. Constructing an optimal design in all respects is a very difficult task and hardly solvable. In this regard, the literature has developed formal criteria for determining the design effectiveness (described in detail in [8]). Let's consider the main ones:

- A — optimal design (from "*average variance*"). This is a design that provides the minimum mean variance of the regression equation coefficients.
- D — optimal design (from "*determinant*"). This design is characterized by the minimum determinant value of the correlation-covariance matrix, which provides the most reliable information. Practitioners find this design type as the most appropriate for the work.
- E — optimal design. This design minimizes the maximum variance of the regression equation coefficients.
- G — optimal design (from "*general variance*"). This design minimizes the maximum variance of one of the regression equation coefficients (almost always b_0).
- Rotatable design — the approximation error does not depend on the direction, but depends only on the distance to the center of the design.
- Orthogonal design — provides a relatively easy calculation of the regression equation coefficients with "manual" counting (without using a computer).
- B_n — design, close in properties to the D — optimal design. In practice, this design is convenient because the "star shoulder" is equal to one.

In reality, all these designs differ from each other both in the location of points in the factor space (in particular, in the size of the "star shoulder"), and in the tests number.

Often, the main designs diagonals have additional test in the center. Let consider such deviation feasibility from the principle of minimizing the cost of experiment conducting. Without additional test, the matrix $X*X$ of the design with a "star" shoulder 1.414 cannot be inverted, since it has a singularity. Accordingly, it is impossible to calculate the regression equation coefficients. An increase in the number of experiments to 9 allows a fairly noticeable decrease in the errors of the most regression equation members.

Adding one test to the design with shoulder 1 does not change the main diagonal of the correlation-covariance matrix and, accordingly, the accuracy of the coefficients calculating.

3. Results and discussion

In practice, in the alcohol industry, evolutionary operation (EVOP) [11], which is a kind of factor designing, shows itself well. It is used to find the optimum in conditions of strong "noise" and a small interval of factors variation, which is typical for the identification criteria of the beverage industry, expressed in limited scores and unstable interval estimates of laboratory studies. Under such conditions, it is very difficult to obtain a regression equation that contains significant terms.

When EVOP implementing, a factorial design for a linear model (with a central point) is used, rightly believing that with a small interval of variation, the investigated dependence may well be approximated by a straight line. When conducting a study, several such designs are built in a certain sequence, each of which is called a phase. Each replication of the design (phase) is called a cycle. The cycles are repeated until a significant effect is obtained at least for one factor. After that, a new design is built, taking as the central point the point of the previous design at which the optimal response was obtained. The search for the optimum continues until the desired result is achieved or until the maximum permissible values of the factors are reached.

The algorithm for finding the optimum is as follows (using the example of a two-factorial experiment):

1) Construct an experimental design, classical or variable, according to Table 1. For the case with three factors, a matrix of the form shown in Table 2 should be used. For convenience, Figure 1 depicts software written in the Wolfram Language to automate the experimental designs development.

```
plan[i] = Join[Tuples[{{doseMin[i], doseMax[i]},
                       {tempMin[i], tempMax[i]},
                       {timeMin[i], timeMax[i]}}],
  {{doseMin[i], tempAvg[i], timeAvg[i]},
   {doseMax[i], tempAvg[i], timeAvg[i]},
   {doseAvg[i], tempMin[i], timeAvg[i]},
   {doseAvg[i], tempMax[i], timeAvg[i]},
   {doseAvg[i], tempAvg[i], timeMin[i]},
   {doseAvg[i], tempAvg[i], timeMax[i]}}];

Grid[Join[
  {Prepend[factors, "№"]},
  MapThread[Prepend, {plan[i], Range[Length[plan[i]]]}]
 ], Alignment → Right, Dividers → {{False, True}, {False, True}},
 ItemSize → {{2, 7, 7, 7}}]
```

a)

№	Доза	Температура	Время
1	1	0	5
2	1	0	50
3	1	25	5
4	1	25	50
5	7	0	5
6	7	0	50
7	7	25	5
8	7	25	50
9	1	12.5	27.5
10	7	12.5	27.5
11	4.	0	27.5
12	4.	25	27.5
13	4.	12.5	5
14	4.	12.5	50

b)

Figure 1. Software-generated three-factor experimental design for the introduction of preparations based on polyvinylpolypyrrolidone [12]: (a) program code, (b) full-factor design

At each point of the design, carry out the test in duplicate. Calculate the average for each test. Do not sort the data in order to exclude anomalous results.

Table 2

Three-factor design for evolutionary operation

No. of the test	Factors value						
	x_1	x_2	x_3	x_1x_2	x_1x_3	x_2x_3	$x_1x_2x_3$
1	0	0	0	0	0	0	0
2	−1	−1	+1	+1	−1	−1	+1
3	+1	+1	+1	+1	+1	+1	+1
4	+1	−1	+1	−1	+1	−1	−1
5	−1	+1	+1	−1	−1	+1	−1
6	−1	−1	−1	+1	+1	+1	−1
7	+1	+1	−1	+1	−1	−1	−1
8	+1	−1	−1	−1	−1	+1	+1
9	−1	+1	−1	−1	+1	−1	+1

2) Calculate the values of the regression equation coefficients:

$$b_1 = 0.5\,(y_3 + y_4 - y_2 - y_5),$$

$$b_2 = 0.5\,(y_3 + y_5 - y_2 - y_4),$$

$$b_{12} = 0.5\,(y_2 + y_3 - y_4 - y_5).$$

Essentially, you should first find the arithmetical mean of the responses obtained in those tests, where $x_1 = +1$. From this value, subtract the average value of the responses obtained in those tests, $x_1 = +1$.

3) Calculate the effect of mean changing:

$$\Delta y = 0.2\,(y_2 + y_3 + y_4 + y_5 - 4y_0)$$

This is a simplified general expression

$$\Delta y = \frac{1}{N}\sum_{i=2}^{N}(y_i - y_1)$$

This expression should be used when the number of factors exceeds 2.

4) Calculate the values of the reproducibility variance in each test and the average value of the s_r reproducibility variance.

5) Calculate the errors of the regression equation coefficients. For b_i and b_{ij} (k — the number of replications):

$$\varepsilon_i = \frac{2s_r}{\sqrt{k}}$$

For Δy:

$$\varepsilon_i = \frac{1.78\,s_r}{\sqrt{k}}$$

If all the regression equation members are insignificant, that is, their absolute value is less than or equal to the corresponding error, all tests should be performed in one more replication. Then the calculations should be repeated, repeating the tests. Repeat these actions until the significance of at least one member of the regression equation.

If at least one member of the regression equation is significant, it is necessary to choose the point with the optimal response value and build a new design, taking this point as its center.

Continue actions to achieve significance either until the desired result is achieved, or until the maximum possible values of the factors are reached.

If at least one member of the regression equation is significant, then the value Δy indicates the shape of the response surface:

- if Δy is significant and greater than zero, there is a minimum response within the designing domain;
- if Δy is significant and less than zero, the maximum response takes place within the designing domain;
- if Δy is not significant, then the response surface is either a plane or a saddle.

This technique was implemented when comparing the effectiveness of industrial preparations based on polyvinylpolypyrrolidone with a decrease in the content of phenolic substances during wine materials processing. [12].

Recommended from the point of view of the constructed regression models dosage regimes, exposure time at room temperature (20 °C) for all drugs: Polyclar 10 — dose 3.2 g/dm³, time 70 min; Polyclar VT — dose 4.6 g/dm³, time 25 min; Polyclar V — dose 4.6 g/dm³, time 25 min.

When constructing discriminating equations, a three-factor evolutionary A-optimal design with a cycle of three was used, which made it possible to obtain statistically significant differences in the preparation's effectiveness.

Using the equations, the optimal processing temperature of wine materials was calculated for the minimum and maximum dosages of each preparation recommended by the manufacturer, as well as the recommended processing time for wine materials — 5 minutes. The calculated range of optimum processing temperatures varied within a fairly wide range, from 6.6 °C to 17.2 °C.

After determining a specific experimental design, suitable for a certain optimization criterion, within the framework of experiment designing with the introduction of new identification criteria, the following tasks should be implemented [13, 14]:

— aggregation of new analytical data on modern identification techniques in the beverage industry;
— development of a dynamic system of cluster identification;
— obtaining a metadata set for the most common similar beverages;

— software development for the practical implementation of the cluster identification system for beverage groups;
— development of methodological principles for determining the directions of falsification in the industry, as well as a methodology for adjusting assessment criteria based on historical data.

4. Conclusion

Providing the population with high quality, safe and at the same time affordable food products is one of the socio-economic priorities of the Russian Federation. In connection with these priorities in the field of nutrition, the development of functional products direction is observed, which is the first step towards personalized nutrition. The paper presents an algorithm for constructing a flexible experimental design for the development of new identification criteria, taking into account the peculiarities of laboratory research in the beverage industry. As part of the task implementation of obtaining analytical data using modern identification methods, a method is proposed for constructing experiments designs to identify the main factors and technological modes that have a dominant effect on the qualitative and physicochemical characteristics of beverages. On the basis of experiments, in the future, it is planned to build local models of new developed beverages using statistical methods for subsequent cluster analysis.

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The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

The authors declare no conflict of interest.

Received 23.06.2020 Accepted in revised 26.08.2020 Accepted for publication 15.09.2020

DOI: https://doi.org/10.21323/2618-9771-2020-3-3-8-13

Available online at https://www.fsjour.com/jour
Original scientific article

RETURNABLE BAKING WASTE — A NEW TYPE OF RAW MATERIALS FOR DISTILLATES PRODUCTION (PART II. STAGE OF RAW MATERIALS PREPARATION FOR DISTILLATION)

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KEY WORDS:
returnable baking waste, wort biochemical composition, saccharification, fermentation, yeast

ABSTRACT
The processes that take place upon saccharified wort obtaining from the returnable baking waste and its fermentation was the research subject of this work. The development of operational parameters at the stage of returnable baking waste preparation for distillation, which provides a high-quality product is the purpose of the work. The samples of saccharified and fermented wort obtained from various bread and bakery products types produced by large enterprises in Moscow were the objects of the study. To characterize the composition of saccharified and fermented wort, the indicators to assess the quality of the wort from grain raw materials were used. The mass concentration of individual sugars in the wort was determined using high performance liquid chromatography on an Agilent Technologies 1200 Series device. The qualitative composition and volatile components concentration in the fermented wort was determined using gas chromatography on a Thermo Trace GC Ultra device. It was established that the percentage of solids transition to a soluble state does not depend on a returnable waste type and is in the range from 87.6% to 90.7%, and the starch transition to a soluble state, on the contrary, is determined by the processed raw materials type. It is shown that the use of rye-wheat bread after its preliminary enzymatic treatment with thinning and cytolytic drugs in a mixture with wheat bread in a ratio of 1÷1 to 1÷2 can improve the wort rheological characteristics and transfer from 98.1% to 99.3% starch of raw materials in a soluble state. It has been shown that for the efficient process of saccharified wort fermentation from returnable baking waste, the Fermiol and Turbo-24 alcohol yeast races are most suitable, which ensure a high yield of ethanol and an optimal composition of volatile components in the fermented wort.

FUNDING: The article was published as part of the research topic No. 0585–2019–001 of the state assignment of the V. M. Gorbatov Federal Research Center for Food Systems of RAS

1. Introduction

As you know, the quality of distillates depends not only on the feedstock biochemical composition, but also is formed at all technological stages of their production. When distillates are produced from traditional types of starch-containing raw materials (wheat, rye, barley, corn, etc.), the stage of its distillation preparation involves several successive technological processes: raw materials preparation for cooking (grinding); grinded grain cooking to destroy the cellular structure and starch transfer into a soluble state; starch saccharification with malt enzymes or with the complex enzyme preparations use; sugars fermentation by yeast [1].

To transfer native raw materials starch to accessible state for saccharification and fermentation, various modes of water-heat treatment are used [2,3,4]. Current trends in improving the process of saccharified wort obtaining are aimed at switching to mild conditions for raw materials starch preparing for fermentation without excessive pressure and temperature above 100 °C use [5,6]. In the case of mechanical-enzymatic method of raw materials processing in alcohol production with the enzyme's participation of microbial origin, at each process stage, the optimal conditions are created for the action of α- and glucoamylases necessary for the starch hydrolysis. Moreover, this technology allows to take into account both the specificity of the processed raw materials and the spectrum of enzyme preparations that can be used [7,8,9].

For starch raw materials saccharification and plant cell biopolymers destruction in order to prepare it for fermentation, enzyme preparations of various spectrum of action are used [10,11]. Thus, preparations containing cytolytic enzymes, while ensuring optimal conditions for their action, allow grain non-starch polysaccharides hydrolysis, which are potential sources of fermentable carbohydrates [12,13]. This technique allows to increase the alcohol yield by 3–5%, as well as to facilitate the process by reducing the processed media viscosity.

It should be noted the development of enzyme preparations use containing proteases of fungal and bacterial origin [14,15]. The main purpose of their introduction into the technological process of alcohol production is the accumulation in the wort of low molecular weight nitrogen-containing compounds necessary for yeast feeding, improving their vital activity, and, as a result, intensifying alcohol fermentation [16,17].

Wort fermentation is one of the most important stages in the fermentation technology. At this production stage due to the enzymatic yeast cells apparatus, qualitative indicators of the final product are formed, which include ethanol and aromatizing secondary fermentation products [18]. Saccharified wort from starch-containing raw materials is a complex system that includes, in addition to fermentable sugars (maltose and glucose), intermediate starch hydrolysis products that are not involved in the yeast metabolism [1,19,12,14].

Saccharomyces cerevisiae yeasts of various races are used in fermentation industry, and their living conditions are determined by the composition and technological parameters of the wort fermentation. It is known that in many respects the efficiency of the fermentation process depends on the dextrins

FOR CITATION: **Krikunova L. N., Dubinina E. V.** Returnable baking waste — a new type of raw materials for distillates production (Part II. Stage of raw materials preparation for distillation). Food systems. 2020; 3(3): 8–13. https://doi.org/10.21323/10.21323/2618-9771-2020-3-3-8-13

conversion contained in saccharified wort to ethanol, since most yeast races are not able to ferment them. For this reason, the use of special yeast races with glucoamylase activity (*S. cerevisiae* Y — 717) is required [20,21].

Features of the structural-mechanical and biochemical composition of the returnable baking waste, consisting in an increased starch content, its better accessibility to hydrolysis, fewer non-starch polysaccharides, in a higher sugars concentration and high molecular weight dextrins [22,23] compared to traditional raw materials, allow grinding and cooking operations to be excluded. Thus, the use of returnable baking waste in the distillates production will reduce the cost of the product and increase the efficiency of the whole production process.

The use of a new raw material type in any production requires a thorough study of the processes occurring at each stage. This approach is also fully justified in the wort from returnable baking waste production and fermentation.

The purpose of this work was to develop regime parameters at the stage of preparing returnable baking waste for distillation, providing a high-quality product.

2. Materials and methods

The objects of the study were samples of saccharified and fermented wort, obtained from various feedstock types. As a raw material, industrial samples of returnable baking waste were used, obtained both from wheat flour and from a mixture of rye and wheat flour in different proportions, in addition, sugar, vegetable oil and complex additives were included in the recipe of individual products. Returnable waste samples were obtained from bakeries in Moscow (bakery No. 24, "Peko" bakery, "Cheryomushki" bakery, Moscow bakery and confectionery "Kolomenskoye", "Nizhegorodsky khleb OOO" (Limited Liability Company), "Russky khleb OOO" (Limited Liability Company), "Cherkizovo OAO" (Open Joint-stock Company)). Samples were prepared as follows: the product was manually cut into cubes 1cm x 1cm in size, the cubes were dried under mild conditions (temperature not more than 100 °C) until a moisture content of not more than 8.0% was reached and then crushed manually until a sufficiently uniform groat size was obtained in accordance with the requirements of GOST 28402–89 [24].

To characterize the saccharified wort composition obtained using returnable baking waste, indicators were used, adopted for assessing the quality of wort from grain raw materials: solids mass concentration, reducing sugars mass fraction, total fermentable carbohydrates mass concentration [2]. Additionally, the individual sugars mass concentration was determined using high performance liquid chromatography on an Agilent Technologies 1200 Series device (Agilent, USA) using a standardized method [25].

When studying processes at the fermentation stage of saccharified wort, the dynamics of carbon dioxide evolution by the gravimetric method has been determined [26], the ethyl alcohol mass concentration and the actual extract content [27], the volatile components composition and content by gas chromatography on a Thermo Trace GC Ultra device (Thermo, USA) with a flame ionization detector. Chromatographic column — HP FFAP: length 50 m, internal diameter 0.32 mm with a film thickness of the stationary phase 0.5 μm. Sample volume — 1 mm^3. [28]. When analyzing samples of fermented wort, they were previously freed from extractive components by distillation and the volatile components composition in the distillate was determined. In order to conduct a comparative analysis of the studied samples, the volatile components concentration was expressed in mg/dm^3 of absolute alcohol (mg/dm^3 of a. a.).

Research results processing was carried out using statistical methods. Illustrative material presents the average values of three dimensions.

3. Results and discussion

As a method for saccharified wort production, in this work, at the first stage, the previously developed regime parameters for the wheat bread processing provided for the ethyl alcohol technology were used [22]. They included mixing the raw material with warm water (at a temperature 70 °C) at a hydromodule of 1:3.5, introducing a diluting enzyme preparation with mesaphilic alpha-amylase with a dosage of 0.5 units of AA/g (amilase activity/g) of conventional starch of raw materials, holding the mixture at the specified temperature for 90 minutes, raising the temperature to 95–98 °C and mixture processing for 30 minutes, cooling the resulting mass to a temperature of 56–58 °C, introducing the saccharifying enzyme preparation with dosage of 6.0 units of GlA/g (glucoamylase activity/g) of conventional starch raw materials, saccharification for 30 minutes.

It was established (Table 1) that the returnable baking waste processing at the accepted operating parameters makes it possible to obtain a fairly concentrated wort, this indicator varies between 17.0–21.3%. A tendency to increase the wort concentration was revealed when using samples obtained from wheat flour as raw materials against samples from rye-wheat flour.

Analysis of total fermentable carbohydrates (TFC) showed that this indicator correlates with the starch content in raw materials. The maximum TFC value corresponds to sample 3 (15.7%), in which the starch content exceeds its value for other samples. The minimum concentration of TFC was detected in

Table 1

Comparative characteristics of wort samples from various returnable baking waste types

Samples	The feedstock composition	The concentration in wort,%			Purity, %
		Solids	Total fermentable carbohydrates	Reducing sugars	
1	Hearth wheat bread, piece	21.0	14.9	3.7	71.1
2	Wheat panloaf, peace	18.9	13.8	3.9	72.9
3	White bread	21.3	15.7	4.5	73.8
4	Sliced long loaf I	20.5	15.0	4.8	73.0
5	Sliced long loaf II	20.1	15.2	4.8	75.7
6	Sliced long loaf III	19.8	14.8	2.7	74.6
7	Hearth wheat-rye simple bread, piece	19.8	14.3	2.6	72.3
8	Rye-wheat hearth simple bread, piece	18.5	12.2	3.1	66.1
9	Rye-wheat simple panloaf, peace I	19.5	12.8	3.3	65.9
10	Rye-wheat simple panloaf, peace II	17.0	11.0	2.7	64.5
11	Rye-wheat simple panloaf, peace III	19.2	12.8	2.7	66.6
12	Rye-wheat scalded panloaf, peace	20.1	11.8	3.0	58.5

sample 10 (11.0%), characterized by a reduced starch content (47.7%). A clear dependence on the content of reducing sugars, depending on the type of returnable waste, has not been identified. Purity, by contrast, depends on the composition of the raw materials used to make bakery products. Processing of rye-wheat bread is characterized by a decrease in the quality indicator against samples obtained from wheat flour, i. e. as soluble components, the former contains an increased content of non-fermentable carbohydrates.

It was found that samples 1–6, obtained from wheat bread and sliced long loafs, contained more glucose and maltose, compared with samples that used rye flour (Table 2). The noted difference can be related both to the increased starch content in the samples from wheat flour, and to its greater tolerance to enzymatic hydrolysis.

Table 2

The influence of returnable waste type on the individual sugars content

Sample	Mass concentration, %				
	Fructose	Glucose	Sucrose	Maltose	Maltotriosis
1	0.38	2.22	0.13	1.11	1.76
2	0.45	2.31	0.09	1.33	1.61
3	0.54	2.97	0.12	1.15	1.36
4	0.73	2.97	0.11	1.25	1.32
5	0.65	2.98	0.12	1.31	1.41
6	0.44	2.42	—	1.12	1.46
7	0.20	1.43	—	0.95	1.45
8	0.40	1.97	—	0.68	1.18
9	0.41	1.79	0.11	1.01	1.53
10	0.38	1.57	—	0.58	1.25
11	0.39	1.45	—	0.69	1.46
12	0.47	1.68	0.20	0.80	1.34

In addition, the proportion of solids and starch transition into the liquid phase, i. e. in a soluble state. The liquid phase was an extract obtained by adding excess water to the wort (dilution 5 times) with its further filtration. The obtained result made it possible not to take into account the structure of bread, which is characterized by porosity and capillarity in comparison with traditional starch-containing raw materials (grain), and to establish the maximum transition of solids and starch into a soluble state.

The data presented in Table 3, led to the following conclusions:

- the transition of solids percentage in the soluble state does not depend on the type of returnable waste and is in the range of 87.6–90.7%;
- the starch transition into a soluble state is determined by the processed raw materials type. The use of samples from wheat flour with the accepted regime parameters of raw material processing is characterized by a high value of this indicator (97.9–99.5%); from a mixture of rye and wheat flour — lower values (85.0–89.4%).

In general, summarizing the obtained data, it can be noted that the use of returnable baking waste makes it possible to obtain saccharified wort with a high dry matter content, including total reducing substances.

Table 3

The influence of the returnable waste type on the transition of solids and starch into a soluble state

Sample	Concentration in the extract, %		Transition to a soluble state, % of the feedstock	
	Solids	Total reducing substances	Solids	Starch
1	4.1	3.1	87.6	99.2
2	4.2	3.0	88.6	98.1
3	4.2	3.3	88.1	99.5
4	4.3	3.2	90.7	98.1
5	4.2	3.2	88.9	97.9
6	4.2	3.1	88.4	98.6
7	4.3	2.9	90.0	96.3
8	4.2	2.3	88.9	88.1
9	4.2	2.5	88.5	89.4
10	4.3	2.1	89.9	88.0
11	4.2	2.2	88.6	87.1
12	4.2	2.1	89.1	85.0

At the same time, it was found that when using samples obtained from rye-wheat bread, the wort is characterized by a lower value of purity and the starch transition to a soluble state, which necessitates the adjustment of the regime parameters of its production. To increase the content of reducing substances and the wort purity index, a variant of using a mixture of wheat and rye-wheat bread as a raw material for distillation can be considered.

At the next research stage, saccharification processes using enzyme preparations of cytolytic action were studied. It was found that obtaining wort from rye-wheat bread with the adopted hydromodule 1:3.5 with the additional use of microbial cytases (enzyme preparation Cellulase Cl. 14) even at an increased dosage (0.1–0.2 units of the main activity (MA) of raw materials, recommended by the manufacturer) does not allow to completely starch transition into a soluble state (Figure 1).

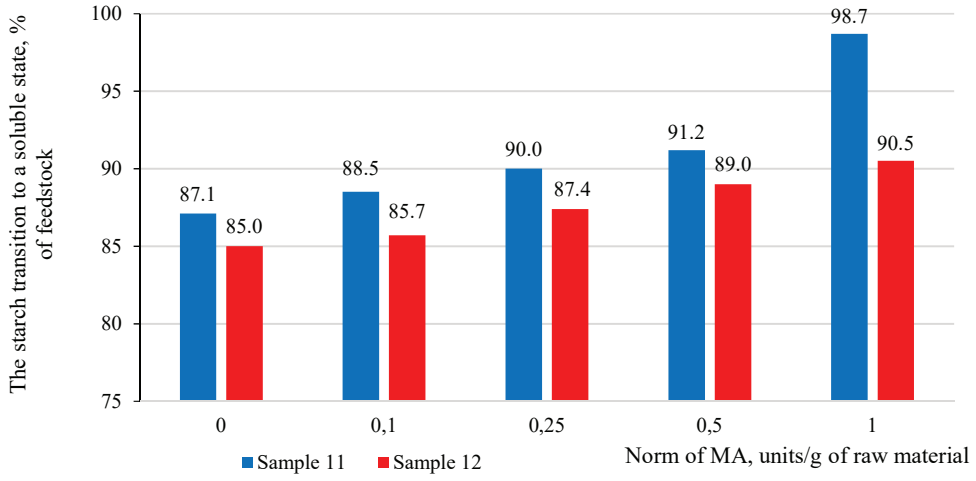

Figure 1. The influence of the MA carrying norm when receiving wort from rye-wheat bread on the transition of starch to a soluble state

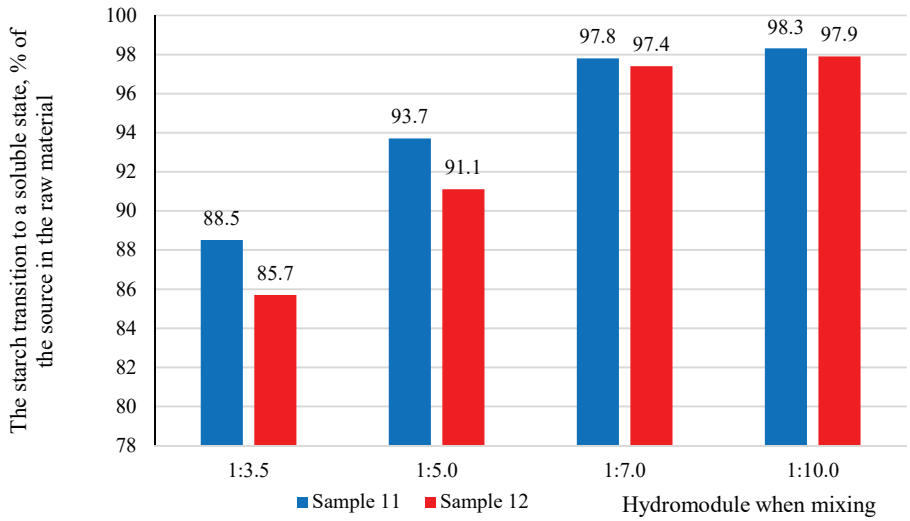

Figure 2. The hydromodule influence upon receipt of the wort from rye-wheat bread on the starch transition a soluble state

It was established that the hydromodule affects the saccharification process to a greater extent than the dosage of the enzyme preparation (Figure 2).

