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Monitoring of frozen fish by histamine content and microbiological parameters

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ABSTRACT

As a result of microbiological decomposition of fish raw materials, especially during putrefactive spoilage or when storage parameters are violated, the muscle tissue of fish characterised by an elevated L-histidine content may accumulate the biogenic amine histamine. The purpose of the study was to investigate the safety indicators of frozen fish sold in supermarkets in Odesa (Ukraine) by histamine content and microbiological criteria (number of mesophilic aerobic and facultative anaerobic microorganisms, *coliform* bacteria, coagulase-positive staphylococci, *Salmonella*, *Listeria monocytogenes*, sulphite-reducing Clostridia, *Vibrio parahaemolyticus*). Histamine content in frozen fish samples was determined by photometric method according to DSTU 4894:2007. Microbiological parameters were studied using the culture method. According to the findings of the study, 55.3% of the samples showed histamine in the range from 11.0 ± 1.13 mg/kg to 216.0 ± 3.24 mg/kg with a total average value of 45.7 ± 13.2 mg/kg. The highest concentrations of histamine were found in samples of longtail southern cod (177.0 ± 2.15), sprat (188.0 ± 2.01), horse mackerel (192.0 ± 5.05), herring (202.0 ± 2.11), and mackerel (216.0 ± 3.24 mg/kg). Histamine content of more than 100.0 mg/kg was detected in 12.1% of positive samples, more than 200.0 mg/kg – in 1.9%, histamine content that was at the limit of an acceptable concentration of 200.0 mg/kg – in 1.9% of samples, respectively. Exceeding the maximum permissible levels of mesophilic aerobic and facultative anaerobic microorganisms (MAFAnM) was detected in samples of "Blakytna" mackerel (Spain) in 10.7 % of the total number of samples with a maximum level of 6.7 x 10^4 CFU in 1 g; herring – in 6.7% of samples with a maximum level of 6.6 x 10^4 CFU in 1 g. *Coliform* bacteria were found in samples of mackerel (Norway) – 9.4% of the total number of samples; "Blakytna" mackerel (Spain) – 7.1%, herring – 13.3%, longtail southern cod – 5.9%, tilapia – 14.3%, sprat – 5.3%, horse mackerel – 5% of the total number of samples, respectively. All the above-mentioned samples had the highest histamine levels, indicating an association between this indicator and the presence of sanitary-indicative microflora. The findings confirmed the danger of some samples of frozen fish sold in the retail network of Odesa, and pointed out the need to strengthen monitoring of fish for histamine content.

Keywords: biogenic amine, L-histidine, fish product, safety, logistic

INTRODUCTION

High global demand for fish requires stronger controls to protect consumer health. The human body may be exposed to toxic substances, such as heavy metals and persistent organic pollutants, due to high consumption of fish and fish products. In addition, a number of factors, such as the conditions for catching, storing, and transporting fish and fish products, can lead to the formation of an organic compound such as histamine **[1]**. According to UIFSA, in 2023, fish and seafood took the first place among all food products imported to Ukraine. According to the results of 2023, the consumption and import of fish and seafood amounted to 330,000 tonnes,

and in 2022 the average annual consumption of marine products in Ukraine was 13 kg per person **[2]**. Consuming fish and seafood is good for your health because they contain significant amounts of protein, lipids, polyunsaturated fatty acids (especially omega-3), and important vitamins and minerals. However, due to their high free-amino-acid content, such products are prone to rapid spoilage by microorganisms **[3]**. As a result of microbial decomposition of fish (especially during putrefactive spoilage or after disruption of certain technological processes, such as storage time and temperature), biogenic amine histamine can accumulate in its muscles, mainly in fish species with high L-histidine content. These fish include mackerel, sardines, anchovies, tuna, and other species belonging to the families *Scombridae*, *Clupeidae*, *Engraulidae*, *Coryphaenidae*, *Pomatomidae* and *Scombresosidae*. There are also fish species with the potential to form histamine belonging to the orders *Istiophoriformes* and *Scombriformes*, including the families *Carangidae* and *Gempylidae*.That is, there are many fish species that have a potential danger to the consumer due to the development of histamine due to the content of elevated levels of free L-histidine in their muscles. Salmonids can sometimes cause histamine poisoning, but this is quite rare, as high levels of histamine accumulate in this species of fish only when they decompose **[1]**.

Determination of histamine levels in fish and fish products is an important task for maintaining consumer health, determining the quality of fish and the state of the food industry **[4]**. Microbiological control is a key step in ensuring food safety, especially in the context of an unstable logistics chain and storage changes, which is also relevant for monitoring the quality of frozen fish **[5]**.

The intensity of histamine development in fish products is significantly affected by temperature, pH, oxygen availability, processing conditions, packaging, and storage duration. Histamine-forming bacteria can synthesise high concentrations of histamine in a wide temperature range (5-37 °C), and the maximum activity of histidine decarboxylase is observed around 30 °C. Violations of the temperature regime and storage time after fishing contribute to rapid microbial growth and excessive histamine accumulation, which significantly compromise the safety and quality of fish products **[6]**. The presence of histamine can be detected not only in frozen or fresh fish, but also in foods for infants and young children. Histamine concentrations in baby food increased with increasing storage time and temperature. After 48 hours of storage at 22 °C, histamine levels were 1.8 times higher than at 2 °C. Dishes containing tuna and sea fish contained more histamine than dishes containing pollock **[7]**. The results highlighted the importance of monitoring storage conditions and the need to raise awareness of histamine risks for particularly sensitive groups – infants and young children.

Scientific Hypothesis

The histamine content in frozen fish samples is associated with the presence of sanitary indicator microflora.

Objectives

The primary objective is to monitor frozen fish sold in supermarkets in Odessa according to safety indicators. Additional objectives: to establish the level of histamine in samples of frozen fish; to determine the number of mesophilic aerobic and facultative anaerobic microorganisms (MAFAnM), coliform bacteria, coagulase-positive staphylococci, *Salmonella, L. monocytogenes*, sulphite-reducing clostridia, and V. parahaemolyticus in the test samples; to determine the compliance of the tested frozen fish with safety indicators in accordance with Order of the Ministry of Health of Ukraine No. 548.

MATERIAL AND METHODS

Samples

Samples description: For the study, samples of frozen fish were purchased from eight supermarkets belonging to the largest retail chains in Odessa (Ukraine). The study covered 67 batches of products from various importers and market operators.

