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Original Scientific Paper

VERIFICATION AND ASSESSMENT OF THE MEASUREMENT UNCERTAINTY OF THE HPLC METHOD FOR DETERMINING THE AMOUNT OF HISTAMINE IN FISH AND FISHERY PRODUCTS

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Summary

The aim of the study is to present the experimental results obtained during the verification of the HPLC method for determining the histamine content in fish and fishery products for three types of fish and fishery products and to estimate measurement uncertainty of the method. The method for determining the amount of histamine in fish and fishery products is verified according to BAS EN ISO 19343:2017. The following parameters of the verification method were evaluated: influence of the matrix/linearity, limit of quantification, accuracy and precision. Measurement uncertainty of the method was estimated for three types of fish (tuna, mackerel and sardines). Linearity was confirmed in the concentrations range from 0 mg kg^{-1} to 500 mg kg^{-1} with correlation coefficients from 0.9925 to 0.9999. The limit of quantification for all three types of fish is 10 mg kg^{-1}. The accuracy was evaluated by analyzing the control materials through recovery and the average value is 96.61%. The precision was evaluated in terms of repeatability and reproducibility at three concentration levels for three types of fish and was expressed as the repeatability limit r and the reproducibility limit R. The reproducibility limits R for tuna are 15.96 to 24.89%, for mackerel 12.80 to 23.04%, and for sardines from 10.25 to 32.17%. The measurement uncertainty was estimated at three concentration levels 25 mg kg^{-1}, 100 mg kg^{-1} and 220 mg kg^{-1} and was 17.78 mg kg^{-1}, 11.40 mg kg^{-1} and 13.88 mg kg^{-1} for tuna, 16.46 mg kg^{-1}, 12.56 mg kg^{-1} and 9.14 mg kg^{-1} for mackerel and 22.98 mg kg^{-1}, 12.70 mg kg^{-1} and 7.32 mg kg^{-1} for sardines. The obtained results of the verification parameters are in accordance with the prescribed and recommended acceptance criteria, and confirm that the test method is sufficiently reliable and the laboratory can use it in routine work.

Key words: verification, histamine, chromatography, fish, HPLC.

INTRODUCTION

The formation of histamine in fishery products is directly correlated with the histidine concentration in the tissue and the microorganisms level in the product (Etiene, 2016). The amount of histamine produced depends on the type of bacteria, temperature and time of exposure of the fish (Jakšić et al., 2017). Improper storage, poor handling and manipulation of fish and fishery products leads to food spoilage, which, in humans, causes symptoms similar to an allergic reaction, known as "scombroid poisoning" (histamine or scombrotoxin). That is why the histamine content is of great importance for the assessment of food toxicity and has been proposed as an indicator of hygienic correctness (Ghazi et al., 2015). The risk of histamine poisoning is kept under control by applying good hygiene and manufacturing practices, linked to a system of risk analysis and critical control points (Shimoji and Bakke, 2019).

The Regulation on Microbiological Criteria for Food of Animal Origin (Regulation, 2019) prescribes the maximum permitted amounts of histamine in fishery products from fish species associated with a large amount of histidine, in the range of 100 to 200 mg kg^{-1}, which are estimated by analyzing representative 9 units from each series of samples. The sample is adequate, that is, it meets the provisions of the Regulation if: the mean value of the results from 9 units does not exceed 100 mg kg^{-1}; two units exceed the value of 100 mg kg^{-1} but do not exceed 200 mg kg^{-1} and if no unit exceeds 200 mg kg^{-1}. Exceptions are fishery products processed by enzymatic maturation in brine produced from fish species associated with a large amount of histidine, where the allowed range is from 200 to 400 mg kg^{-1} per 9 product units and fish sauce obtained by fermentation of fishery products, which must not have a histamine concentration greater than 400 mg kg^{-1} in one product unit (Regulation, 2019).

Separation and quantification of biogenic amines is most often achieved by ion-exchange chromatography, thin-layer chromatography, gas chromatography, liquid chromatography, and enzymatic and radioimmunological methods (Malle et al., 1996). HPLC is most commonly used due to its operational advantages (cost and reproducibility) (Chen et al., 2016).

