Enzymatic color test for the determination of histamine in fish and wine
Test combination for 55 determinations

For *in vitro* use only
Store between 2 – 8 °C (36 – 46 °F)

This test was validated using selected samples of the following matrices: fresh fish (tuna, mackerel, sardines, herring, swordfish, cod, salmon, sea bass, tilapia), canned fish in oil or brine, fish meal, and red and white wine.

Detailed results and information regarding associated validation data are found in the Validation Report.

The test may be used with other foods or samples material, provided that these are subjected to individual validation by the user.

1. Test principle

The enzymatic assay is based on the enzyme histamine dehydrogenase, which catalyzes the oxidative deamination of histamine in the presence of an electron carrier:

Histamine + PMS + H_2O –HDH→ 2-(4-imidazolyl)acetaldehyde + $PMSH_2$ + NH_3

In this reaction, histamine is converted to 2-(4-imidazolyl)-acetaldehyde, while the electron carrier 1-methoxy-phenazinium methyl sulfate (PMS) is reduced; ammonia (NH_3) and protons are also released. The reduced $PMSH_2$ transfers the electrons it has accepted to WST. This converts WST (oxidized) into the intensely colored WST-formazan (reduced):

$PMSH_2$ + WST (ox) ⟶ PMS + WST (red)

The amount formed, or the color intensity of the formazan, is directly proportional to the histamine concentration and is measured at a wavelength of 450 nm.

To account for non-specific reactions in the system, a matrix or sample blank is additionally performed by **omitting the histamine dehydrogenase in Reagent 2 *Blank***.

2. Reagents

2.1. Content & composition

The test was designed for manual processing. All reagents required to perform the enzymatic test are included in the test kit and are ready to use. When used as instructed, the reagents are sufficient for 55 determinations.

Description	Quantity	Content	Cap
Reagent 1	2 x 110 mL	buffer, WST	brown
Reagent 2 *Blank*	2 x 14.5 mL	buffer	blue
Reagent 2 *Sample*	2 x 14.5 mL	buffer, HDH	green

2.2. Reagent preparation

The reagents are ready-to-use and be allowed to reach room temperature (20 – 25 °C / 68 – 77 °F) before use. Do not vortex or shake reagents! Do not interchange components between kits from different lots.

2.3. Storage & stability

If stored as directed and between 2 – 8 °C (36 – 46 °F), reagents remain stable until the printed expiration date, even after opening. Reagents must not be frozen.

Caution: Reagent 1 contains the light- and oxidation-sensitive raw materials WST and PMS. Close bottles immediately after use and do not expose them to direct sunlight.

2.4. Safety & disposal

The test is intended solely for the intended use as described. The provided Instructions for Use must be strictly followed.

Follow standard chemical safety procedures when handling this product. Do not swallow. Avoid contact with skin or mucous membranes.

Detail safety information for individual components is available in the corresponding Safety Data Sheets (SDS).

Dispose of used reagents as laboratory waste in compliance with all relevant regulations. Packaging materials are to be recycled according to local regulations.

3. Sample preparation

3.1. Additional supplies, reagents and apparatus

The following materials, reagents, and equipment are also required for sample preparation:

(1) Spectrophotometer for 4-mL cuvettes (450 nm filter)
(2) Disposable acrylic cuvettes (4 mL)
(3) Mixer or tissue homogenizer
(4) Vortex mixer
(5) Centrifuge for 50-mL centrifuge tubes, optional: benchtop centrifuge
(6) 50-mL centrifuge tubes (PP; conical tubes, Falcon®)
(7) Optional: water bath (100 °C)
(8) Distilled or deionized water
(9) Balance
(10) Paper filters (e.g., Whatman No. 1 or comparable quality; optional: PES syringe filter, e.g., VWR Art. No. 514-7010)
(11) 1 M perchloric acid (for raw fish without cooking)
(12) For wine analysis: RIDA® Sample Decolorant (Art. No. R1699)

3.2. General sample preparation

- Store sample extracts at 2 – 8 °C (36 – 46 °F). Alternatively, store samples at -20 °C (-4 °F) for longer storage periods.
- Frozen samples should be thawed before use in the refrigerator (alternatively at 20 – 25 °C / 68 – 77 °F).
- The histamine level continue to rise even in refrigerated foods; therefore, it is important to perform laboratory analyses immediately after sampling.
- Use only plastic tubes (e.g., polypropylene, PP) for sample preparation – **no glass containers**! Histamine adsorbs to glass surfaces.
- Homogenize semi-solid and pasty samples sufficiently; completely grind solid samples.
- Filtrates may occasionally exhibit slight cloudiness or coloration. This is compensated for by the sample blank.

