CERTIFICATION

AOAC Research Institute

Performance Tested Methods[SM]

Certificate No.

072001

The AOAC Research Institute hereby certifies the method known as:

BioSystems® Y15 HISTAMINE Dehydrogenase Kit

manufactured by

BioSystems S.A.
Costa Brava 30
08030 Barcelona
Spain

This method has been evaluated in the AOAC Research Institute *Performance Tested Methods*[SM] Program and found to perform as stated in the applicability of the method. This certificate indicates an AOAC Research Institute Certification Mark License Agreement has been executed which authorizes the manufacturer to display the AOAC Research Institute *Performance Tested Methods*[SM] certification mark on the above-mentioned method for the period below. Renewal may be granted by the Expiration Date under the rules stated in the licensing agreement.

Scott Coates

Scott Coates, Senior Director
Signature for AOAC Research Institute

Issue Date	December 21, 2023
Expiration Date	December 31, 2024

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METHOD NAME
BioSystems® Y15 HISTAMINE Dehydrogenase Kit

CATALOG NUMBER
12829

INDEPENDENT LABORATORY
ANFACO-CECOPESCA
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36310, Pontevedra, Spain

APPLICABILITY OF METHOD
Analyte(s) – Histamine (2-(4-Imidazolyl)-ethylamine); CAS Registry No. 54-45-6.

Matrixes – (5 g samples) fresh tuna, frozen tuna, water-canned canned tuna, oil-canned tuna, raw salmon, raw sardines, oil-canned sardines, semi-preserved anchovy fillets

Performance claims – (1) Precision - Recovery within 80–110%. (2) Accuracy - <10% RSDr of averages at different concentrations tested in all matrixes (above LOQ)

REFERENCE METHOD

International Organization for Standardization (ISO) 19343:2017, Microbiology of the food chain: Detection and quantification of histamine in fish and fishery products – HPLC method (ISO 19343:2017) Geneva, Switzerland. (2)*

***ISO 19343:2017 HPLC/UV-VIS is based on the Duflos et al. reference method.**

ORIGINAL CERTIFICATION DATE
July 07, 2020

CERTIFICATION RENEWAL RECORD
Renewed annually through December 2024.

METHOD MODIFICATION RECORD
1. **December 2020 Level 1**
2. **November 2021 Level 1**
3. **November 2022 Level 1**

SUMMARY OF MODIFICATION
1. **Editorial/formatting changes.**
2. **Editorial changes.**
3. **Editorial changes.**

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NONE

PRINCIPLE OF THE METHOD (1)
The enzyme histamine dehydrogenase (HDH) catalyzes the oxidation of histamine to 4-amidazolylaldehyde in the presence of 1-methoxy-5-methylphenazinium methyl sulfate (PMS) a photochemically stable electron mediator and a water-soluble tetrazolium salt (WST). When WST is reduced, the corresponding formazan dye is formed and can be measured at 420 nm. The increase of absorbance is proportional to the histamine concentration (Figure 1). Once the sample has been extracted, the necessary actions to measure histamine (dispensing of reagents and sample, absorbance readings, calibration and calculation of results based on the weight of the sample) are performed automatically in the random access analyzer BioSystems Y15.

DISCUSSION OF THE VALIDATION STUDY (1)

The BioSystems Y15 HISTAMINE method evaluated in this validation following the protocols established by the AOAC is applicable for the quantification of histamine in samples of raw fish and canned fish. Automation of the measurement with an analyzer allows measurements to be obtained quickly, easily and with high precision, accuracy and robustness since user intervention is minimized upon extraction. The extraction protocol compared to other methods (HPLC, Fluorometry or ELISA) has been shown to be simple, fast and does not require hazardous solvents since it is done with water.

The method developer validation included linearity, selectivity and interference studies, recovery, accuracy, precision, comparison to reference methods for fishery products, proficiency tests data, estimates of LOD and LOQ, matrix-specific confirmation of LOQ, robustness studies, lot-to-lot consistency and stability testing for reagent and standards.

Linearity in the measurement range (0-200 mg/Kg) has been confirmed according to the regression statistics. This range is adequate to be able to quantify whether or not the fish samples comply with current legislations and with the quality criteria. The measuring range can be increased according to the user's need by diluting the sample made automatically by the analyzer.

Of the 11 substances like histamine used in the selectivity study, only positive interference with agmatine was observed. This interference occurs independently of the matrix and is 6.3% maximum, of the amount of agmatine in the sample. This interference is considered very insignificant since it would have relevance only in cases in which the ratio between histamine and agmatine was very low and the fish is unlikely to contain high concentrations of agmatine. Nevertheless, the BioSystems HISTAMINE instructions for use supplied with each kit, warns the user that in the presence of agmatine, there may be positive interferences.