Samples collection: Sample collection was carried out evenly throughout 2023-2024, taking into account the seasonal distribution of product sales: 70 samples were collected in spring, 65 in summer, 74 in autumn and 75 in winter. Only individual product samples were included in the study. Repeat samples from the same batch, as well as repeat samples from the same manufacturer, selected within the same supply batch, were not included. For each sample, a separate production date, batch number and manufacturer's marking were taken into account, which allowed to avoid duplication of study results. The following types of frozen fish were selected: mackerel (Norway) – 32 samples; “Blakytna” mackerel (Spain) – 28 samples, herring – 30 samples; North American hake – 24 samples; Argentine hake – 25 samples; Alaska pollock – 19 samples; longtail southern cod – 17 samples; tilapia – 21 samples; perch – 15 samples; pangasius – 21 samples; sprat – 19 samples; horse mackerel – 20 samples; wolffish – 13 samples. The samples were transported in a portable isothermal refrigerator container at a temperature of 0 °C to 10 °C within 4 hours of packing, ensuring the preservation of their physicochemical and microbiological properties until laboratory tests were carried out.

Samples preparation:

Fish samples were prepared after defrosting according to DSTU 4894:2007 **[8]**. Prior to the analysis, the experimental material was removed from the refrigerator and kept at room temperature. Next, the samples were homogenised in a laboratory shredder. The crushed material was mixed for several minutes.

Preparation of frozen fish samples for microbiological studies was carried out in accordance with DSTU EN ISO 6887-3:2022 **[9]**. The samples were thawed at room temperature (18-27 °C) for approximately 60 minutes, but not longer than 3 hours. After partial defrosting, pieces of fish were selected with sterile tweezers and a knife for further preparation. If necessary, the samples were left to thaw further until they were sufficiently thawed to grind. The resulting sample fragments were crushed and homogenised in a rotary homogeniser using a suitable diluent to prepare a suspension in a ratio of 1:10.

Number of samples analysed: A total of 284 frozen fish samples were examined: mackerel (Norway) – 32 samples; "Blakytna" mackerel (Spain) – 28 samples; herring – 30 samples; North American hake – 24 samples; Argentine hake – 25 samples; Alaska pollock – 19 samples; longtail southern cod – 17 samples; tilapia – 21 samples; perch – 15 samples; pangasius – 21 samples; sprat – 19 samples; horse mackerel – 20 samples; and wolffish – 13 samples.

Chemicals

The following reagents and consumables were used to conduct the study: trichloroacetic acid (chemically pure); caustic soda (pure for analysis); sodium carbonate (chemically pure); n-butanol (pure); 0.1 normal hydrochloric acid solution (chemically pure); ethyl acetate (pure for analysis); anhydrous sodium sulphate (chemically pure); sodium nitrate (pure for analysis); paranitroaniline (pure for analysis). All reagents were from Khimlaborreaktyv LLC (Ukraine). To calibrate the spectrophotometer, a standard sample of histamine (pure preparation H7125 with a mass fraction of the main substance of at least 97%) was used, by Sigma-Aldrich (USA).

Animals, Plants and Biological Materials

Mackerel (Norway) (*Atlantic mackerel*); "Blakytna" mackerel (Spain) (*Atlantic chub mackerel*); herring (*Atlantic herring*); North American hake (*Silver hake*); Argentine hake (*Merluccius hubbsi*); Alaska pollock (*Gadus chalcogrammus*); longtail southern cod (*Patagonotothen ramsayi*); tilapia (*Nile tilapia*); perch (*European perch*); pangasius (*Striped catfish*); sprat (*Sprattus sprattus*); horse mackerel (*Trachurus trachurus*); wolffish (*Anarhichas lupus*). Fish from various market operators of the Odesa supermarket chain.

Instruments

The following accessories were used for the study the histamine content: ULAB 102 UV spectrophotometer (China) with cuvettes with a working length of 5 mm; water thermostat (water bath) WNB7-45 (Germany); analytical scales, accuracy class 2 OHAUS (China,); mercury glass laboratory thermometer with a measurement range from 0 °C to 100 °C; laboratory chopper; low-temperature electric furnace SNOL 58/350 (Lithuania); Shaker LAB-PU 01 (Ukraine); household refrigerator; laboratory glass tableware.

For microbiological tests, dry-air thermostats TC-160 MIZ-MA (Ukraine) and a laboratory homogenizer BagMixer 400W (China), laboratory glassware were used according to the methods.

Laboratory Methods

Histamine content in samples was determined by photometric method in accordance with DSTU 4894:2007 **[8]**. This method was based on measuring the absorbance of the colored solution obtained during the reaction between histamine and a diazoreagent. The measurement range of histamine content is from 10 mg/kg to 400 mg/kg. The limit of quantitation (LOQ) of the method was 10 mg/kg. The recovery percentage (RP) during the studies was within 70-120% with RSD not > 20%. The measurement uncertainty was not determined. The confidence limits of the absolute measurement error in determining the histamine content are A = ±2.5 % at a confidence level of P = 0.95.

Quantity of MAFAnM was measured in accordance with DSTU EN ISO 4833-1:2014 using PCA (Plate Count Agar) **[10]**; incubation in a thermostat was performed at 30 °C, 72 hours. Studies for the presence of *coliform* bacteria were performed in accordance with DSTU GOST 30726:2002 using McConkey medium with bromocresol purpurea at an incubation temperature of 37 °C, 24-48 hours **[11]**. In the presence of growth, the samples were transplanted into Endo Agar and incubated at 37 °C for 24 hours. Studies for the presence of coagulase-positive staphylococci were conducted in accordance with DSTU EN ISO 6888-1:2022 using salt broth **[12]**; incubation was performed in a thermostat at 37 °C, 48 hours. The transfer was performed on a Baird-Parker medium at a thermostat temperature of 37 °C for 48 hours. Testing for the presence of *Salmonella* in samples was performed in accordance with DSTU EN ISO 6579-1:2022 **[13]**. Determination of this indicator was carried out by a series of successive stages of enrichment and re-seeding on selective media: peptone-buffer water (incubation at 37 °C, 18 hours); Muller-Kaufman medium with novobicin (incubation at 37 °C, 24 hours); Rappaport-Vassiliadis liquid medium (incubation at 37°C, 24 hours); xylose-lysine deoxycholate Agar (XLD-Agar) at incubation temperature 37 °C, 24 hours; Brilliant Green Red Phenol/Agar (at an incubation temperature of 37 °C,

24 hours). Testing for *L. monocytogenes* was carried out in accordance with DSTU ISO 11290-1:2003 by four consecutive stages of enrichment and re-seeding **[14]**. Used: half milling broth (incubation in a thermostat at 30 °C, 18 hours); full milling broth (incubation in a thermostat at 37 °C, 48 hours). Next, re-seeding was carried out on two selective dense media: Oxford-Agar and PALKALM-Agar, incubation was carried out at a temperature of 37 °C, 48 hours. Studies for the presence of sulfite-reducing Clostridium were conducted in accordance with DSTU ISO 7937:2006 (Wilson Blair Agar was used, incubation was carried out at a temperature of 36 °C, 72 hours) **[15]**. Research for the presence of *V. parahaemolyticus* was performed in accordance with DSTU EN ISO 21872-1:2022 (differential diagnostic agar was used for vibrios, incubation was carried out at a temperature of 36 °C, 24 hours) **[16]**.

