MICROBIOLOGICAL METHODS

Validation of the Biofish-300 HIS Enzymatic Biosensor for the Detection of Histamine in Fishery Products

AOAC *Performance Tested Method*[SM] 051604

Abstract

The Biofish-300 HIS method is a simple, reliable, and specific enzymatic biosensor for the detection of histamine. This technology is highly specific and selective and allows quantification of histamine in fishery products (fresh/frozen and processed) in a short time frame (2–3 min). Histamine in raw tuna, raw mackerel, raw sardine, raw anchovy, boiled tuna, canned tuna in water, canned tuna in oil, canned mackerel in tomato sauce, canned pickled sardine, and canned salted anchovy was analyzed using a water-based extract. Matrix-specific assay procedures and calibration curves were used to enable analyses to be carried out across multiple sample types. The performance of this assay was examined using samples that were naturally contaminated (reference materials and interlaboratory studies) and spiked with histamine. All data were judged against previously established acceptance criteria. Performance measures were evaluated for linearity, selectivity, matrix, lot consistency, and robustness. Results produced in all performance measures, except robustness, were within acceptable ranges. Out-of-range robustness results reflected deviation in sample volume compared to the standard assay procedures. Positive interferences from the presence of agmatine were shown.

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The method was independently tested, evaluated, and certified by the AOAC Research Institute as a *Performance Tested Method*[SM]. *See* http://www.aoac.org/testkits/steps.html for information on certification.

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Scope of Method

(a) *Target analyte*.—Histamine.

(b) *Matrixes*.—Raw tuna, raw mackerel, raw sardine, raw anchovy, boiled tuna, canned tuna in water, canned tuna in oil, canned mackerel in tomato sauce, canned pickled sardine, and canned salted anchovy. The samples were divided for the study as follows: (*1*) *Fish*.—Raw tuna, raw mackerel, raw sardine, raw anchovy, boiled tuna, canned tuna in water, canned tuna in oil, canned mackerel in tomato sauce, and canned pickled sardine.

(*2*) *Canned salted fish*.—Canned salted anchovy.

Note: Samples with high levels of agmatine may cause positive interferences.

(c) *Claims*.—Quantification of histamine from 10 to 200 mg/kg in raw, cooked, and canned fish and from 30 to 150 mg/kg in canned salted fish.

Intended Users

Food laboratories.

Technical staff of fish and aquaculture companies, including primary and processing sectors.

Definitions

(a) *Linearity*.—(*1*). Linearity defines the ability of the method to obtain test results proportional to the concentration.

(b) *Lot-to-lot consistency and stability study.*—The product consistency and stability study is a study or set of studies designed to ensure that the performance of the product is consistent from lot-to-lot and over time under normal storage conditions for the shelf life of the product.

(c) *Robustness.*—A study which tests the capacity of a method to remain unaffected by small but deliberate variations in method parameters and which provides an indication of its reliability during normal usage.

(d) *Precision.*—Closeness of agreement between quantity values obtained by replicate measurements of a quantity, under specified conditions. Precision is usually expressed as the standard deviation or relative standard deviation.

(e) *Selectivity*.—Selectivity is the ability of the method to detect the analyte without interference from the matrix or other components of similar behavior.

(f) *Recovery*.—Recovery is the ratio of the mean candidate method result to the true value, expressed as a percentage: ($mean_{cand}$/known spike) × 100.

(g) *Relative recovery*.—Relative recovery is the ratio of the mean candidate method result to the reference method result, expressed as a percentage: ($mean_{cand}/mean_{ref}$) × 100.

(h) *Bias*.—Bias is the difference between the candidate method mean result and the true value: $mean_{cand}$ − known spike.

(i) *Relative bias*.—Relative bias is the difference between the candidate method mean result and the mean reference method result: $mean_{cand} - mean_{ref}$.

(j) s_r.—Calculated using the following equation:

$$s_r = \sqrt{\frac{\sum_{i=1}^{n}\left(X_i - \bar{X}\right)^2}{n-1}}$$

(k) RSD_r.—Calculated as ($s_r/mean_{cand}$) × 100.

(l) *LOD*.—Calculated using the following equation:

$$\text{LOD} = \frac{\bar{X}_0 + 3.3\left(s_b\right)}{1 - 1.65m}$$

(m) *LOQ*.—Estimated as 3 × LOD.

General Information

Histamine is a common biogenic amine produced by bacterial action in fish families that have a high level of the amino acid histidine, such as Scombridae, Clupeidae, Engraulidae, Coryphaenidae, Scombresosidae, and Pomatomidae. Bacterial amino acid decarboxylase enzymatic action catalyzes the decarboxylation of histidine, producing histamine. Histamine content may vary depending on the degree of time/temperature exposure, and can, therefore, be considered a spoilage indicator.

In higher concentrations (>200 mg/kg), histamine is toxic and leads to symptoms similar to severe allergic reactions (1, 2). Commission Regulation No. 2073/2005/EC (3) established that for nine samples taken from each batch, the mean histamine value must not exceed 400 mg/kg, and no sample may have a value exceeding 200 mg/kg for fishery products that have undergone enzyme maturation treatment in brine manufactured from fish species associated with a high amount of histidine. The same regulation states that when nine samples taken from a lot of fish are analyzed for histamine, the mean value must not exceed 100 ppm; two samples may have a value of more than 100 ppm, but less than 200 ppm; and no sample may have a value exceeding 200 ppm. This statement covers a large portion of the fish and fishery products produced in the world today, including raw, frozen, and canned fish that this test is intended to analyze.

Principle

The Biofish-300 HIS is an enzymatic biosensor for the specific detection of histamine. The test method consists of the extraction of histamine in aqueous solution and subsequent quantification by the biosensor after previous calibration. The enzymatic biosensor is a system that incorporates a biochemical sensing element (based on a specific oxidative enzyme) in intimate contact with or in close proximity to a transducer system that relates the concentration of an analyte to a measurable signal. The addition of the analyte causes sequential redox reactions that involve a release of electrons proportional to the concentration of the analyte. Thus, these biosensors combine the specificity and the selectiveness of enzyme–analyte reactions with highly sensitive electrochemical transduction (Figure 1).

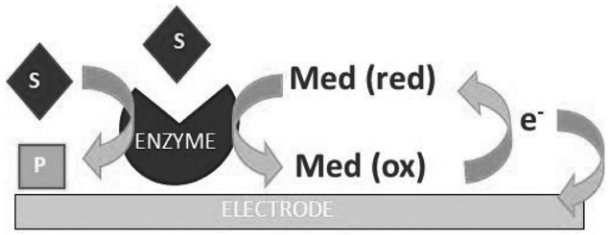


Figure 1. Electrochemical biosensor scheme

Test Kit Information

(a) *Kit name*.—Biofish-300 HIS.

(b) *Cat. No*.—BF300.

(c) *Ordering information*.—Biolan Microbiosensores S.L., Parque Tecnológico de Bizkaia, Laida Bidea Edificio 409, 48170 Zamudio, Vizcaya, Spain; Tel.: (+34) 946 57 41 61; Web site: http://www.biolanmb.com; e-mail: biolanmb@biolanmb.com.

(d) *Test Kit components*.—(*1*) *Biotest*.—BTFHIS5 (five analyses), BT FHIS10 (10 analyses), BT FHIS20 (20 analyses), BT FHIS50 (50 analyses), and BT FHIS100 (100 analyses).

(*2*) *HIS Calibration Kit.*—KITCFHIS, including eight tubes of dehydrated calibration reagent (histamine dihydrochloride) and an empty tube for preparation of the calibration standard for 24 working days. Stable for up to 6 months at 3–8°C.

(*3*) *HIS1 Measurement Kit.*—KITMFHIS1, including eight tubes of measuring reagent for preparing Measurement Solution (MS) for 160 analyses. Stable for up to 6 months at room temperature.

(*4*) *HIS2 Measurement Kit.*—KITMFHIS2, including eight tubes of measuring reagent for preparing MS for 640 analyses. Stable for up to 6 months at room temperature.

(*5*) *Reference electrode*.—ELEREFN.

(e) *Additional supplies and reagents*.—Distilled water.

Apparatus

(a) *Biofish-300 biosensor.*—BF300, an amperometric measurement device, including a measuring cuvette two reference electrode (ELEREFN).

(b) *Analytical balance*.—Capable of measurement of grams to two decimal places.

(c) *Sample homogenizer*.—ULTRA-TURRAX® T 25 (IKA Staufen, Germany)

(d) *Adjustable pipets*.—Capable of delivering 20–200, 100–1000, and 500–5000 µL.

Reference Materials

(a) *Canned fish*.—Food Analysis Proficiency Assessment Scheme (FAPAS; York, United Kingdom; http://www.fapas.com) T27132QC, containing 126.7 mg/kg histamine.

(b) *Canned fish*.—FAPAS T27120QC, containing 173.3 mg/kg histamine.

(c) *Canned fish*.—FAPAS T27126QC, containing 26.8 mg/kg histamine.

