

Justification and Development of the Method for Differentiation of “Frozen-Thawed” Cycles of Fish Based on Differential Scanning Calorimetry

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Abstract: The goal of this study was to justify and develop a new rapid method for differentiating the frozen-thawed cycles of fish at the stages of the life cycle of fish products based on Differential Scanning Calorimetry (DSC). The object of research was the rainbow trout (*Oncorhynchus mykiss*) of the genus *Oncorhynchus* of salmon family (Salmonidae). For the analysis, a sample of muscle tissue was selected from the spinal part of the trout weighing 24 mg. The thermal analysis was started 1 h after the catch. To perform thermal analysis of fish in the heating-cooling process, a synchronous thermal analysis instrument STA 449 F3, Jupiter by NETZSCH was used. The device simultaneously captures the Differential Scanning Calorimetry (DSC) curves and the Weight Loss (WL). The analysis of the fish sample was carried out in a copper furnace with the connection of Dewar's vessel in oxidized aluminum crucibles in helium atmosphere. The accuracy of the temperature measurement was $\pm 0.3^{\circ}\text{C}$. A sample of fish (trout) was continuously cooled-heated at a rate of 5 K/min, according to the developed temperature program. The DSC curves show a maximum corresponding to the course of the endothermic process of sample thawing. Melting of the sample water component is observed in a fairly wide temperature range with the presence of large premelting which indicates a high viscosity of the sample. As the number of frozen-thawed cycles increases, the peak area of the DSC curve decreases from 195.9-118.6 kJ/mol, the temperature of the melting peak also decreases, especially between the second and third melting (from $5.12-2.87^{\circ}\text{C}$) and the melting interval is shifted to the negative region. The expediency and the development of the DSC temperature program with recommendations for its use have been proved in order to monitor the processing and storage of fish accompanied by phase change of water. For the check samples, it is necessary to fix thermoanalytical curves of successive double freezing-heating in the temperature range from $25-30^{\circ}\text{C}$ by cooling with nitrogen at a rate of 5 K/min. As a criterion for the thermal state of the trout check sample the exothermic effect of the second freezing serves for the control sample.

Key words: Differential Scanning Calorimetry (DSC), fresh and frozen-thawed fish, freezing, thawing, rainbow trout

INTRODUCTION

Due to the geographical peculiarities of the customs union countries, the best way to preserve the quality and safety of fish is cold treatment. This method simultaneously can process a large amount of fish raw materials both ashore and directly in the fishery. To properly organize the storage of chilled and frozen fish and reduce losses, it is necessary to know and take into account factors that affect the preservation of its native properties during production and storage. According to the mentioned, it is necessary to implement some plans in way of use of nature of clean energies with approach of sustainable development and create some powerful

foundations for this purpose through an overview of Iran's traditional architecture which has paid specific attention to climate and the designations and constructions have been based on climatic approaches (Poul, 2002; Omachonu and Ross, 2004).

Chilled fish has the best consumer qualities but a much shorter shelf life which with the traditional methods of cooling and storing chilled fish, limits its use to areas near catching or growing in aquaculture. Today, there are new technologies for storing chilled fish with the use of inert atmosphere, “liquid ice”, conserving agent which allow to extend the shelf life of this product to 20 and even 40 days (Aleksandrov, 2011; Artemov and Kharenko, 2010; Poul, 2002).

At the same time, the existence of such technologies increases the risk of falsification of fresh and chilled fish by its thermal state and by the number of frozen-thawed cycles. Depending on the type of raw material and the way it is pretreated, the temperature range for storing frozen fish ranges from -18 to -30°C.

Studies have shown that the shelf life of frozen fish at a temperature of -30°C is 1.5 times higher than at -18°C. To ensure the same duration of frozen fish storage, it is necessary to use additional means such as vacuum packing, glazing, use of inert gases, etc. (Debevere and Boskou, 1996; Fagan *et al.*, 2004). These treatments protect the product from drying out, changing the color, oxidizing but not slowing down the enzymatic processes that occur in the product not only on the surface layer. The results of these processes are denaturational and hydrolytic changes in fish biopolymers in particular, loss of myosin secondary and tertiary structure (Tahmassebpour, 2016, 2017; Seyedhosseini *et al.*, 2016).