Description of the Experiment

Study flow: In the first phase of the experiment, we collected frozen fish samples. After being purchased from retail chains, the fish samples were transported to the laboratory within 4 hours. Subsequently, fish samples were prepared. To determine the histamine content, the sample weight of 10 g (weighted with an accuracy of 0.01 g) was placed in a conical flask with a section with a capacity of 100 cm^3, added 40 cm^3 of trichloroacetic acid solution with a mass concentration of 50 g/dm^3 was added, and the mixture was placed for 5 minutes in a device for shaking liquids. The flask was fitted with a reflux condenser and immersed in a water bath at 60 °C for 15 minutes. Next, the flask was removed and cooled to 20 °C. The cooled mixture was quantitatively transferred to a measuring cylinder with a capacity of 50 cm^3 (the original flask was rinsed in 3 cm^3 portions of trichloroacetic acid solution) and the volume in the cylinder was adjusted to 50 cm^3. The resulting mixture was filtered into a flask with a grinding capacity of 50 or 100 cm^3 through the filter "red tape" (filtrate 1). Filtrate 1, 5 cm^3 in volume, transferred to a test tube and added 1 cm^3 of sodium hydroxide solution with a mass concentration of 200 g/dm^3. Then, anhydrous sodium carbonate was added to the test tube with a spatula, and the mixture was mixed until a saturated solution was obtained. Then, 5 cm3 of water-saturated butanol-1 was added to the test tube and shaken for 30 seconds. The test tube was left until the phases were separated. After stratification, 3 cm^3 of the upper butanol layer was selected and transferred to another test tube, into which 3 cm3 of a hydrochloric acid solution with a molar concentration of 0.1 mol/dm3 had previously been added. The test tube was shaken for 30 seconds, settled until the layers were separated, and 2 cm^3 in the lower water layer was taken and transferred to another test tube, 2 cm^3 solution of sodium carbonate with a mass concentration of 40 g/dm^3 was added, kept in the freezer for 5 minutes. 2 cm^3 of diazo reagent was added to the cooled solution and kept in the freezer for 5 minutes. The solution after adding diazoreactive acquired a pink-crimson colour. Then, 4 cm^3 of acetic acid (ethyl acetate) was added to the test tube and shaken for 30 seconds. The test tubes were left in a tripod until the phases were separated. Next, the top layer was selected and transferred to test tubes, into which 1 g of anhydrous sodium sulfate had been added previously. The stained solutions were alternately transferred to the cuvette of a spectrophotometer with a working length of 5 mm, and the absorption of the solution was measured at a wavelength of 490 ± 10 nm. Ethyl acetate was used as a comparison solution. The same actions were performed for calibration solutions (3 parallel dilutions were prepared for each concentration). Based on the data obtained when measuring the absorption of calibration solutions, a calibration graph was constructed. An example of a calibration graph is shown in Figure 1.

The histamine content in the test sample, in mg/kg, was calculated using the equation (1):

$$\omega = \frac{C*V}{m} * F\ , \quad (1)$$

Where: C – histamine concentration found on the calibration chart, $\mu g/cm^3$; V – volume of filtrate 1, cm^3; m – weight of the sample suspension, g; F – dilution factor calculated by equation (2):

$$F = V + V_1 / V, \quad (2)$$

Where: V – volume of filtrate 1, cm^3; V_1 – volume of trichloroacetic acid solution added to filtrate 1 during dilution, cm^3.

The arithmetic mean of the results of 3 parallel definitions was taken as the final test result. Calculations were performed up to an integer. The permissible difference between the results of parallel determinations obtained in the same laboratory in the same series of tests did not exceed 20% of the arithmetic mean value. According to DSTU 4894:2007, the reproducibility of studies should not exceed 25% of the arithmetic mean value.

Microbiological studies of frozen fish samples were carried out using the culture method in accordance with generally accepted protocols.

In the last phase, we processed the obtained results, subjected them to statistical analysis, and verified the validity of our hypotheses.