Safety Precautions

There are no major risks. Histamine dihydrochloride may cause skin, eye, or respiratory irritation. Avoid contact or inhalation. The reconstitution of the standard (histamine dihydrochloride) is possible without contact with the powder because it is delivered in a tube with a punched cap.

General Preparation

(a) *Biotest storage and use*.—Biotests are shipped at room temperature, vacuum-packed with silica gel crystals to protect them from atmospheric humidity. Upon receipt, store for a maximum of 6 months at 3–8°C. At least 12 h before use, hydrate the Biotest by removing silica gel crystals from inside the tube and filling the tube with MS until the base of the Biotest is submerged. Once hydrated, store the Biotest vertically at 3–8°C for a maximum of 15 days.

(b) *HIS Measurement Solution (MS)*.—For all analyses (except canned salted fish), dissolve contents of one tube of KITMFHIS1 in 250 mL distilled water or one tube of KITMFHIS2 in 1 L distilled water. For canned salted fish analyses, dissolve contents of 2 tubes of KITMFHIS1 in 250 mL distilled water or 2 tubes of KITMFHIS2 in 1 L distilled water. Mix well. Store solutions in bottle with dispenser at room temperature for up to 15 days.

(c) *HIS Calibration Reagent (CR)*.—Add 10 mL MS to 1 tube of dehydrated calibration reagent from the HIS Calibration Kit and mix. To prevent loss of reagent, introduce the pipet tip through the perforated stopper when adding MS. Store at 3–8°C and use within 3 days.

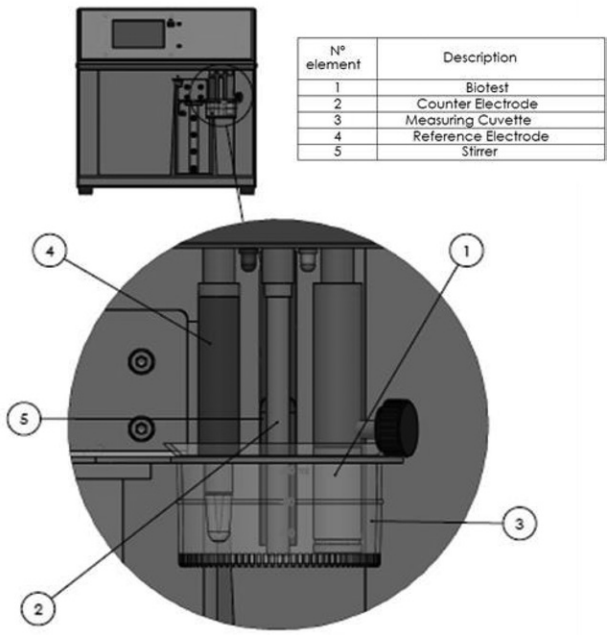


Figure 2. Configuration of the electrochemical cell

(d) *HIS Calibration Standard (CS)*.—Prepare CS in the empty tube provided in the HIS Calibration Kit by adding 19.9 mL MS and 0.1 mL CR. Mix well. Store at 3–8°C. Make fresh daily.

(e) *Reference electrode (ELEREFN)*.—Store in 4 M KCl at room temperature in the dark and in an upright position until use.

(f) *Biosensor setup*.—(*1*) Connect both the Biotest (first place it in the Biotest carrier and screw it into place) and the reference electrode (ELEREFN) to their respective socket connectors (Figure 2).

(*2*) Fill the measuring cuvette with 10 mL MS and put it in the cuvette holder, making sure that all the components of the electrochemical cell are submerged.

(*3*) Connect the device to a 100–240 volts alternating current socket and press the ON switch that is beside the screen, and hold down for 2 s (Figure 3).

(*4*) First press the BIOTEST option: select HISTAMINE, press », enter the Biotest code (printed on the Biotest ID label), press », and lastly, select the type of sample (FISH or SALTED FISH) to be used, and press OK (Figure 4).

(*5*) Select ANALYSIS to go to the main analysis menu: ACTIVATE, CALIBRATE, STANDARD measurement, and SAMPLE measurement (Figure 5).

(g) *Activation and calibration of the Biotest*.—Activate and calibrate the Biotest once a day before performing analyses or when the device has been shut down for more than 2 h. Ensure that the measuring cuvette contains 10 mL MS and that all components of the electrochemical cell are submerged.

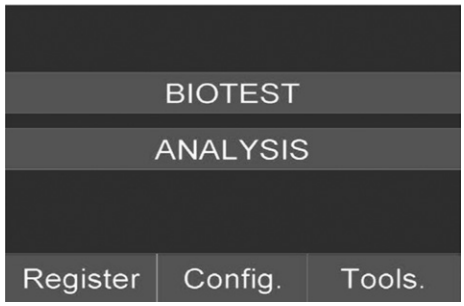


Figure 3. Main menu screen

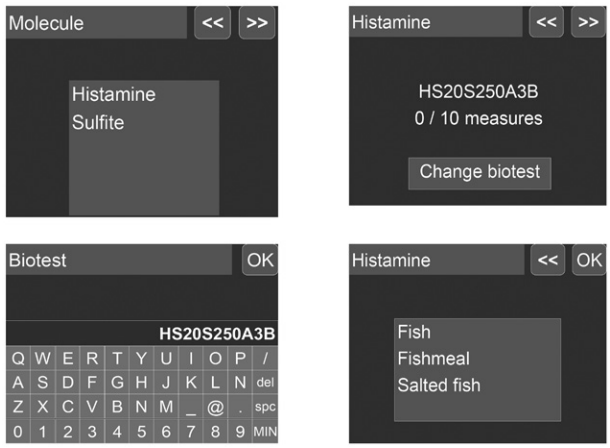


Figure 4. Screens of the BIOTEST option.

Figure 5. Screen of the ANALYSIS option.

(*1*) *Activation*.—(*i*) Press ACTIVATE in the BIOTEST option. Check that the cuvette is correctly fitted, and then press CONTINUE to begin the activation process.

(*ii*) Wait until the device records the first measurement and emits a sound signal. Press CONTINUE again and then pipet 2.0 mL CS into the measuring cuvette for fish or canned salted fish analyses.

(*iii*) Wait until the process ends (the message "Biotest activated. Ready for calibration" will appear).

(*iv*) Replace the cuvette contents with 10 mL MS.

(*2*) *Calibration*.—(*i*) Press CALIBRATE in the BIOTEST option.

(*ii*) Check that the cuvette is correctly fitted, and then press CONTINUE to begin the calibration process.

(*iii*) Wait until the device records the first measurement and emits a sound signal. Press CONTINUE again and then pipet 2.0 mL CS into the measuring cuvette for fish or canned salted fish analyses.

(*iv*) Wait until the device records the second measurement and emits another sound signal.

(*v*) Press CONTINUE a third time and then pipet 2.0 mL CS into the measuring cuvette for fish or canned salted fish analyses.

(*vi*) Wait until the process ends.

Note: It is important to add the CS immediately after pressing CONTINUE to record the correct measurement. The linear regression results of the calibration will appear on the screen. Values of r ≥0.9990 and m ≥150 000 indicate a valid calibration and must be obtained before proceeding with analyses. After every 15 analyses or when the device has not been used for 2 h, test the calibration status using the STANDARD option. Press CALIBRATION STANDARD, fit the cuvette correctly, press CONTINUE, and wait until the device issues a sound signal. Press CONTINUE and pipet 2.0 mL CS into the cuvette. The message "Standard OK" or "Calibrate again" will appear on the screen.

Sample Preparation

(a) *Preparation of the sample*.—(*1*) *Canned fish and canned salted fish*.—Drain 3 min through a sieve with a mesh size of 3 mm to remove the liquid content, and blend using a mincer to obtain a homogeneous sample.

(*2*) *Raw thawed or fresh fish*.—Clean and gut the fish. Cut off an approximately 100 g representative sample from different parts of the edible flesh without skin, and blend using a blender.

(*3*) *Cooked fish*.—Using a mincer, blend an approximately 100 g representative sample taken from different parts of the cooked fish sample.

(b) Extraction.—(*1*) Put 10 g blended sample into a 200 mL plastic bottle, which should be clean and free of residual histamine and other potentially interfering contaminants.

(*2*) Add distilled water: 90 mL for fish (10–200 mg/kg histamine); or 140 mL for canned salted fish (30–150 mg/kg histamine).

(*3*) Close the bottle and shake vigorously for 15–20 s and leave to rest until particulates settle (in the case of raw/fresh fish, an extra blend step is recommended after the water is added to the sample).

(*4*) If particulates remain in suspension, filter the liquid before analysis.

(*5*) Store extracts at 3–8°C (for a maximum of 4 h) unless they are going to be used within the first hour. Bring to room temperature before analysis.

Analysis

(*1*) Press SAMPLE in the BIOTEST option. Choose the measuring range of analysis (10–100 mg/kg or 100–200 mg/kg in the case of fish analysis).

(*2*) Press CONTINUE to begin. Wait for the device to issue a sound signal.