The hydromodule increase to 1:7.0 ÷ 1:10.0 made it possible to almost completely transit starch of rye-wheat bread to a soluble state. However, such a strong dilution of the technological environment is not economically justified, as it will significantly reduce the total wort concentration and the content of total reducing carbohydrates, as a result, the fermented wort strength.

In connection with the identified factor, it is proposed to use rye-wheat bread as a mixture with wheat bread in a ratio of 1÷1 to 1÷2, after preliminary processing of the first (hydromodule from 1÷7.0 to 1÷10.0, the introduction of enzyme diluent and cytolytic drugs, holding the mixture at a temperature of 50–55 °C for 30 minutes). At the same time, wheat bread was introduced into the mixture until the total hydromodule reached 1÷3.5. It was established that such a new technical solution allows almost completely transit raw materials starch to a soluble state (Table 4).

Table 4

The influence of regime parameters for the wort from the returnable baking waste production on the starch transition to a soluble state

Name	Control	Test 1	Test 2
Raw material type	*Rye-wheat bread*	*Mixture 1:1*	*Mixture 1:2*
Hydromodule on mixing stage	1:3.5	1:7.0	1:10.0
Total Hydromodule	1:3.5	1:3.5	1:3.5
The starch transition to a soluble state, % of the source in the raw material	88.5	98.1	99.3

The use of the hydromodule 1:7.0÷1:10.0 instead of the hydromodule 1:3.5 at the first stage of rye-wheat bread preparation and the carrying of the entire estimated amount of enzyme preparations of a diluting and hemicellulase action contributes, firstly, to improving the rheological characteristics of the mix for due to a deeper hydrolysis of the hemicellulose raw materials, secondly, an increase in the enzymatic starch attackability due to the destruction of its complexes with hemicelluloses and proteins, thirdly, it increases the percentage of protein water-soluble fractions.

Fermentation is one of the main stages in the distillates production, during which, under the action of the yeast enzyme complex, the initial formation of product quality indicators occurs. In this regard, special attention should be paid to the selection of the yeast race, which provides high fermentation efficiency and the synthesis of valuable volatile components. The objective was to select the optimal yeast race for fermenting the wort from the returnable baking waste.

Five races of dry alcoholic yeast of foreign manufacture were tested: Fermiol (USA), Turbo-24 (Great Britain), Alcotec Whiskey Turbo (Great Britain), Parmaya Cristal (Turkey) and Angel (China). The yeast application rate was 100 mg/100 g of the wort. Saccharified wort from a mixture of wheat and rye-wheat bread in a ratio of 1:1 was used as a raw material. It was found that the most intensive fermentation process, characterized by the dynamics of carbon dioxide evolution, takes place using Fermiol and Turbo-24 races (Figure 3).

These races were also the most effective in the accumulation of ethyl alcohol, and, consequently, in the yield of alcohol from a unit of starch raw materials (Table 5).

Table 5

The influence of yeast race on the quality indicators of fermented wort and alcohol yield

Indicators	Yeast race				
	Fermiol	Turbo Vaast	Turbo-24	Parmaya Cristal	Angel
Strength, % vol.	8.11	7.74	8.15	8.07	7.63
Valid extract, % wt.	5.5	6.0	5.9	6.2	7.0
The yield of alcohol, dal/t of conventional starch of raw materials	63.25	60.36	63.56	62.94	59.51

At the final work stage, the qualitative and quantitative composition of volatile components in the samples of fermented wort was determined. Analysis of the obtained data showed that the use of yeast Fermiol and Turbo-24 allows to get fermented wort with a minimum concentration of acetaldehyde (Table 6). When using the yeast Parmaya Cristal and Angel, the content of this component, which adversely affects the distillates organoleptic characteristics, increases by 1.4–3.8 times.

It was also found that the higher alcohols content, which form the basis of the final product aroma, is determined by the race of used yeast.

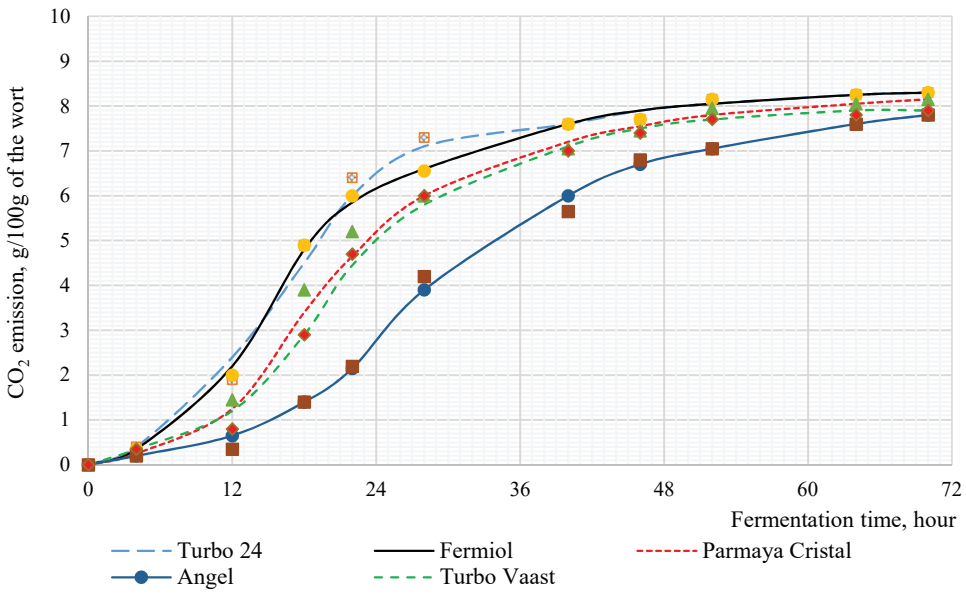

Figure 3. The influence of yeast race on the wort fermentation

Table 6

The influence of yeast race on the composition of the fermented wort volatile components

Name of volatile components	The volatile components content, mg/dm³ of A.A.				
	Fermiol	Turbo Vaast	Turbo-24	Parmaya Cristal	Angel
Acetaldehyde	275	382	295	667	1034
Ethyl acetate	112	101	150	177	107
Methanol	8	10	13	25	12
Σ of higher alcohols, including:	4619	4315	5200	5575	3567
— 1-propanol	446	438	477	486	380
— isobutanol	1244	1081	1311	1372	1060
— isoamylol	2929	2796	3412	3717	2127
Enanthic ethers	19	14	23	16	19
Phenylethyl alcohol	334	456	400	390	702
Σ of volatile components*	5417	5322	6081	6850	5497

* In the sum of volatile components, all identified substances were taken into account, some of them are not presented in this Table

The maximum concentration of higher alcohols was detected in samples of fermented wort obtained using Parmaya Cristal yeast, the minimum — Angel. When using the latter, the maximum accumulation of phenylethyl alcohol was also revealed. Phenylethyl alcohol is formed during the hydrolysis of the phenylalanine amino acid, which is present in saccharified wort from recyclable baking waste in a much higher concentration than in wort from grain raw materials. Usually, during distillation, phenylethyl alcohol is isolated with a tail fraction and remains in the distillers' spent grains, but only a small part of it transit directly to the distillate. The highest concentration of ethers, including enanthic ethers, which are the most valuable aroma-forming components, is noted in the sample obtained using the Turbo-24 race.

In general, the analysis of the obtained data gave reason to recommend the Fermiol and Turbo No. 24 races for fermentation of wort from returnable baking waste products, the use of which allows one to obtain fermented wort with maximum strength and high rates of valuable volatile components.

4. Conclusion

Based on the results of studies, it is recommended to use a mixture consisting of wheat and rye-wheat bread as raw materials to obtain high-quality distillates from returnable baking waste. To obtain saccharified wort from this raw material type, a new technological method has been developed based on preliminary enzymatic hydrolysis of rye-wheat bread with a high hydromodule and the use of enzyme preparations of the cytolytic complex.

For the efficient process of saccharified wort from the returnable baking waste fermentation, the selection of the Fermiol and Turbo-24 yeast races is scientifically justified, allowing not only a high ethanol yield, but also an optimal volatile components composition.

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The authors declare no conflict of interest.

Received 25.06.2020 Accepted in revised 05.08.2020 Accepted for publication 15.09.2020

DOI: https://doi.org/10.21323/2618-9771-2020-3-3-14-19

Available online at https://www.fsjour.com/jour
Original scientific paper

TECHNOLOGICAL SCHEMES FOR THE PROCESSES OF PREPARATION AND MILLING BINARY GRAIN MIXTURES AND BIOCHEMICAL EVALUATION OF PRODUCED PRODUCTS

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KEY WORDS:
wheat, flax, binary grain mixture, grinding technological scheme, wheat-flax flour, biochemical assessment.

ABSTRACT

A study of the preparation and milling of a grain mixture containing 7% of flax seeds has been carried out in order to obtain a composite wheat-flax flour, in which the entire biopotential of flax seeds was preserved. It was revealed that the preparation of the components of the grain mixture should be carried out independently, in parallel flows. During the wheat grain preparation the cold conditioning was carried out, the modes of which were the following: humidity — 15.5%, dwell time in the water — 24 hours. The optimal conditions for milling the wheat-flax mixture have been determined, which are the following: yield (%) / ash content (%) in 3 break systems (in terms of the 1st break system — grain) for the first break system — 53.5 / 1.00; for the second break system. — 22.2 / 1.11; totally for the first and the second break systems — 75.7 / 1.035; totally for the first, the second and the third break systems — 81.0 / 1.1. The technological schemes have been developed and the new varieties of wheat-flax flour with predetermined technological properties and increased nutritional value have been formed. The approximate indices of yield and quality of the new wheat-flax flour varieties are the following: Flour A — yield 45–50%, lipids 3.6–4.0%, protein 13–13.5%, ash 0.55–0.70%, whiteness — 50 conventional units; Flour B — yield 20–25%, lipids 5.5–6.0%, protein 14–14.5%, ash 0.9–1.25%, whiteness — 22 conventional units; Flour C — yield 70–75%, lipids 4.5–5.0%, protein 13.6–14.0%, ash 0.75–0.90%, whiteness — 36 conventional units. It was indicated that the total lipids content in flour from two-component mixtures increases by about 4 times, and the total protein content in the studied samples increases by 1–2%. The content of linoleic acid (ω-6) in wheat-flax flour samples is 1.6...3.3 times higher than in wheat flour; the content of linolenic acid (ω-3) in wheat-flax flour samples is 36.8...57.2 times higher than in wheat flour (taking into account the total lipids content in the samples). The enrichment of wheat flour due to flax seeds allows to make up the deficiency of PUFA family in the diet of a modern person and to obtain products on a grain basis of a balanced composition.

FUNDING: The article was published as part of the research topic No. 0585–2019–0002 of the state assignment of the V. M. Gorbatov Federal Research Center for Food Systems of RAS

1. Introduction

The enrichment of the products of wheat grain processing with proteins, minerals, and dietary fiber is achieved by introducing the milling products of some cereal crops into wheat flour. This solution has found a wide application in the bakery production in the form of so-called composite flour mixtures. However, in recent years the demand of grain products enrichment with essential fatty acids, especially with linolenic acid (the ω-3 family of fatty acids), the deficiency of which negatively affects human health, has emerged [1,2,3,5,6].

Analysis of the lipid composition of various oilseeds shows that linseed oil, as a source of unsaturated fatty acids of the ω-3 family, has an absolute advantage. The main fatty acid of the oil from flax seeds is linolenic acid, the relative content of which varies according to different sources from 47.5% to 68.1% [3,5,7].

The problem of flour enrichment with essential fatty acids is currently solved by using crushed linseed cake (flax meal). The use of linseed cake has several disadvantages. First of all, according to various authors' studies the content of such flax meal in the composite mixture should be 15–25% to provide essential fatty acids in the required amount, but such content significantly degrades the consumer properties of bread [4,5]. The direct use of flax seeds will allow one to significantly (3–4 times) reduce the content of products of flax seeds processing in the composite mixture at maintaining the amount of unsaturated fatty acids, primarily of essential linolenic acid, necessary in terms of composition balance [4,8].

The introduction of flax seeds in the mixture sets the task of developing technology for processing mixtures. First of all, it is necessary to determine the milling conditions of such mixtures (parameters and milling modes), each component of which has its own specific features.

The aim of the research is the development of technological schemes for the preparation and grinding of binary grain mixtures based on wheat and flax seeds and a biochemical assessment of the newly formed varieties of wheat-flax flour obtained by joint grinding of a wheat-flax mixture.

2. Materials and methods

The object of the study was wheat grain and seeds of white and brown seed flax. Table 1 and Table 2 show the technological properties and chemical composition of the initial components of the grain mixture.

The components of the grain mixture are characterized by an average level of values both in chemical composition and technological properties and can be considered as quite representative. The content of flax seeds in the mixture was determined based on the recommended levels of consumption of food and biologically active substances [9,10] and averaged about 7%.

To study the milling and production of wheat and wheat-flax flour, the RSA-5 reduction and sorting unit with corrugated roll-

FOR CITATION: **Pankratov G. N., Meleshkina E. P., Vitol I. S., Kechkin I. A., Nagainikova Yu. R.** Technological schemes for the processes of preparation and milling binary grain mixtures and biochemical evaluation of produced products. *Food systems.* 2020; 3(3): 14–19. https://doi.org/10.21323/2618-9771-2020-3-3-14-19

ers for break systems (P-10 $^{1}/_{cm}$) and microrough surface rollers for reduction systems, the laboratory plansifter and the laboratory bran finisher were used. The whiteness of flour (WF) was determined by measuring the reflectivity of a densely smoothed flour surface using a photovoltaic device (GOST 26361–2013), ash content (Z) — by burning flour and bran, followed by determination of the fireproof residue mass (GOST 27494–2016). The total protein content was determined by the Kjeldahl method (N×6.25) (GOST 10846–91); lipids content — according to Soxhlet (GOST 29033–91); fiber content — according to Kuschner and Hanek; starch content — according to Evers (GOST 31675–2012); reducing sugars according to the Bertrand method; soluble protein — according to the Lowry method. Determination of the fractional composition of proteins according to Osborne: albumins were isolated using distilled water, globulins — using a 10% NaCl solution, prolamines — using 70% ethanol, and glutelins — using a 0.2% NaOH solution. Enzymatic activity of proteases was determined by the modified Anson method using bovine serum albumin as a standard substrate; amylase activity — by the colorimetric method according to the amount of starch hydrolyzed based on an assessment of the color intensity of the starch-iodine complex; the activity of alkaline and acid lipases was determined by the titrometric method by the amount of fatty acids formed [11]. Fatty acid composition — by gas chromatography (gas chromatograph 6890N with mass-selective detector Agilent 5975C, USA).

Table 1

Technological properties of the initial components of the grain mixture

Agricultural crop	Moisture, %	Mass of 1000 seeds, g	Test weight, g/l	Vitreousness, %	Medium geometric grain sizes, mm a-width, b-thickness, l-length
Wheat	12/2	44.66	769	52	a = 3.6 b = 2.9 l = 6.5
Flax seeds: white	5.2	8.40	668	—	a = 2.5 b = 1.2 l = 5.2
Flax seeds: brown	5.1	8.37	667	—	a = 2.5 b = 1.2 l = 5.1

Table 2

The chemical composition of the initial components of the grain mixture

Agricultural crop	Protein, %	Fat, %	Starch, %	Cellulose, %	Gluten, %
Wheat	13.43	1.83	66.8	2.2	24.7
Flax seeds: white	24.68	39.80	5.2	15.0	–
Flax seeds: brown	24.42	37.33	5.1	15.1	–

The analyses were performed in the samples of wheat-flax flour, presenting the results as average arithmetic ones. The discrepancy between parallel assays did not exceed 3% of the average arithmetic value with the confidence probability P = 0.95.

3. Results and discussion

Processing of grain mixtures, the components of which have significant differences in physical and chemical properties, is a rather complicated task [12,13,14,15].

The study of the processes of preparation and milling of a grain mixture containing flax seeds was carried out using 93% wheat grain and 7% flax seeds content. The conditions for the joint processing of wheat grain and flax seeds are the separate preparation and thorough mixing of the components immediately before milling. The content of flax seeds in the mixture was determined in accordance with the "Recommended levels of consumption of food and biologically active substances", it averaged about 7%. During the wheat grain preparation the cold conditioning was carried out, the modes of which corresponded to "The rules of organization and process control at flour mills", humidity — 15.5%, dwell time in the water — 24 hours.

Analysis of the geometric sizes of flax seeds and wheat grain shows the impossibility of their joint cleaning. The preparation scheme should consist of independent preparation flows.

The scheme of the two-factor experiment for determining the optimal conditioning parameters is presented in Table 3.

Table 3

Estimated and actual moisture content of the original wheat grain

Milling number	Estimated moisture, %	Actual moisture, %	Dwell time in the water, hour
1 (control)	16.0	14.7	24
2	16.5	15.2	24
3	16.5	14.9	12
4	15.0	14.4	18
5	14.5	13.6	12
6	14.5	13.7	24

The results of grain mixtures milling are presented in Table 4 and Table 5.

Table 4

Yield (Y) flour and bran,%

Technological system	Yield (Y), %				
	Milling № 2	Milling № 3	Milling № 4	Milling № 5	Milling № 6
Break I	8.4	11.9	8.3	8.3	9.5
Break II	10.8	11.9	7.7	7.7	8.8
Break III	3.6	3.3	4.3	4.0	3.8
Reduction system 1	35.6	35.4	31.4	30.7	32.1
Reduction system 2	5.2	2.5	11.0	11.7	8.4
Reduction system 3	2.4	2.9	5.0	6.0	3.8
∑ flour	66.0	67.9	67.7	68.4	66.4
Bran from break systems	23.2	18.5	19.0	16.3	19.5
Bran from reduction systems	10,8	13.6	13.3	15.3	14.1
Bran from break systems / bran from reducti on systems	2.15	1.36	1.43	1.07	1.38
∑ bran	34.0	32.1	32.3	31.6	33.6

Table 5

The results of laboratory grinding on the whiteness of flour, units

Technological system	Whiteness (WF), units				
	Milling № 2	Milling № 3	Milling № 4	Milling № 5	Milling № 6
Break I	73	69	69	67	66
Break II	56	52	53	51	51
Break III	37	31	31	30	29
Reduction system 1	55	51	50	50	50
Reduction system 2	36	29	36	35	33
Reduction system 3	28	23	24	21	23

Statistical analysis of laboratory milling results using the MINITAB14 program revealed statistically significant linear regression equations. The result of statistical processing of the dependence of flour whiteness on conditioning parameters (Table 3) is presented below.

Regression Analysis: WF2 versus Y2
The regression equation is
WF2 = 72,45–0,2759 Y2
S = 2,39544 R-Sq = 91,0%
R-Sq(adj) = 88,8%

Regression Analysis: WF5 versus Y5
The regression equation is
WF5 = 67,16–0,3066 Y5
S = 1,92102 R-Sq = 95,3%
R-Sq(adj) = 94,2%

Regression Analysis: WF3 versus Y3
The regression equation is
WF3 = 69,93–0,2675 Y3
S = 1,95253 R-Sq = 93,2%
R-Sq(adj) = 91,5%

Regression Analysis: WF6 versus Y6
The regression equation is
WF6 = 67,16–0,3051 Y6
S = 2,26688 R-Sq = 93,1%
R-Sq(adj) = 91,3%

Regression Analysis: WF4 versus Y4
The regression equation is
WF4 = 69,30–0,3255 Y4
S = 1,84777 R-Sq = 96,1%
R-Sq(adj) = 95,1%

Regression Analysis: WF1 versus Y1
The regression equation is
WF1 = 75,00–0,09554 Y1
S = 0,369080 R-Sq = 98,8%
R-Sq(adj) = 98,3%

Based on the obtained equations, the yield indices of top-grade flour were calculated.

The optimal values of the conditioning parameters were determined using the method of contour-graphical analysis, where the following optimization criteria were used: the estimated yield of top-grade flour; maximum value of whiteness of flour; the ratio of the bran yield of break systems to the bran yield of reduction systems, as a characteristic of the grit formation efficiency; they amounted to moisture content of at least 15% and dwell time in the water of at least 18 hours, which corresponds to the recommendations. Thus, the introduction of flax seeds into the mixture does not affect the choice of conditioning parameters.

Mixing wheat grain with flax seeds during the processing of a grain mixture is a difficult task and is possible only immediately before milling, as they have significant differences in physical and chemical properties. In addition, this is based on the separate preparation of the components and self-sorting of the mixture during movement [12,13].

To determine the necessary conditions for the formation of a binary grain mixture, the main mixing methods were modeled:

- active — with a high relative speed of components movement, which are based on a convective movement mechanism (paddle mixers);
- passive — based on the movement of layers sliding relative to each other (drum mixers).

Evaluation of the quality of the mixture by the heterogeneity coefficient (coefficient of variation) was carried out according to the formula:

$$V = \frac{100}{\overline{c}} \sqrt{\frac{\sum_{1}^{n} (c_i - \overline{c})^2}{n-1}}, \% \qquad (1)$$

where

c — the arithmetic mean value of the key component concentration;
c_i — current concentration value;
n — the number of measurements.

The number of samples and the mass of the sample were determined in accordance with the recommendations [13] and amounted to — the number of samples — 8, the mass of the sample according to calculation — 5 g, in fact — 50 g.

Comparison of the mixing methods showed that the passive method is significantly inferior to the active one. So with equal mixing cycles, the coefficient V is 37.4% for the drum mixer and 15.9% for the screw mixer. Subsequently, the active mixing method was used, which ensured satisfactory quality. The basic scheme for the grain mixture preparing for milling includes: separate cleaning of wheat grain flow and flax seeds flow from impurities, cleaning of the surface (shelling) of wheat grain, wheat grain conditioning, wheat grain and flax seeds mixing, forming of a grain mixture flow.

The program of experimental studies of milling modes in the first, the second and the third break systems provided for a wide range of yield indices, which was achieved by a corresponding variation of the roll space: for the first break system from 0.75 mm to 0.20 mm, providing yield index from 25 to 70%, for the second break system from 0.20 mm to 0.05 mm, which corresponded to yield indices from 48 to 66%; for the third break system from 0.05 mm to 0.00, and the yield indices range was from 22 to 45%.

Analysis of the grain-size composition shows that the better part of the intermediate products lies in the size range of 600–150 microns (Figure 1).

The fractional composition of grains is shown in Figure 2.

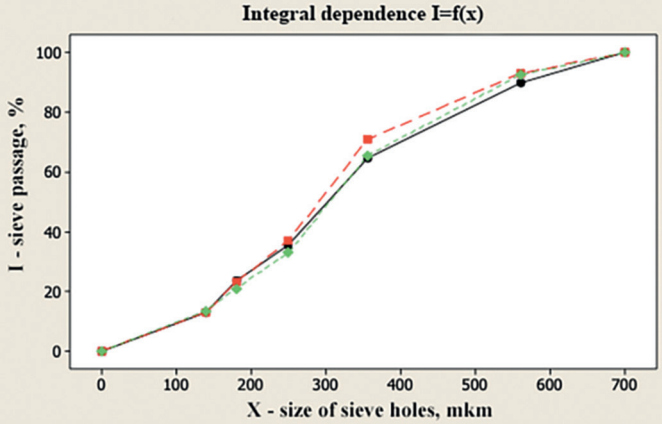

Figure 1. Grain-size composition of dunst products and flour obtained from I — III break systems with varying degrees of extraction: black color — 66%; red color — 72%; green color — 79%

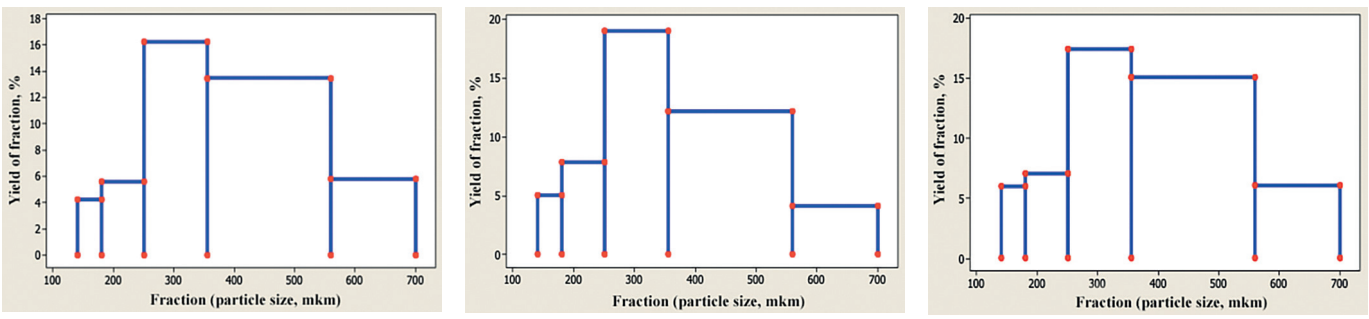

Figure 2. Fractional composition of grains obtained with I — III break systems with a total grains yield: a) — 43%: b) — 48%, c) — 52%

The bulk of the grains is characterized by a size of 250–560 microns, according to the classification [13] — this is a mixture of small and medium grains.

The optimal zone of the milling mode is determined, first of all, by the maximum endosperm content (minimum ash content) in the grains of break systems (Figure 3).