The ability to maintain high relative air humidity (95-99%) is one of the significant advantages of low-temperature storage of frozen fish. This makes it possible to eliminate shrinkage and “cold burns”.

The most noticeable damages to the quality of frozen fish and its food safety are caused by temperature fluctuations both on various channels of goods distribution (in ports, dispatching cold stores, railways, trucks, ships, retail trade) and in the process of realization. On that basis, the regulatory documents set the deadlines for storage and sale of frozen fish, differentiated by temperature regimes (GOST, 2010).

Multiple thawing of frozen fish and temperature fluctuations lead to ice recrystallization. As a result, along with a decrease in the number of crystals their dimensions increase, leading to a disruption in the integrity of muscle fibers, denaturation of proteins and large losses of moisture. Accordingly, the consistency of the fish meat becomes flabby and dry. In addition, with high temperature, oxidative processes are more active, the rate of which is 2-3 times higher for example at a temperature of -9°C than at -18°C, resulting in a rancid taste and odor. Also at temperatures above -10 ... -12°C the mold is in a viable state, some bacteria assimilating the half-life of proteins and causing the formation of ammonia, hydrogen sulfide.

For fish processing enterprises and trade enterprises, the possibility of rapid objective control of the factors that form and preserve the quality of fish products at various stages of production, storage and sale in the trade network is an urgent problem. The solution is connected with the development of instrumental-methodological

support for operations of objective rapid control of unauthorized and undeclared facts of the change in the thermal state of fresh and chilled fish.

The reason for falsification is the much lower market price of frozen fish compared to fresh and chilled one. In this connection, there is an urgent task of instrumentally establishing the facts of undeclared thawing and freezing of fish, determining the number of cycles (operations) of repeated freezing which is indirectly evidenced by signs of denaturation, partial hydrolysis of protein and lipid fractions, the decrease in the moisture-retaining capacity of the fish muscle tissue as a result of changing moisture binding energy and form with the biopolymer structures of muscle proteins.

The goal of the research is to justify and develop a new rapid way of establishing the number of frozen-thawed cycles at the stages of the product life cycle based on monitoring the fish processing and storage processes accompanied by phase change of water.

MATERIALS AND METHODS

Objects and methods of research: The rainbow trout (*Oncorhynchus mykiss*) of the genus *Oncorhynchus* of salmon family (Salmonidae) was used as an object of the study. The trout was grown in the aquaculture of the trout farm “Rosa” (v. Trudovoe, Voronezh Region, Private Enterprise Head of Farm I.A. Alimenko). Private Enterprise Head of Farm I.A. Alimenko implements management system that conforms to the requirements of the standard EN ISO 9001: 2008 for the following scope: growing and sale of fish. According to the data (Artemov and Kharenko, 2010) trout is characterized by the largest content of nutrients and the smallest content of water, respectively it has the largest food density in comparison with other types of commercial fish such as sea perch, spotted catfish, sea flounder, Atlantic cod.

For the analysis, a sample of muscle tissue was selected from the spinal part of the trout weighing 24 mg. The thermal analysis was started in 1 h after catch. To perform thermal analysis of fish in the heating-cooling process, a synchronous thermal analysis instrument STA 449 F3, Jupiter by NETZSCH was used. The device simultaneously captures the Differential Scanning Calorimetry (DSC) curves and the Weight Loss (WL). The analysis of the fish sample was carried out in a copper furnace with the connection of Dewar’s vessel in oxidized aluminum crucibles in helium atmosphere. The accuracy of the temperature measurement was $\pm 0.3^\circ\text{C}$. A sample of fish (trout) was continuously cooled-heated at a rate of 5 K/min, according to the developed temperature program that shown in Table 1.