(*3*) Press CONTINUE and pipet the extract into the measuring cuvette: use 2.0 mL extract for 10–100 mg/kg and canned salted fish; use a 500 µL sample size for 100–200 mg/kg.

(*4*) Wait until the process ends and the device displays the result of the analysis.

Test Result Report

The biosensor expresses the analysis result in milligrams of histamine per kilograms of fish, taking into account the dilution involved in the extraction of the sample.

(*1*) To save the result of an analysis, press SAVE.

(*2*) To view the saved results, go to the RECORDS option. Saved results can be downloaded to a computer.

(*3*) To delete the saved results, go to RECORDS and press DELETE ALL.

Validation Study

This validation study was conducted under the AOAC Research Institute *Performance Test Method*℠ program. The Biofish-300 HIS method was validated for fish (raw tuna, raw mackerel, raw sardine, raw anchovy, cooked tuna, canned tuna in water, canned tuna in oil, canned mackerel in tomato sauce, canned pickled sardine) and for canned salted fish (canned salted anchovy). The method developer studies included linearity, LOD, LOQ, bias, recovery and precision, selectivity, lot-to-lot consistency, stability, and robustness. The independent laboratory study included LOD, LOQ, bias, recovery, and precision. Statistical analyses were performed using the Microsoft Excel 2010 and SPSS Statistics 23 programs.

Linearity Study

(a) *Methodology*.—A calibration line with six concentrations (including the blank) of histamine standard (histamine

dihydrochloride, ≥99%; Sigma-Aldrich, St. Louis, MO) and four replicates per concentration level were analyzed.

An aqueous stock solution of 1000 ppm histamine was prepared. Histamine dihydrochloride standard (167.3 mg) was dissolved in 100 mL distilled water. Working solutions were prepared from this stock standard solution in blended matrix, as follows:

Fish calibration [0, 10, 50, 100, 150, and 200 ppm (μg/mL)]:

0 μg/mL.—Distilled water only.

10 μg/mL.—Pipet 0.10 mL histamine dihydrochloride standard into a 10 mL volumetric flask and dilute to volume with distilled water.

50 μg/mL.—Pipet 0.50 mL histamine dihydrochloride standard into a 10 mL volumetric flask and dilute to volume with distilled water.

100 μg/mL.—Pipet 1.00 mL histamine dihydrochloride standard into a 10 mL volumetric flask and dilute to volume with distilled water.

150 μg/mL.—Pipet 1.50 mL histamine dihydrochloride standard into a 10 mL volumetric flask and dilute to volume with distilled water.

200 μg/mL.—Pipet 2.00 mL histamine dihydrochloride standard into a 10 mL volumetric flask and dilute to volume with distilled water.

Canned salted fish calibration [0, 30, 50, 75, 100, and 150 ppm (μg/mL)]:

0 μg/mL.—Distilled water only.

30 μg/mL.—Pipet 0.30 mL histamine dihydrochloride standard into a 10 mL volumetric flask and dilute to volume with distilled water.

50 μg/mL.—Pipet 0.50 mL histamine dihydrochloride standard into a 10 mL volumetric flask and dilute to volume with distilled water.

75 μg/mL.—Pipet 0.75 mL histamine dihydrochloride standard into a 10 mL volumetric flask and dilute to volume with distilled water.

100 μg/mL.—Pipet 1.00 mL histamine dihydrochloride standard into a 10 mL volumetric flask and dilute to volume with distilled water.

150 μg/mL.—Pipet 1.50 mL histamine dihydrochloride standard into a 10 mL volumetric flask and dilute to volume with distilled water.

These solutions were stored at refrigerated temperature (3–8°C) for 2 weeks.

The standard solutions were measured in the Biofish-300 HIS system according to the steps listed in the *Analysis* section.

(b) *Results.*—The results are presented in Table 1. Linearity and residual plots for the unsalted and canned salted fish calibration ranges are presented in Figure 6. The results show that the method is linear; however, the canned salted fish data are inaccurate. Results appear 16–18 ppm higher than the actual spike level in the range 0–75 ppm. As can be seen, the presence of salt causes an alteration in the measurement of the histamine because the conductivity of the system is altered.

The linearity study was based on the equipment response calibrated with standards in 0.1 M buffer against standards with more salt solution (standards in 0.2 M buffer), in which it was shown that a specific calibration is necessary for these types of samples. The study determined that the linearity of the samples, as well as the prepared standards, in a saline solution of higher concentration show a greater response. It is for this reason that a special option for canned salted fish available on the Biofish-300 biosensor allows the customer to prepare the calibration standards in 0.2 M buffer and calibrates for the increased sensitivity of the system due to the salted samples. Thus, overestimation is taken into account by the biosensor when the salted samples are measured.

The residual plots in the case of canned salted fish seem to show a pattern with a linear relationship between waste and predictions. The points do not look random; however, the size of the residuals is relatively small (Figure 6).

Selectivity

(a) *Methodology.*—Potential interfering compounds were tested in each matrix at 1000 ppm in the presence and absence of histamine to evaluate both positive and negative interferences. Potential interfering compounds included 3-methylhistamine, tyramine, L-phenylalanine, L-histidine, L-tyrosine, tryptamine, L-tryptophan, cadaverine, putrescine, anserine, carnosine, and agmatine (Sigma-Aldrich, St. Louis, MO). Stock solutions of 5 mg/mL of each compound were prepared as follows:

Agmatine sulfate salt (Sigma-A7127-1g).—87.6 mg in 10 mL distilled water.

L-Histidine (Sigma-Aldrich-H800-25g).—50 mg in 10 mL distilled water.

L-Phenylalanine (Sigma-Aldrich P2126-100g).—50 mg in 10 mL distilled water.

Putrescine dihydrochloride (Sigma-P5780-5g).—91.44 mg in 10 mL distilled water.

Cadaverine dihydrochloride (Aldrich-C8561-1g).—85.7 mg in 10 mL distilled water.

Tryptamine hydrochloride (Aldrich-246557-5g).—61.4 mg in 10 mL distilled water.

Tyramine hydrochloride (Sigma-T2879-1g).—63.3 mg in 10 mL distilled water.

Methylhistamine dihydrochloride (Sigma-Aldrich-M7780).—39.6 mg in 5 mL distilled water.

L-Tyrosine disodium salt (Sigma-T1145-25g).—62.1 mg in 10 mL distilled water.

L-Anserine nitrate salt (Sigma-Aldrich-A1131).—31.7 mg in 5 mL distilled water.

L-Carnosine crystalline (Sigma-Aldrich-C9625).—50 mg in 10 mL distilled water.

Samples of each matrix (10 g portions) were weighed, and each portion was spiked with the stock solution (5 mg/mL) of one compound to obtain the final concentration of 1000 ppm in the matrix. In addition, 10 g portions of each matrix were weighed, and each portion was spiked with the stock solution (5 mg/mL) of one compound to obtain the final concentration of 1000 ppm plus 50 ppm histamine in the matrix. The samples were extracted and measured with the Biofish-300 HIS system according to the steps in the *Analysis* section.

(b) *Results.*—The results obtained for the selectivity study are shown in Table 2. Due to solubility problems, L-tryptophan could not be evaluated. It does not solubilize in water, and the use of other solvents such as alcohol interfered with the histamine dehydrogenase in the biosensor. The data were analyzed by the Grubbs' test to determine individual outliers. When an outlier

Table 1. Linearity in salted and unsalted fish ranges

Unsalted fish range, ppm					Canned salted fish range, ppm				
Histamine standard	Biofish-300 result	Mean	SD	Residual	Histamine standard	Biofish-300 result	Mean	SD	Residual
0	0.0	0.5	0.58	0.55	0	21.0	18.0	2.94	−2.87
	0.0					14.0			
	1.0					19.0			
	1.0					18.0			
10	12.0	11.0	1.41	0.59	30	49.0	47.0	4.00	−0.37
	9.0					41.0			
	11.0					49.0			
	12.0					49.0			
50	51.0	51.3	2.63	−0.95	50	70.0	67.0	2.58	1.96
	50.0					68.0			
	55.0					66.0			
	49.0					64.0			
100	107.0	103.3	6.24	−1.26	75	92.0	91.0	4.16	3.87
	105.0					86.0			
	107.0					90.0			
	94.0					96.0			
150	165.0	157.8	6.08	0.94	100	108.0	109.0	1.83	−0.21
	155.0					107.0			
	160.0					111.0			
	151.0					110.0			
200	216.0	209.3	10.44	0.13	150	151.0	151.0	4.08	−2.38
	211.0					156.0			
	216.0					146.0			
	194.0					151.0			