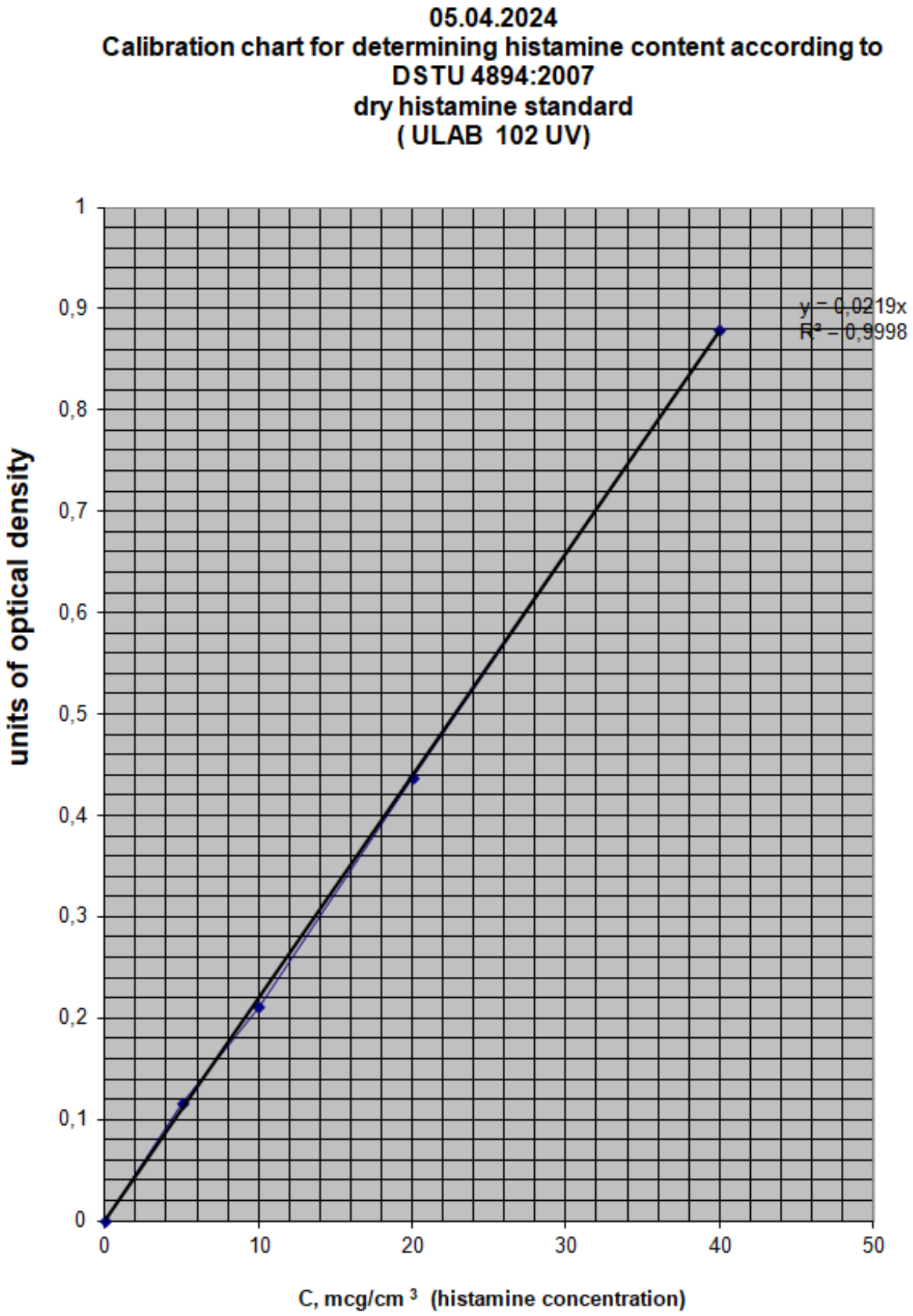

Figure 1 Example of a calibration graph.

Quality Assurance

Number of repeated analyses: All measurements were performed in triplicate (technical replicates).
Number of experiment replication: 1.
Reference materials: -.
Calibration: The following solutions were prepared for calibration of the spectrophotometer: basic standard histamine solution with a concentration of 40 μg/cm^3 and working solutions that were prepared in 5% trichloroacetic acid from the main standard solution at the following concentration: working solution No. 1 (20 μg/cm^3), working solution No. 2 (10 μg/cm^3), working solution No. 3 (5 μg/cm^3). Ethyl acetate was used as the comparison solution. Based on the data obtained when measuring the absorption of calibration solutions, a calibration graph was constructed.
Laboratory accreditation: The research was conducted at the Odesa State Agrarian University (the multidisciplinary laboratory of Odesa State Agrarian University) and at Mykolaiv National Agrarian University. The laboratories are accredited in accordance with DSTU EN ISO/IEC 17025:2019.

Data Access

The data supporting the results of this study are not publicly available.

Statistical Analysis

Statistical processing of experimental data was carried out using the software packages Statistica 13.0 and Microsoft Excel 365. The statistical unit of analysis was a single sample of frozen fish. Since the samples came from different fish species and market operators, each sample was treated as an independent observation, ensuring the correct application of degrees of freedom for the data structure. The study design did not involve repeated independent experiments, since each sample underwent a single laboratory analysis after the completion of the research complex. At the same time, all analytical measurements were performed in three repetitions (technical replicates). In this regard, statistical analysis methods for repeated measurements or mixed models were not used. Preliminary data preparation included checking the data set for outliers using the Grubbs criterion at $\alpha = 0.05$, assessing data completeness, and verifying logical consistency. Descriptive statistics parameters were calculated for quantitative indicators, in particular, histamine content and microbiological characteristics. The results are presented as the arithmetic mean and standard deviation ($x \pm SD$). Statistical analysis was aimed at assessing the variability of histamine concentrations in fish samples and establishing relationships between histamine levels and indicators of sanitary-indicative microflora. To analyze the relationships among the studied indicators, statistical methods were used, selected based on the characteristics of the data distribution. The level of statistical significance for all analytical procedures was set at $p \leq 0.05$. Given the research nature of the work, as well as the heterogeneity of the fish species studied and the sources of origin of the samples, statistical methods were used primarily to identify trends, assess the nature of relationships, and interpret the results scientifically, without building predictive models. Additional covariates were not included in the statistical models.

Reporting and transparency statement

The sampling of fish was carried out in accordance with the Resolution of the Cabinet of Ministers of Ukraine No. 833 (2002) **[17]**, DSTU 7972:2015 **[18]**, and scientific and methodological recommendations **[19]**. The main criteria for sampling were as follows: the origin of the product (selected fish of different market operators, presented in the retail network of supermarkets of the city of Odesa to assess the quality of products of different production and geographical origin); the condition of the product (for the study, frozen fish was selected with undamaged packaging, without signs of defrosting, spoilage, or foreign odour, samples with mechanical damage, ice crystals in the thickness of meat or violation of the tightness of packaging were not allowed); the range and species diversity (the sample included the most common types of fish that are sold on the consumer market, which allowed conducting comparative assessment by species characteristics); representativeness of the sample (the selection was carried out from different retail outlets and lots, which ensured the representativeness and reliability of the study results); shelf life and storage conditions (samples were selected in which the shelf life was not expired, and the temperature regime of storage corresponded to the marking on the manufacturer's label). Randomization and blinding were not applicable. All samples were included in the analysis.

RESULTS AND DISCUSSION

The results of the analysis of histamine content in frozen fish are shown in Table 1. According to the studies, histamine was detected in 157 experimental samples, accounting for 55.3% of the total. No histamine content was detected in 127 (44.7%) samples.

Table 1 Histamine content in frozen fish, x ± SD.

Type of fish	Number of samples studied	Number of samples containing histamine	Histamine content range, mg/kg, min-max
Mackerel, Norway	32	25	34.0 ± 2.18 – 216.0 ± 3.24
Mackerel "Blakytna", Spain	28	19	22.0 ± 2.03 – 208.0 ± 4.12
Herring	30	22	18.0 ± 1.82 – 202.0 ± 2.11
North American hake	24	15	18.0 ± 1.17 – 48.0 ± 2.15
Argentinian hake	25	13	24.0 ± 2.05 – 53.0 ± 1.28
Alaska pollock	19	6	11.0 ± 1.13 – 56.0 ± 1.75
Longtail southern cod	17	8	19.0 ± 1.45 – 177.0 ± 2.15
Tilapia, fillet	21	12	21.0 ± 2.13 – 91.0 ± 3.23
Perch	15	5	13.0 ± 0.95 – 82.0 ± 4.03
Pangasius, filet	21	12	19.0 ± 2.07 – 68.0 ± 3.18
Sprat	19	11	31.0 ± 2.47 – 188.0 ± 2.01
Horse mackerel	20	7	39.0 ± 2.01 – 192.0 ± 5.05
Wolffish	13	2	24.0 ± 1.89 – 75.0 ± 3.45
Total	284	157	–

As shown in Figure 2, the prevalence of histamine-positive samples varied considerably between fish species, ranging from 15.4% (wolffish) to 78% (mackerel, Norway). Species such as mackerel and herring demonstrated consistently high prevalence rates (>70%), whereas lower values were observed for Alaska pollock and perch (<35%). This variability suggests that both intrinsic factors (species-specific biochemical composition) and extrinsic factors (storage and handling conditions) influence histamine formation.