Recovery studies showed excellent results with all types of matrixes studied throughout the entire sample range and even with histamine concentrations below the quantification limit of the method. These results were confirmed in the comparison study against the accredited reference method based on HPLC-UC/VIS conducted in ANFACO. The data showed a very good correlation with the ISO 19343:2017 HPLC method based the Duflos et al. method. The good results with FAPA's certified reference materials and quality control materials and the data obtained from the participation in FAPA's proficiency test schemes, all with a $|z\text{-score}| \leq 2$, ensure a good accuracy of the method.

The repeatability data are very good (at 50 mg/Kg the worst case is 3.44% in the semi-preserved anchovy matrix and the best 1.26% in oil-canned tuna). This data can be obtained thanks to the ease of the extraction protocol and the little intervention of the user in the handling of the reagents and in the measurement protocol.

The study of the LOD/LOQ estimates was performed according to the basis of blank samples and the LOQ was confirmed by spiking experiments. According to the data obtained, a LOQ of 10 mg/Kg was established. In the verification carried out with the repeatability study with real samples spiked with 10 mg/Kg of histamine, RSDr less than 5% and recoveries close to 100% were obtained in all cases, indicating that the LOQ of 10 mg/Kg is valid and it could even be lower.

The stability testing of 13 independent lots showed high lot-to-lot reproducibility and that the control carried out during the manufacture of the kit components ensures that there are no differences in results regardless of the lot used. Stability studies of both reagent and calibrators proved that kits are stable over the claimed shelf-life of two years from the manufacturing date.

A thorough robustness testing scheme was performed. None of the conditions that were altered with respect to the protocol described, caused significant variations in the result demonstrating that the BioSystems Y15 HISTAMINE method is robust.

An evaluation of the BioSystems Y15 HISTAMINE was performed by an independent laboratory and consisted on the analysis of spiked fresh raw and canned fish, including precision, recovery and verification of LOQ. The data obtained revealed that the kit works with the same precision in minimally trained hands as with expert method developers. The recovery was very similar to that obtained by the developer method. The estimated LOQ obtained for the raw tuna sample was equivalent to that obtained by the method developer, but for water and oil-canned tuna, a higher estimated LOD was obtained which generates a LOQ of 20 mg/Kg. In the validation of the LOQ by spiking each matrix at or near the estimated LOQ and testing 10 replicates to demonstrate acceptable precision, very low RSDr were obtained: less than 5% for raw tuna (10 mg/Kg) and less than 2% for the two samples of canned fish (20 mg/Kg). The recovery is within the established tolerances (80-110%). These data suggest that the LOQ could have been overestimated.

The data of LOD and LOQ studies by the method developer were conducted using samples with the lowest possible histamine contamination. The histamine content of the samples in the independent laboratory studies, despite meeting the criteria of having a concentration lower than the LOQ of the method used, were higher, causing that the LOD and LOQ values calculated could still be overestimated.