3.3. Determination of histamine in canned tuna and precooked fish (without a cooking step)

- Blend the sample thoroughly in a blender until homogeneous; if using canned fish, use the entire contents of the can, including the liquid (juice, oil).
- Weigh 5 g of the sample into a 50-mL centrifuge tube and add 20 mL of distilled water.
- Mix on a vortex mixer or by shaking vigorously until a homogeneous suspension forms in the tube.
- Add another 20 mL of distilled water.
- Extract for 20 minutes at room temperature on a roller mixer.
- Filter the extract through a pleated filter into a new 50-mL centrifuge tube; if necessary, centrifuge the filtrate in a bench-top centrifuge.
- Use the filtrate as specified in the test procedure (section 4. Test Procedure).
- When calculating the histamine content (section 5. Calculation of results), use a preparation factor of F = 9.

3.4. Determination of histamine in frozen, fresh, and uncooked fish (with a cooking step)

- Homogenize the sample thoroughly in a blender.
- Weigh 5 g of the sample into a 50-mL centrifuge tube and add 20 mL of distilled water.
- Mix on a vortex mixer or by vigorous shaking until a homogeneous suspension has formed in the tube.
- Add another 20 mL of distilled water.
- Cook for 20 minutes at 100 °C (212 °F) in a water bath.
- Filter the extract through a pleated filter into a new 50-mL centrifuge tube; if necessary, centrifuge the filtrate in a bench-top centrifuge.
- Use the filtrate as specified in the test procedure (section 4. Test Procedure).
- When calculating the histamine content (section 5. Calculation of results), use a preparation factor of F = 9.

3.5. Determination of histamine in frozen, fresh, and uncooked fish (without a cooking step)

- Homogenize the sample thoroughly in a blender.
- Weigh out 5 g of the sample into a 50-mL centrifuge tube, add 2 mL of 1 M perchloric acid, and seal the tube.
- Vortex the homogenate in the perchloric acid until the entire sample matrix has turned whitish.
- Then add 18 mL of distilled water and shake or vortex the sample suspension thoroughly.
- Centrifuge the sample suspension until a clear supernatant can be collected (recommended: 3200 – 3450 x *g* for 15 – 20 minutes).
- Use the supernatant as specified in the test procedure (section 4. Test Procedure).
- When calculating the histamine content (section 5. Calculation of results), use a preparation factor of F = 5.

For very fatty fish (e.g., mackerel), the volume of perchloric acid can be increased to up to 10 mL. The volume of distilled water must be reduced in proportion to the volume of perchloric acid used so that the total extraction volume does not increase.

3.6. Determination of histamine in fish meal (without a cooking step)

- Homogenize the sample in a blender. If using fine flour, mixing may be sufficient.
- Weigh 1 g of the sample into a 50-mL centrifuge tube and add 40 mL of distilled water.
- Mix on a vortex mixer or by shaking vigorously until a homogeneous suspension has formed in the tube.
- Add another 20 mL of distilled water.
- Extract for 20 minutes at room temperature on a roller mixer.
- Filter the extract through a pleated filter into a new 50-mL centrifuge tube; if necessary, centrifuge the filtrate in a bench-top centrifuge.
- Use the filtrate as specified in the test procedure (section 4. Test Procedure).
- When calculating the histamine content (section 5. Calculation of results), use a preparation factor of F = 40.

3.7. Determination of histamine in wine

Wines (red wine, white wine, rosé) contain anthocyanins and polyphenols, which must be removed using the RIDA® Sample Decolorant (Art. No. R1699) prior to enzymatic analysis.