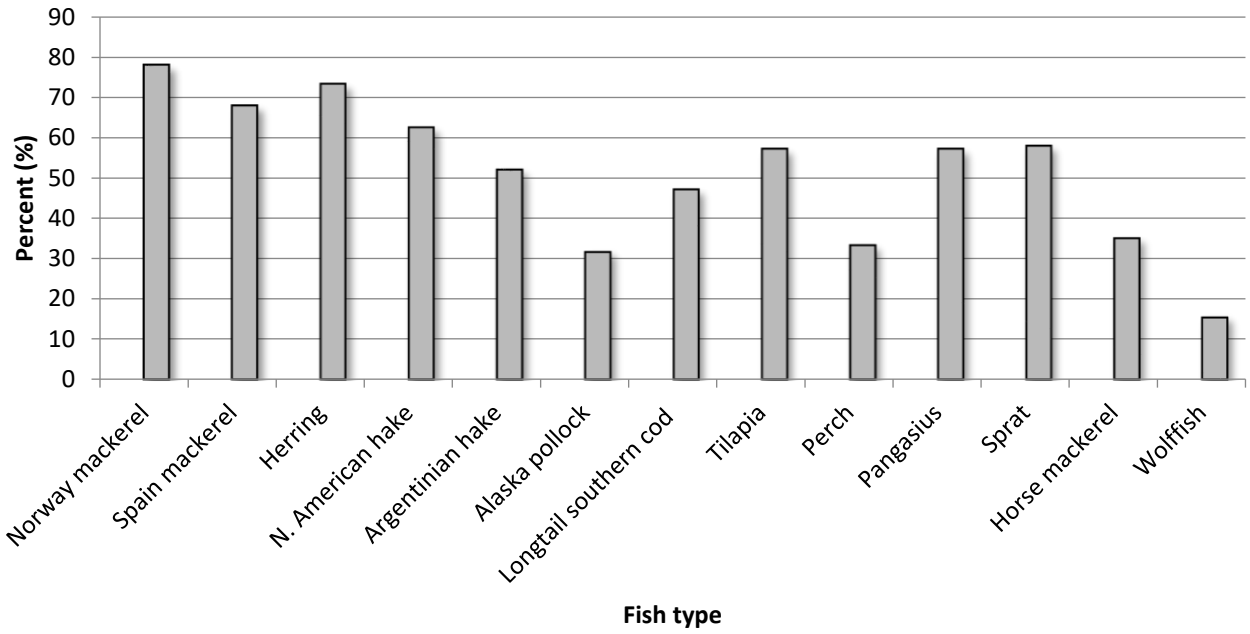

Figure 2 Prevalence of histamine occurrence in frozen fish samples by species (%).

Histamine concentrations in all samples ranged from 11.0 ± 1.13 to 216.0 ± 3.24 mg/kg, with an overall mean of 45.7 ± 13.2 mg/kg. The highest concentrations were found in samples of longtail southern cod (177.0 ± 2.15), sprat (188.0 ± 2.01), horse mackerel (192.0 ± 5.05), herring (202.0 ± 2.11), and mackerel (216.0 ± 3.24 mg/kg).

These findings confirm that histamine is commonly present in fish and fish products and may reach significant levels under improper storage conditions. This is consistent with previous studies indicating that histamine accumulation is associated with bacterial activity and violations of temperature regimes during storage and handling **[20]**, **[21]**.

The work of other scientists also proved that many fish samples taken from manufacturing plants, supermarkets, and restaurants contained histamine **[22]**, **[23]**. According to the researchers, 59 (19.5%) of the 303 batches showed histamine concentrations below the maximum limits set by Commission Regulation (EC) No.

2073/2005 (2005), while another 18 (5.9%) were non-compliant. The findings of this team of authors suggest that special attention should be paid to raw material quality, storage temperature, and hygiene practices in both handling and processing of fish and fish products, which is consistent with the findings of the current study. Moreover, histamine is commonly considered one of the indicators of fish freshness and spoilage **[24]**, and **[25]**.

According to the results of the study, the average histamine concentration for samples of mackerel was 62.6 ± 20.3 mg/kg; "Blakytna" mackerel – 45.0 ± 12.2 mg/kg; herring – 54.4 mg/kg; North American hake – 31.8 ± 5.0 mg/kg; Argentine hake – 34.9 ± 4.5 mg/kg; Alaska pollock – 29.7 ± 7.9 mg/kg; longtail southern cod – 40.1 ± 8.9 mg/kg; tilapia – 43.8 ± 10.7 mg/kg; perch – 42.8 ± 13.4 mg/kg; pangasius – 39.1 ± 7.2 mg/kg; sprat – 58.5 ± 12.3 mg/kg; horse mackerel – 53.1 ± 13.5 mg/kg; wolffish – 49.5 ± 18.3 mg/kg. Histamine content of more than 100.0 mg/kg was detected in 19 samples of frozen fish (12.1% of the total number of positive samples), histamine content of more than 200.0 mg/kg was detected in three samples (1.9% of the total number of positive samples). The histamine content, which was at the permissible concentration limit of 200.0 mg/kg, was found in three fish samples (1.9% each). The results of the conducted studies are shown in Table 2.

Table 2 Maximum histamine values in frozen fish samples, x ± SD.

