CERTIFICATION

AOAC® *Performance Tested*SM

Certificate No.

031901

The AOAC Research Institute hereby certifies the method known as:

RIDASCREEN® Histamine (enzymatic) Kit

manufactured by

R-Biopharm AG
An der neuen Bergstr. 17
64297 Darmstadt, Germany

This method has been evaluated in the AOAC® *Performance Tested Methods*SM Program and found to perform as stated by the manufacturer contingent to the comments contained in the manuscript. This certificate means that an AOAC® Certification Mark License Agreement has been executed which authorizes the manufacturer to display the AOAC *Performance Tested*SM certification mark along with the statement - "THIS METHOD'S PERFORMANCE WAS REVIEWED BY AOAC RESEARCH INSTITUTE AND WAS FOUND TO PERFORM TO THE MANUFACTURER'S SPECIFICATIONS" - on the above-mentioned method for a period of one calendar year from the date of this certificate (November 21, 2021 – December 31, 2022). Renewal may be granted at the end of one year under the rules stated in the licensing agreement.

Scott Coates

Scott Coates, Senior Director
Signature for AOAC Research Institute

November 21, 2021
Date

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SUBMITTING COMPANY
R-Biopharm AG
An der neuen Bergstr. 17
64297 Darmstadt, Germany

METHOD NAME
RIDASCREEN® Histamine (enzymatic)

CATALOG NUMBERS
R1605
RIDA® Sample Decolorant R1699 (approved for sample extraction of wine only)

INDEPENDENT LABORATORY
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APPLICABILITY OF METHOD
Analyte - Histamine (2-(4-Imidazolyl)-ethylamine); CAS registry number 51-45-6

Matrixes – fresh fish (5 g), canned fish (5 g), fish meal (1 g), cheese (60 g), wine (0.2 mL)

Performance claims - The linear range is from 1 – 20 mg/L in the extracted sample. Limits of quantification are 2 mg/kg for fresh fish, canned fish and cheese, 1.4 mg/L in wine and 10 mg/kg for fish meal.

REFERENCE METHODS
Fish Meal HPLC: German Official Reference Method from the Official Collection of Examination Proceedings according to §64 Foodstuffs and Commodities Act L.10.00-5: Determination of the content of biogenic amines in fish and fish products, high-pressure liquid chromatographic determination (14)
Tamim, N.M., Bennett, L.W., Shellem, T.A., Doerr, J.A. (2002) High-performance liquid chromatographic determination of biogenic amines in poultry carcasses, J. Agric. Food Chem. 50: 5012-5015. (15)
Fresh and canned fish LC methods : Eerola, S., Hinkkanen, R., Lindfors, E., Hirvi, T. (1993) Liquid chromatographic determination of biogenic amines in dry sausages, J. AOAC Int. 76: 575-577; modified by ANFACO and accredited by the national accreditation body ENAC (Entidad Nacional de Accreditation).
***Official Methods of Analysis,* (2016), 20th Edition, AOAC INTERNATIONAL, Gaithersburg, MD, Method 977.13 (16)**
Duflos, G., Dervin, C., Malle, P., Bouqullet, S. (1999) Relevance of Matrix Effect in Determination of Biogenic Amines in Plaice (*Pleuronectes platessa*) and Whiting (*Merlangus merlangus*), J. AOAC Int. 82: 1097-1101. (17)
Wine HPLC and LC: The International Organisation of Vine and Wine (OIV); Compendium of International Methods of Analysis; Method OIV-MA-AS315-18; Analysis of biogenic amines in musts and wines using HPLC.(18)
***Official Methods of Analysis,* (2016), 20th Edition, AOAC INTERNATIONAL, Gaithersburg, MD, Method 977.13 (16)**

ORIGINAL CERTIFICATION DATE
March 12, 2019

CERTIFICATION RENEWAL RECORD
Renewed annually through December 2022.

METHOD MODIFICATION RECORD
1. **November 2021 Level 1**

SUMMARY OF MODIFICATION
1. **Editorial changes for clarity.**

Under this AOAC® *Performance Tested*SM License Number, 091901 this method is distributed by:
NONE

Under this AOAC® *Performance Tested*SM License Number, 031901 this method is distributed as:
NONE

PRINCIPLE OF THE METHOD (1)
Different types of food are extracted by means of boiling water (fish products), water extraction and perchloric acid precipitation (cheese) and polyphenol removal (wine; use of an additional sample preparation test kit RIDA Sample Decolorant). The following enzymatic determination (RIDASCREEN Histamine enzymatic) consists of a microtiter plate (coated with an electron carrier and a dye) and ready to use reagents (buffer, standard solutions, catalase and the enzyme histamine dehydrogenase). The enzymatic determination is based on the histamine dehydrogenase which catalyzes the oxidative deamidation of histamine in the presence of an electron carrier that converts a dye to a color product. The color intensity is directly proportional to histamine concentration and is measured at 450 nm. The linear range is from 1 – 20 mg/L in the extracted sample.