According to the Regulation on Microbiological Criteria for Food of Animal Origin (Regulation, 2019), the prescribed reference method for determining the amount of histamine in fish and fishery products is the HPLC method. Since the method is standard, it has already been subject to validation and its suitability for the intended purpose has been established. Having the resources that are a required for performing the method, the laboratory implemented a standard method, and in order to confirm

the ability to achieve accurate and reliable test results, a verification study was created and performed.

The aim of the study is to present the experimental results obtained during the verification of the HPLC method for determining the content of histamine in fish and fishery products for three types of fish and fishery products and to estimate measurement uncertainty of the method.

MATERIALS AND METHODS

In order to verify the method, analyzes were performed on samples of three types of fish: tuna (one sample of raw fish and five products: one canned tuna in brine and four canned tuna in vegetable oil), mackerel (one sample of raw fish and two products: cannded mackerel in vegetable oil) and sardines (one sample of raw fish and one product: a canned sardines in vegetable oil). For the assessment of the verification parameters, in addition to enriched samples, externally ordered control samples were also used: Fapas RM, TYG018RM, Canned fish, Tuna fish in brine, ref. value 220±5 mg kg^{-1} and Fapas QC, T27303QC, Canned fish, ref. value 31.3 mg kg-1 (25.4-37.3 mg kg^{-1}).

The standard histamine dihydrochloride, manufactured by Sigma Aldrich, purity ≥99%, batch no: WXBD1225V, and the internal standard 1,7 diaminoheptane, manufactured by Sigma Aldrich, purity 98%, batch no: STBG2472V, were used. For the analysis, a basic 12.5 mg mL^{-1} histamine solution was prepared from the reference standards by dissolving 1.034 g of histamine dihydrochloride in 50 mL of deionized water (the solution is stable for one year if stored in a freezer (-20±5˚C)) and the stock solution of the internal standard 1,7-diaminoheptane, concentration 6.4 mg mL^{-1} was prepared by dissolving 0.320 g of 1,7-diaminoheptane in 50 mL of deionized water (the solution is stable for three weeks if stored in a refrigerator (5±3˚C)).

Perchloric acid, sodium carbonate, dansyl chloride and L-proline produced by Sigma Aldrich were used for sample preparation and processing. Toluene produced by Honeywell was used for purifying the extract, and ultrapure water and acetonitrile, HPLC grade acetonitrile produced by Fisher and Honeywell were used for chromatographic separation.

PTFE/PP filters with a pore size of 0.2 μm were used for filtering the prepared sample, and nitrogen gas 4.6 was used for evaporation.

Analysis was performed on a liquid chromatograph with a UV/VIS detector, Agilent Technologies, InfinityLab LC Series 1260 Infinity II Quaternary System with

associated software OpenLAB Chem Station Edition Rev. C.01.07 SR3 [465]. A Nucleosil C18 chromatographic column, 5 µm 100Å (25 cm x 4.6 mm), Macherey-Nagel, LOT: 21301076, was used.
The test was performed in accordance with BAS EN ISO 19343 (ISBIH, 2018).
Calibration curves were made on the matrix of the same type of fish as the sample for analysis.

RESULTS AND DISCUSSION

Experiments were performed in 15 batches by two analysts. The following verification parameters were evaluated: influence of the matrix/linearity, limit of quantification, accuracy, precision through repeatability and reproducibility of the method, and the measurement uncertainty of the method was estimated for three species of fish at three concentration levels. Samples of three types of fish (tuna, mackerel and sardines) and samples of their products were included.
The determination of acceptance limits for verification parameters was done on the basis of prescribed (Regulation, 2010) and recommended criteria (ISBIH, 2018), and own experience and laboratory practice.
The chromatogram with the separated peak of histamine and the internal standard with marked retention times in minutes is shown in Figure 1.