Type of fish	Designation of regulatory documents for the test method	Sample number	Maximum histamine content, mg/kg
Mackerel, Norway	DSTU 4894:2007 **[8]**	12	107.0 ± 1.26
		14	119.0 ± 2.11
		20	124.0 ± 1.88
		27	145.0 ± 2.46
		29	131.0 ± 4.18
		32	216.0 ± 3.24
Mackerel "Blakytna", Spain		3	119.0 ± 2.78
		11	208.0 ± 4.12
		18	111.0 ± 2.03
		20	105.0 ± 2.81
		25	114.0 ± 3.56
Herring		4	202.0 ± 2.11
		19	119.0 ± 1.85
		21	117.0 ± 2.45
Sprat		5	113.0 ± 3.21
		17	188.0 ± 2.01
Horse mackerel		2	105.0 ± 1.44
		15	192.0 ± 5.05
Longtail southern cod		5	177.0 ± 2.15
Total		19	–

The distribution of samples with elevated histamine levels is illustrated in Figure 3, which demonstrates that the highest concentrations were predominantly detected in species known to be rich in free histidine or sensitive to microbial spoilage.

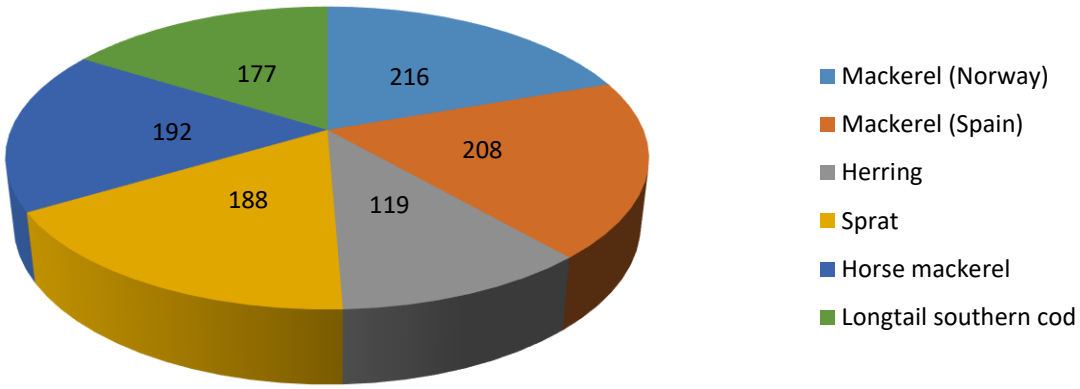

Figure 3 Types of fish that contain the highest amount of histamine, mg/kg.

The highest histamine levels observed in mackerel, herring, and sprat are in agreement with previous findings indicating that fish species rich in histidine are particularly susceptible to histamine accumulation **[20]**, **[26]**. The high histamine content in icefish confirms recent evidence that external factors, especially storage conditions, may play a crucial role in its formation **[22]**, **[23]**. This finding suggests that limiting monitoring to specific fish families could underestimate the actual risk.

Other scientists have highlighted studies of histamine content in raw fish samples **[26]**. According to the data obtained, the prototypes had levels of this biogenic amine exceeding 100 mg/kg. However, the average histamine content in fresh fish samples taken from supermarkets and street vendors in Sicily ranged from 77.67 ± 431.84 mg/kg to 1,318.60 ± 1,565.12 mg/kg **[1]**, which considerably exceeds the maximum values recorded in this study. Such differences may be explained by more pronounced violations of storage conditions and temperature control, as histamine formation is known to intensify under elevated temperatures and improper handling **[27]**, **[28]**.

These researchers also reported that histamine content in fresh fish samples that did not meet the standards ranged from 228.50 to 4,110.00 mg/kg. Studies have shown that histamine levels in fish samples sold through the retail network can reach significant concentrations. Among unsuitable samples, 50% were bluefin tuna fillets. Approximately 25% of the albacore samples under study exceeded EU limits with a maximum value of 533.30 mg/kg. In the current study, the maximum histamine content in the samples ranged from 105.0 ± 1.44 to 216.0 ± 3.24 mg/kg, with an overall mean of 142.7 ± 13.26 mg/kg. Along with determining the level of histamine, microbiological studies of the selected samples were performed, which is reflected in Tables 3, 4.

In experimental samples of some frozen fish species (mackerel, herring, cod icefish, tilapia, sprat, horse mackerel, and wolffish), the presence of MAFAnM, CFU in 1 g of the product was found, which had certain limits, which is reflected in Table 3.

In mackerel samples (Norway), MAFAnM did not exceed the maximum permissible level (MPL), but in one sample, this indicator was at the upper limit of 4.8 x 10^4 CFU in 1 g. The histamine content in this sample was 216.03 ± 3.24 mg/kg. The total number of MAFAnM-positive samples of this fish species was two, which is 6.3% of the total number of samples. In the samples of "Blakytna" mackerel (Spain) for MAFAnM, five samples were found to be positive, accounting for 17.9% of the total number of experimental samples. Of these, three samples exceeded the standards, accounting for 10.7% of the total. The maximum indicator for MAFAnM in this type of fish was 6.7 × 10^4 CFU in 1 g (the histamine level of this sample was 208.0 ± 4.12 mg/kg). Three herring samples were found positive by MAFAnM, accounting for 10.0% of the total number of experimental samples. Of these, two samples exceeded the standards, accounting for 6.7% of the total. The maximum indicator for MAFAnM in this type of fish was 6.6 × 10^4 CFU in 1 g (with an MPL of no more than 5 × 10^4 CFU in 1 g). The histamine level of this sample was 202.0 ± 2.11 mg/kg.

Table 3 Results of determining sanitary indicator microorganisms in frozen fish samples, x ± SD.

Type of fish	MAFAnM, CFU in 1 g	Coliform bacteria in 0.001 g
	Maximum permissible levels according to regulatory documents	
	No more than 5×10^4	Not allowed
Mackerel, Norway	< 1×10^2 to 4.8×10^4	Detected
Mackerel "Blakytna", Spain	< 1×10^2 to 6.7×10^4	Detected
Herring	< 1×10^2 to 6.6×10^4	Detected
North American hake	< 1×10^2	Not detected
Argentinian hake	< 1×10^2	Not detected
Pollock	< 1×10^2	Not detected
Longtail southern cod	< 1×10^2 to 6.7×10^3	Detected
Tilapia	< 1×10^2 to 4.4×10^4	Detected
Perch	< 1×10^2	Not detected
Pangasius	< 1×10^2	Not detected
Sprat	< 1×10^2 to 5.4×10^3	Detected
Horse mackerel	< 1×10^2 to 3.2×10^4	Detected
Wolffish	< 1×10^2 to 2.3×10^3	Not detected

Note: MAFAnM – mesophilic aerobic and facultative anaerobic microorganisms; CFU – colony-forming units.