Table 1: Temperature program of the research

Test procedure number	Process	Process conditions and modes	
		Initial temperature (°C)	Final temperature (°C)
1	Freezing of fresh fish	25 in closed crucible	-30, nitrogen cooling
2	Heating (Thawing)	-30 in closed crucible	25
3	Freezing	25 in closed crucible	-30, nitrogen cooling
4	Heating (Thawing)	-30 in closed crucible	25
5	Freezing	25 in closed crucible	-30, nitrogen cooling
6	Heating	-30 in closed crucible	250

RESULTS AND DISCUSSION

Among the instrumental methods of differentiation between fresh and frozen-thawed fish, spectroscopy of the samples in the visible and near infrared spectral regions, visible and near infrared hyper spectral imaging (400-1000 nm) coupled with classifiers and spectral pre-processing techniques (Uddin and Okazaki, 2004; Cheng *et al.*, 2015) is known. These methods of differentiating the samples were used for horse mackerel and silver carp (Jun-Hu *et al.*, 2015) fluorescent spectroscopy (Karoui *et al.*, 2017) and proteomics (Ethuin *et al.*, 2014) was used for sea bass. A high correlation between fluorescence spectroscopy and traditional measurements based on the evaluation of the color, texture and chemical parameters of the fish was shown in the study (Ethuin *et al.*, 2014).

However, the spectral methods for establishing the fish's correspondence to its declared thermal state are based on the identification of changes in the physical structure of the predominantly surface layer of fish.

Two-dimensional electrophoresis in polyacrylamide gel and mass spectrometry on samples of sea bass fillets revealed biochemical markers of differentiation of fresh and thawed fish. However, proteomics requires complex sample preparation and is not suitable for solving routine tasks. This method could not be applied for online or at-line processing control.

Calorimetric, dilatometric and electrolytic methods can be used to analyze the thermal thawing pattern and determine the proportion of frozen water (Radhakrishnan, 1997; Silva *et al.*, 2008; Kasyanov and Syazin, 2014).

In the study, Silva *et al.* (2008) water activity was calculated for some fish species ($a_w = 0.946-0.974$) by the DSC method and compared with the maximum permissible value of this index for frozen products $a_w = 0.85$ established by the FDA (Food and Drug Administration) according to which water in frozen foods is in a state inaccessible for use by bacterial cells in the course of their vital activity.

Researchers of the study by Wang and Kolbe (1991) determined the specific heat, enthalpy, freezing temperature of water in the composition of pollock tissue fluid by the method of scanning calorimetry. Researcher

noted the great potential of calorimetric studies for obtaining and modeling the thermophysical properties of fish as a bioobject with a tissue level of the organization. The researchers of Radhakrishnan (1997) note that the value of the enthalpy of crystallization of water depends on the fat content of the fish and the moisture content slightly affects this index.

Frozen fish fillets can be considered as a set of structured protein gels (proteins of myofibrils) and protein sols (sarcoplasm of muscle cells). Tissue fluid is a protein sol due to a violation of the native muscle tissue cellular structure, containing dissolved organic and inorganic substances. The process of thawing in this case will first of all, look like a process of melting tissue fluid, i.e., a solution of a relatively small molal concentration. This solution should have a freezing point (cryoscopic temperature) below 0°C (Zaitsev, 1962).

Since, the cryoscopic temperature of the solutions depends on their concentration as the temperature decreases, the freezing temperature of the remaining liquid phase decreases and therefore as the product freezes the moisture freezes out with different binding energy with the product. First of all, mechanically bound moisture is freezing which contained in macro and microcapillaries, then physicochemical (by adsorption and osmotically) bound moisture while the chemically bound part of the water remains unfrozen up to -62- (-65)°C (Zaitsev, 1962).

Multiple thawing and freezing, lead to disruption of cell integrity or denaturation of the protein which is accompanied by a change in the relationship between the forms of moisture binding to the product, the study of which makes it possible to determine whether the fish has been deformed and how many times.