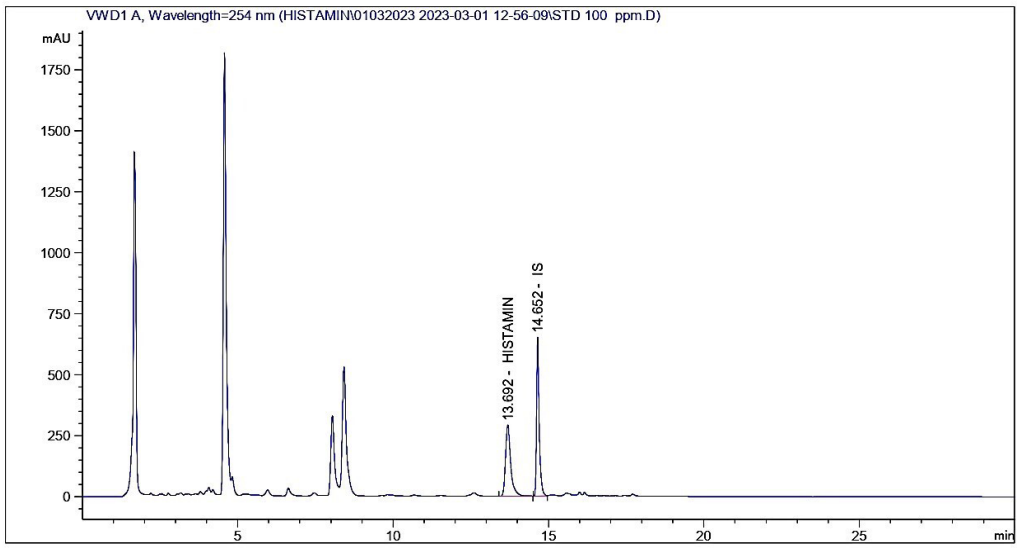

Figure 1 Example of a specific chromatogram for histamine and an internal standard

The Regulation on the Implementation of Analytical Methods and Interpretation of Results (Regulation, 2010) prescribes a general recovery criteria for quantitative methods of 80-110%, while BAS EN ISO 19343 (ISBIH, 2018) specifies an interval of 60-140%. One of the reasons for such a wide acceptance interval is that the BAS EN ISO 19343 method (ISBIH, 2018) was validated based on the results of an interlaboratory study in which nine laboratories from seven countries participated, and this criteria for accuracy verification in one laboratory is not acceptable. Bearing this in mind, the laboratory defined the 80-120% interval as an acceptance criteria for the evaluation of recovery (accuracy of the method) and through experiments confirmed the ability of the method related to this verification parameter. The accuracy of the method was evaluated by analyzing the reference material Fapas TYG018RM Canned fish, Tuna fish in brine and the control material Fapas T27303QC Canned fish, in 6 repetitions and by analyzing enriched samples for three types of fish at a concentration level of 400 mg kg^{-1}. Table 1 shows the results for recovery over the reference and control material. Table 2 shows the results for recovery over enriched samples.

Table 1 Recovery through RM and QC materials

Параметар	Fapas RM, TYG018RM, Canned fish, Tuna fish in brine, ref. value 220±5 mg kg^{-1}	Fapas QC, T27303QC, Canned fish, ref. value 31.3 mg/kg (25.4-37.3 mg kg^{-1})
c_m (mg kg^{-1})	208.1889	30.9996
sdv (mg kg^{-1})	1.5701	2.5217
Rec (%)	94.63	99.04

(c_m): mean value; (sdv): standard deviation; (Rec%): recovery

Table 2 Recovery through the enriched sample at the level of 400 mg kg-1

Parameter	Tuna	Mackerel	Sardines
c_m (mg kg^{-1})	385.6474	378.9199	391.5657
sdv (mg kg^{-1})	1.9049	3.4465	9.5088
Rec (%)	96.41	94.73	97.89

The accuracy of the method was confirmed by participation of laboratory in the PT scheme by the FAPAS provider in the period August/September 2022, with a z-score value of 0.3 and a mean recovery value of 97.6%, which is acceptable in relation to the set criteria. According to Jakšić et al. (2017), the average recovery obtained by the same method by analyzing four PT (FAPAS) samples at different concentrations of histamine was 101.5%, which is close to the average recovery obtained by analyzing the reference and control material at two concentration levels (96.8%) .

The linearity of the method was confirmed in the range of concentrations from 0 mg kg^{-1} to 500 mg kg^{-1} for all three types of fish with correlation coefficients from 0.9925 to 0.9999, and coefficients of direction from 0.0070 to 0.0108. For each analysis, a calibration curve was prepared on a sample of the same type as the test sample. In this way, the influence of the matrix is eliminated and a more reliable result is obtained. An example of the experimentally obtained calibration curve in the matrix is shown in Figure 2.