- Add 200 µL of Reagent 1 from the RIDA® Sample Decolorant to 200 µL of wine (pre-rinse the pipette tips).
- Add 200 µL of Reagent 2 from the RIDA® Sample Decolorant, vortex, and incubate for 5 minutes at room temperature (20 – 25 °C / 68 – 77 °F).
- Then centrifuge for 2 minutes at a minimum of 14.000 x *g* (room temperature, 20 – 25 °C / 68 – 77 °F).
- Transfer 500 µL of the supernatant to a new tube.
- Add 100 µL of Reagent 3 from the RIDA® Sample Decolorant, vortex, and incubate for 5 minutes at room temperature (20 – 25 °C / 68 – 77 °F).
- Centrifuge the sample for 2 minutes at a minimum of 14.000 x *g* (room temperature, 20 – 25 °C / 68 – 77 °F).
- Use the supernatant as specified in the test procedure (section 4. Test Procedure).
- When calculating the histamine content (section 5. Calculation of results), use a preparation factor of F = 3.6.
- The extract is stable for 2 hours at room temperature (20 – 25 °C / 68 – 77 °F) or 1 day at 2 – 8 °C (36 – 46 °F).

4. Test procedure

Wavelength:	450 nm
Temperature (measurement):	37 °C (99 °F)
Photometer alignment:	against air (without cuvette)
Measuring range:	0.8 – 150 mg/L

	Sample blank	**Samples / controls**
Sample / control **Reagent 1**	100 µL 2000 µL	100 µL 2000 µL
Submit sample first, incubate for **3 minutes at 37 °C (99 °F)**. **Read** absorbance **A_1** at **450 nm**, then addition of:		
Reagent 2 *Blank* **Reagent 2 *Sample***	500 µL -	- 500 µL
Incubate for **10 minutes at 37 °C (99 °F)** and **read** absorbance **A_2** at **450 nm**.		

Important notes and recommendations for assay procedure

- When performing the test, a **sample blank** is included using Reagent 2 *Blank* (**without** enzyme). This requires **two** cuvettes per determination, into each of which 100 µL of the sample is pipetted.
- The sample blank (SB) must be determined for **each** sample and subtracted from the respective sample result. The sample blank and the sample must be measured **in the same run** and under the same conditions.
- The specified incubation times were validated at a temperature of 37 °C (99 °F). The test should generally **not** be performed at temperatures **below this value**.
- The test should be performed using disposable acrylic cuvettes (4 mL). A spectrophotometer designed for 4-mL cuvettes with a 450-nm filter is required for absorbance measurement.
- Fat can interfere with the measurement! The sample or filtrate must be taken from below the fat layer. An additional filtration or centrifugation step may be necessary.
- To ensure a high level of analytical reliability, it is recommended that each sample be analyzed as a duplicate. With single determination, there is an increased risk of overlooking errors in the test procedure (e.g., pipetting errors). In addition, greater variation in results is to be expected with single determinations.
- Gas bubbles may form during the test! The bubbles can be removed from the light path by gently tapping or tilting the cuvette.
- For quality control and to verify that the test is performed correctly and without interference, include control samples (see also 5.3. Controls & acceptance criteria).
- To ensure better mixing of the reagent and sample, the use of a multistepper pipette is recommended for the addition of Reagent 1, Reagent 2 *Blank*, and Reagent 2 *Sample*. Use a separate tip for each component.
- Use separate tips for each sample extract and the control solutions to avoid cross-contamination; rinse the tip before pipetting.
- Close reagent bottles immediately after use and do not expose them to direct sunlight.

5. Calculation of results

5.1. Concentration of histamine in sample solutions

To determine the value of one sample, two independent measurements are performed: analysis of the sample using Reagent 2 *Sample* and analysis of the sample using Reagent 2 *Blank* (sample blank).

The extinction difference ΔA must be calculated for each sample:

$$\Delta A = (A_2 - df \times A_1)_{\text{sample or control}} - (A_2 - df \times A_1)_{\text{sample blank}}$$

df: Dilution factor

$$df = \frac{\text{sample volume [mL]} + \text{volume R1 [mL]}}{\text{reaction volume [mL]}} = 0.808$$

The specified df value of **0.808 applies to a sample volume of 100 µL**. An increase in sample volume is not intended.

The histamine concentration is calculated using a batch-specific factor Y. This factor is determined individually for each production batch and is included in the batch-specific certificate of analysis (CoA).