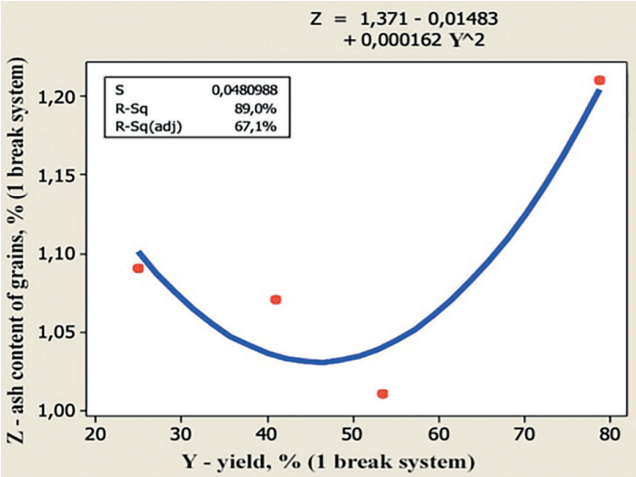

Figure 3. The dependence of the ash content of grains on the total yield (break I system)

The optimal milling conditions were characterized by the following values (yield / ash content): in terms of the first break system — 53.5 / 1.00; the second break system — 22.2 / 1.1; totally the first and the second break systems — 75.7 / 1.03 and the third break system — 5.3 / 2.07. Totally for the first, second and third break systems — 81.0 / 1.10.

Based on the analysis of the grains qualitative characteristics the principle scheme of a two-component grain mixture milling was formed, it included three break systems, one scratch, one sizing systems and five reduction systems. The yield of flour varied from 70 to 75%. The peculiarity of the technological scheme was that the break process was reduced, in fact, the selection of grains was carried out in the first and the second break systems, in the third break system only the dunst was selected. The flow of medium grains was directed to the sizing system, small grains were directed to the 1st reduction system and the dunst from the first, the second and the third break systems — to the 2nd reduction system.

Analysis of quantitative and qualitative characteristics of flour (yield, whiteness, ash) showed that the color of flax seeds has an important role in the market condition of flour and bread (Figure 4).

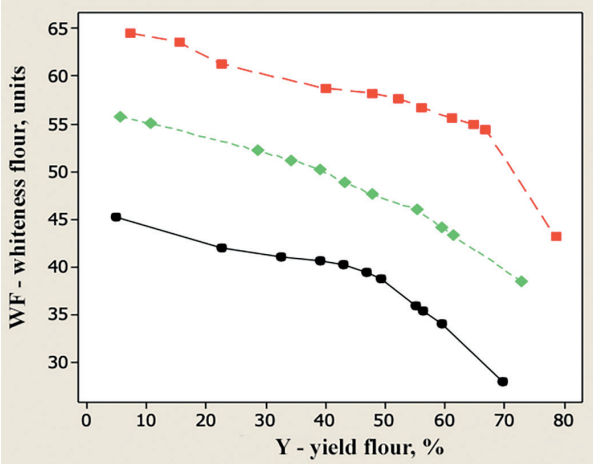

Figure 4. Cumulative curves of whiteness of flour: red color — wheat grain milling, black color — wheat + flax brown, green color — wheat + flax white

In addition, considering the non-uniform lipids distribution between individual flows, namely that the lipids content increases with the turn from the first to the last milling systems, and also taking into account the principle of the formation of flour varieties, which is based on the fact that the individual flows belong to different anatomical parts of the grain, flour varieties A, B and C were formed.

Variety A included flour flows from the central part of the endosperm — the first break system, the second break system, the sizing system, the first reduction system, the second reduction system, the third reduction system, — are characterized by low ash content and high whiteness value. Its yield was 45–50%, whiteness 50 units.

Variety B grade was obtained by mixing flour flows: third break system, the scratch system, the fourth reduction system, the fifth reduction system. It represents the crushed peripheral parts of the grain with a yield of 20–25% and a whiteness of 22 units.

Variety of flour C was obtained as a result of combining all flows of flour with a yield of 70–75% and a whiteness of 36 units.

The chemical composition of the formed flour varieties A, B, C, presented in Table 6, indicates the enrichment of wheat flour with protein and fat components, as well as fiber due to the inclusion of flax seeds in the binary grain mixture.

An analysis of the chemical composition of the formed flour varieties indicates an increase in the mass fraction of protein mass fraction by 1.0–2.0%, fat mass fraction in 1.5–3.5 times; fiber mass fraction by 3.4–4.0 times and a decrease in the mass fraction of starch by about 2–4%.

Table 6

The chemical composition of the formed varieties of flour

Sample	Protein (N × 6.25), %	Lipids, %	Starch, %	Cellulose, %	Reducing sugar, %
Wheat-flax flour, A variety	13.16	3.6	69.52	1.60	0.16
Wheat-flax flour, B variety	14.38	5.6	64.85	1.92	0.18
Wheat-flax flour, C variety	13.58	4.3	68.11	1.86	0.16
Wheat flour top grade (control)	12.65	1.6	72.10	0.46	0.14

The fractional composition of soluble proteins, the ratio of different fractions is important both for evaluating technological properties (gluten formation, its quantity and quality), and for the biological value of proteins, their assimilation degree [4,8,16,17,18,19]. The data presented in Table 7 demonstrate the ratio of soluble proteins fractions in the formed varieties of wheat-flax flour.

Table 7

The fractional composition of soluble proteins of the formed varieties of flour from a grain mixture based on wheat grain and flax seeds

Sample	The fractional composition of soluble proteins, % of the total				
	albumins	globulins	prolamins	glutelins	insoluble part
Wheat-flax flour, A variety	15.8	18.8	30.8	28.6	6.0
Wheat-flax flour B variety	13.2	18.5	29.6	29.8	7.8
Wheat-flax flour C variety	14.8	20.2	28.8	30.2	6.0
Wheat flour top grade (control)	8.4	17.0	35.8	30.8	8.0

The significant increase of the ratio of the albumin-globulin fraction content in wheat-flax flour samples to alcohol and alkali-soluble proteins content, as well as to its content in wheat flour, in which the part of gluten proteins prevails, should be marked.

When grain is processed into flour, the cell structure is destroyed, and as a result, oxidative and hydrolytic processes are enhanced [20]. In this regard, it is of interest to evaluate the activity of the main hydrolytic enzymes in samples of the formed varieties of wheat-flax flour. Thus, the value of proteolytic activity, along with other biochemical parameters, has fundamental importance, as proteinases are able to actively hydrolyze their own proteins, including the gluten ones, which, ultimately, affects the technological process and the finished product. In addition, proteolytic enzymes are involved in the regulation of the activity of other enzyme systems, for example, of amylases.

Amylases also assume major significance in assessing the quality of flour and products made from it. High amylolytic activity negatively affects its baking advantages.

In wheat flour, the substrate for the action of lipases is the flour's own lipids, the content of which can reach up to 1.5–2% of its mass, and in the studied samples of wheat-flax flour up to 3.6–5.6%. It is known that the use of lipase specimen leads to an improvement of the rheological properties of the dough, an increase of the specific volume of products, and an improvement of the crumb structure and color [4,5]. There is also evidence that lipases contribute to the retardation of the bread crumb, which can be explained by the action of hydrolysis products — monoglycerides and fatty acids, which, forming complexes with amylose, slow down its retrograde. It is supposed that lipases modify the interactions between proteins and lipids of flour, improving the gluten quality [19].

Moreover, lipolytic enzymes indirectly affect the oxidation processes in the dough during kneading, which is due to an increase of the availability of unsaturated fatty acids for the action of the lipoxygenase enzyme that is present in flour or introduced into the dough as part of improving agents.

Plant lipases are characterized by an optimum pH: cereal lipases mainly show their activity in the alkaline region — pH 8.0; oilseed lipases — In the acid region — pH 4.7 [21].

The unit activity of the main hydrolytic enzymes in the samples of the formed varieties of flour from a grain mixture based on wheat and flax seeds ispresented in Table 8.

Table 8

The unit activity of the main hydrolytic enzymes in the formed varieties of flour from a grain mixture based on wheat and flax seeds

Sample	UA* protease, units / mg protein		UA amylase units / mg protein	UA lipases, units / g	
	neutral	acid		alkaline	acid
Wheat-flax flour grade A	0.110	0.080	0.45	3.8	5.2
Wheat-flax flour grade B	0.120	0.090	0.60	3.8	6.0
Wheat-flax flour grade C	0.110	0.080	0.55	3.8	5.6
Wheat flour top grade (control)	0.100	0.070	0.50	3.8	0

* UA — unit activity

The unit activity of proteases and amylases in the studied samples of wheat-flax flour changes, but not significantly, and the activity of alkaline lipases (cereal lipases) remains unchanged, while the activity of acid lipases (oilseed lipases) is approximately 1.5 times higher than the activity of alkaline lipases in the studied samples of wheat-flax flour. As noted above, it occurs due to the presence of flax seed milling products and may affect the shelf life of this type of flour. However, the test samples storage in the laboratory at 4–6 °C for 14 weeks led to an insignificant increase of the acid lipases activity by 1.8–2.5%.

The fatty acid composition data (Table 9) of the formed flour varieties from a two-component grain mixture consisting of 93% of wheat and 7% of flax seeds allows us to draw the following conclusion: the content of linoleic acid (ω-6) in the wheat flour sample is 1.6 ... 3.3 times less than in the wheat-flax flour samples (0.93% against 1.51 ... 3.14%, taking into account the total lipids content in the samples); the content of linolenic acid (ω-3) in the wheat flour sample is 36.8 ... 57.2 times less than in the wheat-flax flour samples (0.047% against 1.73 ... 2.69%, taking into account the total lipids content in the samples).

Table 9

The fatty acid composition of the formed varieties of flour from a two-component grain mixture consisting of 93% of wheat grain and 7% of flax seeds

Indicator	The content of high fatty acids, %			
	wheat flour, top grade	wheat-flax flour, grade A	wheat-flax flour, grade B	wheat-flax, flour grade C
C 14: 0 myristic	< 0.1	< 0.1	< 0.1	< 0.1
C 16: 0 palmitic	19.64 ± 1.57	18.79 ± 7.50	12.54 ± 1.00	15.44 ± 1.24
C 16: 1 palmitoleic	< 0.1	< 0.1	< 0.1	< 0..1
C 17: 0 margarine	< 0.1	< 0.1	< 0.1	< 0.1
C 17: 1 margaroleic	< 0.1	< 0.1	< 0.1	< 0.1
C 18: 0 stearic	1.21 ± 0.13	5.79 ± 0.46	4.81 ± 0.53	5.26 ± 0.42
C 18: 1 oleic	17.54 ± 1.40	28.50 ± 1.43	22.54 ± 1.8	25.15 ± 0.02
C 18: 2 linoleic	**57.95 ± 2.90**	**41.21 ± 3.06**	**55.57 ± 2.78**	**49.97 ± 2.46**
C 18: 3 linolenic	**2.95 ± 0.32**	**48.80 ± 0.54**	**39.23 ± 0..43**	**45.10 ± 0.45**
C 20: 0 arachin	< 0.1	0.25 ± 0.03	0.17 ± 0.02	< 0.1
C 20: 1 gondoin	0.73 ± 0.08	0.58 ± 0.06	0.39 ± 0.04	0.31 ± 0.03
C 20: 2 eicosodienoic	< 0.1	< 0.1	< 0.1	< 0.1
C 22: 0 behennaya	< 0.1	0.29 ± 0.03	0.15 ± 0.02	0.15 ± 0.02
C 22: 1 eruca	< 0.1	< 0.1	< 0.1	< 0.1
C 22: 2 docosodienic	< 0.1	< 0.1	< 0.1	< 0.1

Conclusions

Technological schemes for the preparation and milling of two-component grain mixtures based on wheat grain and flax seeds are developed. The patterns of preparation and milling of binary grain mixtures to obtain composite types of flour with specified technological properties and increased nutritional value on account of the enrichment of the traditional types of grain by adding flax seeds with valuable nutritional components such as PUFA, essential amino acids, and other irreplaceable nutritional factors are revealed.

The use of whole flax seed in a binary grain mixture, consisting of 93% of wheat and 7% of flax seeds, allowed to balance the chemical composition of composite wheat-flax flour by the protein and lipids components, and also to enrich it with fiber, which means to use the entire phytopotential of flax seeds. Primarily, as the studies showed, the obtained wheat-flax flour contains the sufficient amount of PUFA in accordance with the recommended standards for consumption of grain-based food products [10], and the products made from it will help to make up the deficiency of ω-3 family PUFA in the diet of a modern person.

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The authors declare no conflict of interest.

Received 06.07.2020 Accepted in revised 25.08.2020 Accepted for publication 15.09.2020

DOI: https://doi.org/10.21323/2618-9771-2020-3-3-20-24

Available online at https://www.fsjour.com/jour
Original scientific article

PRODUCTION OF OLIGOFRUCTOSE SYRUP BY ENZYMATIC HYDROLYSIS FROM INULIN-CONTAINING CHICORY SYRUP

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KEY WORDS:
oligofructose, inulin, chicory, extract, syrup, purification, enzyme preparation, hydrolysis, carbohydrate composition

ABSTRACT

The purpose of the work is to study the enzymatic hydrolysis of inulin-containing syrups of various purification degrees from chicory chips in the production of oligofructose to be used in dietary, diabetic and health-preventive nutrition products. It has been determined that ion exchange purification of the syrup is necessary for hydrolysis. Individual stages of ion-exchange purification are specified using a two-stage scheme: K1-A1-K2-A2 and an additional stage on the "Macronet" sorbent MN200 to stabilize pH and remove bitterness taste. Requirements for the quality of syrup for hydrolysis have been developed: pH value — 4.5–5.0; chromaticity — not more than 0.5 units opt. den.; ash — not more than 0.2%; protein — 0.5%; no bitterness taste. Optimal conditions for hydrolysis of inulin-containing syrup have been established using Novozim 960 endoinulinase (Denmark): temperature — 55–58 °C; pH — 4.7–5.2; DS (dry substance) — 19%; preparation dosage — 0.4 units. INU/g of syrup DS; time 20–24 h. A sample of oligofructose syrup was obtained using the preparation "Novozim 960." The carbohydrate composition of oligofructose after the inulin hydrolysis was determined: fructooligosaccharides (FOS) — 70.12%; oligofructosides — 24.79%; disaccharides — 2.11%; fructose — 2.98%. Requirements for carbohydrate composition of oligofructose obtained by enzymatic hydrolysis of inulin-containing syrup have been developed: sum total of FOS and oligosaccharides — not less than 93%, sum total of di- and monosaccharides — not more than 7%.

FUNDING: The article was published as part of the research topic No. 0585–2019–0030-C-01 of the state assignment of the V. M. Gorbatov Federal Research Center for Food Systems of RAS

1. Introduction

In the modern world one of the main directions of development of food industry is production of products for functional and health-improving nutrition based on inulin-containing types of vegetal raw materials such as chicory and girasol [1,2,3]. A promising source for the production of sugar products for functional and recreational purposes is chicory, which is characterized by a convenient form of roots, unlike tuberous girasol for processing into inulin, and also by a high inulin content.

In world practice, inulin and oligofructose as the product of its partial directed enzymatic hydrolysis, being known as widely used prebiotics for industrial use, occupy a significant place among functional food products [4,5]. Inulin and oligofructose, obtained from chicory roots, have valuable healing properties: they improve the digestive system, ensure the growth of their own intestinal bifidoflora, increase immunity, improve calcium absorption, lower blood cholesterol and even reduce the risk of bowel cancer. Inulin and oligo-fructose are also used to improve nutritional and functional properties of food products [4,5].

Thus, inulin and oligofructose are widely used as low-calorie foods of dietary and diabetic nutrition, structural formers. All this contributed to the widespread development of research on the development of inulin and oligofructose technology. In recent years, in Western Europe, their output has increased tenfold and now it amounts about 150 thousand tons per year and annually experts estimate the market growth at about 10%. Daily inulin consumption in Europe is 2–12 g/person, in the USA it is 4 g/person. [1].

Oligofructose has high solubility, but does not crystallize and does not precipitate. Therefore, it is produced both as a powder and as a syrup containing 75% dry matter. By its technological properties and taste, it is similar to sugar and can partially and completely replace it in various recipes. However, its sweetness level is only a third of the sugar sweetness, so when completely replacing sugar, oligofructose is used in combination with intense sweeteners or fructose. It is also used for diabetic nutrition, since it has an extremely low glycemic index. At the same time, it masks the aftertaste of sweeteners, improves organoleptic indicators, bringing the taste of such products to the taste of traditional products prepared using sugar as much as possible.

In the preparation of oligofructose syrups, both mineral and organic acids as well as enzyme preparations and the so-called water-soluble inulinases [6,7,8], are used as a catalyst for the partial directed hydrolysis of inulin. The advantage of acid hydrolysis is a cheaper catalyst compared to enzyme preparations. There are known studies on the use of enzyme preparations of inulinase of various origins for partial or complete hydrolysis in the production of oligofructose and fructose [9,10,11]. Studies on the use of the immobilized preparation of inulinase for the production of oligofructose are given in this work [12,13,14].

Currently, oligofructose syrups in EU countries are produced by directed partial enzymatic hydrolysis using the water-soluble endoinulinase preparation from "Novozim 960" brand Aspergillus niger ("Novozims", Denmark). According to "Orafti" (Belgium), the carbohydrate composition of "Raftilose" oligofructose in accordance with the quality certificate is as follows: the permissible mass ratio of the sum of fructooligosaccharides and oligofructosides is 93.2–97.5%, the sum of disaccharides, glucose, fructose is 2.5–6.8%. "Kosukra" (Belgium) produces the so-called "Fibrulose" food fiber with an oligofructose content of 95–99%; sum of disaccharides, glucose, fructose is not more than 5%.

According to the "Unified sanitary-epidemiological and hygienic requirements for goods subject to sanitary-epidemiological supervision (control)", the level of FOS consumption is 5–10 g/day.

FOR CITATION: **Puchkova T. S., Pikhalo D. M., Karasyova O. M.** Production of oligofructose syrup by enzymatic hydrolysis from inulin-containing chicory syrup. *Food systems*. 2020; 3(3): 20–24. https://doi.org/10.21323/2618-9771-2020-3-3-20-24

In Russia, inulin and oligofructosis are not produced. The estimated need for inulin and oligofructose only for therapeutic nutrition in the country is at least 10 thousand tons per year [2].

The All-Russian Research Institute of Starch Products is conducting scientific research on the development of a universal technology for the processing of inulin-containing raw materials for inulin and oligofructose. As a result of the studies, the most optimal sequences of technological methods for producing inulin and its derivatives — oligofructose and fructose syrup [15,16,17] were developed. The developed universal technology for processing inulin-containing raw materials into inulin consists of the following stages: purification of raw materials from impurities; washing; scalding; cutting into chips; diffusion; coagulation; mechanical filtration; purification with active carbon and ion exchange resins; cooking and drying of inulin concentrate containing no more than 85% of inulin. Powder-like inulin with higher degree of purification containing at least 95% of inulin is obtained by additional ion-exchange purification, nanofiltration or chromatography, followed by cooking and drying. Methods of purification of inulin-containing syrups using various ion-exchange adsorbents are given in studies of ARRI starch products [18,19,20]. Choice of inulin technology depends on its required purity, as well as economic prerequisites [15,21]. Partial directional enzymatic hydrolysis of inulin is required to produce oligofructose.

The purpose of the present work was to study the hydrolysis of inulin-containing syrups from chicory chips of various degrees of purification using "Novozim 960" endoinulinase enzyme preparation (Novozimes Company) for the production of oligofructose syrups.

2. Materials and methods

Inulin-containing products such as extracts, syrups of inulin oligofructose syrups from raw or dry chicory chips were used as an object of research. The extract was obtained by hot aqueous extraction from chicory chips at a temperature of 80–85 °C. After acid-thermal coagulation and filtration, the extract was purified with active coal of the OU-B brand and on "Pyrolight" ion exchange resins with strong acid cationite C150, weakly basic anionite A103S and additionally with "Macronet" sorbent MN-200. Ion-exchange purification of inulin-containing syrup from chicory was carried out according to a two-stage scheme K1+A1+K2+A2 under the following conditions: temperature — 20–35 °C; transmission speed — 1.0–2 volumes of syrup with one volume of resin per hour. In the extract and syrup purified on ion exchange resins, the pH, chromaticity, protein, ash content, carbohydrate composition, optical density in the UV spectrum (presence of such products as hydroxymethyl furfurfurol — HOMF), as well as transparency and taste (absence of bitterness) were determined. For the studies, "Raftylin" inulin of "Orafti" (Belgium) was used as a control solution for hydrolysis.

Enzymatic hydrolysis of inulin was carried out using endoinulinase preparation "Novozim 960" with activity of 400 units INU/g of the "Novozimes" company (Denmark) under following conditions: T = 55–58 °C; pH 4.7–5.2;% DS=19; dosage — 0.4 units. INU/g DS of syrup. A sample of "Raftylose" oligofructose from "Orafti" chicory (Belgium) was used to compare the results of the studies on hydrolysis of inulin-containing syrups and inulin control solution. According to the company, the carbohydrate composition of "Raftilose" oligofructose in accordance with the quality certificate is as follows: the mass fraction of the sum of fructooligosaccharides (FOS) and oligofructosides is 93.2–97.5%, di- and monosaccharides is 2.5–6.8%.

The following analysis methods and instruments were used in the studies:

- mass fraction of moisture content in inulin and oligofructose on a moisture meter MF-50 (AND company, Japan);
- mass fraction of dry solids in analysed samples of extract, syrup and solution — on a refractometer IRF-454B2M;
- determination of the carbohydrate composition of extract, syrup, inulin and oligofructose on a liquid chromatograph of carbohydrates with a Gilson refractometric sensor;
- determination of the optical density of syrups — on a spectrophotometer SF 2000 (Design Bureau "Spectrum", St. Petersburg);
- chrominance determination — on photoelectrocorimeter KFK-3;
- weight fraction of protein by Kjeldahl method on a K-424 apparatus (BUCHI Labortecknik, Germany);
- determination of ash weight fraction, pH value and other parameters — according to methods adopted in the starch and beet sugar production [22].

3. Results and discussion

The article presents the results of studies on enzymatic hydrolysis of inulin-containing syrups from chicory chips of various degrees of purification to produce oligofructose syrups. Hydrolysis of the solution of inulin and inulin-containing syrups after ion-exchange purification, as well as chicory chips extract, was carried out using the "Novozim 960" enzymatic preparation endoinulinase from Aspergillus niger (Denmark). Enzyme activity is 400 units. INU/g preparation. Hydrolysis results were evaluated by the carbohydrate composition of the products obtained.

3.1. Development of requirements for the purification degree of chicory chips syrup for hydrolysis

In order to study the effect of the degree of purification of the initial inulin-containing syrup on hydrolysis in the production of oligofructose, studies were carried out to elaborate separate steps of ion-exchange purification of chicory extracts and syrups using "Purelight" resins (USA). The original extract obtained by aqueous diffusion and purified by acid-thermal coagulation was characterized by a high content of impurities and chromaticity: the weight fraction of protein is 4.2%; ash — 5.1%, chromaticity — 3.50 comp. units opt. den. Due to the presence of intibin glycoside in it, he had a strong taste of bitterness.

It was established that the bitterness taste of chicory extract during its purification using the "Macronet" sorbent MN200 completely disappears [18]. In this regard, to study the effect of the degree of purification of the initial extract on hydrolysis, it was purified without double ion exchange purification using only the "Macronet" sorbent MN200. The results of studies on the purification of chicory extract on the "Macronet" adsorbent showed that its chromaticity after purification decreased by more than 50%, as evidenced by a decrease of optical density in the ultraviolet region of the spectrum at a wavelength of 275–280 nm. The results of the UV spectrum extract analysis before and after purification are as follows (Figure 1) on the "Macronet" sorbent.

From Figure 1, it follows that the content of compounds like hydrooxymethylfurfurfurol-HOMF type (optical density γ = 275–285 nm), giving high chromaticity to the extract, after purification on the "Macronet" adsorbent MN200, decreased by 2.2 times. The absence of a peak in the purified product at this wavelength indicates a significant decrease of colorants content.

In addition, it should be noted that the mass fraction of protein, ash elements and other impurities in the extract from chicory purified on the "Macronet" adsorbent MN200 remained at a high level, but this stabilizes the pH (4.7–5.0). Therefore, further, in preparing the syrup for hydrolysis, it is not necessary to further adjust the pH to the optimal value for the action of the used enzyme preparation inulinase.

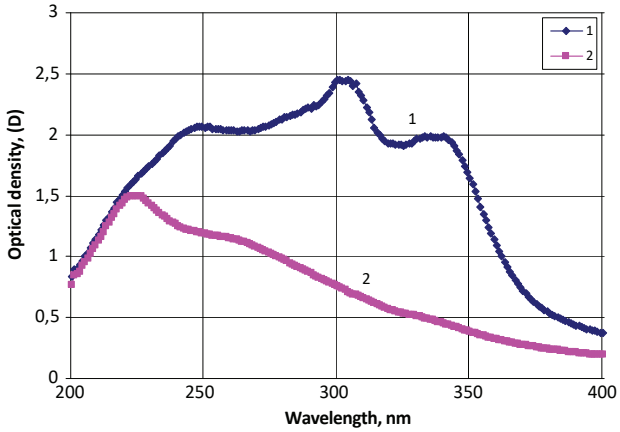

Figure 1. Optical density (D) versus wavelength: 1 — initial chicory extract; 2 — chicory extract after purification on the "Macronet" adsorbent MN200

To improve the quality of chicory extract purification and to remove the above impurities, preliminary double ion-exchange purification of the extract according to the K1-A1-K2-A2 scheme was carried out using resins: strong acid cationite grade C150, weakly basic anionite grade A103S. Despite satisfactory results in the removal of impurities ash content in the product is reduced to 0.2%, and the weight fraction of protein is up to 0.5%, the discoloration effect is 87%, however, there was a bitterness taste in the syrup. To remove the bitterness taste of the syrup, it was additionally cleaned on the "Macronet" adsorbent MN200.

Thus, it has been found that in preparation of chicory syrup for the hydrolysis, with the enzymatic preparation of inulinase it is required obligatory double ion exchange purification. At the same time, it is possible to reduce the mass fraction of impurities such as protein, colorants and minerals by ten times. At the final stage of syrup purification it is necessary to use "Macronet" sorbent MN200 for its additional purification, removal of bitterness taste and stabilization of pH syrup before hydrolysis.