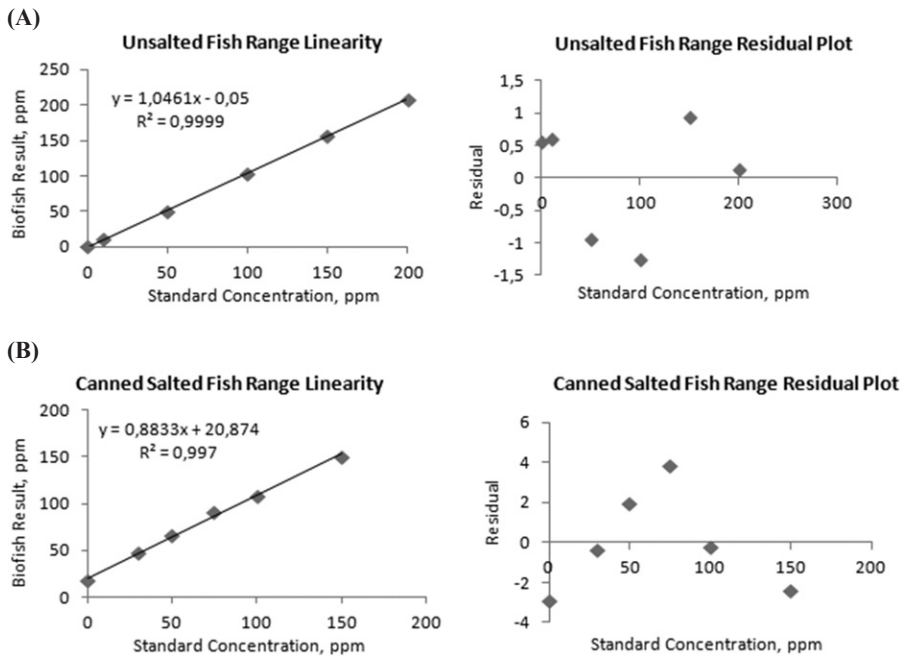


Figure 6. Linearity and residual plots for (A) unsalted fish and (B) canned salted fish calibration ranges.

Table 2. Selectivity results

Histamine concn, ppm	Compound, 1000 ppm	Raw tuna	Raw sardine	Raw mackerel	Raw anchovy	Cooked tuna	Canned tuna in water	Canned tuna in oil	Canned mackerel in tomato sauce	Canned pickled sardine	Canned salted anchovy
0	Water	**3**[a]	4	**5**	3	0	2	5	7	1	19
	3-Methylhistamine	0	9	0	4	0	0	0	4	0	11
	Tyramine	0	1	0	0	0	2	0	0	1	10
	L-Phenylalanine	0	6	0	8	0	2	0	4	0	16
	L-Histidine	0	1	0	4	0	4	3	5	1	13
	L-Tyrosine	1	9	0	2	0	3	5	5	0	19
	Tryptamine	0	0	0	0	1	2	0	0	0	15
	L-Tryptophan	ND[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Cadaverine	1	7	0	5	7	4	1	11	3	18
	Putrescine	1	**26**	**15**	**26**	6	6	1	**17**	**17**	10
	Anserine	1	4	0	3	0	0	1	2	0	13
	Carnosine	1	4	1	2	6	8	3	5	2	19
	Agmatine	**250**	**42**	**49**	**42**	**42**	**39**	**50**	**24**	**190**	**33**
50	Water	51	46	50	45	48	50	50	45	50	**48**
	3-Methylhistamine	50	60	49	56	53	51	53	45	53	63
	Tyramine	47	56	47	52	55	56	48	51	52	64
	L-Phenylalanine	40	57	47	53	54	51	48	54	54	65
	L-Histidine	49	46	46	48	52	55	54	52	53	63
	L-Tyrosine	56	58	47	52	56	58	59	48	56	62
	Tryptamine	36	50	45	**37**	48	49	**36**	40	51	65
	L-Tryptophan	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Cadaverine	49	47	49	55	54	54	50	59	56	65
	P**utrescine**	46	**80**	**54**	**80**	54	55	51	**67**	**66**	69
	Anserine	50	49	48	49	52	51	50	48	54	65
	Carnosine	53	52	46	52	55	58	57	54	54	65
	Agmatine	**>200**	**>200**	**145**	**>200**	**>200**	**>200**	**>200**	**>200**	**>200**	**71**

[a] Bold type indicates statistical outliers by the Grubbs' test.

[b] ND = Not determined.

was found, it was removed and the Grubbs' test was repeated. The process was repeated until no outliers were found.

A positive interference is one in which the added compound increased the method result either in the presence or absence of histamine, presumably by acting as a substrate for histamine oxidase. A negative interference is one in which the compound caused a decrease in the signal in the presence of histamine, presumably by acting as an enzyme inhibitor. Agmatine was found to cause significant positive interference in all matrixes. Putrescine caused positive interference in five of the matrixes (raw mackerel, raw sardine, raw anchovy, canned mackerel in tomato sauce, and canned pickled sardine). Lastly, there were three instances in which the result in the absence of any added compound was significantly different. Thus, putrescine appears to be a weak enzyme substrate, causing positive interference in five of the matrixes. Agmatine appears to be a very strong enzyme substrate, causing positive interference in all matrixes, albeit at a concentration 20-fold higher than histamine.

Matrix study

(a) *Methodology.*—Each matrix was examined at six concentrations of added histamine. For unsalted fish products, 0, 10, 50, 100, 150, and 200 ppm histamine were added. For canned salted anchovy, 0, 30, 50, 75, 100, and 150 ppm histamine were added. Endogenous histamine concentrations in each matrix were determined by the reference HPLC method (4). Each matrix was spiked with the appropriate concentrations of histamine, and five replicate test portions at each concentration were randomized and blind-coded. Each blind-coded test portion was extracted and analyzed according to the instructions of Biofish-300 HIS as detailed in *Sample Preparation*.

(b) *Results.*——The results are presented in Tables 3–12, and the corresponding Figures 7–16 show the regression analysis for each matrix. Recoveries ranged from 82 to 122%, with two instances in which values were outside the range 80–110%. These instances (112 and 122%) were found at the lowest level (10 mg/kg) in canned tuna in water and canned mackerel in tomato sauce, respectively. Although the recoveries in both cases (in this part of the study) were higher than the stipulated level (110%), it established the LOQ for the two matrixes in the 10 mg/kg level, because the results were accurate at that level.

The method bias is generally slightly negative, in agreement with recoveries that are generally below 100%. RSD_r values ranged from 1.4 to 223.6% across all matrixes, demonstrating acceptable r precision. Only four values were above 10% RSD_r, and those were very near the lower limit of the method range.

Table 3. Method developer results for fortified raw tuna

Matrix	Initial histamine concn, mg/kg	Fortification, mg/kg	Total histamine, mg/kg	Biofish-300 results				
				Average, mg/kg	s_r	RSD_r, %	Recovery, %	Bias, mg/kg
Raw tuna	3.4	0	3.4	3.4	1.1	33.5	100	0.0
		10	13.4	13.4	1.5	11.3	100	0.0
		50	53.4	51.6	2.1	4.0	97	−1.8
		100	103.4	100.6	3.2	3.2	97	−2.8
		150	153.4	141.2	3.3	2.3	92	−12.2
		200	203.4	192.6	5.1	2.7	95	−10.8

Table 4. Method developer results for fortified raw mackerel

Matrix	Initial histamine concn, mg/kg	Fortification, mg/kg	Total histamine, mg/kg	Biofish-300 results				
				Average, mg/kg	s_r	RSD_r, %	Recovery, %	Bias, mg/kg
Raw mackerel	4.8	0	4.8	4.8	1.3	27.2	100	0.0
		10	14.8	15.0	1.0	6.7	101	0.2
		50	54.8	54.6	4.0	7.3	100	−0.2
		100	104.8	101.0	6.1	6.0	96	−3.8
		150	154.8	142.8	9.5	6.7	92	−12.0
		200	203.4	196.4	5.5	2.8	96	−8.4

Table 5. Method developer results for fortified raw sardine

Matrix	Initial histamine concn, mg/kg	Fortification, mg/kg	Total histamine, mg/kg	Biofish-300 results				
				Average, mg/kg	s_r	RSD_r, %	Recovery, %	Bias, mg/kg
Raw sardine	4.4	0	4.4	4.4	2.3	52.3	100	0.0
		10	14.4	11.8	1.1	9.3	82	−2.6
		50	54.4	50	1.4	2.8	92	−4.4
		100	104.4	93.6	3.3	3.5	90	−10.8
		150	154.4	142.0	5.4	3.8	92	−12.4
		200	204.4	197.0	6.4	3.2	96	−7.4

Table 6. Method developer data for fortified raw anchovy

Matrix	Initial histamine concn, mg/kg	Fortification, mg/kg	Total histamine, mg/kg	Biofish-300 results				
				Average, mg/kg	s_r	RSD_r, %	Recovery, %	Bias, mg/kg
Raw anchovy	3.0	0	3.0	3.0	2.1	70.7	100	0.0
		10	13.0	12.2	1.3	10.7	94	−0.8
		50	53.0	48.4	1.7	3.5	91	−4.6
		100	103.0	94.2	1.3	1.4	91	−8.8
		150	153.0	149.2	7.5	5.0	98	−3.8
		200	203.0	199.6	4.1	2.1	98	−3.4