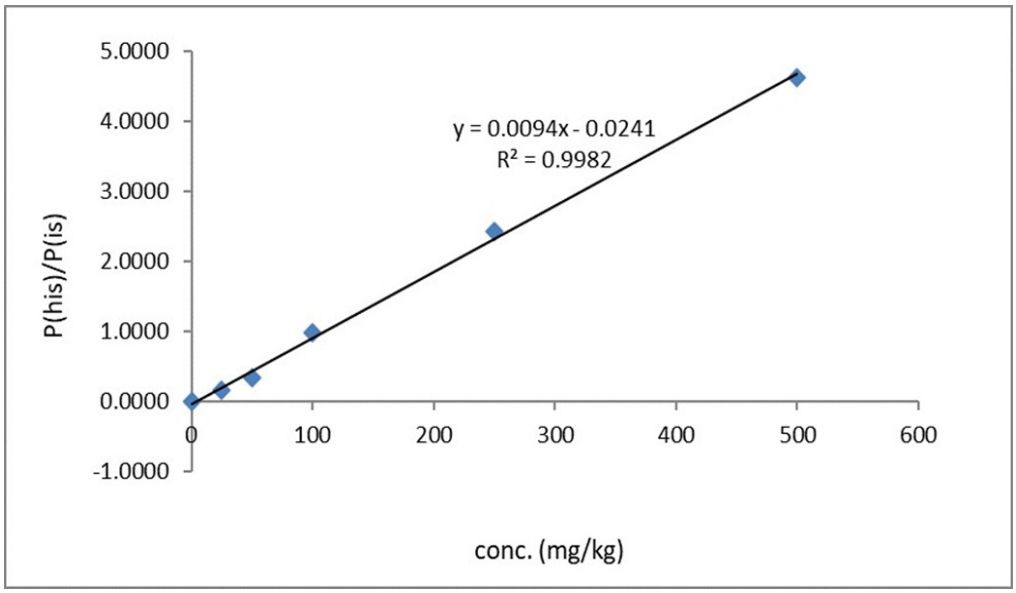

Figure 2 An example of an experimentally obtained calibration curve in a matrix

Based on literature data, for both chromatographic and enzymatic methods, a correlation coefficient higher than 0.9 is considered acceptable. Jinadasa et al. (2016)

evaluated the linearity of the histamine analysis method by liquid chromatography with a correlation coefficient higher than 0.99, which is consistent with the results of our study.

The measurement range of the method is expressed over the limit of quantification. Taking into account the maximum allowed amounts of histamine prescribed by the Regulation (Regulation, 2019), the limit of quantification was selected as ten times lower than the lowest prescribed value. The experiment was set up in a way that, for each of the three types of fish, six samples enriched at a concentration level of 10 mg kg^{-1} were analyzed. The obtained concentrations, their mean value (c_m), standard deviation (sdv) and recovery (Rec%) were calculated. The obtained results are shown in Table 3. The recovery of enriched samples at the level of the limit of quantification is in the acceptable range and the mean value for three types of fish is 105%.

Table 3 Obtained experimental values for the limit of quantification

Parameter	Tuna	Mackerel	Sardines
c_m (mg kg^{-1})	10.2386	9.4321	11.8314
sdv (mg kg^{-1})	0.8908	0.3893	0.9817
Rec (%)	102.39	94.32	118.31

When it comes to the precision of the method, there is no strictly defined criterion for the acceptability of this verification parameter. The precision of the method was evaluated in terms of repeatability and reproducibility according to BAS EN ISO 19343 (ISBIH, 2018), and r and R limits were calculated. The reproducibility of the method was assessed by analyzing enriched samples in six repeated tests at three concentration levels. For each working day and each batch, the mean concentration value (mg kg^{-1}), standard deviation (mg kg^{-1}), repeatability variation coefficient (%), recovery (%) and repeatability limit r (mg kg^{-1}, %) were calculated. The reference material (TYG018RM) was used to evaluate the reproducibility for the tuna at a concentration level of 220 mg kg^{-1}. The result of the repeatability of one working batch for all three matrices is given in Table 4.

