TECHNICAL PRODUCT INFORMATION

MaxSignal® Histamine Enzymatic Assay
Catalog #FOOD-1032-09

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Validation of the MaxSignal® Histamine Enzymatic Assay for the Detection of Histamine in Fish/Seafood

AOAC Performance Tested Method℠ 051701a

General Information

Histamine is a contaminant often found in improperly handled seafood. At elevated temperatures, histamine-producing bacteria convert histidine to histamine[1]. High levels of histamine in seafood are often associated with scombroid poisoning, a bacterially induced illness from spoiled fish. Scombroid poisoning triggers acute allergic response, vomiting, diarrhea, hypotension, rashes, and hives.

Histamine testing is a control strategy used by seafood processors in Hazard Analysis and Critical Control Points(HACCP). The international codex standard for fish includes histamine levels as indicators of decomposition, hygiene, and handling. Good quality fish contains less than 10 ppm of histamine, whereas 30 ppm indicates significant deterioration. The defect action level (DAL) imposed by the US-FDA is 50 ppm[2].

A strong need exists for a histamine detection method that couples simple extraction with high-accuracy quantification. The MaxSignal® Histamine Enzymatic Assay Kit is one such robust enzyme-based assay, using a simple aqueous extraction with high recovery in a variety of fish samples such as fresh/frozen tuna, canned tuna, pouched tuna, and mahi-mahi.

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For the Better

Principle of Method

The MaxSignal® Histamine Enzymatic Assay Kit measures the chemical reduction of a pro-dye molecule using a histamine-specific enzyme. The color change caused by production of the reduced visible dye is directly proportional to the amount of histamine present in the sample. The MaxSignal® Histamine Enzymatic Assay Kit detects and quantifies histamine in foods, such as seafood, using a colorimetric enzyme assay and histamine standards. The resulting absorbance is measured by a spectrophotometric reader. Using the MaxSignal® Enzymatic Assay Analysis Program Excel Workbook, an assay calibration curve is constructed. The absorbance level of each sample is compared to the absorbance levels of the known histamine standards.

Description of Validation Study

Fresh/frozen tuna, canned tuna, pouched tuna and frozen mahi-mahi samples were used for a double-laboratory study under the specific guidelines of AOAC Research Institute Performance Tested Methods℠ Program. Evaluation parameters include precision, accuracy, selectivity, range of detection, ruggedness, lot-to-lot variability, and stability.

Materials and Methods

Details regarding kit contents, storage conditions, precautions, and reagent preparation instructions are provided in the kit insert (Page 3).

I. Reference Materials

Naturally contaminated samples of known concentration were not available, therefore artificially spiked samples were used. The spike solution was prepared from USP histamine dihydrochloride (Certified Reference PHR1357-500 mg; Sigma Aldrich®). To make 1000 ppm spike solution, 82.5 mg ± 0.2 mg of histamine dihydrochloride was dissolved in a 50 mL of distilled water in 50 mL volumetric flask.

II. Sample Preparation

Frozen Tuna and Frozen Mahi-Mahi

1. Thaw product on ice in the refrigerator. Do not thaw in a heated water bath.
2. Cut samples into 2 cm cubes and homogenize in a blender.
3. Aliquot into test portions and spike appropriately.
4. Refreeze samples at -20°C for two weeks to stabilize the matrix.

Fresh Tuna

1. Cut samples into 2 cm cubes and homogenize in a blender.
2. Aliquot into test portions and spike appropriately.
3. Store samples at 4°C for 24 hours until analysis, to stabilize the matrix.

Canned Tuna

1. Process canned tuna according to AOAC 937.07a methodology. Place the contents of the can in a blender for homogenization.
2. Aliquot into test portions and spike appropriately.
3. Store samples at 4°C for 24 hours until analysis, to stabilize the matrix.

Pouched Tuna

1. Place the contents of the pouch in a blender for homogenization.
2. Aliquot into test portions and spike appropriately.
3. Store samples at 4°C for 24 hours until analysis to stabilize the matrix.

III. Sample Extraction

1. Transfer 4.0 g of the sample to a clean 50 mL conical tube. Add 4.0 mL of 1X Enrichment solution per gram of sample. Vortex for 2 minutes at the maximum setting.
2. Transfer 1.0 – 1.5 mL of the suspended homogenate to a micro-centrifuge tube. Heat the sample at 85°C for 10 minutes. Vortex the mix for 5 – 10 seconds at the maximum setting.
3. Centrifuge for 5 minutes at 13,000 rpm. Transfer 0.5 mL of supernatant to a new tube.
4. Add 0.1 mL of 6X Sample Extraction Buffer to the sample. Mix briefly by vortexing. The sample is ready to test.

IV. Assay Procedure

1. Add 100 µL of each of the samples in duplicate to wells of the 96-well microtiter plate.
2. Add 100 µL of each standard in duplicate to different wells of the plate.
3. Add 100 µL of the Reaction Mix to each well. Mix gently.
4. Incubate for 5 minutes at room temperature.
5. Measure absorbance of each well at 450 nm using a plate reader.