As a result of studies, requirements for the quality of chicory syrup after the above purification methods for obtaining oligofructose by enzymatic hydrolysis using inulinase brand "Novozim 960" were developed:

- pH value — 4.5–5.0;
- chromaticity — not more than 0.5 units opt. square;
- ash mass fraction — not more than 0.2%;
- protein weight fraction — 0.5%;
- lack of taste of bitterness.

In the preparation of oligofructose syrup, "Raftylin" inulin of "Orafti" (Belgium) was used as a control solution for the hydrolysis.

As a result of studies for enzymatic hydrolysis in the production of oligofructose syrup, quality indexes of both the control solution of inulin "Raftylin GR" and the inulin-containing products from chicory chips of various purification degrees were determined (Table 1).

The studies showed that the maximum degree of purification and the optimal carbohydrate composition (minimum amount of disaccharides and absence of monosaccharides) has a control solution of inulin "Raftylin GR" (Table 1). After the two-stage ion-exchange purification of extract, weight fraction of impurities in the purified syrup decreases tenfold. However, after ion exchange purification, fructose appears in the syrup, which is undesirable in the production of oligofructose. The extract from chicory chips after purification on the "Macronet" sorbent MN200 has a high content of impurities (ash and protein). Attention should be paid to the carbohydrate composition of the obtained samples. The total weight fraction of di- and monosaccharides in the initial syrups is significantly different from the control sample. In the chicory syrup after ion-exchange purification and, especially in the chicory chips extract, their weight fraction is more than 12%, and in control inulin solution only 5.76%.

Table 1

Characteristics of inulin-containing products of varying degrees of purification for hydrolysis in the production of oligofructose

Sample name	Mass fraction, %		Weight fraction of carbohydrates, %				
	ash	protein	inulin	oligo-fructosides	disacharides	glucose	fructose
Control solution of inulin «Raftylin»	0.03	0.00	89.25	4.99	5.76	0.00	0.00
Chicory extract after cleaning on "Macronet"	3.1	4.5	78.38	9.20	9.72	0.00	2.7
Chicory syrup after purification K_1-A_1-K_2-A_2	0.2	0.5	83.24	4.51	6.85	0.00	5.39

3.2. Hydrolysis studies on chicory chips syrup of various purification degrees

Hydrolysis of the control solution of inulin, the inulin-containing extract and the syrup after the above purification methods was carried out with the enzymatic preparation of endoinulinase "Novozim 960." Hydrolysis of inulin to produce oligofructose was carried out under the following conditions: T = 55–58 °C; pH — 4.7–5.2; DS = 19–20%; dosage of the preparation 0.4 units. INU per DS syrup; time — 24 h. To compare the obtained results after the hydrolysis of inulin, a chicory oligofructose was used as a control sample.

After hydrolysis of the above samples of inulin-containing syrups with various degrees of purification, the carbohydrate composition of oligofructose syrups and the "Raftylose" sample was determined (Table 2).

Table 2

Carbohydrate composition of samples of "Raftylose" oligofructose and oligofructose syrups after hydrolysis

Sample name	Carbohydrate composition of samples after hydrolysis,%						
	FOS	oligofructosides	∑FOS and oligofructosides	disacharides	glucose	fructose	∑ di- and monosacharides
"Raftiloza"	70.12	24.79	94.91	2.11	0.00	2.98	5.09
Syrup from «Raftylin» inulin	73.04	22.79	95.83	1.74	0.00	2.43	4.17
Chicory syrup after «Macronet»	62.23	25.69	87.92	2.87	4.01	5.20	12.08
Chicory syrup after K_1-A_1-K_2-A_2	60.82	31.93	92.75	1.96	2.27	3.02	7.25

Comparison of the carbohydrate composition after hydrolysis of chicory syrup of various purification degrees (on the "Macronet" adsorbent and after K1-A1-K2-A2) with the carbohydrate composition of the control sample of "Raftilose" oligofructose shows a significant effect of impurities of the initial syrup and its carbohydrate composition on enzymatic hydrolysis. The optimal carbohydrate composition (a small amount of disaccharides and the absence of monosaccharides) in the "Raftylin GR" solution of inulin makes it possible to obtain an optimal composition for oligofructose, similar to the "Raftylose" oligofructose.

When using an extract purified only on the "Macronet" adsorbent (Table 2), hydrolysis proceeds to form a small amount of oligofructosides and an increased fructose and glucose con-

tent. The sum total of FOS (fructooligosaccharides) and oligofructosides is below 90% (87.92%), and the sum total of di- and monosaccharides has a high value — 12.08%. This is probably due to the presence in the original extract for hydrolysis of a high content of oligofructosides, disaccharides — a substrate for hydrolysis, as well as fructose. The increased weight fraction of impurities in the extracts (protein — 6.8%, ash — 3.2%), as well as its carbohydrate composition do not contribute to the optimally required carbohydrate ratio during hydrolysis of the inulin-containing extract.

Thus, in the hydrolysis of an extract from chicory with a low degree of purification (only using the "Macronet" sorbent), it is not possible to achieve the required quality of the carbohydrate composition of the oligofructose syrup, both due to the influence of the contained impurities and its carbohydrate composition.

It was found that after a two-stage ion exchange purification (Table 1) content of mono and disaccharides in chicory syrup is higher compared to the control sample of inulin and makes: disaccharides — 6.85% and fructose — 5.39%. Despite this, hydrolysis of chicory syrup after ion exchange purification (Table 2) proceeds with the formation of an optimal carbohydrate composition: the sum total of FOS and oligofructosides is 92.75%, and the sum total of di- and monosaccharides is 7.25% (including disaccharides — 1.68%, glucose — 2.27%, fructose — 3.02%).

The obtained results indicate the need to control the carbohydrate composition of the initial syrup for hydrolysis (absence of glucose, minimum content of mono- and disaccharides, as well as thorough purification of inulin-containing syrup from impurities before hydrolysis).

To obtain oligofructose syrup with optimal ratio of carbohydrate composition for di- and monosaccharides, it is necessary to use initial inulin-containing syrup containing no glucose for hydrolysis, disaccharides — less than 5.5%, as well as the sum total of mono- and disaccharides — less than 7%.

Thus, during enzymatic hydrolysis of chicory syrup, a thorough ion exchange purification according to a two-stage scheme is required to obtain oligofructose: K1-A1-K2-A2 and additionally on the "Macronet" sorbent MN200 to stabilize the pH value and remove bitterness flavor, as well as control over the carbohydrate composition of the initial syrup for hydrolysis.

In laboratory conditions, a sample of oligofructose syrup was obtained using the above scheme for purifying chicory chips syrup before hydrolysis. The following is a carbohydrate composition of the laboratory sample (Table 3) obtained at a dosage of 0.4 INU/g DS, a hydrolysis duration of 20 hours and a control sample of "Raftilose" oligofructose. The laboratory sample of oligofructose syrup differs from the control one by a slight increase in the sum total of di- and monosaccharides — 6.82%, the sum total of FOS and oligofructosides corresponds to the required value — 93%.

Table 3

Carbohydrate composition of oligofructose samples

Sample Name	Carbohydrate composition, %						
	FOS	oligofructosides	∑ FOS and oligofructosides	disaccharides	glucose	fructose	∑ di- and monosacharides
Control sample "Raftylose"	70,12	24,79	95,01	2,11	—	2,98	5,09
Laboratory sample	70,85	22,32	93,17	3,24	—	3,58	6,82

The obtained data correspond to the quality certificate indexes for "Raftylose" oligofructose of "Orafti" Company for the mass fraction of the sum total of FOS and oligofructosides, as well as di- and monosaccharides. That indicates the absence of glucose in both control and laboratory samples.

Based on the studies, the requirements for the carbohydrate composition of oligofructose from inulin-containing syrup were developed: the sum total of high-molecular fructooligosaccharides (FOS) and oligofructosides is at least 93%, the sum of low-molecular saccharides (di- and monosaccharides) is not more than 7%.

The significance of the work is the creation of domestic technology of oligofructose from inulin-containing chicory extract as a component for therapeutic and preventive nutrition of the population and ensuring its import substitution

4. Conclusion

To study the effect of the degree of purification of the initial inulin-containing syrup on hydrolysis in the production of oligofructose, studies were carried out to clarify individual steps of ion-exchange purification of chicory extracts and syrups using "Purelight" resins (USA).

Separate steps of ion-exchange purification of chicory extracts and syrups in production of oligofructose syrup are specified. It was established that in preparation the syrup for enzymatic hydrolysis it is required an obligatory double ion exchange purification and an additional purification are on the "Macronet" sorbent MN200 to remove bitterness flavor and stabilize the pH of the syrup. At the same time, it is possible to reduce the mass fraction of such impurities as protein, colorants and minerals by ten times.

Requirements for the quality of syrup after ion-exchange purification for hydrolysis with the enzyme preparation inulinase have been developed: pH — 4.5–5.0; chromaticity — not more than — 0.5 units opt. square; ash mass fraction — not more than 0.2%; protein weight fraction — 0.5%; lack of bitterness.

Enzymatic hydrolysis of inulin-containing syrup was carried out under the following conditions: T = 55–58 °C; pH — 4.7–5.2; % DS=19; dosage — 0.4 units. INU/g DS syrup, duration 20–24 h. A sample of "Raftylose" oligofructose from "Orafti" chicory (Belgium) was used to compare the hydrolysis results of the control solution of "Raftilin" inulin and inulin-containing syrups.

Comparison of obtained results on hydrolysis of inulin solution of "Raftylin" brand, extract after purification only on "Macronet" sorbent MN200 and syrup after double ion-exchange purification shows significant influence of impurities of initial syrup and its carbohydrate composition on enzymatic hydrolysis.

With the hydrolysis of "Raftylin GR" inulin, its maximum degree of purification and optimal carbohydrate composition allow to obtain the carbohydrate composition required for oligofructose, similar to "Raftylose" oligofructose.

The hydrolysis of chicory syrup after ion exchange purification also proceeds with the formation of an optimal carbohydrate composition: the sum total of FOS and oligofructosides is 92.7%, and the sum total of di- and monosaccharides is 7.25%.

The obtained results indicate the need to control the carbohydrate composition of the initial inulin-containing syrup before hydrolysis (absence of glucose, content of di- and monosaccharides — less than 7%).

Thus, during the enzymatic hydrolysis of chicory syrup, a thorough ion exchange purification according to a two-stage scheme is required to obtain oligofructose: K1-A1-K2-A2 and additionally on the "Macronet" sorbent MN200 to stabilize the pH and remove bitterness flavor.

A laboratory sample of oligofructose from chicory syrup purified according to the proposed scheme is obtained, the carbohydrate composition of which corresponds to the data of the quality certificate for the sample of oligofructose brand "Raftilose" according to the weight fraction of the sum total of FOS and oligofructosides, as well as di- and monosaccharides.

Based on the studies, the requirements for the carbohydrate composition of oligofructose from inulin-containing syrup were developed: the sum total of FOS and oligofructosides — at least 93%, the sum total of di- and monosaccharides — no more than 7%.

In Russia the organization of the production of inulin, oligofructose and food products based on them is an important national economic task. Due to the lack of production of inulin and oligofructose from inulin-containing raw materials in Russia, the development of their technology is very relevant and promising.

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The authors declare no conflict of interest.

Received 30.06.2020 Accepted in revised 26.08.2020 Accepted for publication 15.09.2020

DOI: https://doi.org/10.21323/2618-9771-2020-3-3-25-31

Available online at https://www.fsjour.com/jour
Original scientific paper

IMPACT OF ARTISANAL TECHNOLOGIES ON THE QUALITY INDICES OF THE COZONAC

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KEY WORDS:
cozonac, sourdough, fermentation, artisanal technologies, soriz (Sorghum oryzoidum), nutritional aspects

ABSTRACT
The research focused on studying the impact of sourdough with spontaneous flora (SSF) (obtained also from gluten-free flour) and artizanal technologies on the quality indices of cozonac — a traditional pastry product.
Physico-chemical and microbiological indices were analyzed, as well as glycemic index (GI) of cozonac samples with different fermentation agents. The experimental results showed that the GI of the cozonac samples have similar values, being between GI = 68 and GI = 71. Respectively, cozonac with SSF samples can be classified in the category of foods with moderate GI, and cozonac with commercial yeast (CY) — in the category of foods with high GI. Digestibility indices for all samples reached values between 72% and 76%. The sensory profiles of baked cozonacs were analyzed descriptively by the panel members, and the results showed that all cozonac samples were characterized by slightly acid taste, with specific and pleasant flavors.
However, it seems that a long fermentation of the dough, even if CY is used as a fermenting agent, leads to the formation of quality indices of the dough and cozonac, very close to the products obtained only with SSF.
The use of SSF from sorghum flour would be an alternative in the development of gluten free bakery and pastry products using artisanal technologies.

1. Introduction

Technologies inspired by nature for a sustainable future! These are the trends of the modern consumer: quality products based on "bioinspired" technologies, obtained from natural and quality materials. Trends that are found in the first objectives of the 2030 Agenda for Sustainable Development are a challenge for a healthy future! Consumer concern about traditional artisanal products is on the rise [1]. This situation generates a demand for agricultural or food products with certain identifiable characteristics, in particular as regards their geographical origin. Currently, there is an upward trend of consumers to benefit from products not only with optimal organoleptic properties, but also with increased nutritional values.

Symbol of the fundamental holidays of Christianity, the cozonac also has the symbolism of bread: bread, in its Eucharistic form, refers to active life. And the active principle of fermentation becomes a symbol of spiritual transformation. Cozonac is a sweet bread, traditional in the Republic of Moldova, Romania, Bulgaria (kozunak), being traditionally cooked for Easter or Christmas. A similar sweet is "panettone" in Italy, in Russia "kulichi", in Ukraine and Belarus "paska". According to some sources, the cake in the form and recipe known in the Republic of Moldova and Romania by all of us, is a Romanian invention.

The Bulgarian researcher, ethnologist Donka Sabotinova, says that he arrived in Bulgaria around 1915, brought from Romania by merchants around the Easter holidays, traded under the name of kozunak. It seems that the name cozonac comes from the Greek — κοσωνάκι (kosonáki).

Cozonac in its simplest form is a sugar-sweetened flour mixed with eggs, milk, fat and a fermenting agent, but for every country and region there is a huge variety of recipes.

Currently, sourdough with spontaneous flora (SSF) is used more and more frequently in the production of artisanal products such as: panettone, pandoro, cozonac, typical regional bakery products, bringing rheological, sensory and nutritional benefits, as well as extending the shelf-life of the product. Sourdough is seen as an intermediate transition between the mixture and the final product, in which the active metabolic microorganisms modify the original characters of the initial ingredients (water and flour), a process that stimulated the interests of researchers [2,3].

There are multiple methods of making SSF. In general, this involves a mixture of water and flour and possibly salt and sugar, which is left to ferment for about 24 hours. At this stage the yeasts and lactic bacteria, naturally present in the flour, produce CO2 and organic acids. Reducing the pH activates the flour proteases, which, together with the hydrolytic enzymes of the bacteria, act on gluten, leading to a reduction in the consistency (fluidization) of the mixture. In the second stage, the mixture is refreshed to ensure oxygenation and provide a new nutrient substrate for microorganisms. The refreshments are repeated at certain intervals, until the fermentation capacity is kept constant. It is considered that the optimal time to make the refresh is determined by increasing the volume of the mixture about 3–4 times compared to the initial volume [4].

Regardless of the procedure involved, the microbiological composition of the SSF is represented by a mixture of lactic bacteria and yeasts in a ratio of 100: 1, with respective values of 109 and 107 CFU / g [4,5].

It is considered that the use of SSF in the manufacture of bakery and pastry products has many advantages:

- improving the rheological properties of the dough (by accumulating metabolites, respectively amino acids);
- obtaining products with a better flavor and texture compared to products fermented only with commercial yeast;
- improving the nutritional values of products by increasing the bioavailability of minerals and reducing the glycemic index;
- increasing the shelf life of the products, through the inhibitory effect on molds possessed by organic acids, formed during fermentation;
- low pH inhibits amylase activity, so that starch degradation is avoided;
- fermentation with SSF improves water binding capacity, starch swelling and solubility of pentosans etc.

FOR CITATION: **Siminiuc R., Turcanu D.** Impact of artisanal technologies on the quality indices of the cozonac. *Food systems*. 2020; 3(3): 25–31. https://doi.org/10.21323/2618-9771-2020-3-3-25-31

The aim of the research is to study the impact of artisanal technologies (the use of SSF and the long fermentation time) on the quality indices of cozonac — a traditional product of the Moldovan people.

2. Materials and methods

The quality of finished products depends on several factors, among which the most important are: the quality of the raw material and the technological process. For the preparation of SSF, as well as cozonacs, 2 types of local flour were used:

- high quality wheat flour (origin: Măgdăcești village)
- sorghum flour (*Sorghum Oryzoidum*) (SC „Andigor").

The quality indices of the flours used are presented in Table 1.

Table 1

Physico-chemical indices of flour samples [6,7]

Quality index	Wheat flour	Soriz flour
Acidity, (degrees)	2.8±0.2	3.2±0.3
Dry substance, %	13.45±0.3	12.7±0.2
Wet gluten, (%)	26.18±0.7	—
Dry gluten, (%)	61.0±1.5	—
Hydration capacity, (%)	51.0±0.5	100.0±1.0
Maltose index, g / 100 g	0.5±0.2	2.3±0.1

The research was performed on the following cake samples:

- Cozonac of wheat flour with commercial yeast (CY) — standard sample;
- Cozonac of wheat flour with sourdough with spontaneous flora (SSF) of wheat flour;
- Cozonac of wheat flour with sourdough with spontaneous flora (SSF) of soriz flour.

SSF were obtained in laboratory conditions, by mixing wheat / soriz flour with water in proportions of 50/50. The mixture obtained, being subjected to fermentation under the influence of spontaneous flowering for 66 hours with periodic refreshments (Figure 1).

In order to characterize and evaluate the quality of cozonacs with SSF, the parameters mentioned in Table 2 were identified.

Table 2

Methods for determining the quality indices of cozonac

Quality indices	References
Loss of mass at baking	GOST 21094–75 [7]
Moisture	GOST 21094–75 [9]
pH	26-PH-metru-WTW-Inolab-ph-7110
Porosity and elasticity of the crumb	GOST 5669–96 [10]
Microbiological indices (the total number of yeasts and molds)	GOST 10444.1 2–88 [11]
Glycemic index (*in vivo* method)	ISO 26642–2010 [12].
Protein digestibility *in vitro*	Sudeash, J. et al. [13]
Sensory indices	GOST ISO 6658–2016 [14]

The glycemic index of the tested samples was determined in vivo by monitoring the blood glucose level of the experiment participants until and after the consumption of the researched food products, according to ISO 26642: 2010 [12]. The glycemic response after consumption of each product was compared with that stimulated by glucose consumption as a reference substance [15]. The data obtained were used to construct the glycemic response curves of the participants after consuming the tested samples. The area of the surface under curves was determined by mathematical method with the help of AutoCAD through the program "Inquiry" which calculates exactly the area of the surface. Finally, the glycemic index was calculated according to the formula:

$$GI = \frac{Sa}{Sg} 100; \quad (1)$$

where:

GI — glycemic index of the analyzed food;
Sa — the surface area under the glycemic curve of the studied food;
Sg — the surface area under the glucose curve of glucose.

The blood glucose in the capillary blood of the experiment subjects was determined by the endpoint glucose oxidase method at the biochemical analyzer "STAT-FAX 1904" [16]. Principle of the method: Glucose, under the action of glucosidase, is con-

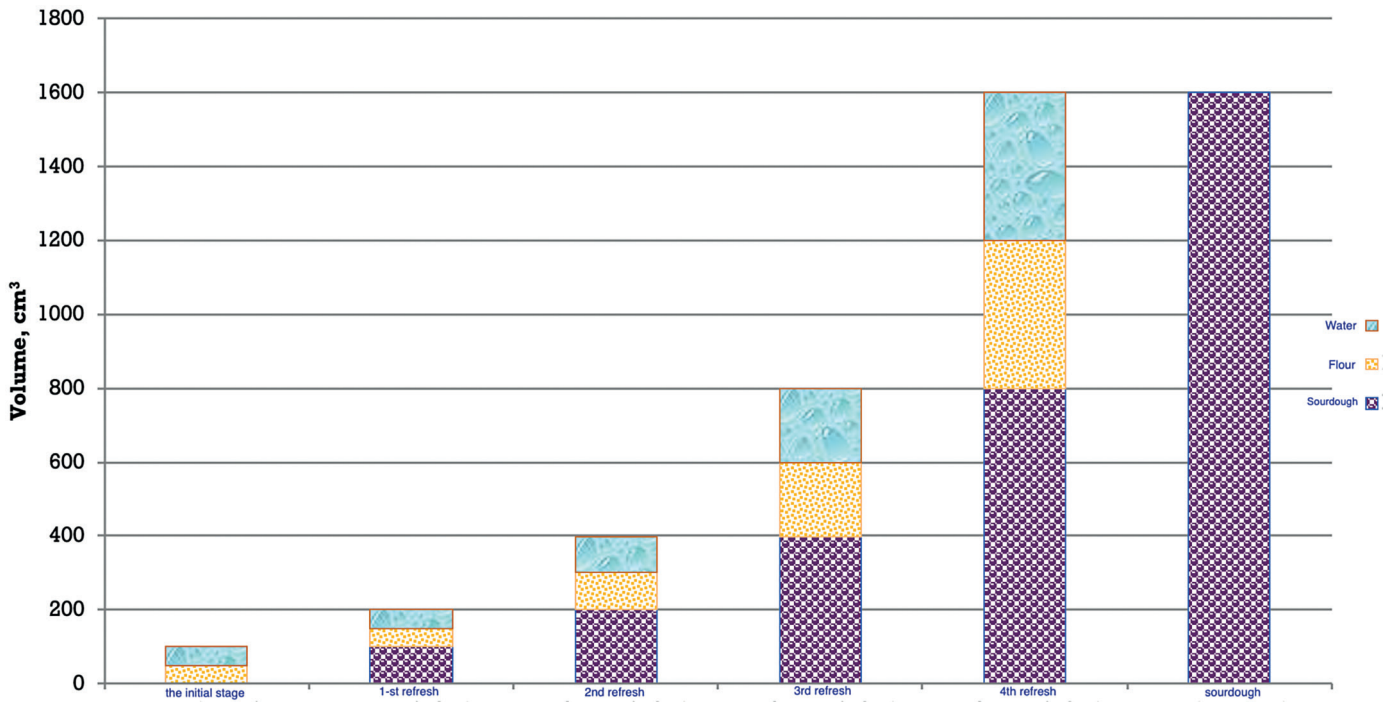


Figure 1. General scheme for obtaining SSF

verted into gluconic acid. The resulting H2O2 will be decomposed by peroxidase, following the reaction in which the Trinder indicator (phenol and 4 amino antipyrine) also participates, resulting in a red-colored condensation product with maximum absorption at λ = 505 nm. Extinction is directly proportional to glucose concentration [17].

3. Results and discussion

Cozonac, a typical traditional product, symbol of Christmas and Easter, once cooked only by artisanal technologies, now broadcast at industrial level, is characterized by a strong spongy core, consistency and flavor unmistakable. The cozonacs were obtained from the culinary technology laboratory of the Food and Nutrition Department, Technical University of Moldova, by the indirect method according to the technological scheme (Figure 2):

3.1. Physico-chemical indices

The average *humidity* values of the cozonac samples were about: 25% for the cozonac with CY, 26% for the cozonac with SSF from wheat flour and 23% for the cozonac with SSF from soriz flour. In the last stage of the technological process, the product undergoes physical / structural and biochemical changes, which are crucial for acquiring rheological, sensory and nutritional characteristics. The samples obtained, after baking, were cooled to t — 18–20 C for 8 hours, to strengthen the structure of the product after which they were subjected to determinations [18].

The values of *mass losses* at baking were close to all samples, being between 10–13% (Figure 3). The smallest being in the samples of soriz flour, probably due to the specific feature of agglutinative flours to retain a larger amount of water. The loss of mass results from the loss of moisture, from the outer layers of the dough. In the literature these losses are between 6–22% [4, 19].