DISCUSSION OF THE VALIDATION STUDY (1)

The RIDASCREEN Histamine (enzymatic) test kit investigated in this validation was proven to be highly applicable for the accurate quantification of histamine in fresh fish, canned fish, fish meal, wine, and cheese. For sample extraction of wine, the sample preparation kit RIDA Sample Decolorant was also validated. The in-house validation included characterization of linearity, side reactions, interferences, estimates of LOD and LOQ, matrix-specific confirmation of LoQ, comparison to reference methods for fish products and wine, an extended precision study, recovery, ruggedness studies for both systems, stability testing, and lot-to-lot comparisons.

Compared to other methods e.g. ELISA or HPLC the sample extraction procedures and the enzymatic test are easy to perform and robust. This is clearly underpinned by precision data for extraction and the stability of sample extracts. The claimed measurement range is linear as proven by residual plots for three different test kit lots. None of the investigated chemically-related substances showed a reactivity of practical relevance and only in cases were the ratio between histamine and the related substance is very low e.g. 0.005 a significant difference will be observed. The method developers decided not just to give an estimate of LoQ on the basis of blank samples or calibration data. Additionally, the matrix-dependent LoQ was confirmed by spiking experiments or naturally contaminated samples. It was revealed that all estimates were correct and that in cases of wine the real LoQ is even lower. As for nearly all enzymatic methods, all different measures of precision were excellent within the measurement range from 1 mg/L to 20 mg/L sample extract. Even dilution of sample extracts with concentration outside of this range resulted in acceptable or excellent results. An important part of this validation study already included the comparison to HPLC reference methods in different labs from the USA, France, Spain, and Germany. All method comparisons for wine, fresh fish, and canned fish revealed that both methods were comparable. One exception was the analysis of fish meal sample with concentrations higher than 1000 mg/kg. In this case it can be speculated that the reference method is not capable to derivatize the high histamine load in the extract. It goes without saying that in-house recovery studies also supported results from method comparison studies and ended up in excellent recoveries not only within the measurement range but often also near the LoQ of the respective method. Possible interferants for the enzymatic procedure are ascorbic acid, sulphite, and polyphenols. While the latter are precipitated using the sample preparation test kit for wine, the influence of sulphite in the normal concentration range in wine was shown to have no influence. Ascorbic acid in white wine does not interfere up the legal limit of 250 mg/L. Ascorbic acid on fresh fish is used in high amount up to 3 g/kg as a preservative and will therefore interfere with the enzymatic reaction. For this reason a special sample treatment using H_2O_2 and catalase is described and was validated. A thorough ruggedness testing scheme was applied to both test kits. Pipetted enzyme volumes, pipetting technique, and pipetting order were tested for the enzymatic system and were proven to be robust. Also incubation temperature was shown to be rugged against changes in the expected range of normal variation (16°C – 30°C). One critical parameter is the incubation time before the first absorbance measurement. In this case a time of 3 min should be strictly followed. The second incubation time of 10 min is also critical for some samples. Therefore, it is strictly recommended to exactly follow the method in this case. Robustness testing of the sample preparation test kit revealed that the procedure is stable against variation of incubation times, volumes, mixing, freezing of the components and transporting the components under harsh conditions. The stability testing of at least three independent lots showed high lot-to-lot reproducibility and proved that both kits are stable over the claimed shelf-life.

An independent evaluation of the RIDASCREEN®Histamine (enzymatic) and RIDA Sample Decolorant was performed by an independent laboratory and consisted of the analysis of spiked fresh cod fillets and red wine at different levels including verification of LOQ. The analytical performance claims of the manufacturer were confirmed.