The MAFAnM content in longtail southern cod samples was determined in one sample, which corresponded to 6.7 × 10^3 CFU in 1 g (5.9% of the total number of samples). The histamine content in this sample was 177.0 ± 2.15 mg/kg. According to MAFAnM content in tilapia samples, a positive result was obtained in two samples (9.5% of the total number of samples). This indicator in one sample was close to MPL – 4.4 × 10^4 CFU in 1 g (the histamine level of this sample was 91.0 ± 3.23 mg/kg). As for sprat samples, the presence of MAFAnM was detected in two samples (the maximum level was 5.4 x 10^3 CFU in 1 g, which was 10.5% of the total number of samples). The histamine content in this sample was 188.0 ± 2.01 mg/kg. One sample containing MAFAnM – 3.2 x 10^4 was found in horse mackerel samples 4 CFU in 1 g (5% of the total number of samples). The histamine content in this sample was 192.0 ± 5.05 mg/kg). In wolffish samples, one sample contained the above indicator (2.3 × 10^3 CFU in 1 g), accounting for 7.7% of the total number of samples. The histamine content in this sample was 75.0 ± 3.45 mg/kg. In samples of North American and Argentine hake, pollock, perch, and pangasius, MAFAnM was less than 1 × 10^2 CFU in 1 g.

According to the *coliform* bacteria indicator in 0.001 g of the product, positive samples were found in the following fish species: mackerel (Norway) three samples (9.4% of the total number of samples); "Blakytna" mackerel (Spain) – two samples (7.1%); herring – four samples (13.3%); longtail southern cod – one sample (5.9%); tilapia – three samples (14.3%); sprat – one sample (5.3%); horse mackerel – one sample (5% of the total number of samples, respectively). All study samples had the highest histamine levels. In experimental fish samples (North American and Argentinian hake, pollock, perch, pangasius, wolffish), no coliform bacteria were detected in 0.001 g of the product. According to the results of microbiological studies, it was established that experimental samples of frozen fish of all species did not contain the following pathogenic microorganisms: *Salmonella* in 25 g, *L. monocytogenes* in 25 g, coagulase-positive staphylococci in 0.01 g, sulfite-reducing clostridia in 0.01 g, and *V. parahaemolyticus* CFU in 1 g (Table 4).

The studies conducted indicate that in all positive samples for MAFAnM and coliform bacteria, histamine levels reached a maximum, suggesting an association between this indicator and the presence of sanitary-indicative microflora.

These findings are consistent with previous studies demonstrating that histamine is formed by the activity of histidine decarboxylase-producing bacteria, including representatives of the Enterobacteriaceae **[20]**, **[24]**. It is known that the rate of histamine accumulation depends on microbial contamination, storage conditions, and duration, as well as the activity of bacteria capable of producing histidine decarboxylase **[25]**, **[28]**.

Other researchers have also confirmed that bacterial contamination is associated with histamine accumulation in fish. Thus, the team of authors examined the histamine content in frozen sardine samples **[29]**. The average histamine value was 8.25 mg/100 g. The types of enterobacteria were identified, as a result of the vital activity of which the specified biogenic amine was formed: *Klebsiella pneumonia*, *Staphylococcus xylosus*, *Escherichia coli* and *Enterobacter cloacae*. Histamine was detected in all sardine samples contaminated with these microorganisms. It is noted that the intensity of histamine formation depends on the microbiological purity of raw materials, storage conditions and duration, and technological processes **[30]**. The rate of its accumulation is affected by the activity of bacteria in the family Enterobacteriaceae and other microorganisms with histidine decarboxylase activity. Bacteria that can form histamine include the following types: *Morganella*, *Klebsiella*, *Hafnia*, *Proteus*, *Enterobacter*, *Serratia*, *Raoultella*, *Providencia*, *Citrobacter*, and individual strains of *Clostridium*, *Vibrio*, *Acinetobacter*, *Plesiomonas*, *Pseudomonas*, *Aeromonas* and *Photobacterium*.

In Ukraine, market operators conduct fish sampling to check compliance with safety criteria in accordance with the Order of the Ministry of Health of Ukraine No. 548 **[31]**. This document states that for fish (families *Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae, Scombresosidae*) individual samples for inspection are selected at the retail trade level and if they meet the criteria specified in this order, the entire batch of fish is safe. According to this paper, histamine levels are considered satisfactory (in fish species with naturally high levels of L-histidine), if the following requirements are met: the average obtained value in experimental samples is less than or equal to 100 mg/kg; the maximum values obtained in the samples are in the range between 100 mg/kg and 200 mg/kg; none of the histamine content obtained in the samples exceeds the permissible limit of 200 mg/kg. The histamine content is considered unsatisfactory if the average value obtained in the samples selected from the batch for the study exceeds 100 mg/kg, or in the samples selected from the batch, those whose histamine level is between 100 mg/kg and 200 mg/kg prevail, or one or more of the histamine content obtained in the samples exceeds the maximum value of 200 mg/kg. Thus, according to the conducted research, frozen fish of various market operators, which was sold in supermarkets in Odesa, namely mackerel (Norway), "Blakytna" mackerel (Spain), and herring, have an excess of histamine content; the histamine content of such fish species as sprat and horse mackerel have critical limits for this indicator (Table 1), which is associated with the presence of sanitary-indicative microflora. This may indicate a possible violation of the cold storage chain and its timing.

Table 4 Results of determination of pathogenic and conditionally pathogenic microorganisms in frozen fish samples, x ± SD.

Type of fish	Coagulase-positive staphylococci in 0.01 g	*Salmonella* in 25.0 g	Sulphitre-ducing clostridium in 0.01 g	*L. mono-cytogenes* in 25.0 g	*V. parahae-molyticus* CFU in 1 g
	MPL according to regulatory documents				
	Not allowed				No more than 100.0
Mackerel, Norway	Not detected				<10.0
Mackerel "Blakytna", Spain	Not detected				<10.0
Herring	Not detected				<10.0
North American hake	Not detected				<10.0
Argentinian hake	Not detected				<10.0
Pollock	Not detected				<10.0
Longtail southern cod	Not detected				<10.0
Tilapia	Not detected				<10.0
Perch	Not detected				<10.0
Pangasius	Not detected				<10.0
Sprat	Not detected				<10.0
Horse mackerel	Not detected				<10.0
Wolffish	Not detected				<10.0

Scheduled monitoring of histamine content applies to frozen fish of the families *Scombridae, Scombridae/Thunnini, Salmonidae*, and *Clupeidae*. However, monitoring found that some experimental fish samples from species that do not form histamine contained measurable levels of histamine. One of the samples (longtail southern cod) had histamine levels almost at the critical limit: 177.0 ± 2.15 mg/kg.

Notably, histamine was detected even in fish species that are not typically classified as histamine-forming. Similar observations have been reported in other studies and are associated with secondary microbial contamination and temperature abuse **[27]**, **[28]**, which confirms the results obtained.