Table 4 Reproducibility assessment for matrices: tuna, mackerel and sardines

Level of enrichment	25 (mg kg^{-1})			100 (mg kg^{-1})			220 (mg kg-$^{-1}$)		
A type of fish	Tuna	Mackerel	Sardines	Tuna	Mackerel	Sardines	Tuna	Mackerel	Sardines
Number of replicates	6	6	6	6	6	6	6	6	6
Mean (mg kg^{-1})	24.95	26.15	28.95	108.42	101.43	107.63	187.78	221.14	229.35
Recovery (%)	99.80	104.61	115.81	108.42	101.43	107.63	85.34	100.52	104.25
Standard deviation,s_r (mg kg^{-1})	1.22	2.09	0.72	2.37	1.86	3.85	4.91	9.97	8.65
Coeficient of variation, CV_r (%)	4.88	7.99	2.50	2.19	1.83	3.58	2.23	2.22	3.77
Repeatability limit (r = 2,8 x s_r) (mg kg^{-1})	3.41	5.85	2.03	6.64	5.21	10.78	13.76	27.92	24.24
Repeatability limit (r = 2,8 x CV_r) (%)	13.66	22.37	7.00	6.13	5.12	10.02	6.24	6.22	10.56

The results of the validation study of the enzymatic method of histamine analysis on the matrix of canned tuna in oil for recovery are in the range of 93.5-124.7%, and the coefficient of variation of reproducibility at all levels of enrichment is < 20% (Shimoji and Bakke, 2019). In our study, the recovery for the same type of matrix was obtained in the interval 85.34-101.43%, and the coefficient of variation of reproducibility < 5% at all three levels of enrichment (Table 4). Such different results are not surprising considering that these are two methods with different determination principles and different measurement techniques.

The reproducibility of the method was evaluated by analyzing spiked samples at three concentration levels for three fish species by two analysts on different days. The results for the reproducibility of the method obtained by one analyst were combined with the results obtained by another analyst for each concentration level and each type of fish and the mean value of the concentration (mg kg^{-1}), standard deviation (mg kg^{-1}), coefficient of variation, reproducibility (%), recovery (%) and

reproducibility limit (mg kg^{-1}, %) were calculated. Table 5 shows the results for tuna, mackerel and sardines.

Table 5 Repeatability limit for matrices: tuna, mackerel and sardines

Level of enrichment	25 (mg kg^{-1})			100 (mg kg^{-1})			220 (mg kg-$^{-1}$)		
A type of fish	Tuna	Mackerel	Sardines	Tuna	Mackerel	Sardines	Tuna	Mackerel	Sardines
Number of replicates	12	18	18	18	16	18	14	18	12
Mean (mg kg^{-1})	27.08	25.99	28.50	108.35	102.17	102.96	201.76	218.33	224.78
Recovery (%)	108.32	103.95	114.01	108.35	102.17	102.96	91.70	99.24	102.17
Standard deviation,s_r (mg kg^{-1})	2.40	2.14	3.27	6.17	6.42	6.53	14.00	9.97	8.23
Coeficient of variation, CV_r (%)	8.89	8.23	11.49	5.70	6.28	6.35	6.94	4.57	3.66
Repeatability limit ($r = 2,8 \times s_r$) (mg kg^{-1})	6.74	5.99	9.17	17.29	17.98	18.31	39.22	27.92	23.06
Repeatability limit ($r = 2,8 \times CV_r$) (%)	24.89	23.044	32.17	15.96	17.584	17.78	19.43	12.80	10.25

The obtained results for mean values of concentration, recovery, standard deviations and coefficient of variation are similar for the tuna matrix, while for the mackerel and sardines matrices the results differ in relation to the average values given in the standard. The coefficients of variation for mackerel and sardines are lower than those stated in the standard, and the recovery is much higher, especially for sardines, which is taken in this study as a fish species similar to herring. Although they differ, the obtained values are not considered inconsistent with those given in the standard. On the contrary, a better precision was determined, which was expected considering the conditions of assessment of this parameter. The standard defined precision based on the results of an interlaboratory study, while precision in our study was evaluated intralaboratory. It should also not be overlooked that the study included three types of fish and three levels of concentration, and therefore the obtained limits may not be applicable to other levels of concentration and other types of fish and their products. Munir et al. (2021) selected mackerel for the assessment of precision and

accuracy at two concentration levels, medium and higher, in six repetitions, during seven days, where the coefficient of variation of reproducibility was 7.38%, and the recovery was 103%, which corresponds to our experimental results for mackerel.
The measurement uncertainty of the method was estimated based on the results of the standard deviation of the histamine test under reproducibility conditions for all three fish species at all three concentration levels using an assurance factor of 2, to reach a confidence level of approximately 95%. The results are shown in Table 6 for tuna, mackerel and sardines.