Table 14. Method comparison at ANFACO for fresh fish and canned fish samples with histamine concentrations less than 300 mg/kg. (1)

Sample		HPLC mg/kg	Enzymatic mg/kg
tuna (spiked)	fresh	109	111.4
tuna	fresh	25	18.5
tuna in sunflower oil	canned	45	43.2
tuna in sunflower oil	canned	25	16.5
tuna in sunflower oil	canned	37	49.6
tuna in sunflower oil	canned	50	50.8
tuna in sunflower oil	canned	28	14.0
tuna in sunflower oil	canned	49	42.0
pilchard	canned	242	287.6
pilchard	canned	242	200.4
yellowfin tuna	canned	17	21.9

Table 15. Method comparison at ANFACO for highly contaminated fresh fish and canned fish samples with histamine concentrations of 1000 mg/kg and more (1)

Sample		HPLC	Enzymatic
		mg/kg	mg/kg
tuna	fresh	>350	1267
tuna	fresh	>350	2392
yellowfin tuna	frozen	1540	2186
yellowfin tuna	frozen	1323	1888
yellowfin tuna	frozen	1111	1547
yellowfin tuna	frozen	975	1335
yellowfin tuna	frozen	1302	1851
yellowfin tuna	frozen	2023	3100
yellowfin tuna	frozen	1684	2391
yellowfin tuna	frozen	1555	2186
yellowfin tuna	frozen	1340	1973

Table 16. Method comparison at ANSES with canned fish. (1)

Sample		HPLC	Enzymatic	Assigned (a) or certified (c) value
		mg/kg	mg/kg	mg/kg
FAPAS 27161	Pilchards in tomato sauce	19	18.8	17 (a)
BIPEA 1-1477	Canned fish	56	40	50.1 (a)
BIPEA 1-1377	Canned fish	< 5	< 2	Blank (a)
FAPAS TET031RM	Canned tuna	134	133	139.8 (c)

Table 17. Results for FAPAS rounds from 2011 to 2015; samples were raw tuna, canned tuna in brine or pilchards in tomato sauce. (1)

FAPAS round	**mean** mg/kg	**min** mg/kg	**max** mg/kg	**R1605** mg/kg	**R1605 recovery**	**Neogen |z|> 2**	**All |z| > 2**
2796	51,9	42,7	61	51,2	99%	26%	26%
27101	311,1	269,1	353,1	327,2	105%	100%	44%
27105	192,6	164,7	220,6	193,8	101%	100%	35%
27110	33,7	27,3	40	35,5	105%	47%	32%
27116	380,4	330,6	430,1	342,4	90%	83%	30%
27126	26,8	21,5	32	28,2	105%	44%	27%
27132	126,7	107,2	146,3	135,5	107%	50%	37%
27155	202	173	231	209,9	104%	33%	34%
27161	17,0	13,4	20,5	18,8	111%	52%	36%

Table 18. Method comparison for different fish meal samples; the last column gives the absolute difference as mg/kg and –in brackets- the relative difference related to the HPLC reference value. (1)

Internal number	ELISA	HPLC (Trilogy)	HPLC (Eurofins)
LM13-2160-12	< 10	< 5	
LM13-2160-14	< 10	< 5	
LM13-2160-15	< 10	< 5	
LM13-2160-16	87		103
LM13-2160-1	743		711
LM13-2160-4	112	123	
LM13-2160-5	229	217	
LM13-2160-13	2038	2198	
LM13-2160-3	3085	3182	

Table 19. Comparison of different red wine samples by the reference HPLC method (OIV method) and the enzymatic method. Values in bold show unexpected differences between both methods. (1)

Wine sample	R1605 mg/L	HPLC mg/L
Red wine D.O Almansa	2.6	3.1
Red wine D.O. Rioja	5.2	6.0
Red wine D. O. Ribera del Duero	6.3	6.8
Red wine D. O . Vinos de Madrid	3.9	5.1
Red wine D. O. Ribera del Duero	3.2	4.8
Red wine D. O. Ribera del Duero	3.3	3.6
Red wine Vino de Tierra de Castilla	**<1.4**	**3.6**
Red wine Vino de Tierra de Castilla	<1.4	1.0
Red wine D. O . Vinos de Madrid	5.3	5.4
Red wine D. O. Navarra	5.1	5.9
Red wine D. O. Navarra	<1.4	<0.5
Red wine D. O. Navarra	<1.4	<0.5
Red wine D. O. Penedés	7.8	8.9
Red wine D. O . Vinos de Madrid	2.0	1.9
Red wine D. O . Vinos de Madrid	1.8	1.4
Red wine D.O. Rioja	5.2	5.4
Red wine D.O. Rioja	6.0	6.6
Red wine NRL PT 2013	5.0	5.2
Red wine NRL PT 2014	9.9	10.3
Red wine D. O. Navarra spike 5 mg/L	4.3	5.3
Red wine D. O. Navarra spike 10 mg/L	9.1	10.3
Red wine D. O. Navarra spike 5 mg/L	4.2	5.5
Red wine D. O. Navarra spike 10 mg/L	9.1	10.2
Red wine Rioja	8.6	10.2
Red wine Rioja	6.7	6.7
Red wine Castilla	3.6	3.6
Red wine Chianti	2.1	2.8
Red wine California	<1.4	<0.5

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