LOD and LOQ

The LOD and LOQ were determined by first plotting s_r against concentration and performing linear regression for each matrix (Figure 17). Next, LOD was calculated as

$$\text{LOD} = \frac{\bar{X}_0 + 3.3(s_b)}{1 - 1.65m}$$

where $\bar{X}_0$ is the mean analytical value of the known negative matrix, s_b is the intercept of the plot, and m is the slope of the plot. LOQ is then estimated as 3 × LOD. These results are presented in Table 13. The estimated LOQ values for unsalted fish ranged from 1.46 to 16.63 mg/kg, with an average estimate of 8.6 mg/kg. For canned salted fish, the estimated LOQ was 35.3 mg/kg. These estimated values are close to the claimed lower limit of the method range of 10 mg/kg for unsalted fish and 30 mg/kg for canned salted fish. Note that the higher LOD and LOQ values for canned salted fish are due to the altered conductivity, as mentioned previously. The LOQ for unsalted fish was validated at 10 mg/kg and for canned salted fish at 30 mg/kg by spiking each matrix at those levels and testing 10 replicate test portions. These results are presented in Table 14. The RSD_r values were all

Table 7. Method developer data for fortified cooked tuna

Matrix	Initial histamine concn, mg/kg	Fortification, mg/kg	Total histamine, mg/kg	Biofish-300 results				
				Average, mg/kg	s_r	RSD_r, %	Recovery, %	Bias, mg/kg
Cooked tuna	0.4	0	0.4	0.4	0.9	223.6	100	0.0
		10	10.4	9.6	1.3	14.0	92	−0.8
		50	50.4	47.8	2.4	5.0	95	−2.6
		100	100.4	95.8	2.4	2.5	95	−4.6
		150	150.4	143.4	4.8	3.4	95	−7.0
		200	200.4	193.0	9.7	5.0	96	−7.4

Table 8. Method developer data for fortified canned tuna in water

Matrix	Initial histamine concn, mg/kg	Fortification, mg/kg	Total histamine, mg/kg	Biofish-300 results				
				Average, mg/kg	s_r	RSD_r, %	Recovery, %	Bias, mg/kg
Canned tuna in water	2.0	0	2.0	2.0	1.6	79.1	100	0.0
		10	12.0	13.4	0.5	4.1	112	1.4
		50	52.0	52.0	1.4	2.7	100	0.0
		100	102.0	97.4	4.4	4.5	100	−4.6
		150	152.0	159.2	5.4	3.4	105	7.2
		200	202.0	196.2	12.7	6.5	97	−5.8

Table 9. Method developer data for fortified canned tuna in oil

Matrix	Initial histamine concn, mg/kg	Fortification, mg/kg	Total histamine, mg/kg	Biofish-300 results				
				Average, mg/kg	s_r	RSD_r, %	Recovery, %	Bias, mg/kg
Canned tuna in oil	4.8	0	4.8	4.8	2.2	45.2	100	0.0
		10	14.8	13.0	0.7	5.4	88	−1.8
		50	54.8	54.4	1.9	3.6	99	−0.4
		100	104.8	99.4	2.2	2.2	95	−5.4
		150	154.8	144.0	6.4	4.4	93	−10.8
		200	204.8	197.8	6.3	3.2	97	−7.0

Table 10. Method developer data for fortified canned mackerel in tomato sauce

Matrix	Initial histamine concn, mg/kg	Fortification, mg/kg	Total histamine, mg/kg	Biofish-300 results				
				Average, mg/kg	s_r	RSD_r, %	Recovery, %	Bias, mg/kg
Canned mackerel in tomato sauce	2.8	0	2.8	2.8	1.3	46.6	100	0.0
		10	12.8	15.6	2.4	15.4	122	2.8
		50	52.8	52.0	3.7	7.2	98	−0.8
		100	102.8	95.6	4.7	4.9	93	−7.2
		150	152.8	145.2	5.0	3.4	95	−7.6
		200	202.8	189.6	8.5	4.5	93	−13.2

<20%, with only 4 values >10%. Thus, the LOQ values were validated.

Reference Materials and Proficiency Tests

(a) *Methodology.*—Three canned fish reference materials obtained from FAPAS were analyzed under intermediate R conditions (on different days by different technicians). The reference material histamine content ranged from 26.8 to 173.3 mg/kg, covering a large part of the Biofish-300 analytical range for unsalted fish. Proficiency testing was organized by the National Association of Manufacturers of Canned Fish and Shellfish and carried out in 2014 and 2015. Each proficiency test consisted of analyzing homogenized tuna in oil with a known concentration of histamine (the assigned values were 115 mg/kg in 2014 and 73 mg/kg in 2015). Each sample was analyzed one

Table 11. Method developer data for fortified canned pickled sardine

Matrix	Initial histamine concn, mg/kg	Fortification, mg/kg	Total histamine, mg/kg	Biofish-300 results				
				Average, mg/kg	s_r	RSD_r, %	Recovery, %	Bias, mg/kg
Canned pickled sardine	0.6	0	0.6	0.6	1.3	223.6	100	0.0
		10	10.6	9.4	0.5	5.8	89	−1.2
		50	50.6	50.2	1.3	2.6	99	−0.4
		100	100.6	96.2	4.3	4.5	96	−4.4
		150	150.6	145.0	2.1	1.5	96	−5.6
		200	200.6	192.4	6.5	3.4	96	−8.2

Table 12. Method developer data for fortified canned salted anchovy

Matrix	Initial histamine concn, mg/kg	Fortification, mg/kg	Total histamine, mg/kg	Biofish-300 results				
				Average, mg/kg	s_r	RSD_r, %	Recovery, %	Bias, mg/kg
Canned salted anchovy	19.4	0	19.4	19.4	4.0	20.8	100	0.0
		30	49.4	46.2	3.9	8.4	94	−3.2
		50	69.4	67.2	2.3	3.4	97	−2.2
		75	94.4	89.6	4.8	5.3	95	−4.8
		100	119.4	111.8	6.5	5.8	94	−7.6
		150	169.4	153.4	3.9	2.5	91	−16.0

time, and the obtained result was statistically analyzed with the results of the rest of the participants. There were 21 participants in 2014 and 20 participants in 2015.

(b) *Results.*—The reference material results are shown in Table 15. Recoveries ranged from 91 to 100%, bias ranged from −0.1 to −2.3 mg/kg, and RSD_r values ranged from 4.62 to 7.64% across the three samples, demonstrating excellent accuracy and r of the method. Proficiency testing resulted in satisfactory results, with *z*-score ≤2 (results not shown).

Lot-to-Lot Consistency and Stability Study

(a) *Methodology.*—The Biofish-300 HIS method consists of a Calibration Kit, a Measurement Kit, and the Biotest electrode. Lot-to-lot data were collected for three lots of each kit. Stability was evaluated over 6 months for the Calibration Kit and Measurement Kit. Eight tubes are supplied in each kit, so it was necessary to use a new tube of each at each of the six time points. At the 1-month time point, two additional lots were tested to evaluate the lot-to-lot consistency. The Biotest stability was examined over a 30-day period in a separate experiment. One lot of electrodes was chosen and tested with one Calibration Kit and one Measurement Kit over the course of 30 days, using a fresh electrode for each time point (0, 2, 5, 10, 20, and 30 days after manufacture). One time point was chosen to test three different lots of electrodes. A single kit for each of KIT CFHIS, KIT MFHIS1, and KIT MFHIS2 using a fresh tube for each time point was used. In both experiments, two replicates of spiked canned tuna per time point or lot were analyzed.

(b) *Results.*—Results for the lot-to-lot testing and the 6-month stability for the Calibration Kit and Measurement Kit are presented in Table 16. Data were analyzed by generalized linear model (GLM) analysis using the SPSS Statistics

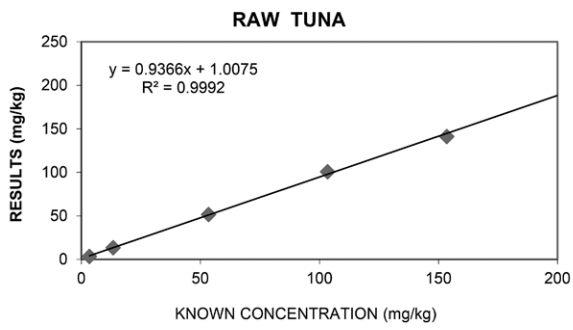


Figure 7. Linear regression of method developer Biofish-300 results for raw tuna.

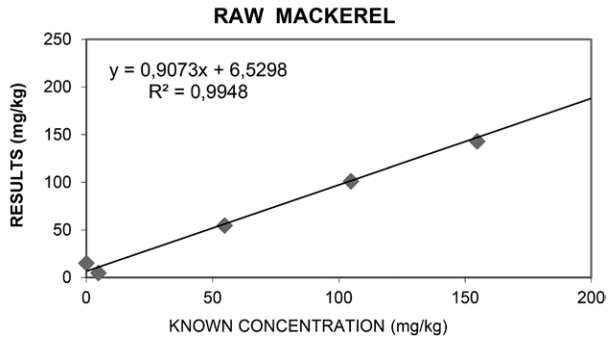


Figure 8. Linear regression of method developer Biofish-300 results for raw mackerel.