V. Interpretation of Test Result Report

1. Use the MaxSignal® Enzymatic Assay Analysis Program Excel Workbook to evaluate the test results.
2. Construct a standard curve by plotting the mean corrected absorbance obtained from each reference standard against its concentration in ppm.
3. Calculate the slope and the y-intercept for the line which fits the standard curve data.

4. The histamine concentration in the well can be determined from the equation:

   Concentration = ((mean absorbance – y-intercept)/slope)*6. Where 6 equals the dilution factor.

   The dilution factor is derived as follows: one part of fish (approximately 90% water) is mixed with four parts of 1X Enrichment Solution (4.0 g + 16 mL) making the dilution factor five. In the subsequent steps, five parts of the fish extract is mixed with one part 6X Sample Extraction buffer (0.5 mL + 0.1 mL). The overall dilution factor is 6 with respect to the ready-to-use standards supplied with the kit.

Internal Validation Study

I. Linearity Study

The linearity study determines the relationship between the signal output of the method and the analyte concentration. Linearity of the method beyond the set of standards supplied with the kit was measured at an additional level of 108 ppm. By using this method, linearity was verified independently of any matrix interference.

Method - Histamine dihydrochloride (Certified Reference PHR1357-500 mg; Sigma Aldrich) was dissolved in water to make a stock standard of 1000 ppm. The stock standard was further diluted to make concentrations of 0, 6, 12, 24, 48, 72, and 108 ppm. The dilutions were measured in duplicates in five separate runs according to the assay procedure described above.

Results - Linear regression was applied plotting the absorbance against spiked concentration. All five runs exhibited excellent linearity, with R2 values exceeding 0.995, and low deviation in slope (Figure 1). Residual analysis of linearity results was performed without multiplying by the dilution factor by plotting results vs concentration (Figure 2). The residual plot shows a random pattern, with random distribution around zero. Non- random patterns were ruled out and a linear regression model was chosen for the data.

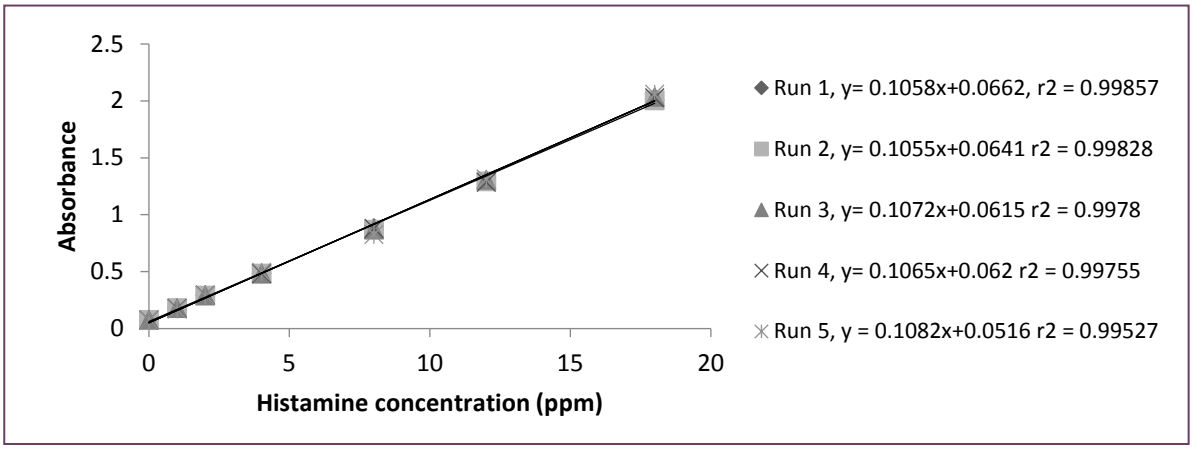

Figure 1. Comparison of the linearity of 5 separate runs of standards at pre-determined concentrations.

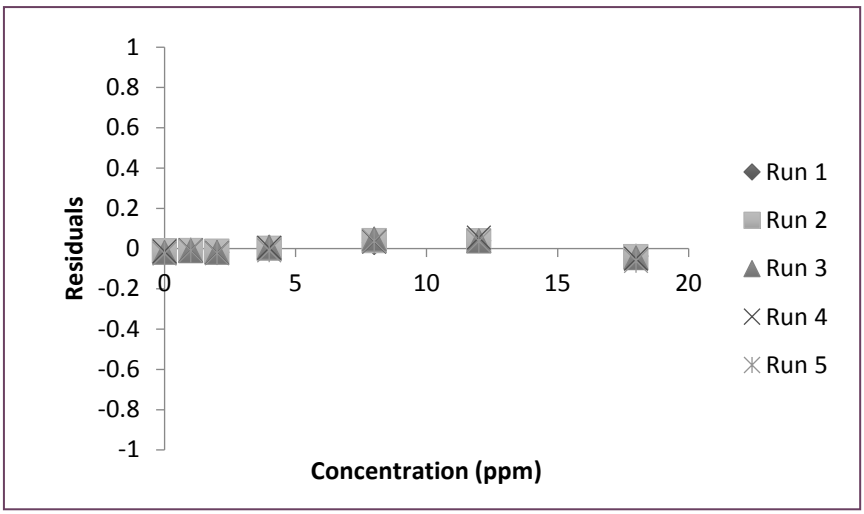

Figure 2. Residual plot of linearity study with mean residuals at each concentration for all five runs.