Table 3. Results of selectivity of enzyme histamine dehydrogenase in matrixes

		Raw tuna			Water-canned tuna			Oil-canned tuna			Raw sardine			Oil-canned sardine			Semi-preserved anchovy fillets		
Histamine *mg/Kg*	Interferent (1000 mg/Kg)	Result *mg/Kg*	D	%	Result *mg/Kg*	D	%	Result *mg/Kg*	D	%	Result *mg/Kg*	D	%	Result *mg/Kg*	D	%	Result *mg/Kg*	D	%
0	No interferent added	3.4	-	-	1.6	-	-	4.2	-	-	5.1	-	-	4.8	-	-	3.6	-	-
	Methylhistamine	3.3	0.0	0.0%	1.5	0.0	0.0%	4.3	0.0	0.0%	5.0	0.0	0.0%	4.6	0.0	0.0%	3.7	0.0	0.0%
	Tyramine	3.4	0.1	0.0%	1.8	0.3	0.0%	4.2	-0.1	0.0%	4.8	-0.2	0.0%	4.2	-0.4	0.0%	4.0	0.3	0.0%
	L-Phenylalanine	3.5	0.2	0.0%	1.7	0.2	0.0%	4.4	0.1	0.0%	4.9	-0.1	0.0%	4.5	-0.1	0.0%	3.9	0.2	0.0%
	L-Histidine	3.2	-0.1	0.0%	1.8	0.3	0.0%	4.2	-0.1	0.0%	4.9	-0.1	0.0%	4.4	-0.2	0.0%	3.8	0.1	0.0%
	L-Tyrosine	3.3	0.0	0.0%	1.8	0.3	0.0%	4.1	-0.2	0.0%	5.0	0.0	0.0%	4.4	-0.2	0.0%	3.8	0.1	0.0%
	Tryptamine	3.4	0.1	0.0%	1.6	0.1	0.0%	4.5	0.2	0.0%	5.1	0.1	0.0%	4.7	0.1	0.0%	3.6	-0.1	0.0%
	Cadaverine	3.4	0.1	0.0%	1.6	0.1	0.0%	4.5	0.2	0.0%	4.8	-0.2	0.0%	4.8	0.2	0.0%	3.7	0.0	0.0%
	Putrescine	5.2	1.9	0.2%	3.3	1.8	0.2%	5.8	1.5	0.2%	4.9	-0.1	0.0%	4.3	-0.3	0.0%	4.8	1.1	0.1%
	Anserine	3.4	0.1	0.0%	1.7	0.2	0.0%	4.4	0.1	0.0%	4.9	-0.1	0.0%	4.6	0.0	0.0%	4.1	0.4	0.0%
	Carnosine	3.5	0.2	0.0%	1.7	0.2	0.0%	4.4	0.1	0.0%	5.0	0.0	0.0%	4.5	-0.1	0.0%	4.0	0.3	0.0%
	Agmatine	65.8	62.5	6.3%	60.2	58.7	5.9%	52.7	48.4	4.8%	56.2	51.2	5.1%	61.2	56.6	5.7%	51.6	47.9	4.8%
25	No interferent added	28.2	-	-	25.6	-	-	28.9	-	-	28.3	-	-	30.1	-	-	28.5	-	-
	Methylhistamine	27.5	0.0	0.0%	25.3	0.0	0.0%	29.1	0.0	0.0%	28.6	0.0	0.0%	30.2	0.0	0.0%	29.0	0.0	0.0%
	Tyramine	26.7	-0.8	-0.1%	25.2	-0.1	0.0%	28.2	-0.9	-0.1%	29.7	1.1	0.1%	30.5	0.3	0.0%	29.2	0.2	0.0%
	L-Phenylalanine	28.6	1.1	0.1%	27.5	2.2	0.2%	28.6	-0.5	-0.1%	29.5	0.9	0.1%	29.9	-0.3	0.0%	28.9	-0.1	0.0%
	L-Histidine	28.1	0.6	0.1%	28.5	3.2	0.3%	29.4	0.3	0.0%	28.0	-0.6	-0.1%	30.5	0.3	0.0%	28.7	-0.3	0.0%
	L-Tyrosine	27.0	-0.5	-0.1%	26.3	1.0	0.1%	28.7	-0.4	0.0%	29.1	0.5	0.1%	30.9	0.7	0.1%	29.1	0.1	0.0%
	Tryptamine	27.2	-0.3	0.0%	29.1	3.8	0.4%	29.3	0.2	0.0%	28.9	0.3	0.0%	31.2	1.0	0.1%	28.1	-0.9	-0.1%
	Cadaverine	28.5	1.0	0.1%	28.7	3.4	0.3%	29.0	-0.1	0.0%	27.9	-0.7	-0.1%	29.9	-0.3	0.0%	29.5	0.5	0.1%
	Putrescine	28.7	1.2	0.1%	29.4	4.1	0.4%	29.8	0.7	0.1%	29.0	0.4	0.0%	30.8	0.6	0.1%	30.7	1.7	0.2%
	Anserine	28.2	0.7	0.1%	26.1	0.8	0.1%	29.9	0.8	0.1%	28.9	0.3	0.0%	30.9	0.7	0.1%	29.9	0.9	0.1%
	Carnosine	28.4	0.9	0.1%	26.0	0.7	0.1%	28.7	-0.4	0.0%	29.1	0.5	0.1%	31.0	0.8	0.1%	28.7	-0.3	0.0%
	Agmatine	90.2	62.7	6.3%	84.7	59.4	5.9%	77.1	48.0	4.8%	79.6	51.0	5.1%	85.9	55.7	5.6%	75.1	46.1	4.6%