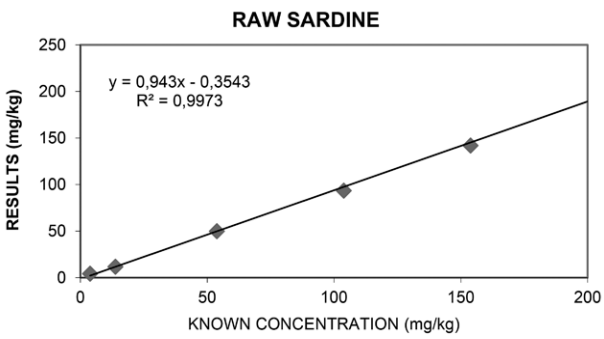


Figure 9. Linear regression of method developer Biofish-300 results for raw sardine.

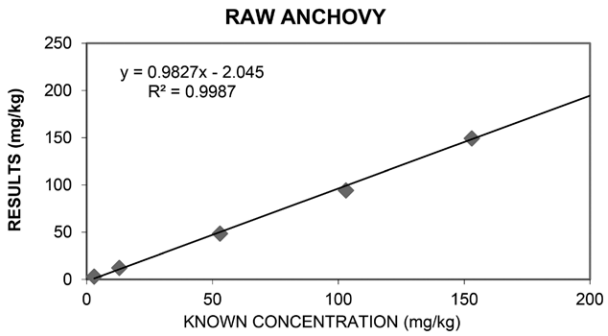


Figure 10. Linear regression of method developer Biofish-300 results for raw anchovy.

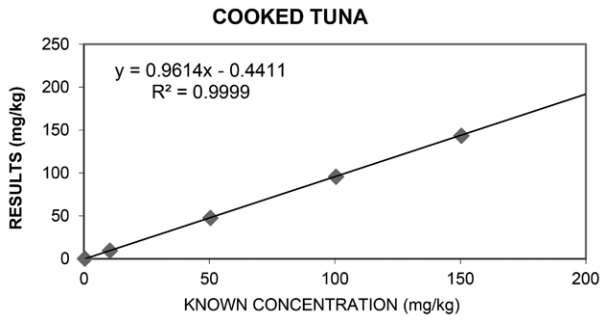


Figure 11. Linear regression of method developer Biofish-300 results for cooked tuna.

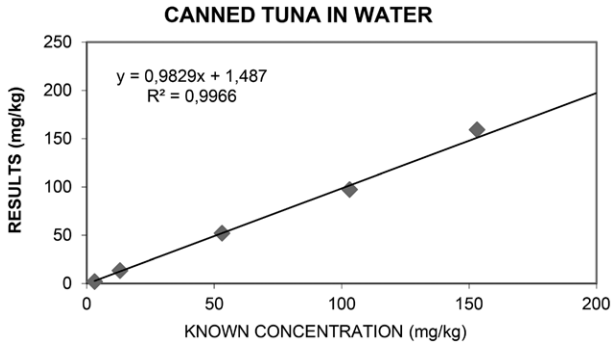


Figure 12. Linear regression of method developer Biofish-300 results for canned tuna in water.

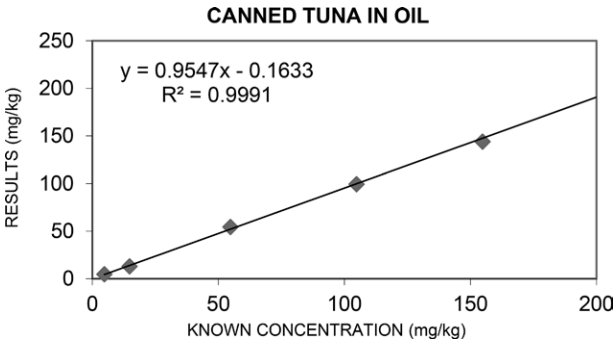


Figure 13. Linear regression of method developer Biofish-300 results for canned tuna in oil.

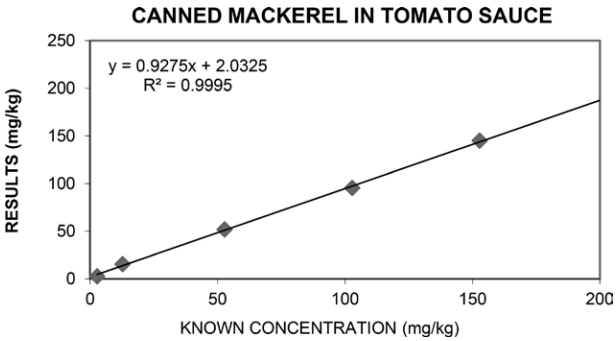


Figure 14. Linear regression of method developer Biofish-300 results for canned mackerel in tomato sauce.

23 program. The GLM results obtained showed significant differences in the content of histamine between the third month and the rest of the evaluated months. The average results obtained with the B4 lot of Biotest used in the third month were slightly higher (54 mg/kg) than the those obtained with the other Biotest lots (50–53 mg/kg) evaluated throughout the different months of study. In this case, the GLM test seems to be too strict because the obtained values are inside the estimated uncertainty of the method (10%). According to the obtained results of the evaluation throughout the 6-month study time, it appears that the cause of the significant differences is this strictness, and not a problem of stability of the Calibration Kit or Measurement Kit.

The results of the 30-day stability of the Biotest electrode are shown in Table 17. GLM analysis showed that there were no significant differences between the different Biotest electrode lots evaluated at day 2, or between the time points over the 30-day period.

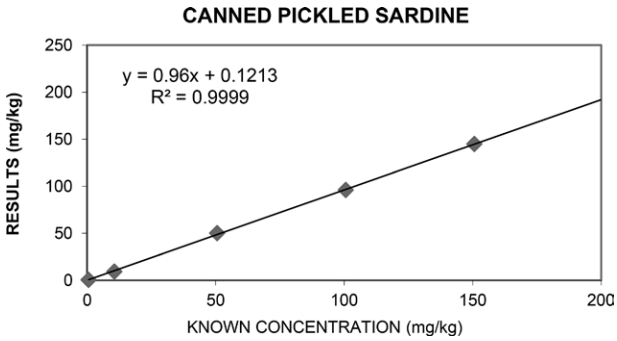


Figure 15. Linear regression of method developer Biofish-300 results for canned pickled sardine.

Robustness

(a) *Methodology.*—Three method parameters were varied, including extraction volume, extraction time, and sample storage time before analysis. The parameter values are shown in Tables 18, and Table 19 shows the factorial design used. Treatment combination 9 shows the normal method parameter values. Treatment combinations 1–8 use either the high or low values for each parameter. For each treatment combination, two replicates of canned tuna in water spiked at 50 mg/kg histamine were analyzed.

(b) *Results.*—The results of the duplicate analyses are shown in Table 19. The factorial design was translated into the Youden design (5) in Table 20, and the Youden test was applied (Table 21).

According to the Youden test results, significant differences were observed in all the studied cases, with the evaluated variations adversely affecting the analytical results. In the case of tuna in water with 50 mg/kg histamine, it was observed that the method deviation was low, and for this reason, the acceptance criterion seems to be too strict. The acceptance criterion is from Commission Decision No. 2002/657/EC concerning the performance of analytical methods and the interpretation of results (6).

To confirm the results obtained by the Youden test, a GLM was applied to determine whether any parameter change affects the results. In this case, significant differences were observed in the results when extraction volume was varied. The rest of the evaluated parameter variations caused no significant differences in the results. Post hoc results confirmed that significant differences were obtained between 80 and 100 mL of extraction volume. No significant differences were observed in the rest of the evaluated variations.

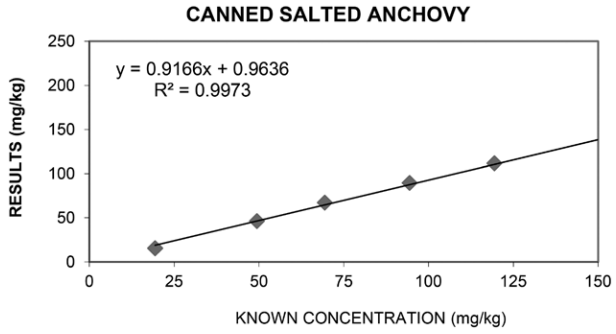


Figure 16. Linear regression of method developer Biofish-300 results for canned salted anchovy.

Independent Validation Study

Independent matrix studies were performed at the Eurofins Steins A/S laboratory (Vejen, Denmark) under the guidance of the AOAC Research Institute.

Methodology

Four matrixes were examined: raw tuna, cooked tuna, canned pickled sardine, and canned salted anchovy. Raw tuna, cooked tuna, and canned pickled sardine were fortified with 0, 10, 50, 100, 150, and 200 mg/kg histamine. Canned salted anchovy was fortified with 0, 30, 50, 75, 100, and 150 mg/kg histamine. Endogenous histamine was determined by HPLC (4) by analyzing four test portions of unfortified matrix and determining the mean result. The total histamine content of the test portions for Biofish-300 analysis, therefore, comprised the endogenous histamine concentration plus the fortified histamine concentration. Five replicate test portions at each concentration were analyzed.