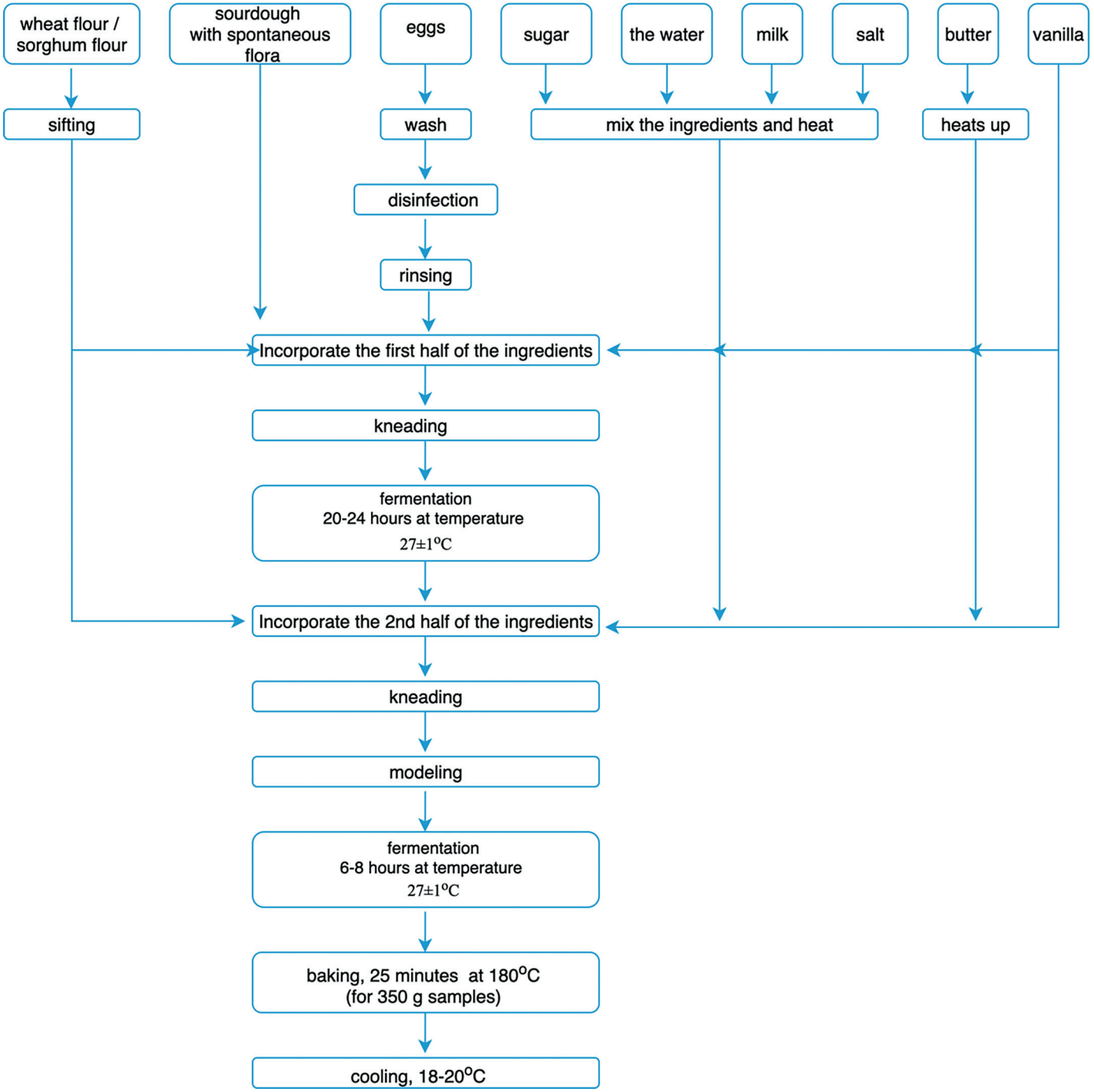


Figure 2. General technological scheme for obtaining the cozonac

The *porosity* of the investigated cozonac samples was between 85% (for the wheat SSF cozonac) and 79% (for the sorghum SSF cozonac) (Figure 4). The porosity of the SSF cozonac sample from wheat flour is 1% higher than the standard sample (CY cozonac) (Figure 4).

The elasticity was between 76 ÷ 96%, the most elastic being the cozonac with SSF from wheat (96%), and the least elasticity was the cozonac with SSF from soriz (76%), probably due to the gluten free properties of the sorghum flour from which the SSF was obtained. However, the differences between values, for all cozonac samples, are not considerable and could be explained by the formation of acids in the fermentation process, and as a result there is swelling and development of gluten proteins, which potentially contributes to a more efficient intercalation within the elastic network (Figure 5).

The pH was 4.46 in the CY cozonac, in the wheat flour SSF cozonac- pH = 4.52 and pH = 4.54, respectively, in the sorghum SSF cozonac and is due to organic acids, in particular lactic acid formed in the fermentation process of SSF and subsequently of the dough.

The acidity values for all samples are close, because even in doughs fermented with commercial yeast, the acidification is due primarily to the lactic acid produced by the lactic microbiota when the fermentation exceeds 8–12 hours and, secondly, to the production of succinic acid by Saccharomyces cerevisiae [20,21,22]. The pH of the cozonac samples decreased slowly during the storage period (30 days) to 4.40, 4.49 and 4.52, respectively. The results obtained are close to the values for artisanal Italian panettone whose pH initially had values of 4.54 and decreased to 4.12 after 180 days of storage [19].

3.2.Microbiological indices

To determine the total number of yeasts and molds, inoculation was performed on Yeast Extract Glucose Chloramphenicol Agar (YGCA), followed by incubation under aerobic conditions at 25oC for 5 days. The results are shown in Table 3 and are characteristic for artisanal bakery products with SSF [19].

Table 3

Microbiological indices of cozonac samples

	Cozonac samples	the total number of yeasts and molds, CFU
1.	cozonac with commercial yeast	0.78±0.8
2.	cozonac with SSF of wheat flour	0.79±1.0
3.	cozonac with SSF of soriz flour	0.78±0.9

3.3.Nutritional indices

In vitro protein *digestibility*. Heat treatment beyond 95 °C is considered to have both a beneficial and inhibitory influence on

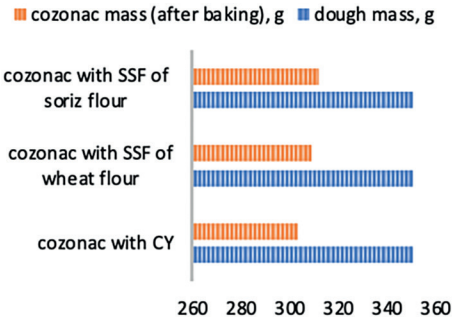


Figure 3. Baking mass losses

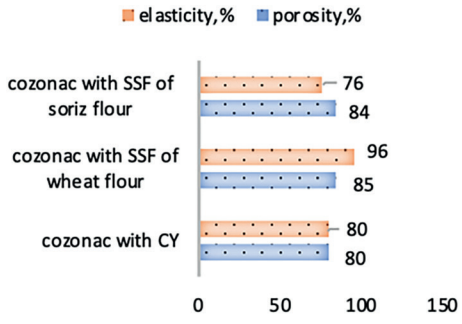
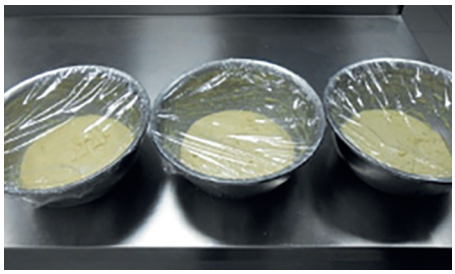


Figure 4. The impact of SSF on the elasticity and porosity of cozonacs

Kneading (40 min) after incorporating the first half of the ingredients (formation of the gluten network)



Initiation of the first fermentation (20–24 hours)

After 24 hours of fermentation

Second fermentation (after 6–8 hours at T = 27 ± °C)

Baking at 180 °C

Cozonac samples after baking

Figure 3. Images of the process of obtaining cozonacs

protein digestibility. Beneficial effects include inactivation of digestive enzyme inhibitors and development of heat-induced protein structure, while aggregation of denatured proteins resulting from new molecular interactions (little or no attack of digestive enzymes) and have the opposite effect. It is also considered that a major impact on digestibility has a microstructure of gels, formed by proteins, starch, etc. which affects the diffusion of enzymes in the food system and therefore the enzymatic degradation of proteins [23,24]. The digestibility values (with trypsin) of the cozonac samples reached values between 72 and 76% (Table 4).

Table 4

Degree of digestibility of cozonac proteins

	Cozonac samples	Digestibility, %
1.	cozonac with commercial yeast	72±1.0
2.	cozonac with SSF of wheat flour	76±1.0
3.	cozonac with SSF of soriz flour	75±1.6

The values of the digestibility indices of the cozonacs could be explained by the fact that all the samples (although they had different fermentation agents), took place in identical conditions of time and environment. In the literature there are indices of protein degistibility in bakery products, with values between 79.96 and 80.62 [25].

Glycemic index. The average pre-prandial glycemia of participants in the experiment was in the optimal range of 3.8±0.8 mmol/l. After consuming the samples examined maximum glycemia was reached over 30 minutes.

The experimental results showed that the GI of the cozonac samples have similar values, being between GI = 68 and GI = 71 (Figure 6). Although GI values do not differ significantly, cozonacs with SSF can be included in the category of foods with moderate GI, and cozonacs with CY — in the category of foods with high GI [15,26,27,28]. However, experiments on GI are required, given the multitude of factors that can influence this index. According to the literature, the values obtained are characteristic of bakery and pastry products: cupcake — GI = 73 ± 12, croissant-GI = 67, bread au lait — GI = 63 ± 10, baguette-GI = 95 ± 15 [26].

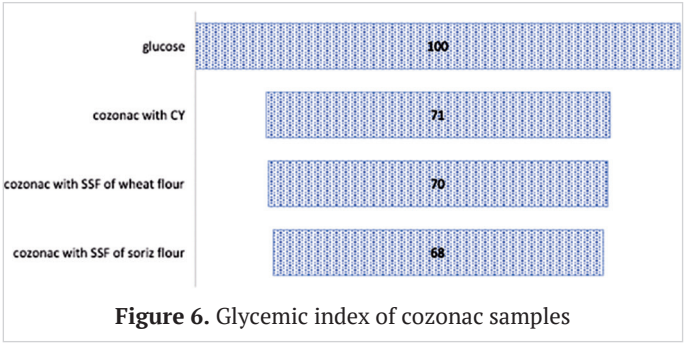


Figure 6. Glycemic index of cozonac samples

3.4. Sensory indices

As mentioned in the literature, any change in the technological process of obtaining cozonacs or in the development of the recipe can lead to changes in their quality. The volatile compounds in the kernel derive, in particular, from the fermentation process of SSF, from the oxidation of lipids in flour and, to a lesser extent, from the Maillard reaction, while the aroma of the crust is mainly due to the Maillard reaction. The sensory profiles of baked cozonacs were analyzed descriptively by the panel members (specialists from the Department of Food and Nutrition, Technical University of Moldova), assessing the intensity of each (Table 6) and a hedonic parameter — as a general assessment [29,30].

Table 5

Evolution of glycemia after glucose and cozonac samples consumption

	Time, min							
Sample	0	15	30	45	60	90	120	180
	Glycemia, mmol/l							
glucose	3.8±0.8	5.6±1.1	6.8±1.2	5.6±0.9	4.5±0.7	4,9±0.9	4.4±0.6	3.7±0.4
cozonac with CY	3.8±0.7	4.8±0.8	6.1±0.8	5.2±0.2	4.5±0.4	4,3±0.4	4.2±0.6	4.1±0.6
cozonac with SSF of wheat flour	3.8±0.6	4.8±0.6	5.9±0.9	5.0±0.4	4.6±0.4	4,4±0.4	4.1±0.6	4.1±0.6
cozonac with SSF of soriz flour	3.8±0.6	4.9±0.8	5.8±0.9	5.5±1.0	4.5±0.5	4.2±0.5	4.1±0.5	4.0±0.5

Table 6

Sensory descriptors used to describe separately the crumb and the crust of cozonac

Sensorial evaluation of cozonac crumb			
Aspect	Smell	Taste	Structure
Intensity of color	Cereals	Sweet	Elasticity
Luminosity	Acetic acid	Acid	Deformability
Density	Hay	Bitter	Resistance to chewing
Porozity	Yeast	Cereals Flavor	Surface moistness
Homogeneity	Rancid	Hay Flavor	Compactness
		Yeast flavor	Cohesiveness
		Astringent	Juiciness
		Aftertaste	
Sensorial evaluation of cozonac crust			
Aspect	**Smell**	**Taste**	**Structure**
Intensity of colour	Intensity of smell	Sweet	Structure regularity
Regularity of colour	Cereals	Acid	Hardness
Tonality of colour (yellow/brown)	Fragrant	Bitter	Friability
	Roasted	Hay flavor	Crispness
	Burned	Yeast flavor	Resistance to detachment (crust/crumb)
		Astringent	
		Aftertaste	

The cozonacs obtained with different fermentation agents (CY, SSF with wheat flour, SSF with sorghum flour) were characterized by close sensory profiles. Cozonacs with SSF from sorghum flour had the weakest sensory profile in terms of structural characteristics, but the best aromatic profile. The wheat flour SSF cozonac showed optimal structural characteristics of the core and crust, and the CY cozonac, despite a good consistency, had an uneven crust both in color and structure. All cozonac samples were characterized by slightly acid taste, with specific, pleasant flavors.

4. Conclusions

- The tendencies of specialists in the field of producing with artisanal technologies are based on the use of local traditional raw materials, which give the products specific texture and sensory characteristics;
- The artisanal processes in the elaboration of cozonacs do not necessarily involve high quality flours, but they require increased amounts of fluids (water, milk etc.) and a long kneading (about 40 minutes) for the formation of the gluten network;
- SSF is the key element in the development of artisanal products due to its ability to improve flavor and potential nutritional aspects;
- Fermentation is the fundamental process in obtaining cozonacs with SSF and is also characterized by specific time and temperature parameters;
- The parameters of the technological process, such as time, temperature and consistency influence the characteristics of the dough and, as a result, of the cozonac;
- The final product has a slightly acidic flavor, obtaining particular organoleptic properties, due to the production of metabolites (products of the metabolism of many microbial species present in SSF), which gives a more complete and richer aroma, but also a longer shelf life compared to industrial products;
- However, it seems that a long fermentation of the dough, even if CY is used as a fermenting agent, leads to the formation of quality indices of the dough and cozonac, very close to the products obtained only with SSF;
- The use of SSF from sorghum flour would be an alternative in the development of gluten free bakery and pastry products using artisanal technologies.

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The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

The authors declare no conflict of interest.

Received 12.08.2020 Accepted in revised 03.09.2020 Accepted for publication 25.09.2020

DOI: https://doi.org/10.21323/2618-9771-2020-3-3-32-41

Available online at https://www.fsjour.com/jour
Review paper

METHODS OF MOLECULAR DIAGNOSTICS FOR FISH SPECIES IDENTIFICATION

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KEY WORDS:
DNA analysis, molecular diagnostics, PCR, sequencing, sampling, species identification, adulteration

ABSTRACT
The growth in demand for fish products as a result of globalization of trade caused a risks and threats of selling poor-quality and falsified fish products. This has become a great problem both for supervising agencies and for consumers.
Many countries have regulations on food labelling and safety. For example, in the Russian Federation, Republic of Belarus and Republic of Kazakhstan has been passed the Technical Regulation of the Customs Union TR CU022/2011 "Food products in part of their labeling" that aims to prevent misinformation of consumers to ensuring realization of consumer rights to reliable information about food products, and Technical Regulation TR EAEU040/2016 "On safety of fish and fish products" requires indication of the zoological name of the species of the aquatic biological resource or the object of aquaculture.
Fish species identification is traditionally carried out based on external morphological traits. However, it becomes impossible to identify species by ichthyological traits upon fish cutting, if the head and fins are removed, and the body is cut on pieces (especially, in case of fillets) and even more so upon technological processing. In this case, objective analytical methods of species identification are used, which are based on ELISA or PCR. However, DNA-based methods have several advantages compared to ELISA methods and complement traditional morphological identification methods. This paper gives a wide overview of the most recent and used methods of fish species identification based on DNA analysis such as single-strand conformation polymorphism (SSCP) analysis, species-specific PCR, real-time PCR, polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP), DNA barcoding, Sanger sequencing and next-generation sequencing (NGS).

FUNDING: The article was published as part of the research topic No. 0437.19.001 of the state assignment of the V. M. Gorbatov Federal Research Center for Food Systems of RAS

1. Introduction

Fish is one of the most saleable goods in the international food market. Globalization of fish trade and an increase in the demand for fish products have led to serious concerns about the risk of selling poor-quality and even adulterated fish products. These concerns were caused by the revealed high level of incorrect labeling of fish products worldwide, which led to deterioration of their quality and safety [1,2,3,4,5].

The risk and threat of adulteration of edible fish products linked with unreliable labeling of species origin of their composition, especially in technologically processed products, became a serious problem both for controlling agencies and for consumers [6,7].

Legislation regarding food safety assessment and labeling was enacted in many countries of the world [6].

In the EU countries, products are labeled according to Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers [8].

In the Russian Federation, Republic of Belarus and Republic of Kazakhstan, Technical Regulation of the Customs Union TR CU022/2011 "Food products in part of their labeling" is in force to prevent misinformation of consumers in reference to ensuring realization of consumer rights to reliable information about food products [9].

On one hand, this Technical Regulation 022/2011 requires reliable indication of finished product composition, while Technical Regulation TR EAEU040/2016 "On safety of fish and fish products" requires indication of the zoological name of the species of the aquatic biological resource or the object of aquaculture; on the other hand, the presence of allergens should be indicated on a label of any food product even if they are contained at a trace level.

Special concerns that led to strengthening measures on quality control of produced and distributed food products are linked with possible consequences of eating food that contains substances causing allergic reactions or intolerability, when these substances are absent on a label of a consumed product [7].

Over the last years, an increase in the number of patients with food allergy has been observed; this problem is more topical for childhood. At present, fish and fish products are among the most frequent causes of food allergy both in children and in adults, which is linked with the widespread increase in consumption of these products. The prevalence of fish allergy widely varies and is about 0.2% in total population [10].

The majority of allergic reactions on fish is caused by the main allergen — sarcoplasmic protein parvalbumin, which is present in many species of river and sea fish. Up to 90% of "fish" allergy patients react exactly on this protein. Parvalbumin is thermally stable and remains in a product even after its cooking. In addition to parvalbumin, other fish allergens were revealed — tropomyosin, collagen, aldolase, enolase, vitellogenin, calcitonin [11,12].

Fish allergy causes pathological reactions, which are based on immune mechanisms — specific IgE-mediated reactions. Clinical manifestations of allergy can be quite various including rhinitis, angioedema, urticaria, gastrointestinal disorders (nausea, vomiting) and also the most severe, life-threatening form — the anaphylactic shock [13,14,11].

Correct labels of fish-based food products can play a part in stimulation of sustainable fishing helping consumers rightly detect fish origin and, therefore, allowing them to make justified and responsible buying decisions [5].

FOR CITATION: **Fomina T. A., Kornienko V. Yu., Minaev M. Yu.** Methods of molecular diagnostics for fish species identification. *Food systems.* 2020; 3(3): 32–41. https://doi.org/10.21323/2618-9771-2020-3-3-32-41

These circumstances underline the necessity to control correspondence of labeling and reveal cases of species adulteration in fish and fish products.

To detect fish species adulteration, it is necessary to identify fish. Identification of fresh and raw fish can be carried out visually by characteristic taxonomic morphological traits, such as shape and pattern of scales, shape of the body and its size, shape and number of fins, location of eyes, specific features of internal organs and so on. However, it becomes impossible to identify species by ichthyological traits upon fish cutting, if the head and fins are removed, and the body is cut on pieces (especially, in case of fillets) and even more so upon technological processing [15, 16].

In this case, other methods of identification are necessary — instrumental. In the world practice, raw material and product identification is based on species specificity of both protein molecules and DNA. These methods allow avoiding adulteration when expensive species are replaced with cheaper ones [15,17,18].

DNA-based methods have several advantages compared to protein analysis. First of all, the DNA molecule is more stable to an impact of high temperatures and even if DNA is partly destroyed due to product technological processing (cunning, cooking), it can still be used for investigations, for example, by PCR, which allows amplification of small DNA fragments with sufficient information for species identification. Secondly, DNA is present in all tissue types and it can be extracted from any organic material. In addition, methods of DNA analysis are preferable due to larger variability of the genetic code. For example, the mitochondrial genome is characteristic by pronounced instability — the evolution rate of mtDNA exceeds that of nDNA by 10–20 times. The evolution rates of the mitochondrial genome of mammals are estimated as 5.7×10–8 substitutions per synonymous site per year. This instability ensures intra- and interspecies polymorphism, which allows the most effective use of mtDNA to differentiate closely related species of animals, fish and birds [7,16,19,20,21,22,23].

This paper discusses currently available methods for fish species identification.

2. Main part

2.1. Single-strand conformation polymorphism (SSCP) analysis

SSCP (single-strand conformation polymorphism) — analysis of conformation polymorphism of single-stranded DNA (ssDNA) — is a method for detection of differences in ssDNA electrophoretic mobility due to mutations by spatial organization (conformation) of molecules. Conformation of small ssDNA depends on the composition of nucleotides; therefore, substitution of even one nucleotide leads to changes in the spatial structure (Figure 1) [24,25].

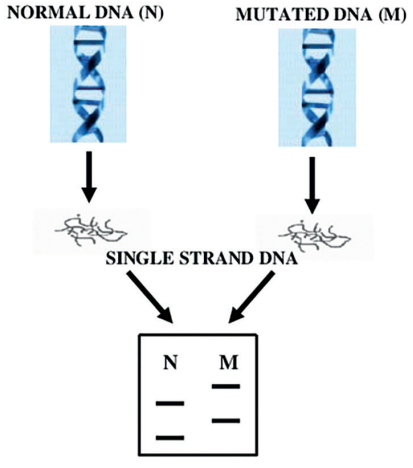

Figure 1. Schematic presentation of SSCP analysis [26]

Thus, detection of the changed pattern of ssDNA fragment migration in different samples in the SSCP analysis allows suggesting species differences, even if species are closely related [16].

The application of this method is described in several studies. For example, Weder, J. et al (2001) [27] used the SSCP method, which had been initially applied to identify tuna of genera *Katsuwonus* and *Thunnus*, to study other species of fish and animals. The 148 bp amplicon obtained using PCR of the fragment of cytochrome b gene (cytb) was used for the study. The ssDNA fragments of blue ling, carp, haddock, mackerel, mackerel shark, saithe, catfish, Alaska pollack, and skipjack gave two to four clear patterns; however, they were different from those obtained with ssDNA samples from tuna (*Thunnus*). The ssDNA fragments of other fish species showed weak bands (cod, spined dogfish) or their absence (Atlantic salmon, halibut, herring, pike-perch, plaice, redfish, sprat, trout).

Rehbein (1997) used the SSCP analysis for identification of different fish species from the family *Acipenseridae*. The length of the amplicon of the cytochrome b gene (cytb) fragment was 358 bp (Figure 2) [28].

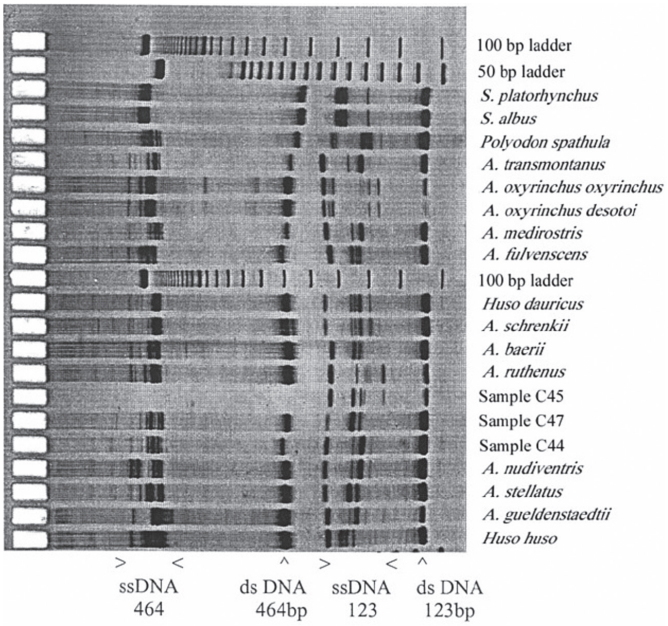

Figure 2. Differences in sturgeon species based on conformation polymorphism of single-stranded DNA (ssDNA) of cytb fragment [28]

SSCP analysis is rapid and easy to use; nevertheless, this method shows three main disadvantages: a) it is necessary to run a reference sample and test sample simultaneously on the same gel, b) intraspecies variation can lead to different conformations, which, in turn, can lead to incorrect identification, c) sometimes two bands with different intensities can be seen. The reason for this can be the fact that ssDNA exists in several states of conformation depending on electrophoretic conditions [16].

2.2. Species-specific PCR

Species-specific PCR is one of the most common variants of using PCR (polymerase chain reaction) method for the diagnostic purpose. The prerequisite in this method is the knowledge of nucleotide sequence of the gene, on which basis the species identification will be carried out; that is, primers will be designed [29,30].

Upon corresponding reaction conditions, such primers generate a fragment that is visualized by agarose gel electrophoresis only in the presence of DNA of this species (Figure 3). This

procedure is applicable only when some previous knowledge about material analyzed is available and identification is to be made [16].

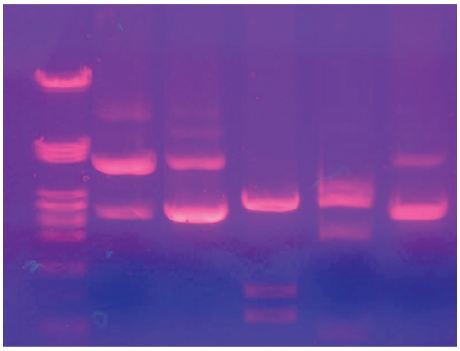

Figure 3. Photo of gel containing template DNA (photo from the authors' archive)

This method has been applied for quite a long time. For example, Vadim J. Birstein et al. [31] describe the use of species-specific PCR for identification of black caviar from beluga sturgeon (*H. huso*), sevruga (*A. stellatus*) and Russian sturgeon (*A. gueldenstaedtii*). For each of these species, a set was developed, which consisted of a pair of specific primers complementary to the regions of template DNA, between which the target sequence was located. In these studies, the occurrence of an amplification product obtained using primers, was assumed to enable correct identification of species under investigation [28,31].

More recent studies of the same groups of scientists showed that species-specific PCR did not allow accurate differentiation of Russian sturgeon (*A. gueldenstaedtii*) from closely related species (*A. baerii*, *A. naccarii* and *A. persicus*) due to overlapping mitochondrial DNA profiles [32].

Although this method has its advantages being easy to use, inexpensive and rapid for species identification of beluga sturgeon (*H. huso*) and sevruga (*A. stellatus*), it failed to differentiate between caviar obtained from Russian sturgeon (*A. gueldenstaedtii*) and Persian sturgeon (*A. persicus*) [28].

There is also a possibility to use together several pairs of species-specific primers in a single reaction tube for simultaneous amplification of DNA of different species. This modification was given the name multiplex PCR, which is one of the variants of species-specific PCR [33]. Michelini E. et al. [34] describe the development of one-step analysis based on triplex-polymerase chain reaction (PCR) to discriminate between three tuna species, yellowfin (*Thunnus albacares*), bigeye (*Thunnus obesus*), and skipjack (*Katsuwonus pelamis*), even in highly processed food samples such as canned or cooked tuna. In this analysis, amplification of the specific regions occurs only in the presence of template DNA, and the species origin of the template DNA is assessed by the size of the PCR product: yellowfin has 245 bp, bigeye 262 bp and skipjack 113 bp [34].

Species-specific PCR is based on unique interspecies nucleotide differences; nevertheless, there are aspects that can lead to false positive or false negative results, which require inclusion of reference samples in each analysis [28,16,33].