Table 5. Method developer results for spiked matrixes

Matrix	Naturally contaminated histamine, *mg/Kg*	Spiking *mg/Kg*	Total histamine *mg/Kg*	BioSystems Y15 HISTAMINE results				
				Mean (n=5) *mg/Kg*	Sr	RSDr (%)	Recovery (%)	Bias *mg/Kg*
Raw tuna	1.2	0	1.2	1.2				
		10	11.2	10.3	0.09	7.81	92%	-0.8
		50	51.2	48.9	0.33	3.16	96%	-2.3
		100	101.2	97.0	1.94	3.96	96%	-4.2
		150	151.2	147.5	2.74	2.83	98%	-3.7
		200	201.2	192.1	6.93	5.04	95%	-9.1
Water-canned tuna	2.1	0	2.1	2.1				
		10	12.1	12.9	0.10	4.78	107%	0.8
		50	52.1	54.4	0.39	3.00	105%	2.4
		100	102.1	102.4	1.11	2.04	100%	0.3
		150	152.1	150.5	0.99	0.97	99%	-1.6
		200	202.1	203.1	1.84	1.22	101%	1.0
Oil-canned tuna	4.2	0	4.2	4.2				
		10	14.2	14.2	0.04	0.90	100%	0.0
		50	54.2	55.6	0.18	1.26	103%	1.4
		100	104.2	105.2	0.81	1.46	101%	1.0
		150	154.2	154.0	1.13	1.07	100%	-0.1
		200	204.2	203.5	1.39	0.90	100%	-0.7
Raw sardines	5.1	0	5.1	5.1				
		10	15.1	15.2	0.25	4.95	101%	0.1
		50	55.1	55.3	0.27	1.76	100%	0.2
		100	105.1	102.6	1.43	2.59	98%	-2.5
		150	155.1	151.2	1.83	1.78	98%	-3.9
		200	205.1	206.1	1.38	0.91	101%	1.0
Oil-canned sardines	5.0	0	5.0	5.0				
		10	15.0	14.8	0.19	3.83	99%	-0.2
		50	55.0	54.0	0.28	1.89	98%	-1.0
		100	105.0	98.7	0.54	1.00	94%	-6.3
		150	155.0	146.6	1.37	1.39	95%	-8.4
		200	205.0	200.7	1.99	1.36	98%	-4.4
Semi-preserved anchovy fillets	3.3	0	3.3	3.3				
		10	13.3	13.1	0.14	4.26	98%	-0.2
		50	53.3	54.7	0.45	3.44	103%	1.4
		100	103.3	101.5	0.80	1.46	98%	-1.8
		150	153.3	147.5	4.08	4.02	96%	-5.8
		200	203.3	197.7	4.97	3.37	97%	-5.6
Raw salmon	5.1	0	5.1	5.1				
		10	15.1	15.2	0.20	3.95	101%	0.1
		50	55.1	55.3	0.32	2.10	100%	0.2
		100	105.1	102.6	0.83	1.50	98%	-2.5
		150	155.1	151.2	0.91	0.89	98%	-3.9
		200	205.1	206.1	1.48	0.98	101%	1.0

Table 15. Independent laboratory results for spiked matrixes

Matrix	Endogenous histamine, *mg/Kg*	Spiking *mg/Kg*	Total histamine *mg/Kg*	BioSystems Y15 HISTAMINE results				
				Mean (n=5) *mg/Kg*	Sr	RSDr (%)	Recovery (%)	Bias *mg/Kg*
Raw tuna	1.1	0	1.1	1.1	0.3	31.0	100%	0
		5	6.1	6.3	0.60	10.0	104%	0.2
		10	11.1	10.6	0.60	6.0	96%	-0.5
		50	51.1	51.2	0.90	1.8	100%	0.1
		100	101.1	98.8	0.20	0.2	98%	-2.3
		200	201.1	188.7	1.80	1.0	94%	-12.3
Water-canned tuna	5.5	0	5.5	5.5	0.30	5.0	100%	0
		5	10.5	10.1	0.20	1.9	96%	-0.4
		10	15.5	14.4	0.50	3.4	93%	-1.1
		50	55.5	50.8	0.70	1.3	92%	-4.7
		100	105.5	93.7	2.60	2.7	89%	-11.8
		200	205.5	176.1	1.10	0.7	86%	-29.4
Oil-canned tuna	6.6	0	6.6	6.5	0.20	3.1	98%	-0.1
		5	11.6	7.6	0.30	4.2	66%	-3.9
		10	16.6	12.4	0.70	5.5	75%	-4.1
		50	56.6	49.7	0.50	0.9	88%	-6.8
		100	106.6	99.9	0.80	0.8	94%	-6.6
		200	206.6	191.8	2.70	1.4	93%	-14.7

REFERENCES CITED

1. Dueñas, S., Boix, M., and Tobeña, A., Validation Study of BioSystems® Y15 Histamine Enzymatic Assay for the Detection of Histamine in Fish and Fishery Products, AOAC *Performance Tested Methods*℠ certification number 072001.
2. International Organization for Standardization (ISO) 19343:2017, Microbiology of the food chain: Detection and quantification of histamine in fish and fishery products – HPLC method (ISO 19343:2017) Geneva, Switzerland.