Therefore, the presence of histamine in these fish species may indicate that the cooling chain of these fish has been interrupted. It may also indicate a deterioration in the quality of these samples, even in the absence of sensory signs of fish unsuitability.

Histamine is formed by bacterial enzymatic activity and is thermally stable, meaning it is not destroyed during freezing or cooking **[24]**. Therefore, its presence indicates spoilage even in the absence of sensory changes. Thus, histamine content can be used as an indicator of deterioration in fish freshness. This suggests that histamine can be considered a universal indicator of fish freshness, regardless of species.This is also a reason to believe that not only marine fish, which belong to families with elevated levels of L-histidine in the muscles, need control. It is necessary to control all types of fish sold in supermarkets, since in wartime conditions it is not always possible to ensure proper storage, leading to spoilage. According to international guidelines, histamine levels above 200 mg/kg may pose a health risk **[25]**, and **[32]**, while levels above 50 mg/kg may already indicate the onset of spoilage, which emphasizes the importance of monitoring even moderate concentrations of this biogenic amine.

As is known, histamine can be formed as a result of the action of a bacterial enzyme of microorganisms of the *Enterobacteriaceae* family, etc. **[33]**. Therefore, to evaluate fish for safety indicators, including histamine content, it is advisable to examine fish using microbiological criteria such as the presence of MAFAnM, coliform bacteria, L. monocytogenes, and microorganisms of the genus Salmonella. After examining the selected samples for the above microbiological indicators, an association was observed between histamine content and the presence of MAFAnM and *coliform* bacteria. These microorganisms were detected in fish samples with histamine content exceeding 100 mg/kg. This association confirms that histamine accumulation is directly related to microbial contamination and can intensify under improper storage conditions **[24]**.

Therefore, exceeding these indicators, along with the presence of histamine, may indicate the onset of fish spoilage, which in turn can lead to an even greater increase in histamine levels, especially if storage temperature conditions are violated. Even short-term temperature abuse can significantly accelerate histamine formation, highlighting the critical role of proper storage conditions **[27]**, **[34]**.

To reduce histamine formation, it is advisable to focus on factors that promote its formation and to prevent them during fish processing. Therefore, strict adherence to temperature control and maintenance of the cold chain

are essential measures to prevent histamine accumulation. These measures will prevent scombrotoxicosis in consumers. Such preventive approaches are crucial for ensuring the safety of fish products and reducing the risk of histamine poisoning in consumers.

Limitations

Among the main limitations of the study are the relatively small sample size and the limited representativeness of the analysed samples, which may affect the generalisability of the results. In addition, the study was conducted under controlled laboratory conditions that do not fully reflect the actual conditions of fish storage, transportation, and retail distribution. Another limitation is the lack of detailed information on storage history and temperature fluctuations along the supply chain prior to sampling, which limits interpretation of factors influencing histamine accumulation and microbiological parameters. The analytical method used in this study (photometric determination of histamine according to DSTU 4894:2007) has established limits of detection and quantification, which may have resulted in the underestimation of low histamine concentrations in some samples. Furthermore, the method does not allow identification of other biogenic amines that may be present in frozen fish. The microbiological analysis included general hygiene indicators and selected pathogenic microorganisms; however, histamine-producing bacterial strains were not identified. Therefore, the observed relationship between microbial contamination and histamine levels should be interpreted as associative rather than causal. In addition, the results may have been influenced by methodological uncertainties related to sample preparation, laboratory precision, environmental conditions during analysis, and the biological variability and uneven distribution of microorganisms in fish samples. Future studies employing more sensitive and selective analytical techniques, such as high-performance liquid chromatography, combined with detailed monitoring of storage and transportation conditions, could provide a more comprehensive understanding of the factors contributing to histamine accumulation in frozen fish.

CONCLUSION

According to the results of the conducted research, samples of frozen fish (mackerel (Norway), “Blakytna” mackerel (Spain), herring) of various market operators, which were sold in the retail chain of supermarkets in Odesa, did not meet regulatory requirements for the content of histamine. Some samples of mackerel and herring had an excess of the MPL of histamine, which according to the Order of the Ministry of Health of Ukraine No. 548 is equal to 200.0 mg/kg. The histamine content of the herring and horse mackerel samples, although not exceeding the maximum permitted concentration, approached the maximum permitted concentration in some samples. Along with fish species that have a naturally high content of L-histidine in the muscles (mackerel, herring, sprat) and fish species that can potentially form histamine (horse mackerel), the content of histamine is determined in fish species that do not belong to the histamine-forming group (hake, pollock, longtail southern cod, tilapia, perch, pangasius, wolffish). The level of histamine in samples of fish that do not belong to histamine-forming does not exceed MPL, but its presence indicates the advisability of further monitoring of this indicator for all types of fish sold in the retail network, and not just those that have a naturally elevated content of L-histidine in the muscles. According to the results of determination of microbiological indicators in frozen fish, it was established that 10.7% of “Blakytna” mackerel (Spain) samples and 6.7% of herring samples did not meet the requirements of regulatory documents for MAFAnM; according to the content of *coliform* bacteria in 0.001 g of the product, the discrepancy was found in the following types of fish: mackerel (Norway) – 9.4% of samples; “Blakytna” mackerel (Spain) – 7.1%; herring – 13.3%; longtail southern cod – 5.9%; tilapia – 14.3%; sprat – 5.3%; horse mackerel – 5% of the total number of samples, respectively. The obtained data may indicate the presence of a certain relationship between the histamine content and the presence of sanitary-indicative microflora. Such pathogenic microorganisms as *Salmonella* in 25 g, *L. monocytogenes* in 25.0 G, coagulase-positive staphylococci in 0.01 g, sulphite-reducing clostridia in 0.01 g, and *V. parahaemolyticus* CFU in 1 g was not isolated in any frozen fish sample. The obtained results indicate the feasibility of further monitoring of frozen fish for histamine content and microbiological parameters. This may serve as a basis for considering the possibility of improving approaches to monitoring the safety of fish products in accordance with current regulatory requirements. Prospects for further research include systematic monitoring of histamine content in a wider range of fish products, including species that are not traditionally classified as histamine-forming, in order to identify potential risks and clarify regulatory approaches; conducting studies that will justify the feasibility of making changes to the Order of the Ministry of Health of Ukraine No. 548 on strengthening control over histamine content, and assessing the effectiveness of the implemented corrective measures in the future; establishment of scientifically based recommendations for market operators to reduce the risk of histamine accumulation, improve storage conditions and logistics.

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