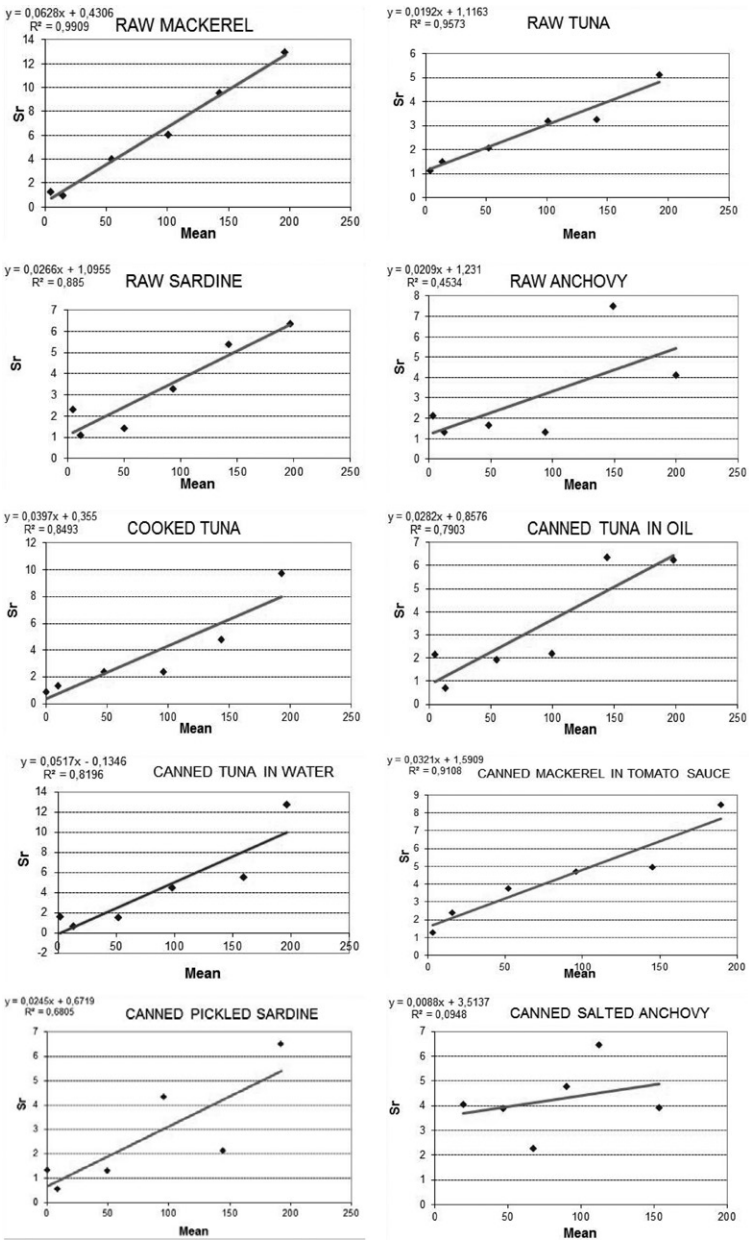
Results

Biofish-300 results are presented in Table 22. Recoveries ranged from 73.1 to 138%, with five instances of values outside the range 80–110%. Three of these instances were at the lowest level, with no added histamine, in raw tuna, canned pickled sardine, and canned salted anchovy. These levels are below the claimed range of the method for unsalted and canned salted fish. The other two instances were at the second lowest level of cooked tuna (10.7 mg/kg total histamine, at the low end of the claimed range of the method) and at the second highest level of canned pickled sardine. These values were outside the 80–110% range because the recoveries were calculated by taking into account the HPLC value instead of the Biofish-300 value of matrix without addition.

The method bias is generally slightly negative, in agreement with recoveries that are generally below 100%. RSD_r values ranged from 1.1 to 149.1% across all matrixes; however, dropping the data points that fell below the range of the method for unsalted and canned salted fish resulted in an RSD_r range of 1.1–12.6%, demonstrating acceptable r precision. Only two values were >10% RSD_r, and those were very near the lower limit of the method range.

LOD and LOQ were estimated as in the method developer study. The estimates are presented in Table 23. The unsalted fish matrixes yielded estimated LOQ values near 10 mg/kg, and those of the canned salted fish matrix were near 30 mg/kg, consistent with the claimed lower range of the method. These LOQ values were validated by spiking the unsalted matrixes at 10 mg/kg and the canned salted anchovy at 30 mg/kg. Ten replicate test portions were analyzed, and the precision results are presented in Table 24. The RSD_r values were all below 10%, validating the LOQ for unsalted fish at 10 mg/kg and for canned salted fish at 30 mg/kg.

Method Developer Data:



Independent Laboratory Data:

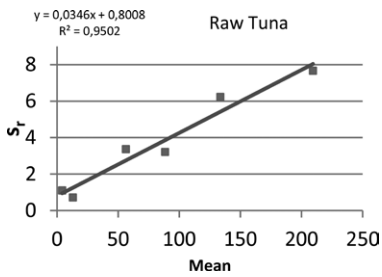


Figure 17. Plots of s_r versus mean result for estimating LOD.

Table 13. Method developer estimation of LOD and LOQ

Linear regression parameters	Raw tuna	Raw mackerel	Raw sardine	Raw anchovy	Cooked tuna	Canned tuna in water	Canned tuna in oil	Canned mackerel in tomato sauce	Canned pickled sardine	Canned salted anchovy
X_0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S_b	1.1163	0.4306	1.0955	1.231	0.355	0.1346	0.8576	1.5909	0.6719	3.5137
m	0.0192	0.0628	0.0266	0.0209	0.0397	0.0517	0.0282	0.0321	0.0245	0.0088
LOD	3.80	1.59	3.78	4.21	1.25	0.49	2.97	5.54	2.31	11.77
LOQ = 3 × LOD	11.41	4.76	11.34	12.62	3.76	1.46	8.90	16.63	6.93	35.30

Table 14. Method developer validation of LOQ by matrix

Matrix	Histamine concn, mg/kg	Mean result, mg/kg	S_r	RSD_r, %
Raw tuna	10	7.9	0.99	12.6
Raw mackerel	10	11.3	0.95	8.4
Raw sardine	10	10.0	0.94	9.4
Raw anchovy	10	9.2	1.75	19.0
Cooked tuna	10	11.8	1.03	8.8
Canned tuna in water	10	10.7	0.95	8.9
Canned tuna in oil	10	9.2	0.79	8.6
Canned mackerel in tomato sauce	10		1.25	11.2
Canned pickled sardine	10	9.6	1.26	13.2
Canned salted anchovy	30	29.3	0.82	2.8

Table 15. Method developer results from reference material testing

Organizer	Code	Matrix	Analyte	Assigned value, mg/kg	Biofish-300 result, mg/kg	Average, mg/kg	Recovery, %	Bias, mg/kg	s_r	RSD, %
FAPAS	T27132qc	Canned fish	Histamine	126.7	128	126.6	100	−0.1	7.4	5.82
					123					
					123					
					134					
					127					
					125					
					129					
					138					
					112					
FAPAS	T27126qc	Canned fish	Histamine	26.8	27	24.5	91	−2.3	1.9	7.64
					24					
					25					
					26					
					22					
					23					
FAPAS	T27120qc	Canned fish	Histamine	173.3	180	171.4	99	−1.9	7.9	4.62
					164					
					176					
					175					
					162					

Table 16. Lot-to-lot results and 6-month stability for the Calibration Kit and Measurement Kit

Study time, months	Calibration Kit lot	Measurement Kit lot	Biotest lot (different lot each month of the study)	Biofish-300 results in canned tuna in water + 50 mg/kg histamine, mg/kg	Average, mg/kg	s_r	RSD, %
0	C1	M1	B1	53	53	0.71	1.35
				52			
1	C1	M1	B2	52	51	1.41	2.77
				50			
	C2	M2		52	53	1.41	2.67
				54			
	C3	M3		51	52	0.71	1.37
				52			
2	C1	M1	B3	50	50	0.00	0.00
				50			
3	C1	M1	B4	53	54	0.71	1.32
				54			
4	C1	M1	B5	52	52	0.00	0.00
				52			
6	C1	M1	B6	51	51	0.71	1.40
				50			

Discussion

The aim of the present work was to receive *Performance Tested Method* certification for the Biofish-300 HIS method for the detection and quantification of histamine in fishery products. This technology is highly specific and selective and allows quantification of histamine in fishery product matrixes, including raw tuna, raw mackerel, raw sardine, raw anchovy, boiled tuna, canned tuna in water, canned tuna in oil, canned mackerel in tomato sauce, canned pickled sardine, and canned salted anchovy. Histamine in all the studied matrixes was extracted in water in a short time (15–20 s). Aqueous extraction removed the need for hazardous solvents.