Table 6 Measurement uncertainty for matrices: tuna, mackerel and sardines

Level of enrichment	25 (mg kg^{-1})			100 (mg kg^{-1})			220 (mg kg^{-1})		
A type of fish	Tuna	Mackerel	Sardines	Tuna	Mackerel	Sardines	Tuna	Mackerel	Sardines
Coeficient of variation, CV_R (%)	8.89	8.23	11.49	5.70	6.28	6.35	6.94	4.57	3.66
Expanded measurement uncertainty U = 2 x RSD (%)	17.78	16.46	22.98	11.40	12.56	12.70	13.88	9.14	7.32

Ghazi et al. (2015) determined the expanded measurement uncertainty (combined measurement uncertainty x 2) for tuna and other types of fish, which was 26.4%. This value can be compared with the value for the expanded measurement uncertainty for sardines at the lowest concentration level, which is 22.98% and is the highest value obtained experimentally. The average value for the extended measurement uncertainty for all three types of fish is much lower than the measurement uncertainty of the method of the mentioned authors.

CONCLUSION

Based on the obtained results of the verification parameters test for fish and fishery products, and taking into account the acceptance criteria for linearity, repeatability, reproducibility, accuracy, recovery, limit of quantification (LOQ), the method was successfully verified and can be implemented in routine work.
Differences in results for the same verification parameters between laboratories

are not surprising considering that these are methods with different determination principles and different measurement techniques. The differences also originate from different approaches to the estimation of the verification parameters in terms of matrix selection and experiment design.

The extent to which the laboratory will carry out verification in its working conditions depends on many factors. The challenge is to find a compromise between the possibilities (time, finances) and the optimal number of experiments on the basis of which it is possible to determine the characteristics of the method and evaluate them in relation to the prescribed and/or recommended acceptance criteria.

Conflict of interest statement: The authors declare that there is no conflict of interest.

REFERENCES

Chen R., Deng Y., Yang L., Wang J., Xu F. (2016): Determination of Histamine by High-Performance Liquid Chromatography After Precolumn Derivatization with o-Phtalaldehide-Sulfite. *Journal of Chromatografic Science*, 54(4):547-553.

Etiene M. (2016): Methodology for histamine and biogenic amines analysis. Seafoodplus Traceability, Ifremer.

Ghazi A. A., Mohamed M. M., Gad Alla S. A., Kennedy G., Atalla E. R. (2015): Optimization and Validation of Analytical Method for Determination of Histamine in Fish using HPLC-FLD. *Egyptian Journal of Pure and Applied Science*, 53(2):33-42.

ISBIH (2018): Microbiology of the food chain – Detection and quantification of histamine in fish and fishery products – HPLC method. Institute for standardization of Bosnia and Herzegovina, BAS EN ISO 19343.

Jakšić S., Baloš M. Ž., Mihaljev Ž., Prodanov Radulović J., Nešić K. (2017): Comparison of analytical methods for determination of histamine in reference canned fish samples. *IOP Conference Series: Eart and Environmental Science Sci.*, 85 012066:1-6.

Jinadasa B. K. K. K., Jayasinghe G. D. T. M., Ahmad S. B. N. (2016): Validation of high performance liquid chromatography (HPLC) method for quantitative analysis of histamine in fish and fishery products. *Cogent Chemistry*, 2(1):1156806.

Malle P., Vallé M., Bouqelet S. (1996): Assay of Biogenic Amines Involved in Fish Decomposition. *Journal of AOAC International*, 79(1):43-49.

Munir M. A., Mackeen M. M. M., Heng L. Y., Badri K. H. (2021): Study of Histamine Detection using Liquid Chromatography and Gas Chromatography. *ASM Sc. J*., 16:1-9.

Regulation (2010): The Regulation on the Implementation of Analytical Methods and Interpretation of Results. Official Gazette of the Bosnia and Herzegovina, 95/10.

Regulation (2019): The Regulation on Microbiological Criteria for Food of Animal Origin. Official Gazette of the Republic of Srpska 69/19.

Shimoji K., Bakke M. (2019): Validation Study of Histamine Test for the Determination of Histamine in Selected fish Products. *Journal of AOAC International*, 102(1):164-180.

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