The factor is based on Lambert-Beer's law. The histamine concentration can then be calculated using the specified lot-specific factor with a simplified formula as follows:

$$C_{\text{Histamine}}\ [g/L] = Y_{\text{batch-specific factor}} \times \Delta A$$

$$C_{\text{Histamine}}\ [mg/L] = Y_{\text{batch-specific factor}} \times \Delta A \times 1000$$

Important Note: This formula applies only under the conditions specified by the following variables and the test conditions described in this document. **These must not be altered.**

V:	Test volume [mL]	= 2.600
MW:	Molecular weight histamine [g/mol]	= 111.15
d:	Optical path [cm]	= 1.00
v:	Sample volume [mL]	= 0.100

5.2. Content of histamine in solid samples

When analyzing solid or semi-solid sample matrices (fresh or cooked fish and canned fish) that are weighed for sample preparation, the analytical result is expressed in [mg/kg] relative to the initial weight and calculated using the following factors:

- the weighed sample quantity (W) in [kg]
- the sample preparation volume (V) in [L]
- the calculated histamine concentration (C) in [mg/L]
- the sample-preparation-dependent dilution factor (preparation factor F) (see Section 3. Sample Preparation)
- if the filtrate is further diluted, take the additional filtrate dilution factor (f) into account (for undiluted filtrate, f = 1).

$$\text{Content}_{\text{Histamine}}\ [mg/kg] = \frac{C_{\text{Histamine}}\ [mg/L] \times V\ [L]}{W_{\text{Sample}}\ [kg]} \times F \times f$$

5.3. Controls & acceptance criteria

Control or reference samples should be included in each run for quality control purposes. For this purpose, we recommend:

- FAPAS Reference Material TYG018RM; Tuna in brine
- FAPAS QC material T27340QC; Canned fish
- Histamine dihydrochloride, e.g., C. Roth, Art. No. 4017

The expected recovery rates for *extracted* fish and wine samples range from 90 – 110 %.

Examples of spiking experiments are provided in the validation report on request.

6. Performance data

6.1. Specificity & side activities

The specificity of the Enzytec™ *Liquid* Histamine test was determined by measuring the side activity toward the corresponding substances in the buffer system.

The enzyme histamine dehydrogenase is specific for histamine. Side activities for cadaverine, L-histidine, spermidine, 4-imidazoleacetic acid, L-lysine, L-tyrosine, L-tryptophan, putrescine, trimethylamine, spermine, L-carnosine, serotonin, and tyramine are less than 1 %. Agmatine is determined at approximately 9 %, Nα-methylhistamine dihydrochloride at 78 %, and 3-methylhistamine dihydrochloride at 1 %.

6.2. Interferences

SO_2 and ascorbic acid showed no interference at concentrations up to 2.5 mg/L and 7.5 mg/L, respectively.

6.3. Linearity, measuring range & sensitivity

Linearity is given up to 150 mg/L histamine with a recommended measuring range of 0.8 – 150 mg/L for a sample volume of 100 µL

The limit of detection (LoD) was determined according to the method DIN 32645:2008-11 in an aqueous solution. For a sample volume of 100 µL, the calculated LoD is 0.22 mg/L.

The limit of quantification (LoQ) was determined based on a precision profile and is 0.8 mg/l histamine for a sample volume of 100 µl.

7. Supporting documents

On request, we offer the following documents:

- Enzytec™ *Liquid* Histamine Validation report
- Enzytec™ *Liquid* Histamine Excel template for results
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* Troubleshooting guide

Safety data sheets (SDS) and certificates of analysis (CoA) are available in digital form, quoting the batch number, via the following link:

https://eifu.r-biopharm.com/

8. Limits of this method

Test results may vary depending on the sample matrix, specific test implementation, and laboratory environmental conditions. Detection and quantification limits are dependent on respective sample matrices extraction procedures. Refer toto the current Validation Report for details.

For this test, only the matrices explicitly listed in the documentation were validated, due to the wide variety of food products and other potential sample materials.

When analysing non-validated matrices results should be verified by performing spiking (fortification) experiments. If appropriate or necessary, a suitable sample preparation procedure for the respective matrix must be developed and validated.

The responsibility for validating non-validated matrices and for ensuring the suitability of the assay for its intended use lies solely with the user.

9. Services & technical support

On request, we offer the following services:

- Customized troubleshooting
- Workflow analysis
- Data & results analysis
- Customer workshops & webinars

10. Disclaimer

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