Figure 1 and Table 1 show the results of the DSC study of the single trout sample after a 3 time freeze (test procedures No. 1, 3, 5 of Table 1). The DSC curve show a minimum corresponding to the exothermic freezing of the sample. As the number of "frozen-thawed" cycles increases, the peak area of the DSC curves decreases from -199.7 to -113.1 J/g (Fig. 1) that is directly proportional to the change in the enthalpy of the process which is associated with a decrease in the number of free water in the sample.

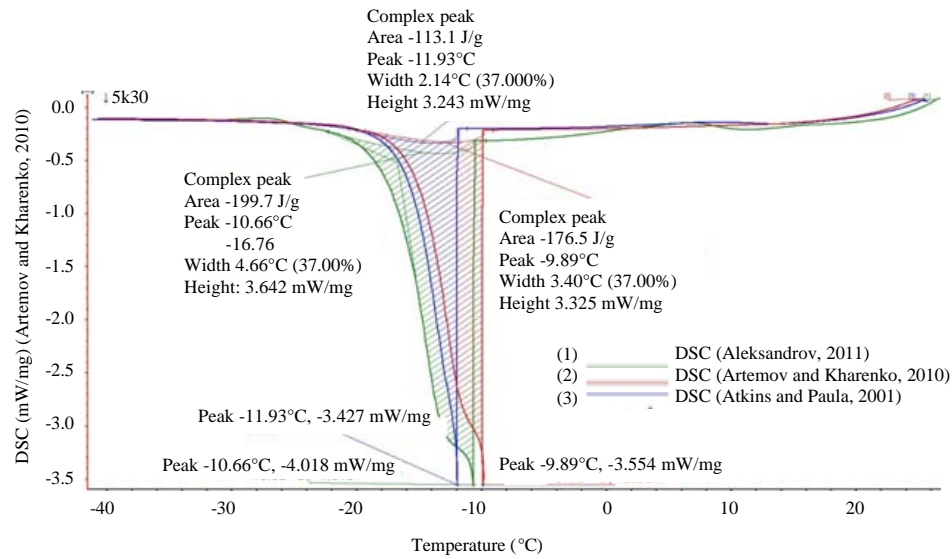


Fig. 1: Thermoanalytical curves of Differential Scanning Calorimetry (DSC): 1) first freezing; 2) second freezing and 3) third freezing

Table 2: Parameters of DSC curves during freezing and water activity in a trout sample

Test procedure, process number according to table 1	Cryoscopic temperature (°C)	Peak area (J/g)	Water activity
First freezing (No. 1)	-10.66	-199.7	0.925
Second freezing (No. 3)	-9.89	-176.5	0.911
Third freezing (No. 5)	-11.93	-113.1	0.894

According to Raoult's laws, the freezing point of the solution is always below the freezing point of the pure solvent (Atkins and Paula, 2001) and since in fish during the freezing crystallizes the tissue juice containing a certain amount of dissolved substances; the temperature of water crystallization in the fish is below the freezing point of pure water.

It should be noted that with a monotonic increase in the enthalpy, there is no regularity in the change of water crystallization temperature in the product. Since, the difference between the crystallization temperatures of the first and second, second and third does not exceed freezing of 1°C (Table 2) the absence of regularity is not significant. In this case, the magnitude of enthalpy change is more important for determining the quality of fish.