2.3.Real-time PCR

Real-time PCR is a modification of conventional PCR, where accumulation of amplification products is analyzed by a special apparatus, which distinctive feature is a possibility to excite and detect fluorescence that reflects accumulation of amplicons in each amplification cycle [35].

In this variant, the fluorescent signal comes from another important component added to the reaction mixture — the DNA fragment that contains a fluorescent dye and a fluorescence quencher (a probe) attached at the 5' and 3' ends of the oligonucleotide, respectively. The probe is complimentary to one of the chains within the amplicon and in the course of copying by polymerase of the DNA fragment specified by the primer, the probe is degraded due to 5′-3′-exonuclease activity of polymerase. The dye and fluorescence quencher are spatially separated and fluorescence occur. Therefore, occurrence of one amplicon is linked with fluorescence of one molecule of free (not quenched) fluorophore. A probe for the marker gene is made in one color, for example, fluorescein — FAM, a probe for a gene under investigation is made in another color, for example, introducing rhodamine (R6G, ROX and others) into oligonucleotide and so on. Thus, the fluorescent signal over the course of PCR increases proportionally to the quantity of an amplification product and allows observing the process of product accumulation over the course of the reaction using computer software. A moment of pronounced increase in a signal and its separation from the baseline, the so-called threshold cycle (log-phase), depends on the initial quantity of target DNA. The higher the quantity of DNA in a sample, the earlier the beginning of an increase in the fluorescence signal is observed [29,36,37,38].

Real-time PCR, also known as quantitative real-time PCR (qPCR), is used for measuring gene copies or a level of gene expression [29]. Hird et al. describe the study of the variation in the proportion of muscle tissue to the numbers of a single copy gene in haddock (*Melanogrammus aeglefinus*) and, then, the development of a haddock-specific quantitative assay using TaqMan technology and real-time PCR for a single copy gene. The study shows that the calibration curve was able to quantify model samples with the accuracy of up to 7% of the true percentage [39].

The accuracy of PCR quantification to a large extent depends of the reference material used in construction of the standard curve [16].

Sánchez A. et al. [40] describe the use of real-time PCR for absolute, absolute-relative and relative quantification of the most valued hake species in European markets, European hake (*Merluccius merluccius*). The authors compared two systems, nuclear and mitochondrial. The research showed that the best quantification results for this species in binary mixtures with non-target species (*Merluccius capensis*) and using the species-specific real-time PCR system were achieved with a relative quantification approach. It was demonstrated that absolute quantification using the nuclear system was appropriate for the quantification of the *Merluccius genus* in food model samples [40].

Real-time PCR is the most common technology to use for species identification. Continuous measurement of fluorescence allows eliminating stages, which usually are necessary after performing PCR, that is, electrophoresis and gel staining. Moreover, in case of real-time PCR, potential risks of contamination are significantly reduced as samples with the reaction mixture are sealed throughout the analysis [29].

2.4.Polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP)

PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism analysis) is a method, where a fragment of the studied gene carried the recognition site for endonuclease is amplified with its following cutting with the corresponding enzyme leading to appearance of several smaller fragments with different sizes (restriction fragments). Restriction fragment sizes are analyzed by gel-electrophoresis [41,42].

PCR-RFLP allows identifying different meat types originated from mammals, birds or fish [43] and has been widely used in many countries worldwide.

Georgina L. Hold et al. [44] describe the study on application of the PCR-RFLP based method for identification of salmon species in food products. A 464 bp portion of the cytochrome B gene was used as a target sequence for amplification; restriction fragments were obtained using the following enzymes: Dde I; Nla III; Hae III; Bsp 1286I; Eco RII; Sau 3AI. The reliability and practicality of the method were tested in the inter-laboratory study, in which five European laboratories took part: 1. Rowett Research Institute, Greenburn Road, Aberdeen, AB21 9SB, Scotland, UK; 2. Bundesforschungsanstalt für Fischerei, Institut für Biochemie und Technologie, Palmaille 9, 22769 Hamburg, Germany; 3. Departamento de Bioquimica y Biologia Molecular, Facultad de Biologia, Universidad de Santiago de Compostela, 15706 Santiago de Compostela, Spain; 4. Instituto de Investigaciones Marinas (CSIC), Eduardo Cabello 6, 36208 Vigo, Spain; 5. Instituto de Investigacao das Pescas e do Mar, Avenida de Brasilia, Lisbon, Portugal. Ten samples of morphologically identified salmon species (*Salmo salar, Oncorhynchus keta, Oncorhynchus kisutch, Oncorhynchus gorbuscha, Oncorhynchus nerka, Oncorhynchus tschawytscha, Oncorhynchus mykiss, Salvelinus alpinus, Salvelinus fontinalis, Salmo trutta*) and two encoded samples (1 — *S. salar* (commercial product), 2 — a mixture of two salmon species *O. keta* and *O. gorbuscha*) were used as the control samples. All results (including those for encoded samples) showed 100% agreement and were correctly identified. In addition to the inter-laboratory study, larger scale investigations of UK commercial products were also carried out covering the whole range of available salmon products. In almost all cases, the declared salmon species was confirmed; a trout species was detected in one product, which label declared only the presence of salmon. The performed research confirmed the reproducibility of the method in different laboratories as well as its applicability for analysis of commercial products [44,45].

Lin and Hwang [46] used this method to detect eight species of the family Scombridae: bluefin tuna (*T. thynnus*), albacore (*T. alalunga*), bigeye tuna (*T. obesus*), yellowfin tuna (*T. albacares*), skipjack (*Katsuwonus pelamis*), eastern little tuna (*E. affinis*), frigate mackerel (*Auxis thazard*) and oriental bonito (*Sarda orientalis*) in samples of canned tuna. Two sets of primers were designed to amplify 126 bp and 146 bp fragments of mitochondrial cytochrome B gene, and five restriction enzymes (Bsp1286I, HincII, RsaI, ScaI and MboII) were used to analyze short length fragments. The method was successfully applied for authentication of 18 samples of canned tuna [46].

Ya-Jung Wu [47] used the PCR-RFLP method to identify filefish species (*Monacanthidae*), which are a food delicacy in Taiwan. The cytochrome B gene region with the molecular weight of 465 bp was chosen for primers. The obtained results showed that when using this method, six commercial filefish species could be identified not only in fresh but also in thermally processed products [41].

The PCR-RFLP method is regarded as robust and easy to use for identification of fish species; however, problems with analysis robustness due to the use of individually prepared and non-optimized components as well as the manual nature of the analysis can potentially affect the reliability of results [48].

Dooley J. J. [49] and his colleagues presented an optimized PCR-RFLP approach for fish species identification. The scientists replaced the gel-electrophoretic steps for fragment separation, detection and analysis with a chip-based capillary electrophoresis system with the use of the Agilent 2100 Bioanalyser. The presented solution reduces analysis duration and allows obtaining a result with a minimum impact on a sample. Figure 4 presents the scheme of the analysis.

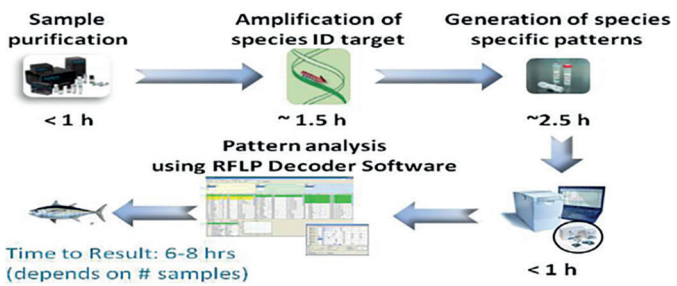

Figure 4. Scheme of the PCR-RFLP analysis with chip-based capillary electrophoresis using the Agilent 2100 Bioanalyser [48]

The main steps include extraction of genomic DNA, amplification of the target DNA — the region of the cytochrome B gene, cleavage of the PCR product with three different restriction enzymes (Dde I; Nla III; Hae III) and separation on the bioanalyser; then, the stage of the instrumental analysis is applied using software with extensive database of experimental profiles of fish species Agilent RFLP Decoder for RFLP pattern analysis.

Software analyzes the obtained result for a test sample and compares it with profiles of authentic fish species in the database using standard calculation methods to reveal the most probable matches. The flexibility of software allows easy addition of users' profiles to extend the number of species that can be identified based on the experimental models. In addition, analysis of mixed products is envisaged, including different fish species as well as detection of the presence of pork, beef, mutton and turkey. At present, the further work on adaptation of the method to specific tasks for fish species identification has been carried out, but even now the reagent kit and consumables for detection of fish species Agilent DNA Fish Species ID Ensemble are available for purchase including the territory of the Russian Federation [49,48,50].

2.5. DNA barcoding

The main idea of DNA barcoding resides in the fact that some short DNA region can play a role of a marker that allows definitive identification (or almost definitive, as there is intraspecies variability) of species origin of an organism similarly to the work of a barcode on a label, which is read by a scanner upon payment for goods [51,52].

Figure 5 presents the technology of DNA barcoding. As can be seen from the scheme, the DNA barcoding basis includes two methods of molecular diagnostics: first of all, the polymerase chain reaction, which due to the DNA ability to replicate allows accumulation (amplification) of a chosen fragment of its molecule in quantities suitable for further analysis; secondly, the method for determination of nucleotide sequence in DNA molecules (its sequencing) [51,52].

In 2003, Hebert et al. [42] introduced the term DNA barcoding for the first time in the paper "Biological identifications through DNA barcodes" and proposed to use the 648 bp portion of the mitochondrial cytochrome c oxidase I (COI) gene as a marker DNA sequence to create the global system for animal bio-identification [53,42,54]. Hebert's study caused mixed reactions varied from enthusiasm, especially in ecologists [55] to criticism, mainly regarding identification of closely related species using single gene [56,57,58,16]. However, the DNA barcoding method is long-established; its advantages and limitations became obvious [53].

Different tasks are accomplished using the DNA barcoding technology, for example, identification of a plant only by its leaves when its flowers and fruits are unavailable; identification of insect larvae, which have less diagnostic traits than adult individuals; determination of an animal diet by the stomach content, saliva or feces [56,60].

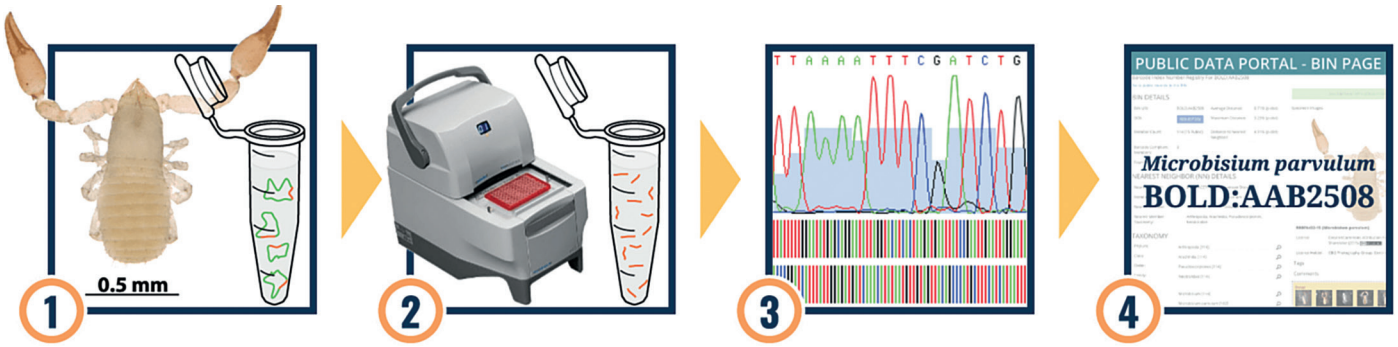

Figure 5. Step 1: DNA isolation; Step 2: Amplification of the target DNA barcode region using PCR; Step 3: Sequencing the PCR products; Step 4: Comparing the resulting nucleotide sequences with reference databases to find the matching species [52].

DNA barcoding is also used as a molecular tool for determination of food product mislabeling and revelation of species adulteration [61].

The DNA barcoding technology is inextricably linked with Sanger sequencing — a gold standard of DNA typing.

Reading a nucleotide sequence began with the development of the method for sequencing RNA obtained from the DNA template using RNA polymerase. In 1976, therefore, the sequence of the most part of the genome of DNA virus SV40, which length is more than 5000 base pairs, was determined [62,63]. Then, the methods for direct DNA sequencing were developed.

In 1975, F. Sanger and A. Coulson developed the method of direct enzymatic DNA sequencing that is also called plus and minus method. A fragment of single stranded DNA served as a template in the polymerase chain reaction and synthetic complementary sequences or short DNA regions obtained by the action of restriction endonucleases were used as primers [64].

The method consisted of two steps. First, under limiting conditions, the polymerase reaction was performed in the presence of all four dNTP types (one of them was labeled on the alpha position of phosphate), obtaining, in the end, a set of products of incomplete copying of a template fragment. The mixture was then purified from unbound deoxynucleoside triphosphates and divided into eight parts. After that, in the plus system, four reactions were carried out in the presence of each type of nucleotides and in the minus system in the absence of each of them. As a result, in the minus system termination occurred before dNTP of the given type, and in the plus system after it. Eight samples obtained in such a way were separated by electrophoresis, the signal was read off and the sequence of the initial DNA was determined. Using this method, phage ϕX174 short DNA, which consisted of 5386 bp, was sequenced [64,65].

Genome investigations enable solving many applied and fundamental tasks. Using these methods, new drugs and products have been developed; they also allow penetrating the long human history or understand the cause of mass extinctions of species [66].

Due to these projects, the international genome base NCBI was formed. With its use, it is possible to select nucleotide sequences of interest and develop diagnostic test-systems on their basis. Since then, the PCR method have gradually come into routine laboratory practice and ceased to belong only to the fundamental science.

2.6. Sanger sequencing

Sanger sequencing allows reading off sequences of up to 1000 base pairs and is used for small fragments of genome/genes. In particular, it is used for sequencing individual genome regions to analyze mutations and polymorphisms; identify viruses and organisms (bacteria, rickettsia, plants, fungi and animals); validate data obtained on the platforms of next generation sequencing (NGS); microsatellite analysis; analysis of deletions and insertions (small and long) [66].

Since 2015, Sanger sequencing has also been used for identification of fish species composition. From July 1, 2018, the interstate standard GOST 34106–2017 came into force as a national standard of the Russian Federation. This standard regulates the method for sequencing the fragments of the mitochondrial genome of animals and fish to determine species origin in one-component products. The essence of the method described in GOST consists in determination of the nucleotide sequences of the mitochondrial genome region of different animal and fish species and their comparison with known sequences to identify their species origin. The analysis by this method includes: DNA extraction and purification; PCR with primer pairs flanking the site of the mitochondrial genome in the region of cytB gene; detection of PCR products by the method of electrophoresis in agarose gel to reveal the specific band of amplified DNA and assessment of the concentration of the PCR product; sequencing the PCR product purified from unbound primers and dNTP by the dideoxynucleotide method with fluorescent dyes; identification of a nucleotide sequence by separating products of the sequencing reaction purified from the excess of dNTP, fluorescently labeled ddNTPs, primer and salts, by the method of capillary electrophoresis and comparison of obtained nucleotide sequence of the genome fragment extracted from the analyzed sample with known sequences from databases for its identification [67].

At present, sequencers are produced also in Russia; the best known model is the genetic analyzer “Nanofor 05”.

The main advantages of Sanger sequencing are high accuracy of reading (many PCR methods are validated by its use) and low primary cost of analysis. However, Sanger sequencing has a serious limitation — it is impossible to carry out species identification in multicomponent products by this method.

Only when using multiplex next generation sequencing (NGS), it is possible to identify complete species composition of multicomponent products in one reaction.

2.7. Next generation sequencing

Next generation sequencing began with the principal discovery of the possibility of clonal amplification of fragmented DNA on the solid surface. If in Sanger sequencing, information was read off from one amplified DNA fragment, in next generation sequencing, there is parallel reading from tens of thousands of amplified fragments, where every fragment is cloned and read in its own cell.

Contrary to Sanger sequencing, the NGS methods are used for deep (multiple) read of genetic material which is necessary, for example, for re-sequencing and assembly of novel genomes (*de novo*), transcriptomic and epigenomic investigations. In addition, next generation sequencing (NGS) is much more efficient allowing reading millions and even billions of short fragments.

Such growth in performance led to a possibility to simultaneously determine sequences of tens of genomes (depending on their sizes) in a single run [66].

Next generation sequencing gave an opportunity to assess metagenome of mixed microbial populations and reveal previously unknown and uncultivable microbial forms. This became possible with the development of the algorithms for assembly and analysis of genomes, software and high-performance workstations.

New mathematical and information technologies allow genomics to develop quicker and use more complex algorithms. These algorithms can include simultaneously several applications and programs, and enable working with very large volume of data [66].

When analyzing whole genomes, two approaches are used: analysis by alignment against the reference genome or re-sequencing, or genome assembly from zero or sequencing de novo.

2.8. Basic next generation sequencing methods

The first technology of next generation sequencing is the 454-sequencing technology (high-throughput DNA pyrosequencing or pyrophosphate sequencing) developed by 454 Life Sciences. On the basis of this technology, the methodical recommendations on species identification of fish and fish products based on sequencing of amplified DNA fragments were approved and presented in Russia in 2015 [68]. The amplified DNA fragment with a length of 350–500 base pairs is obtained in the course of two PCR rounds using fusion primers consisted of several parts: the A and B adaptors, four-nucleotide 'key' sequence as well as the sequences specific to a target gene. Optionally, the multiplex identifier (MID) sequence is included in the primer sequence. Then, the obtained amplification products are subjected to emulsion PCR (ePCR), during which amplified fragments of the DNA library and special spherical beads are incorporated into water droplets of emulsion, which are amplification microreactors. Clonal parallel amplification in emulsion droplets ensures obtaining millions of copies of each fragment from the DNA library on each individual bead. Using centrifugation, beads are loaded onto a plate containing several hundreds of thousands of microscopic wells. Each bead gets into an individual well of the plate; then, the plate is placed into an instrument along with reagents and sequencing begins. During the work of the instrument, nucleotides are successively flowed through the plate wells. Incorporation of a nucleotide complementary to the template on each individual bead leads to a chemiluminescent signal in a certain well that is recorded by a camera of the instrument.

This technology allows reading simultaneously several genome regions, which makes it possible to identify fish species in the composition of multicomponent products [68].

The obtained nucleotide sequence of the specific DNA fragment is analyzed in the international database NCBI [69].

Figure 6 presents a fragment of a file with the results of analysis by 454 sequencing; the file contains a pool of consensus reads in the FASTA format marked by the identifier clusterid=X. The obtained sequences are copied into the memory buffer and entered into the dialog window "Nucleotide BLAST" of the database NCBI (Figure 7). After that, the analysis program starts and the result window appears showing reference sequences that match those under investigation in the descending order of matching (Figure 8).