The linearity and matrix studies demonstrated linear dose responses from 0 ("nondetect") to 200 mg/kg histamine. As a whole, RSD_r calculations and within-range results indicate that the kit provides accurate and precise histamine quantification for all the evaluated matrixes (except for canned salted fish data, which was linear but inaccurate), confirming that sample preparation and assay procedures produce acceptable results.

Agmatine was found to cause significant positive interference in all matrixes, and putrescine caused significant positive

Table 17. Lot-to-lot results and 30-day stability for the Biotest

Study time, days	Calibration Kit (different lot every day)	Measurement Kit (different lot every day)	Biotest lot	Biofish-300 results in canned tuna in water + 50 mg/kg histamine, mg/kg	Average, mg/kg	s_r	RSD, %
0	C1	M1	B1	52	52	0.00	0.00
				52			
2	C2	M2	B1	51	52	0.71	1.37
				52			
				49	51	2.83	5.55
				53			
				51	51	0.71	1.40
				50			
5	C3	M3	B1	53	52	2.12	4.12
				50			
10	C4	M4	B1	52	50	2.83	5.66
				48			
20	C5	M5	B1	52	53	0.71	1.35
				53			
30	C6	M6	B1	54	52	2.83	5.44
				50			

Table 18. Robustness parameters of canned tuna in water spiked with 50 ppm histamine

Robustness parameters	Value 1	Value 2	Value 3
A: Extraction volume, mL	80	90	100
B: Extraction time, s	10	20	30
C: Sample storage before analysis	None	2 h at 3–8°C	4 h at 3–8°C

Table 19. Factorial design and results of robustness testing of canned tuna in water spiked with 50 ppm histamine

Robustness parameters and results	Treatment combination								
	1	2	3	4	5	6	7	8	9
Extraction volume, mL	80	80	80	80	100	100	100	100	90
Extraction time, s	10	10	30	30	10	10	30	30	20
Sample storage before analysis	None	4 h	None	4 h	None	4 h	None	4 h	2 h
Biofish-300 results, mg/kg	56	57	56	61	49	50	51	50	55
	62	59	65	58	56	44	54	43	53

Table 20. Youden factorial design

		Determination combination								
F-factor	Value of *F*-factor	1	2	3	4	5	6	7	8	9
Extraction volume, mL	A: 80	A	A	A	A	a	a	a	a	a′
	a:100									
	a′: 90									
Extraction time, s	B:10	B	B	b	b	B	B	b	b	b′
	b: 30									
	b′: 20									
Time of sample storage, h	C: none	C	c	C	c	C	c	C	c	c′
	c: 4									
	c′: 2									
Result, mg/kg	Average data	59	58	61	60	53	47	53	47	54

Table 21. Youden test results

Variables comparison[a]	D_i[b]	SD_i[c]	SD of the method	Acceptance criterion	Compliance
Δ A, a	9.50	4.75			No
Δ A, a′	5.50	2.75			No
Δ a, a′	4.00	2.00			No
Δ B, b	6.00	3.00			No
Δ B, b′	4.25	2.13	1.4	$SD_i \leq SD$	No
Δ b, b′	5.25	2.63			No
Δ C, c	6.63	3.31			No
Δ C, c′	3.50	1.75			No
Δ c, c′	6.00	3.00			No

[a] Variables comparison is calculated as $X - x$.

[b] D_i = Average differences.

[c] SD_i = SD of the differences.

Table 22. Independent laboratory matrix study results

Matrix	Endogenous histamine, mg/kg[a]	Fortification, mg/kg	Total histamine, mg/kg[b]	Biofish-300 results				
				Average, mg/kg	s_r	RSD_r, %	Recovery, %	Bias, mg/kg
Raw tuna	5.45	0	5.5	4.2	1.1	26.1	76.4	−1.3
		10	15.5	13	0.7	5.4	83.9	−2.5
		50	55.5	56.4	3.4	6.0	101.6	0.9
		100	105.5	88.4	3.2	3.6	83.8	−17.1
		150	155.5	133.6	6.2	4.7	85.9	−21.9
		200	205.5	209.4	7.7	3.7	101.9	3.9
Cooked tuna	0.68	0	0.7	0.6	0.9	149.1	85.7	−0.1
		10	10.7	14.2	1.8	12.6	132.7	3.5
		50	50.7	53.2	3.3	6.1	104.9	2.5
		100	100.7	82.2	1.3	1.6	81.6	−18.5
		150	150.7	123.4	2.4	2.0	81.9	−27.3
		200	200.7	209.4	7.7	3.7	104.3	8.7
Canned pickled sardine	3.21	0	3.2	3.6	1.1	31.7	112.5	0.4
		10	13.2	10.6	1.1	10.8	80.3	−2.6
		50	53.2	52.2	2.3	4.4	98.1	−1.0
		100	103.2	92	2.5	2.8	89.2	−11.2
		150	153.2	112	4.7	4.2	73.1	−41.2
		200	203.2	184.2	3.6	1.9	90.7	−19.0
Canned salted anchovy	14.21	0	14.2	19.6	3.4	17.2	138.0	5.4
		30	44.2	38.2	2.6	6.8	86.4	−6.0
		50	64.2	59.4	1.8	3.1	92.5	−4.8
		75	89.2	74.4	3.4	4.6	83.4	−14.8
		100	114.2	96.2	1.8	1.9	84.2	−18.0
		150	164.2	134.6	1.5	1.1	82.0	−29.6

[a] Determined by HPLC according to Duflos et al. (5).

[b] Total histamine is endogenous histamine plus fortified histamine.

Table 23. LOD and LOQ estimates from the independent laboratory data

Matrix	LOD, mg/kg	LOQ, mg/kg
Raw tuna	2.8	8.4
Cooked tuna	2.4	7.2
Canned pickled sardine	4.4	13.2
Canned salted anchovy	10.9	32.7

Table 24. Validation of LOQ estimates

Matrix	Histamine concn, mg/kg	Mean result, mg/kg	s_r	RSD_r, %
Raw tuna	10	12.8	1.2	9.6
Cooked tuna	10	14	1.3	9.5
Canned pickled sardine	10	10.6	1.0	9.1
Canned salted anchovy	30	40.3	2.9	7.2

interference in five of the evaluated matrixes. In the case of agmatine, levels of this biogenic amine increase during the spoilage of some fish products. The Biofish-300 package insert notifies the user that in presence of agmatine, there may be positive interferences.

In case of putrescine, several scientific reports as those cited by Rawles et al. (7) showed that the formation of putrescine during fish product decomposition is usually much lower than that of histamine. Therefore, it is expected that high levels of putrescine would not be found in the presence of low levels of histamine. Nevertheless, levels could vary depending on the fishery products being tested.

The analysis of reference materials during participation in proficiency testing has shown that the results obtained for histamine were adequate in all the analyzed cases.

Data produced in the lot-to-lot consistency and stability study show good results for the Calibration Kit and Measurement Kit components during 6 months in dry storage conditions, and during 30 days for the Biotest electrodes.

The robustness study showed that variation of the extract volume produced unacceptable results. The package insert defines the correct protocols; the procedure must be performed according to these instructions to avoid errors.

The results obtained in the study were similar to those performed by the independent laboratory. It appears that the recoveries found by the independent laboratory were, in general, lower, and the obtained results for LOD and estimated LOQ were similar to the method developer results.

Conclusions

In summary, the Biofish-300 HIS method precisely and accurately evaluated samples of fish product matrixes in a short time. The LOQ was established at 10 mg/kg for unsalted fish and at 30 mg/kg for canned salted fish. The method is selective, except in the presence of agmatine and putrescine in some studied matrixes. The method is robust, consistent, and stable in most of the studied circumstances.

References

(1) Food and Agriculture Organization (2013) Expert meeting on the Public Health Risks of Histamine and other Biogenic Amines from Fish and Fishery Products, Meeting report, July 23-27, 2012, Rome, Italy

(2) U.S. Food and Drug Administration (2011) Fish and fishery products hazards and controls guidance, Ch 7, Scombrotoxin (Histamine) Formation, 4th Ed, pp 113-152

(3) Commission Regulation No. 2005/2073/EC (2005) *Off. J. Eur. Comm.* **L338**, 1–26

(4) Duflos, G., Dervin, C., Malle, P., & Bouquelet, S. (1999) *J. AOAC Int.* **82**, 1357–1363

(5) Youden, W.J., & Steiner, E.H. (1975) *Statistical Manual of the AOAC, Association of Official Analytical Chemists*, AOAC INTERNATIONAL, Washington, DC, p. 35 ff

(6) Commission Decision No. 2002/657/EC (2002) *Off. J. Eur. Comm.* **L221**, 8–36

(7) Rawles, D.D., FlickRoy, G.J., & Martin, E. (1996) In Adv*ances in Food and Nutrition Research*, Vol. **39**, S.L. Taylor (Ed.), Academic Press, San Diego, CA, pp 329–365