The value of the cryoscopic temperature can be determined from the water activity in the fish (Yuzov, 2009). The indicator "water activity" a_w is defined as the ratio of the partial pressure of water vapor over the product to the partial pressure of water vapor over pure water at a given temperature and reflects the degree of active water participation in various processes occurring in the food product:

$$a_w = P_w / P_0 = \text{POB} / 100 \quad (1)$$

Where:

P_w = Water vapor pressure in the food system

P_0 = Vapor pressure of clean water (at the same temperature)

ERH (Equilibrium Relative Humidity) relative humidity in a state of equilibrium (in which the product does not absorb moisture and does not emit it into the environment). Activity can also be calculated as the mole fraction of water according to Eq. 2 for an ideal system:

$$a_w = n_w / (n_w + n) \quad (2)$$

Where:

n_w = Amount of water in the product (mole)

n = Amount of dissolved substances (mole)

There are a number of equations connecting the magnitude of the cryoscopic temperature and water activity obtained by thermodynamic analysis of the moisture crystallization processes (Wang and Kolbe, 1991; Atkins and Paula, 2001; Kalatsevich and Murashev, 2012; Tahmassebpour, 2017). To calculate the water activity, the method used by the researcher by Wang and Kolbe (1991) was implemented, based on the results of scanning calorimetry and results of Raoult's law. It is known that the decrease in the freezing point of solutions is proportional to their concentrations:

$$\Delta t_{3aM} = C \cdot c_m \quad (3)$$

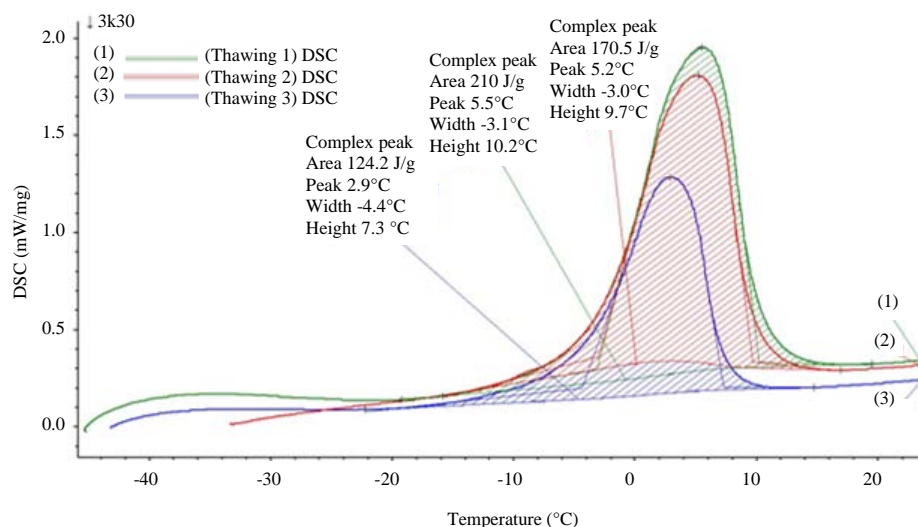


Fig. 2: Thermoanalytical DSC curves: 1) first thawing; 2) second thawing and 3) third thawing

Table 3: Parameters of DSC curves during thawing of a trout sample

Test procedure, process number according to table 1	The peak temperature of the melting (°C)	The temperature interval of melting (°C)	Area of peak of DSC curves (J/g)
Melting after the first freezing (No. 2)	5.55	-2.8 ... -10.20	195.9
Melting after the second freezing (No. 4)	5.12	-3.40 ... -9.76	176.8
Melting after the third freezing (No. 6)	2.87	-4.42 ... -7.26	118.6

Where:

$C = 1.86$ = Cryoscopic water constant ($^{\circ}\text{C}\cdot\text{kg}/\text{mole}$)

c_m = Molal concentration of solution (mol/kg)

The results of calculations by using Eq. 2 and 3 for three cryoscopic temperatures are presented in Table 2. As the freeze increases, water activity decreases from 0.925-0.894. This fact can be explained by the fact that during the multiple freezing processes the sample loses some of the free moisture and the water vapor pressure in the food system decreases (Eq. 1).