2.9. Ion semiconductor sequencing technology

The technology was developed by Ion Torrent Systems, Inc and was launched in 2010 [70]. The principle of the method resides in recording a released hydrogen ion by a ion sensor ISFET.

```
>JCY5JSI01C3T4I length=456 clusterid=1 members=3239
GCCTCTGCCCACAATGGTACGGCCGCGGTATTTTGACCGTGCGAAGGTAGCGCAATCACT
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AAGTCAATGAAATTGATCTGCCCGTGCAGAAGCGGACATAAAACACATAAGACGAGAAGA
CCCTATGGAGCTTTAGACACCAGGCAGATCACGTCAAGTAACCTTGAATTAACAAGTAAA
AACGCAGTGACCCCTAGCCCATATGTCTTTGGTTGGGGCGGACCGCGGGGGAAAACAAAG
CCCCCATGTGGACTGGGGGCACTGCCCCCACAACCAAGAGTCACAACTCTAAGTACCAGA
ATTTCTGACCAAAAATGATCCGGCATCACGCCGATCAACGGACCGAGTTACCCTAGGGAT
AACCAGCGCAATCCTGAGAGCTTCCAACCACTTCTT
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TTAATCAATCAATCACTCAATGCAAAAATGCAAATAGTACATGCAAAAACACAGATATTA
AAAACAATTCCAAAAAATATACCTGTAGTAGAGCATGCTGGGTAAAAAGTTAATTTGTTA
TAACTTAAAAAAATTGCATTAATGTGACTCATTTAGATTTGAGCCAGTACCACATAGACA
TTTTAAGTAATAATGACTTTTCATTGACTGAGTTCAACTTACTAGAAGTATTTGAGTTAA
AAATGCCTTGTGGTTTACAGTGCACAAAGTGCGGCACCACAAATGGTCATTGTTCTCCCT
CCTTTCTTTACCTTGATGAGCAGATTGATGCCCACTTTGAGGTCGCTGAGCTTCTCAGAG
ATCTGGTTGGAGTTTCTGACCATTAGGCTGA
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CAGCGACCTCAAAGTGGGCATCAATCTGCTCATCAAGGTAAAGAAAGGAGGGAGAACAAT
GACCATTTGTGGTGCCGCACTTTGTGCACTGTAAACCACAAGGCATTTTTAACTCAAATA
CTTCTAGTAAGTTGAACTCAGTCAATGAAAAGTCATTATTACTTAAAATGTCTATGTGGT
ACTGGCTCAAATCTAAATGAGTCACATTAATGCAATTTTTTTAAGTTATAACAAATTAAC
TTTTACCCAGCATGCTCTACTACAGGTATATTTTTGGAATTGTTTTTAATATCTGTGTTT
TTGCATGTACTATTTGCATTTTTGCATTGAGTGATTGATTGATTAATTTTATGCTTCACA
CAGATATATAACATACATTTTTCTACGTTTTCACAAAGATAAATAACATACCGGAATTTT
GCAAACCTACTTGCAGGCCTGAT
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Figure 6. A file fragment with the results obtained in the course of sequencing of amplified DNA fragments using the 454-sequencing technology (Figure from the authors' archive)

It is released when an introduced nucleotide is complementary to the single-stranded fragment of amplified DNA. The reaction and recording of the event occur on a special semiconductor chip with different capacities. In contrary to other systems, this allows scaling performed investigations selecting a chip with a corresponding capacity without buying a more efficient sequencer.

However, compared to the 454 Life Sciences technology (pyrosequencing), the semiconductor technology does not yet allow reading fragments more than 400 bp. Although, in 2018, the company released chips with the possibility to read fragments of up to 600 bp, this immediately doubles the cost of analysis compared to the standard 400 bp reads.

Also, a huge plus of this technology is a possibility of full automation of the sequencing process. A minus, probably, is closeness of this technology compared, for example, with Illumina.

At present, methods based on next generation sequencing have begun to be used in fish species identification as only they are capable of complete revelation of species composition in multicomponent fish products. The only disadvantage of these methods is the price of analysis.

3. Conclusion

Methods based on DNA analysis are often used for fish and fish product species identification. Therefore, they can be used in controlling fish products for correspondence to the species composition indicated on a label.

The most promising are PCR-based methods, which allow identifying meat of different, even closely related fish species. The potential for using the PCR method proceeds from the possibility to identify meat of different, even closely related fish species. This identification can be performed on the biological material obtained from raw tissue, as well as on muscles subjected to different technological treatments. Relative simplicity and rapidness of analyses allow suggesting that these methods consisted in DNA analysis will find wide application in the future for food quality control.

However, despite undoubted advantages of PCR-based methods, we should not overlook their disadvantages.

Generally speaking, SSCP analysis is not recommended to use for the identification of products consisting of several species

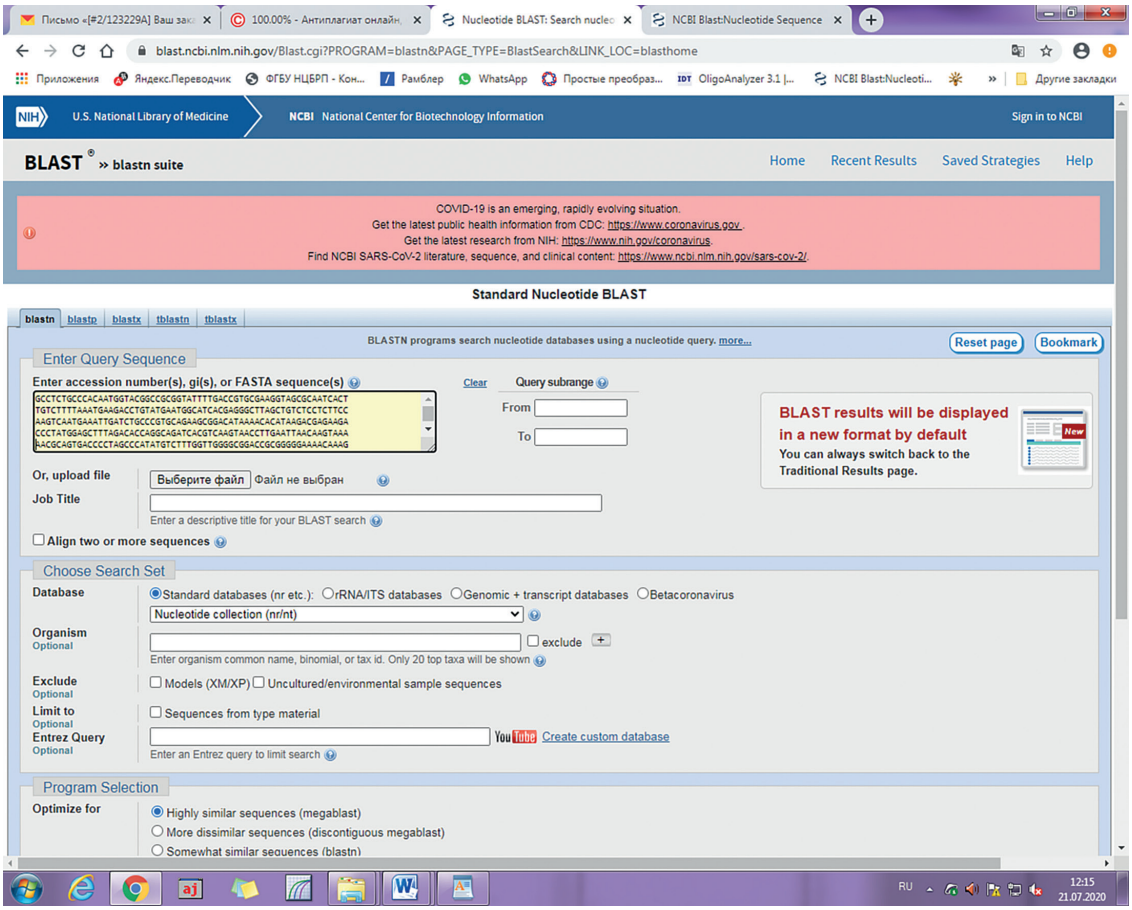

Figure 7. The dialog window "Nucleotide BLAST" of the NCBI database [69]

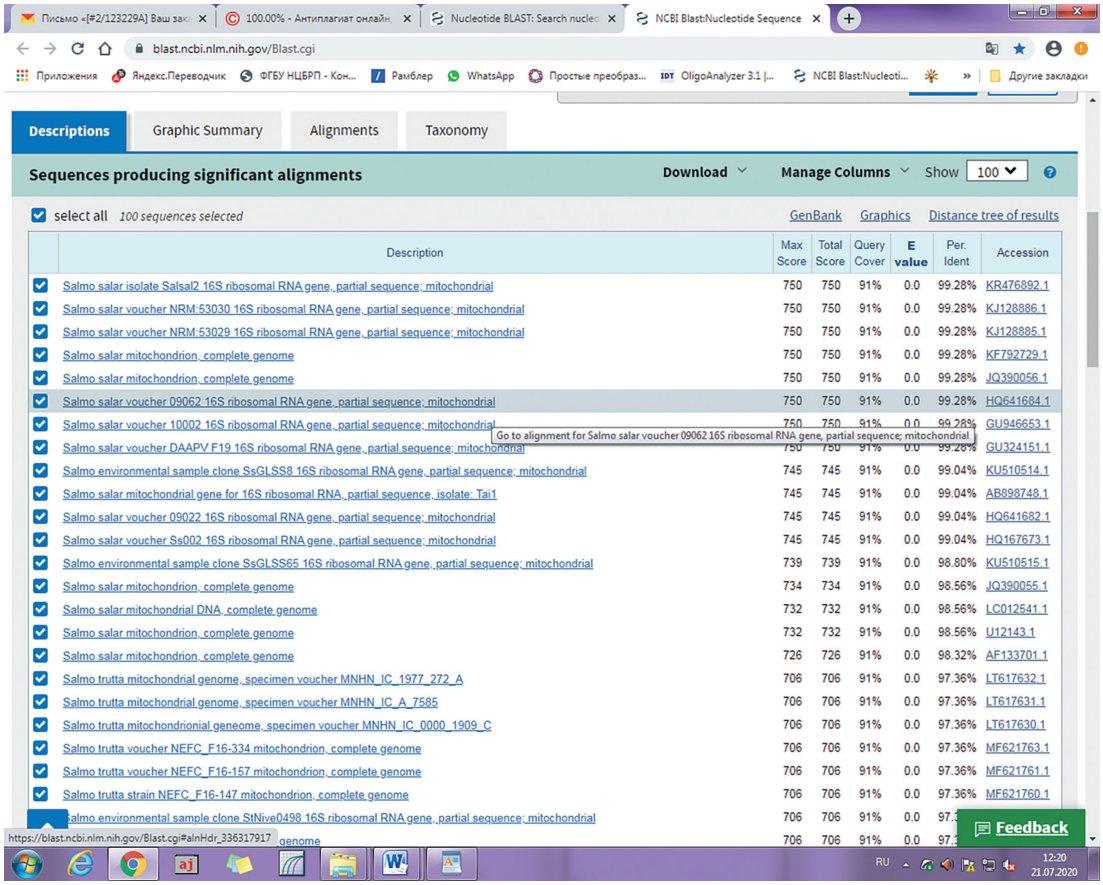

Figure 8. The window with the results of the analysis of the nucleotide sequence obtained in the course of sequencing of amplified DNA fragments using the 454 sequencing technology [69]

of fish and in products subjected to intense heating (sterilization). In addition, a prerequisite for species identification using the SSCP analysis is the presence of reference samples and test samples on the same electrophoretic gel. The repeatability of obtained results in the SSCP analysis is influenced by analysis conditions, that is, a temperature and concentration of used reagents (for example, buffers).

According to several researchers, the PCR-RFLP method is also not recommended for verification of species composition in fish mixtures, as obtained results not always reflect the true composition of a mixture. In case of the PCR-RFLP method, there is a risk of incomplete restriction of a region or intraspecies differences, which can facilitate deletion or extension of restriction sites.

Species-specific PCR in the conventional variant envisages the use of the step of electrophoresis and gel staining to detect the amplification results, which can lead to contamination and, consequently, to unreliable results. In this case, the modification of conventional PCR — real-time PCR — can be considered the most promising method.

Real-time PCR is the most common technology to use for species identification. Continuous measurement of fluorescence allows eliminating the stages (electrophoresis and gel staining) that usually are necessary to perform after PCR. In addition, in case of real-time PCR, the potential risks of contamination are significantly reduced as samples with the reaction mixture remain to be sealed throughout analysis. At present, real-time PCR is the most common technology to use for species identification in all product types ranging from raw materials to thermally processed products including products with the multicomponent composition from different fish types.

The DNA barcoding method with the use of next generation sequencing is also quite promising. It can detect the presence of all fish species contained in the test sample over one analysis, and the ability to carry out analyses of any fish products makes this method universal. The main disadvantage of the DNA barcoding method using next generation sequencing is its high cost, which is made up from high cost of equipment for analysis, reagents and diagnostic test-systems.

It is necessary to emphasize that PCR methods are only those methods that allow determining product species composition; however, complex quality and safety assessment of fish products also includes analyses by other methods, which simultaneously can confirm or do not confirm correspondence of the producer declaration to what consumer purchased.

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The authors declare no conflict of interest.

Received 28.06.2020 Accepted in revised 15.09.2020 Accepted for publication 25.09.2020

DOI: https://doi.org/10.21323/2618-9771-2020-3-3-42-50

Available online at https://www.fsjour.com/jour
Original scientific paper

THE INFLUENCE OF MILK-CLOTTING ENZYMES ON THE FUNCTIONAL PROPERTIES OF PIZZA-CHEESES

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KEY WORDS:
pizza-cheese, rhizomucor miehei, camel chymosin, proteolysis, microstructure

ABSTRACT

The effect of the type and dose of milk-clotting enzymes (Chy-max® M based on recombinant camel chymosin, Fromase® TL based on Rhizomucor miehei protease) on the physicochemical, functional properties and shelf life of pizza-cheeses was studied. When using a low dose of milk-clotting enzymes (MCE) for milk coagulation (250–1100 IMCU per 100 kg of milk), cheeses were obtained with an increased moisture content (55–57%), excessive acidity (pH 4.8–4.9) and texture defects (incoherent, crumbly, with separation of free moisture). This is due to the formation of a weak curd, which releases moisture poorly during processing. The use of an increased dose of MCE makes it possible to obtain a denser curd, better releasing moisture. Cheese produced with a high dose of milk-clotting enzymes (2000–2800 IMCU per 100 kg of milk) had a lower moisture content (52–53%) and lower acidity (pH 5.0–5.1). The protein matrix is more hydrated in these cheeses, which ensures its better water holding capacity and a more homogeneous and cohesive texture. The use of an increased dose of MCE with a high total proteolytic activity (Fromase) gives undesirable consequences in the form of accelerated proteolysis of cheese mass proteins, rapid loss of functional properties of the cheese, and a decrease in the shelf life of cheese (less than 60 days). Cheese production using an increased dose of MCE with a low level of total proteolytic activity (Chy-max M) allows achieving a low level of proteolysis during cheese ripening and increasing its shelf life.

FUNDING: The article was published as part of the research topic No. 0585–2019–0011 of the state assignment of the V. M. Gorbatov Federal Research Center for Food Systems of RAS.

1. Introduction

The production of pizza-cheeses in the world is growing every year. Pizza-cheeses are a marginal product for a manufacturer and are in high demand in the market.

Pizza-cheese is a product with its own specific properties that differ from those of other cheeses. Unlike other types of cheese, pizza-cheese is a semi-finished product and is intended for final consumption in a heated state after baking, therefore it must have certain properties in a normal and heated state.

There are a number of mandatory requirements for pizza-cheeses. Cheese should have a clean taste associated with the taste of young cheeses and a coherent, elastic, moderately dense texture, convenient for cutting and grating (this is important for grinding cheese using industrial machines). Once thawed, the cheese should maintain a cohesive texture without separating free moisture, which is required for cheeses used for frozen pizza. When baked on pizza, the cheese should acquire a liquid flowing texture, not form a burn on the surface, not release incoherent fat and moisture, maintain a coherent fibrous structure that allows the formation of long strands when stretched [1].

The cheese should retain its flavor, texture and properties when baked on pizza for a long shelf life. The fibrous structure, capable of stretching when heated, is a feature of pizza-cheeses ("pasta filata", "string cheese"). To give cheeses a similar structure, limited decalcification of the paracaseine-calcium phosphate complex of the cheese is required, followed by its structuring by mechanical stretching in a heated state [2]. In the process of stretching, the cheese mass is heated in hot water at a temperature of 65–80 °C with simultaneous mechanical action (stretching), which allows the curd mass to be stretched into long strands. During the pasta-filata process, the internal structure of the cheese becomes fibrous, since initially shapeless protein particles are stretched into fibers, between which fat globules are embedded [3].

Fresh pizza-cheese has a distinct fibrous structure and, when heated, can be stretched into strands up to several meters long, but such cheese is not suitable for use in baked goods. Insufficient hydration of proteins in unripened cheese when baked on pizza leads to the separation of free moisture and fat and strong protein burn. To give the cheese mass a sufficient degree of hydration, the cheese is kept for 2–3 weeks (the cheese is "ripened"). During maturation, under the action of milk-clotting enzymes and ferments of the starter culture, limited proteolysis of caseins occurs, as a result of which their hydration increases. When ripe, pizza-cheese acquires resistance to high temperatures during baking of pizza: free moisture and fat are not melted, a desired slight browning of the cheese occurs [4,5].

The problem is that, in view of the sufficiently high amount of moisture (42–52%), proteolysis of proteins proceeds at a high rate and pizza-cheeses overripe quickly. A high degree of proteolysis increases the degree of protein hydration, which, in combination with the high moisture content of the cheese mass, leads to the formation of a viscous, sticky cheese texture, making it impossible to cut and grate it on the industrial devices. During maturation, proteolysis occurs, accompanied by an increase in protein hydration, the formation of cross-linkages between casein submicelles, and a loss of fibrous structure. As a result, after baking on pizza, overripe cheese acquires an excessively fluid (liquid) texture and does not form characteristic strands of the required density and length when lifted [6,7].

A serious requirement from retail chains and pizza producers is a long shelf life of cheeses. In practice, this can be achieved by reducing the rate of proteolysis during storage of cheese. To increase the shelf life, cheese is stored at low temperatures (freezing). This

FOR CITATION: **Myagkonosov D. S., Mordvinova V. A., Delitskaya I. N., Abramov D. V., Ovchinnikova E. G.** The influence of milk-clotting enzymes on the functional properties of pizza-cheeses. Food systems. 2020; 3(3): 42–50. https://doi.org/10.21323/2618-9771-2020-3-3-42-50

method has disadvantages in the form of increased energy consumption and labor costs for subsequent defrosting.

Other ways to limit proteolysis can be:

- the use of MCE with low proteolytic activity [7,8,9,10];
- the use of a starter culture with low proteolytic activity [11];
- reducing the dose of the starter culture (with preliminary acidification of milk with organic acids (citric, lactic) or glucono-delta-lactone) [12,13];
- reducing the dose of MCE incl. by creating conditions that increase its milk-clotting activity (increase in temperature and decrease in milk pH) [14,15];
- reducing the dose of MCE by increasing the content of casein in milk (normalization of milk by protein by adding milk powder or milk protein concentrate) [16,17];
- deactivation of MCE and starter by conducting thermomechanical processing of cheese mass at elevated temperatures (62–66 °C) [2,3].

The use of MCE and a starter culture with low proteolytic activity is the simplest method that does not require adjusting the parameters of the technological process. Based on a literature review [9,18,19,20,21], the Chy-max® M preparation based on the recombinant camel chymosin was chosen as the MCE with low proteolytic activity, and the Fromase® TL preparation based on proteases of the microorganism Rhizomucor miehei was chosen as the MCE with high proteolytic activity. The culture Str. thermophilus was chosen as the starter culture, which has low proteolytic activity [10, 22]. The use of a thermophilic streptococcus starter assumes the use of a higher temperature (36–38 °C) at the stage of making cheese in the vat than using a mesophilic lactococcus starter culture (30–33 °C). This made it possible to test a method for reducing the dose of MCE, based on increasing the activity of milk-clotting enzymes at an elevated coagulation temperature [23].

The aim of the research was to establish the effect of the type and an introduced dose of milk-clotting enzymes on the functional properties of pizza-cheeses and on their shelf life.

2. Materials and methods

2.1. Materials

The studies used cow's milk from one supplier-manufacturer — AgriVolga LLC (Yaroslavl Region, Uglichsky District, Burmasovo village). In the production of cheeses, we used lactic acid culture of *Streptococcus thermophilus* based on the bacterial concentrate BK-Uglich-TNV (FGBNU Experimental Biofabrika, Russia) with preliminary activation of the culture on sterilized milk at 30 °C for 18 h. For milk coagulation, MCE brands Fromase® 2200 TL (DSM Food Specialties, France) and Chy-max® M 1000 (Chr Hansen A / S, Denmark) were used.

2.2. Methods

2.2.1. Methods for studying the properties of milk-clotting enzymes

The determination of the total proteolytic activity was carried out in accordance with the Russian state standard (GOST) method (GOST «34430–2018»). The method is based on the hydrolysis of a substrate from bovine hemoglobin in a weakly acidic zone (at pH 5.3) by the studied enzyme preparation to low molecular peptides and free amino acids, followed by termination of the enzyme action by precipitation of the unhydrolyzed protein with trichloroacetic acid (TCA) and determination of the resulting peptides and free amino acids.

The one unit (U) of total proteolytic activity (PA) is the amount of enzyme that, in 1 min at a temperature of 30 °C, brings hemoglobin into a non-precipitated TCA state in an amount corresponding to 1 μmol of tyrosine. Activity is expressed in U/g of enzyme preparation.

The amount of protein converted into low molecular peptides and amino acids is determined by the reaction of free amino acids with Folin-Ciocalteu reagent and further determination of the optical density of the resulting blue solutions on a LEKI model SS1207UV photoelectrocolorimeter (MEDIORA OY, Finland) at a light wavelength λ = 670 nm.

2.2.2. Cheese production process

The technological regulations for the cheese production are shown in Table 1.

The moment of completion of cheddarization was determined by the readiness of the curd mass for thermomechanical treatment by its ability to stretch when heated in hot water.

After cheddarization, thermomechanical treatment of the cheese mass («stretching») was carried out manually until a coherent homogeneous texture was obtained. After stretching, the cheese mass was formed into blocks in the form of a low cylinder weighing 1.0±0.1 kg and a height of 10±2 cm and sent to aging

Table 1

Technological regulations for the cheese production

Stage of the technological process	Process parameters	
Mass fraction of fat in milk mixture	2.4–2.5%	
Milk pasteurization	10 sec at 72 °C	
Calcium Chloride Dose	Based on 35 g of anhydrous salt per 100 kg of milk	
Dose of lactic acid starter culture	1,5% (v/w)	
Milk acidity before coagulation	pH 6.2±0.1	
Milk-clotting enzyme dose, g/100 kg of milk *	Fromase® 2200 TL	Variant "Low" — 0.125
		Variant "High" — 1.25
	Chy-max® M 1000	Variant "Low" — 1.15
		Variant "High" — 2.30
Coagulation temperature of milk	37.5±0.5 °C	
Size of the grain edges when cutting	1.0±0.2 cm	
Grain processing temperature	37.5±0.5 °C	
Active acidity of the curd mass at the end of processing	pH 5.10±0.5	
Mass fraction of salt in solution during thermomechanical treatment	5%	
Thermomechanical treatment modes	Brine temperature — 80 °C Duration — 10 min Cheese curd temperature after processing — 50–55 °C	

Note: the maximum application rate was taken in accordance with the recommendations given in the documentation for the MCE. The minimum dose was determined based on the rennet test result [23].

for 16–18 hours at a temperature of 8–10 °C to separate free whey and form a closed surface. After aging, the cheeses were packed on a Henkelman Boxer 42 machine (Henkelman Vacuum Systems, The Netherlands) under vacuum (negative pressure 1 Bar; vacuuming time 20 s) into bags made of Amivak CH-B polymer film (Atlantis-Pak, Russia) and sent for storage at a temperature of 3 ± 1 °C.

2.2.3. Methods for studying the properties of cheeses

Determination of active acidity — 10 g of grated cheese was ground in a pouder with 10 ml of deionized water; active acidity was determined using a pH-150MI pH-meter (Measuring equipment, Russia).

Determination of the mass fraction of moisture was carried out by drying at a temperature of 102 ± 2 °C according to the Russian state standard GOST 3626–73.

The degree of proteolysis in cheeses was expressed as a percentage of the absolute content of soluble nitrogen from the absolute content of total nitrogen.

Determination of the mass fraction of total and soluble nitrogen was carried out by the Kjeldahl method according to the Russian state standard GOST R54662–2011.

The extraction of water-soluble nitrogen was carried out according to the method [24] in the modification [25]: 20 g of grated cheese was mixed with 40 ml of deionized water, homogenized on a high-speed FSH-2A homogenizer (Jiangsu Jinyi Instrument Technology Company Limited, China) for 1 min. The homogenized mixture was kept at 40 °C for 1 h with continuous stirring for 100 min^{-1} on a SK-O180-E orbital shaker (DLAB Scientific Co., Ltd, China). The samples were cooled to 4 °C and centrifuged at 3000 g for 30 min. The upper fat layer was removed, and the supernatant was filtered on cellulose acetate filters with a pore size of 0.2 μm (Vladipor, Russia).