a_w level influences the intensity of the lipid oxidation, melanoidin formation, enzymatic, microbiological and other processes occurring in the product (Wang and Kolbe, 1991; Zaitsev, 1962). For the most pathogens, minimal levels of water activity are determined, below which they cannot develop which is very important for forecasting food safety. For example, in the activity area 1.00-0.95, the following microorganisms can grow in fish: *Pseudomonas*, *Escherichia*, *Proteus*, *Shigella*, *Klebsiell*, *Bacillus*, *Clostridiumperfringens*, some yeast. Effective means to prevent microbiological damage and some chemical reactions that reduce the quality of food products during storage is the reduction of water activity in food products. On the one hand, decrease in water activity leads to inhibition of microorganisms growth and

this is a positive process but decrease in water activity as a result of repeated frozen-thawed will worsen the organoleptic and physico-chemical properties of the product which will adversely affect its quality. By controlling the functional and technological indicators in the product and in particular, a_w indicator, it is possible to predict its storage capacity which will create “stability charts” of products and determine the optimal conditions for their storage.

Figure 2 and Table 3 show the results of the tissue fluid melting in the sample during 3 time freezing (Test Procedures No. 1, 3 and 5 of Table 1). The DSC curves show maximum correspondence to the course of the endothermic process of the sample thawing. Melting of the sample water component is observed in a fairly wide temperature range with the presence of large pre-melting which indicates a high viscosity of the sample. As the number of frozen-thawed cycles increases, the peak area of the DSC curve decreases from 195.9-118.6 kJ/mol, the temperature of the melting peak also decreases, especially between the second and third melting (from 5.12-2.87°C) and the melting interval is shifted to the negative region.

It should be noted that the crystallization enthalpy and melting of water (Table 2 and 3) are numerically practically equal to each other only for the second

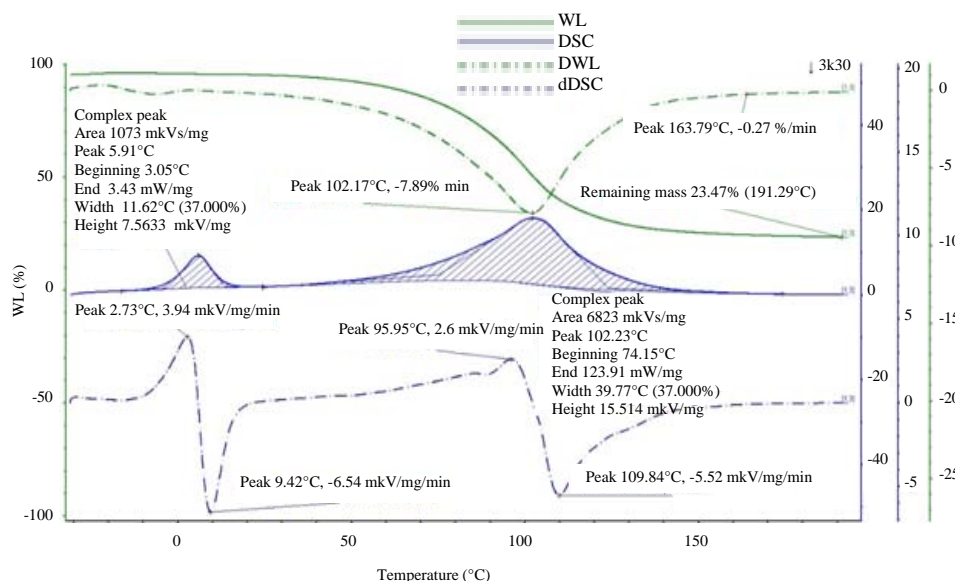


Fig. 3: Thermoanalytical curves of a fish sample when heated from minus 30-250°C (test 6, Table 1)

Table 4: Parameters of thermal effects when heating trout from -30°C to +250°C

Substance	Thermal effect	Enthalpy (kJ/mol)	Beginning temperature of the effect °C	Peak temperature (°C)	End temperature (°C)	Mass loss (%)	Event
Frozen trout	Endothermic	118.6	-3.05	5.91	13.43	-	Ice melting
	Endothermic	754.2	75.15	102.20	123.90	76.53	Dehydration

freezing and cooling (-176.5 and 176.8 kJ/mol). This is similar to the congruent melting of crystalline substances, when the composition of the melt coincides with the composition of the solid phase. The difference between the enthalpies of crystallization and melting of water after the first and third freezing indicates about complexity of the processes in the system, in this case the solid phase of ice is transformed into a melt and a solid phase of a different composition.

In determining the forms of water binding in a food sample, an experimental difficulty arises that is associated with loss of moisture during sample preparation and weighing it at room temperature. After the analysis of the obtained DSC curve is carried out it is difficult to determine the beginning of endothermic effect peak of water evaporation and therefore to calculate the enthalpy of thermal transformation (Peregonychaya *et al.*, 2009).

When the sample is heated from negative temperature (Test 6, Table 1) the possibility of water mass loss is excluded, the peaks are separated, it is easier to identify them, determine the beginning and end of the endothermic dehydration effect (Fig. 3 and Table 4). The first endothermic effect on the DSC curve (Fig. 3) corresponds to the melting of ice, the second effect to the evaporation of water. In the process of dehydration, the mass,

detected by the WL curve (Fig. 3) falls by 76.53% which coincides with the data by Radhakrishnan (1997) on the moisture content in trout (76-82%). In the process of dehydration, 754.2 kJ/mol of heat is absorbed. The method of freezing a sample and heating it to a high temperature can be useful to researchers to determine the forms of moisture binding in the food sample.

The advantages of differential scanning calorimetry over the known spectral methods of differentiation of fresh and frozen fish consist in obtaining a set of data on the state of water, the forms and energy of water binding with the biomaterial and the kinetics of the dehydration of fish samples. This is the basis for calculating the content of kinetically free and bound water in a fish sample, predicting its functional and technological properties and directions of expedient use in accordance with the principles of bioobjects controlled hydration as well as revealing the number of "frozen-thawed" cycles which limits the use of such fish raw materials.

CONCLUSION

The developed temperature program for conducting fresh fish research on the example of the rainbow trout fillet (*Oncorhynchus mykiss*) makes it possible to

determine the difference in the quantitative characteristics of multiple freezing processes in a closed oxidized aluminum crucible in a helium atmosphere according to the temperature-dependent thermoanalytical curves of differential scanning calorimetry ranging from 25 to -30°C by the following factors: cryoscopic temperature, peak area numerically equal to the exothermal effect of the freezing process, water activity value.

The obtained results can be used to establish the falsification of the thermal state of rainbow trout by its undeclared freezing. To implement the developed method of establishing the fact of an undeclared freezing, it is necessary to prepare samples of muscle tissue from the spinal part of the trout, equal in weight to the reference sample of a similar biomaterial (24 mg). The reference sample in the developed method for identifying the thermal state of the trout was taken from fresh fish 1 h after the catch. For the check samples, it is necessary to fix thermoanalytical curves of successive double freezing-heating in the temperature range from 25 to -30°C by cooling with nitrogen at a rate of 5 K/min. The exothermic effect of the second freezing for the reference sample serves as a criterion for the thermal state of the trout check sample. In this case, if the discrepancy between the values of the criterial index for the test and reference samples exceeds the error of the tool ($\pm 0.5\%$) then it testifies that preliminary freezing of the rainbow trout sample was carried out.

One of the principles of HACCP (Gitlow, 2000) is the control of Critical Points (CP) in which strict control over the quality of products makes it possible to prevent the occurrence of risks. For this purpose, observation systems in CP and various inspections are established through regular analysis and testing (Krishnamoorthi and Krishnamoorthi, 2011; Omachonu and Ross, 2004; Clute, 2008; Tokusoglu and Swanson, 2014). Thermal analysis to determine the temperatures and the freezing-melting enthalpy for multiple freezing-thawing as well as calculate the activity of water can be used in the CP observation system. The results can be applied to rainbow trout fillets of the following thermal conditions: fresh chilled, frozen-thawed once or repeatedly.

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