The determination of the molecular weight distribution of soluble nitrogenous substances was carried out by high resolution gel filtration using a Superose 12 10/300 GL column (GE Healthcare, Sweden). The eluent was an aqueous solution of 0.05 M Na_2HPO_4 + 0.15 M NaCl, the flow rate of the eluent was 0.5 ml/min; the detector wavelength was 280 nm.

Determination of the ability of cheese to stretch when heated was carried out by a commercial method by the ability of cheese to stretch when lifting the melted cheese with a fork with an estimation of the length of the resulting threads [26].

The microstructure of the cheeses was investigated by the method of light microscopy in transmitted light, on micro-sections of cheese with a thickness of 100 ± 10 μm.

The experiments were repeated three times.

Mathematical processing was performed using Microsoft Excel and Statsoft Statistica software packages.

3. Results and discussion

In the process of cheese production, the following indicators were studied:

- the duration of the individual stages of the processing in the cheese vat;
- dynamics of changes in the active acidity of milk and milk curd.

The introduced dose of different types of MCE according to the rennet test results and according to the recommendation of the MCE manufacturer (the minimum recommended application dose) are shown in Table 2.

Table 2

Application dose and activity of MCE used in the experiment

Indicator	Type and dose of MCE			
	Fromase		Chy-max M	
	Low	High	Low	High
Application dose, g/100 kg	0,125	1,25	1,15	2,3
Milk-clotting activity, IMCU/g	2200	2200	910	910
Introduced dose into milk, IMCU/100 kg	275	2750	1045	2091
Proteolytic activity of MCE, U/g *	118,6	118,6	0,68	0,68
Application dose of proteolytic activity, U/100 kg	14,82	148,25	0,78	1,56
The degree of retention of MCE in the cheese mass, %	3%	3%	30%	30%
Proteolytic activity in cheese mass, U/ kg **	0,44	4,45	0,23	0,47

Note:
* actual value determined by the method according to GOST 34430–2018.
** calculated proteolytic activity based on the degree of MCE retention in the cheese mass according to [21]: for Fromase — ⩽ 3%, for Chy-max M — ⩾ 30%.

In the option when the rennet test was used to calculate the dosage of MCE, it was possible to reduce the introduced dose into milk for MCE Fromase by 10 times, for MMCE Chy-max M — by 2 times. This should lead to a significant decrease in the rate of proteolysis in the cheeses of Fromase Low variant. An even greater effect of reducing proteolysis in cheeses with Fromase can be expected due to the low retention of this type of MCE in the cheese mass. The expected level of proteolytic activity in cheeses with a low level of Fromase application has been reduced to the level of cheeses with a high level of Chy-max M.

Differences in MCE dosage resulted in minor differences at the stage of cheese production in the cheese vat. The duration of the processing operations at the stage of cheese production in the vat is shown in Table 3.

Differences in the introduced dose of MCE affected the duration of coagulation. When using a high dose of MCE, the coagulation time was reduced on average: for Fromase — by 3 times, for Chy-max M — by 2.7 times. The differences between the variants

Table 3

Duration of operations at the stage of cheese production in the cheese vat

Stage of technical process	Type and dose of MCE			
	Fromase		Chy-max M	
	Low	High	Low	High
Fermentation	100,00 ± 16,97	100,00 ± 16,97	100,00 ± 16,97	100,00 ± 16,97
Coagulation	35,67 ± 10,06 a)	11,67 ± 2,99 б)	35,33 ± 8,84 a)	13,33 ± 2,99 б)
Curd processing before cheddarization	22,67 ± 18,21	18,67 ± 4,53	25,67 ± 17,68	23,67 ± 10,06
Cheddarization	45,00 ± 25,93	71,67 ± 5,66	50,00 ± 9,80	66,67 ± 11,32
Total processing time	203,33 ± 58,88	202,00 ± 20,37	211,00 ± 16,75	203,67 ± 10,06
Thermo-mechanical treatment	10,00 ± 0,00	10,00 ± 0,00	10,00 ± 0,00	10,00 ± 0,00

Note: The mean values and confidence intervals of variation are given for the level of statistical significance p = 0.05. The values in the table row with the same indices a) and b) have statistically significant differences (p < 0.05). The values in the table row that are not marked with indices do not have statistically significant differences (p < 0.05)

Table 4

Dynamics of changes in the acidity of cheeses at the stage of production in the cheese vat and during storage

Stage of technical process	Type and dose of MCE			
	Fromase		Chy-max M	
	Low	High	Low	High
Milk before starter introduction	6,53±0,11	6,50±0,12	6,53±0,11	6,50±0,12
Before coagulation	6,22±0,16	6,19±0,06	6,22±0,16	6,19±0,06
After cutting the curd	5,86±0,08 [a)]	6,13±0,04 [б)]	5,83±0,13 [a)]	6,13±0,07 [б)]
Before cheddarization	5,65±0,13	5,98±0,02	5,62±0,08	5,95±0,17
After cheddarization	5,07±0,04	5,16±0,09	5,10±0,13	5,12±0,09
After thermo-mechanical treatment	5,08±0,04	5,11±0,08	5,05±0,08	5,06±0,13
1 day	4,80±0,09	5,04±0,11	4,73±0,13	4,98±0,14
8 day	4,89±0,17	5,07±0,08	4,78±0,11	5,03±0,09
30 day	4,90±0,20	5,08±0,13	4,82±0,06	5,05±0,09
60 day	4,87±0,27	5,06±0,09	4,81±0,11	5,03±0,08

Note: The mean values and confidence intervals of variation are given for the level of statistical significance p = 0.05. The values in the table row with the same indices a) and b) have statistically significant differences (p <0.05). The values in the table row that are not marked with indices do not have statistically significant differences (p <0.05)

of cheeses with different types and doses of MCE in terms of the duration of other operations and the total duration of processing in the cheese vat were statistically insignificant (p > 0.05).

The dynamics of changes in the acidity of cheeses at the stage of production in the cheese vat and during storage is shown in Table 4.

The data in Table 4 shows that the duration of the processing operations affected the dynamics of pH changes in the cheese vat. Differences in pH between the variants of cheeses produced with low and high doses of MCE were noted only at the moment after cutting the curd, which was associated with the different duration of coagulation in these variants. Despite the fact that there were no significant differences in the pH level between the variants of cheeses after the completion of the pasta-filata process, on the 1st day of storage, the pH level in the cheeses acquired different values during storage.

In cheeses produced with different doses of MCE, differences in pH were noted at the beginning of the shelf life, which was maintained until its completion. Despite the fact that the differences between the cheese variants in terms of the average pH level were not statistically significant (p > 0.05), there were clear differences in the texture of the cheeses. The cheeses with a lower pH, produced with a low dose of MCE (Fromase Low and Chy-max M Low variants), had a layered, kind of incoherent, granular texture with the release of free moisture on the cut. Cheese with a higher pH level, produced with a high dose of MCE (Fromase High and Chy-max M High variants) had a homogenic layered texture without separation of free moisture.

Data analysis was carried out to determine the factors influencing the pH of the cheeses. Since the duration of processing of curd in the cheese vat does not differ between the variants of cheeses (table 3), this factor cannot influence the pH of the cheeses. Other factors affecting the pH of the cheeses may be the pH of the curd after the completion of processing in the cheese vat and the moisture content of the curd. The dependence of the pH of the cheese at the beginning of storage on these factors is shown in Figure 1.

Fig. 2 a) it follows that there is a weak relationship between pH at the end of processing and pH of the cheese at the beginning of storage ($R^2 \approx 0.3$). To a greater extent, the pH of cheeses depends on their moisture content. The dependence shown in Fig. 2 b) shows that there is a strong positive relationship between these indicators ($R^2 > 0.9$).

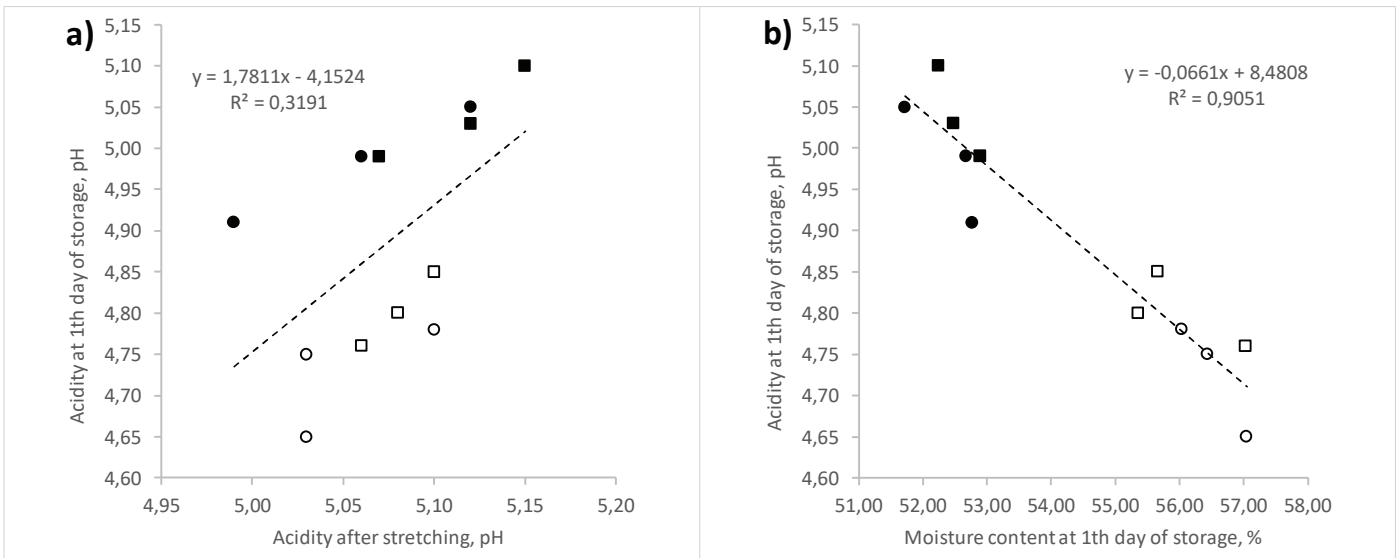


Figure 1. Dependence of the pH of the curd mass on the 1st day of storage on (a) the pH of the curd mass after the completion of processing in the cheese vat, (b) the moisture content in the curd mass on the 1st day of storage for the following cheeses: □ — Fromase Low; ■ — Fromase High; ○ — Chy-max M Low; ● — Chy-max M High; R^2 — coefficient of determination of the regression equation.

In addition, fig. 1 shows that according to the acidity level at the beginning of storage, cheeses are divided into 2 groups: cheeses with a low and high dose of MCE. Cheeses with low dose of MCE have a higher moisture content and lower pH than cheeses with high dose of MCE. This confirms the data of other researchers. In particular, Creamer, et al [27] found that the use of a very low dose of MCE results in a weaker curd, less moisture release and results in a more moist curd.

Consequently, milk coagulation with a low dose of MCE results in a more moist curd mass than using a high dose of MCE. The lactose contained in the aqueous phase of the cheese serves as a source for the formation of lactic acid by microorganisms of the starter culture and a drop in pH. In turn, a low pH level leads to a decrease in the degree of hydration of caseins, a decrease in the water-binding capacity of the cheese mass and the formation of an incoherent texture. When using a high dose of MCE, cheese is obtained with a lower moisture content, with a higher pH and cohesive texture. Since the duration of treatment in the cheese vat does not differ for the variants of cheeses with different doses of MCE, it can be concluded that a high dose of MCE contributes to the production of a denser curd having better syneresis properties.

Figure 2 shows the dynamics of changes in the mass fraction of moisture in cheeses during storage.

The data obtained shows that the pH and mass fraction of moisture in cheeses remains at an approximately constant level from the 1st day until the end of the shelf life. Therefore, the choice of the type and dose of MCE affects the properties of the resulting cheeses already at the stage of processing in the cheese vat.

Another important factor influencing the texture and other functional properties of cheeses is the proteolytic activity of MCE. Studies have shown that a multiple (two or more) increase in the dose of milk-clotting enzyme (rennet) led to an increase in the proteolysis of alpha-S1-casein, the appearance of a more plastic texture and an increase in the severity of the bitter taste in Gouda and Meshanger cheeses [28,29].

Figure 3 shows the dynamics of proteolysis during storage of cheese.

The degree of proteolysis in cheeses during storage depended on the MCE activity in the cheese mass (data in Table 2). The highest rate and final content of proteolysis products was observed in cheeses produced with a high dose of Fromase. Differences in the dynamics of proteolysis between cheeses made with low dose Fromase and cheeses made with Chy-max M (regardless of dose) were not statistically significant.

Milk-clotting enzymes of different origins (Fromase, based on R. miehei protease and Chy-max M based on camel chymosin), apart from differences in the depth of proteolysis, can also give qualitative differences in the composition of proteolysis products. The molecular weight distribution of water-soluble nitrogenous substances (amino acids and peptides) formed as a result of proteolysis by the end of the storage period (60 days) is shown in Figure 4.

From the data obtained on the form of the molecular weight distribution of proteolysis products in cheeses with different doses of MCE (Fig. 4), the following conclusions can be drawn: with a low dose of MCE, there are insignificant differences in the qualitative composition of proteolysis products between MCEs of different origins; with a high dose of MCE, the differences in the nature of proteolysis between MCEs of different origins increase. Fromase with an increased introduced dose forms an increased amount of peptides with a molecular weight >10 kDa. Peptides with such a high molecular weight are tasteless [30]. Proteolysis, accompanied by splitting into large fragments, leads to the rupture of a small number of peptide bonds in a large number of casein molecules that form a protein matrix. This leads to a decrease in the number of spatial bonds at many points of the protein matrix and a decrease in its strength. As a result, the degree of plasticity of the curd is increased. The peptides formed during proteolysis have a higher degree of hydrophilicity than the initial proteins, which leads to an increase in the degree of hydration of the cheese mass.

The cheeses made using Fromase had various degrees of bitter taste: with a low dose –slightly bitter taste, with a high dose — moderately expressed. The cheeses made with Chy-max M did not have a bitter taste at any dose. According to [31], peptides formed during hydrolysis of casein with a molecular weight of less than 6 kDa have a bitter taste. At the same time, with a decrease in molecular weight, the degree of bitter taste of peptides increases. According to [32], peptides with a mass of 2–5 kDa isolated from cheeses have a mild bitter taste, while peptides with

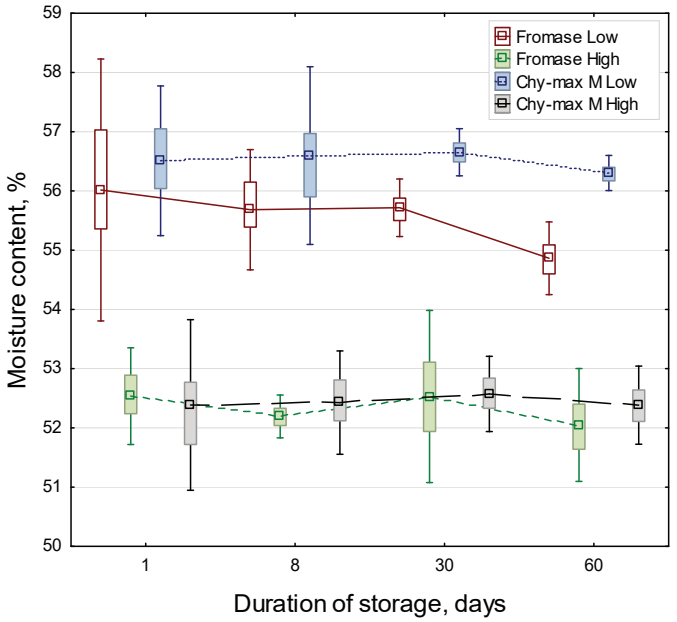


Figure 2. Dynamics of changes in the mass fraction of moisture in cheeses during storage.
Point: Mean; Box: Min-Max; Whisker: Mean ± 0,95 Conf. Interval

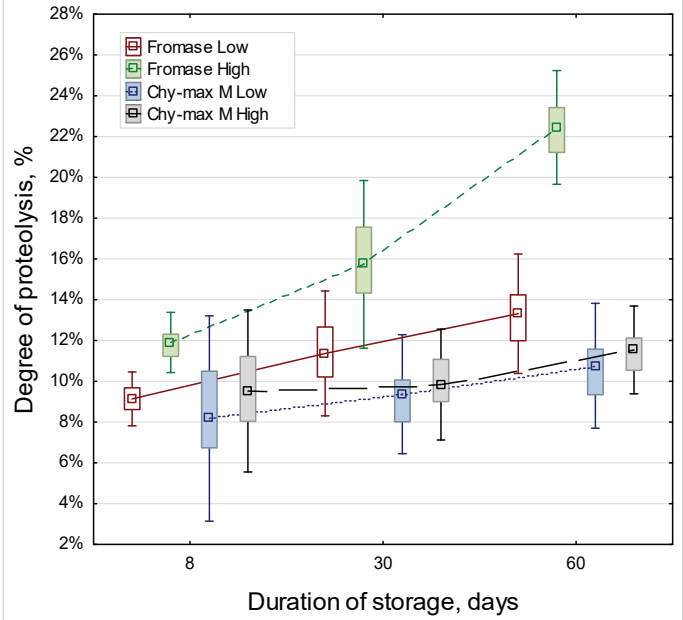


Figure 3. Dynamics of proteolysis (proportion of soluble nitrogen in total nitrogen) in cheeses during storage.
Point: Mean; Box: Min-Max; Whisker: Mean ± 0,95 Conf. Interval

a mass of less than 1 kDa have the most pronounced bitter taste. The fraction of peptides with a molecular weight of 0.5 kDa to 3.0 kDa had a bitter taste (peptides weighing less than 0.5 kDa did not have a bitter taste) [33]. Peptides with a molecular weight in the range of 3–10 kDa had a less pronounced bitter taste [34].

Cheeses made with Fromase have an increased content of peptides with molecular weights in the range of 0.5–1.0 kDa and 2.0–5.0 kDa (Figure 4). The presence of these fractions can be the source of the bitter taste in cheeses making with Fromase.

Differences in the degree of proteolysis of cheeses caused differences in the microstructure of cheeses produced with different types and doses of MCE. Photographs of the microstructure of cheeses after 60 days of storage are shown in Figure 5.

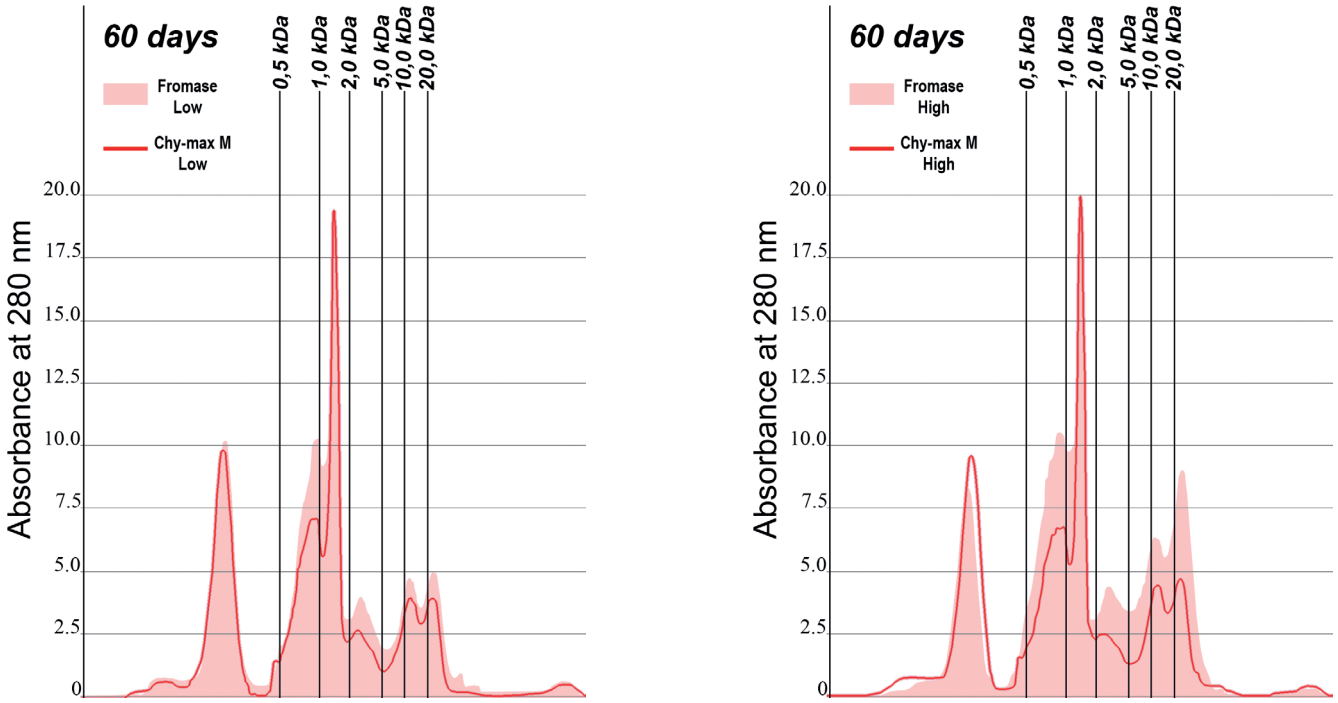
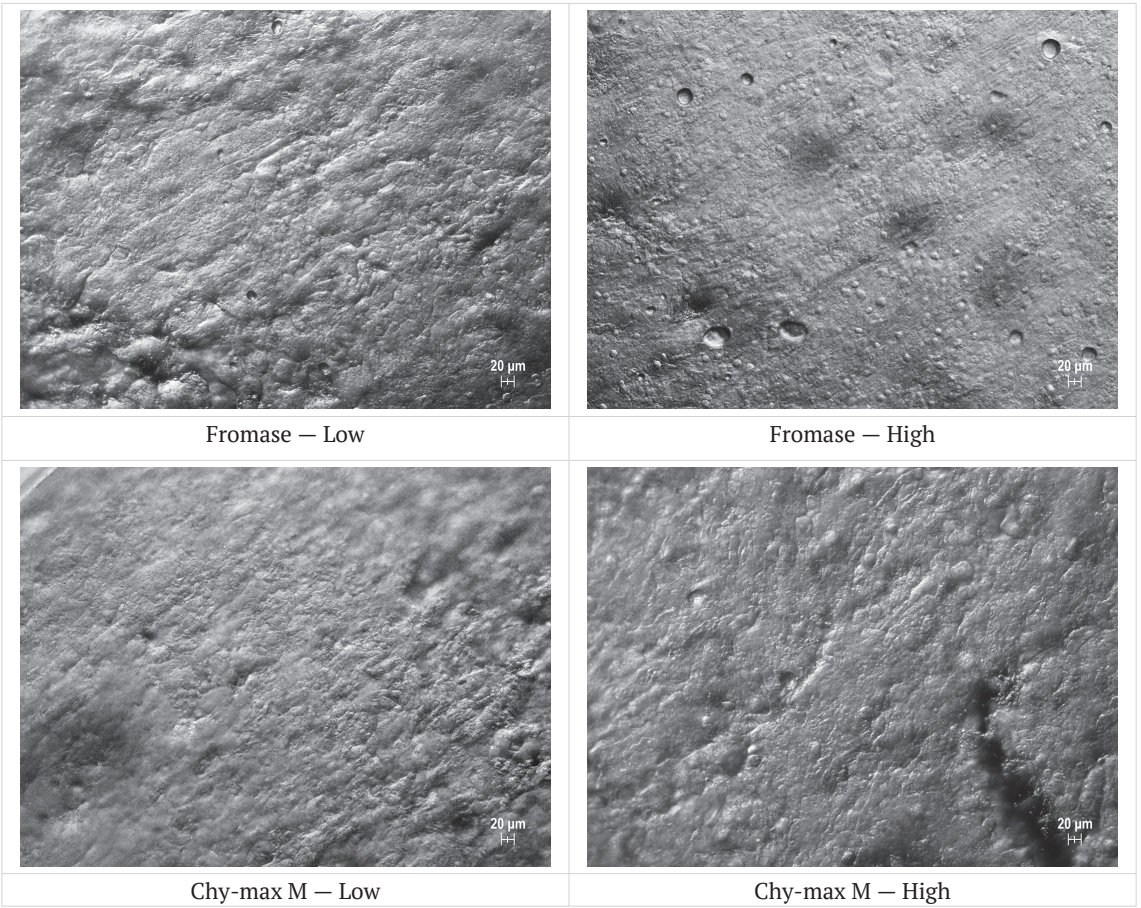


Figure 4. Molecular weight distribution of proteolysis products in cheeses after 60 days of storage



Fromase — Low | Fromase — High

Chy-max M — Low | Chy-max M — High

Figure 5. Microstructure of cheeses after 60 days of storage

After stretching processing, the cheese mass has the form of parallel elongated protein fibers with clusters of fat globules and bacteria packed between them [3]. In the process of proteolysis, which occurs in cheese under the action of milk-clotting enzyme and proteolytic enzymes of the starter culture, para-casein decomposes with the formation of water-soluble nitrogenous substances (peptides). As a result of the cleavage of a part of the polypeptide bonds in para-casein molecules, the degree of their hydration increases, and cross-linking between para-casein aggregates increases with the formation of new bonds under the action of the forces of hydrophobic and electrostatic interaction. This leads to the transformation and weakening of the protein matrix of the cheese and a change in the rheological properties of the cheese, which is expressed in an increase in the cohesion and elasticity of the cheese mass. Protein "capsules" surrounding the fat globules are degraded, which leads to the release of fat globules from the casein matrix [3,35].

In the cheese mass, which has undergone strong proteolysis, protein conglomerates, initially in the form of elongated "strands", swell and the unidirectional fibrous structure of the cheese disappears. This can be seen in the photographs of the microstructure of the cheeses. Cheeses made with Chy-max M and a low dose of Fromase have a low degree of proteolysis and are characterized by a layered structure with pronounced borders between the cheese grains. Cheeses made with a high dose of Fromase have a high degree of proteolysis and are characterized by a more homogeneous, finely dispersed structure, which is associated with the disappearance of large grains as a result of their hydration.

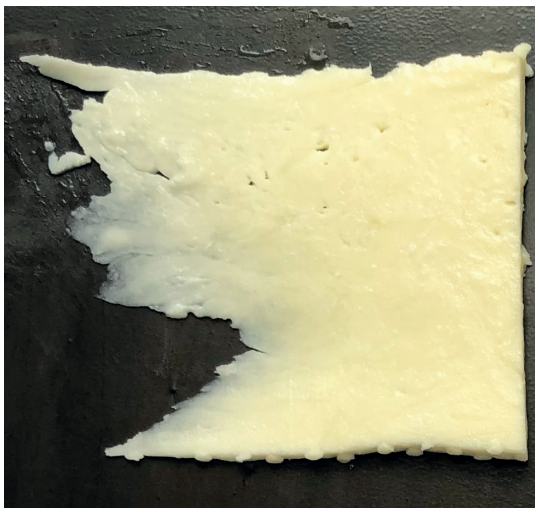
Differences in the degree of proteolysis in cheeses with different types and dosages of MCE resulted in differences in the structure and texture of these cheeses.

In cheeses with a low dose of MCE, which had a low degree of proteolysis until the end of the storage period, there were no noticeable changes in texture. They retained a pronounced fibrous structure. The disadvantage of the low level of proteolysis in such cheeses was the low degree of protein hydration, due to which the cheeses had an insufficiently coherent texture and contained unbound moisture that was released on the cut.

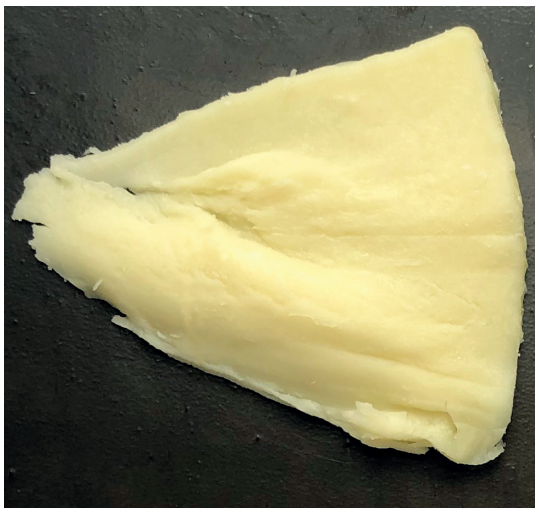
Cheese with a high level of Chy-max M had a coherent, homogeneous, moderately dense texture. Cheese made with a high level of Fromase, which had the highest degree of proteolysis, acquired a viscous and sticky texture. The use of such cheese for making pizza on a commercial scale is impossible: when sliced, the cheese sticks to the knife, when shredded, cheese chips stick together into lumps.

The appearance of the cheese cut after 60 days of storage is shown in Figure 6.

Tests of the functional properties of cheeses in a heated state were carried out. The results of the fork test of the melted cheese mass are shown in Figure 7.



Fromase — Low

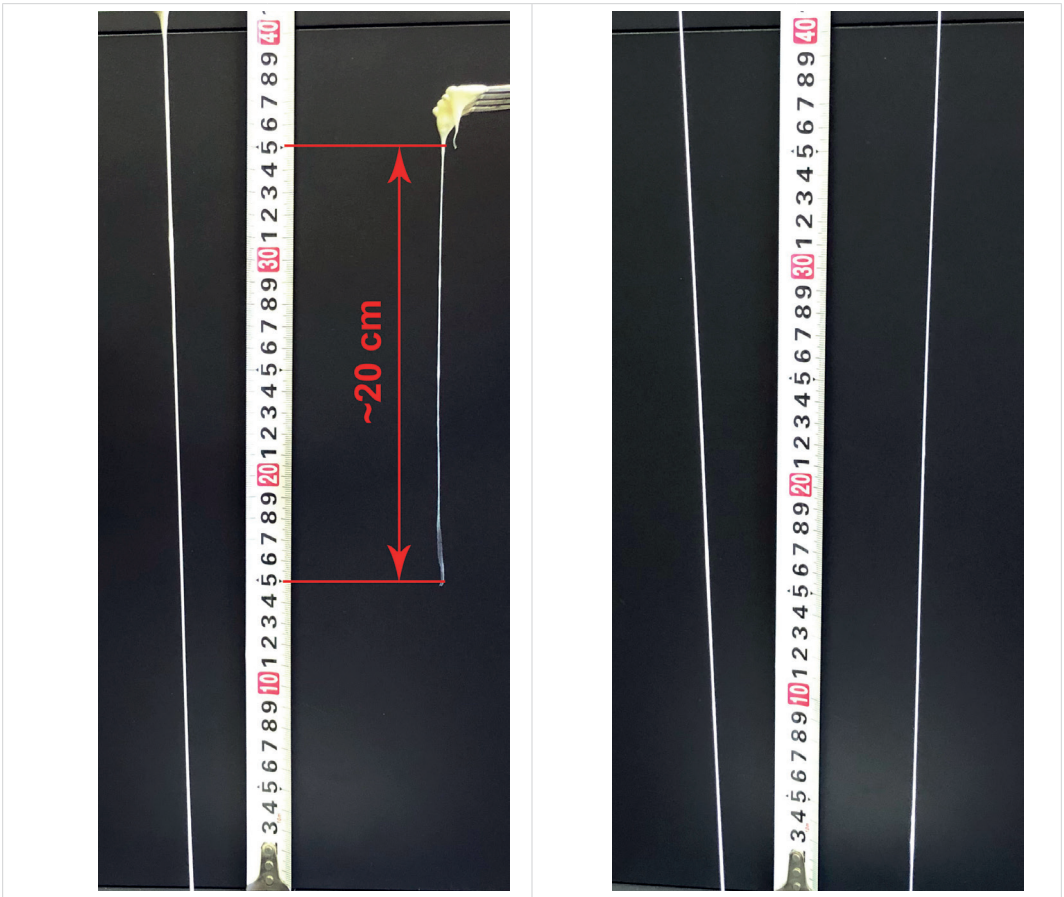


Fromase — High

Chy-max M — Low

Chy-max M — High

Figure 6. Appearance of the cheese cut after 60 days of storage



Chy-max M — High	Fromase — High	Chy-max M — Low	Fromase — Low

Figure 7. The results of the "fork" test for assessing the ability of the heated curd to stretch

As follows from the data provided in the analytical review, cheese made with a high dose of Fromase and having the highest degree of proteolysis, when heated, acquired an unnecessarily liquid and fluid consistency. The length of the threads formed during the stretching of such cheese did not exceed 20 cm. Cheese that had a low degree of proteolysis (with a low dose of Fromase and with Chy-max M, regardless of the dose), after heating, retained a coherent elastic texture and gave when stretching a thread about 100 cm.

4. Conclusions

When a low dose of milk-clotting enzymes (250–1100 IMCU per 100 kg of milk) was used for milk coagulation, cheeses were obtained with a high moisture content (55–57%), excessive acidity (pH 4.8–4.9) and texture defects (incoherent, crumbly, with separation of free moisture). This is due to the formation of a weak curd, which releases moisture poorly during processing.

The use of an increased dose of MCE makes it possible to obtain a denser curd, better releasing moisture. Cheeses produced with a high dose of milk-clotting enzymes (2000–2800 IMCU per 100 kg of milk) have a lower moisture content (52–53%) and lower acidity (pH 5.0–5.1). The protein matrix is more hydrated in these cheeses, which ensures its better water holding capacity and a more homogeneous and cohesive texture.

The use of an increased dose of a milk-clotting enzyme with a high level of total proteolytic activity (Fromase) gives undesirable consequences in the form of accelerated proteolysis of cheese mass proteins, rapid loss of functional properties of the cheese and a decrease in the shelf life of cheese (less than 60 days). Cheese production using an increased dose of a milk-clotting enzyme with a low level of total proteolytic activity (Chy-max M) allows achieving a low level of proteolysis during cheese ripening and increasing its shelf life.

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All authors bear responsibility for the work and presented data.

All authors made an equal contribution to the work.

The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

The authors declare no conflict of interest.

Received 15.08.2020 Accepted in revised 01.09.2020 Accepted for publication 25.09.2020