II. Selectivity

Selectivity and cross reactivity determines the ability of the method to detect an analyte without interference from the matrix and/or other components of similar behavior. Potential cross reactive or interfering compounds were selected based on structural similarity to histamine. A few of the selected biogenic compounds such as putrescine, cadaverine, spermine, and spermidine are also associated with seafood decomposition[3].

Method - Potential cross-reactive compounds tested include 3-methylhistamine, tyramine, L-phenylalanine, L-histidine, L-tyrosine, tryptamine, L-tryptophan, cadaverine, putrescine, anserine, carnosine, agmatine, spermine, and spermidine (Sigma-Aldrich). Each potential cross-reacting compound was prepared at a concentration of 1000 ppm in water by weighing on an analytical balance with 0.2 mg precision. Frozen tuna was the selected matrix for this study, where all samples were analyzed in duplicate. Two sets of frozen tuna samples per compound were tested in the absence and presence of 25 ppm of histamine. Cross reactivity of potential cross-reactive compounds was tested in frozen tuna samples that were spiked at 250 ppm in the absence of histamine. Frozen tuna spiked at 25 ppm histamine and un- spiked samples were run as controls. Interference of potential interfering compounds was tested in the frozen tuna samples spiked at 250 ppm in the presence of 25 ppm of histamine. The absorbance of potential cross-reacting compounds was corrected by background subtraction from the unspiked samples. The results were analyzed by normalizing the absorbance of corrected cross-reactive compounds to histamine concentration (25 ppm). The percentage activity was calculated based on the assumption of histamine activity to be 100%. Interference data was processed in a routine manner and percentage recovery was calculated.

Results - Less than 1% cross reactivity is seen in all the compounds except agmatine, where a negligible 4% cross-reactivity is seen (Table 1). This data is consistent with the literature reference of cross reactivity of the enzyme Histamine Dehydrogenase to agmatine and putrescine[4]. No interference is observed in the compounds tested, with recoveries falling in the normal range of 80-120% (Table 2). Agmatine shows a higher recovery rate of 129%, owing to its cross-reactivity.

Compound	A_{450}	Normalized	% Activity
Histamine	0.55	0.408	100.00
Tyramine	0.15	0.001	0.17
Anserine	0.16	0.001	0.24
L-Histidine	0.16	0.002	0.40
Spermine	0.16	0.001	0.24
L-Tyrosine	0.15	0.001	0.12
L-Tryptophan	0.15	0.001	0.12
Agmatine	0.32	0.017	4.10
Cadaverine	0.15	0.001	0.12
Tryptamine	0.15	0.001	0.12
Carnosine	0.15	0.000	0.02
3-Methyl Histamine	0.15	0.000	0.02
Putrescine	0.19	0.004	0.93
Spermidine	0.17	0.002	0.50
L-Phenyl Alanine	0.16	0.001	0.34
Unspiked	0.15	0.000	0.00

Table 1. Summary of cross-reactivity testing with related biological amines. No significant cross-reactivity was observed except with Agmatine.

Compound	Recovery
Tyramine	98.0
L-Histidine	99.1
L-Tyrosine	99.4
L-Tryptophan	98.1
Tryptamine	93.0
3-Methyl Histamine	98.4
Spermidine	99.1
L-Phenyl Alanine	99.4
Anserine	96.0

Spermine	98.0
Agmatine	129.0
Cadaverine	100.0
Carnosine	96.2
Putrescine	105.0
Water + Histamine	95.0

Table 2. Summary of interference testing with related biological amines. No interference observed with other biological amines.

III. Accuracy and Precision

Accuracy and precision of MaxSignal® Histamine Enzymatic Assay Kit was tested for frozen tuna, fresh tuna, canned tuna, frozen mahi-mahi, and pouched tuna spiked at five concentrations spanning the analytical range of the method. Five replicates of each concentration were analyzed. The matrix was spiked and stabilized according to the specified conditions. Endogenous histamine concentration of all samples was determined by the OMA 977.13 method[5].

Method - Five samples of each matrix were spiked at 0, 6, 20, 50, and 72 ppm of histamine. Sample preparation was carried out as described in the sample preparation section. Sample homogeneity and background histamine levels were determined with the AOAC fluorometric method (977.13) before spiking. The mean concentration, standard deviation, repeatability precision, bias, and recovery were calculated. The measured concentrations were corrected for the endogenous histamine contamination by subtracting mean concentrations of native samples from measured concentration of spiked sample. The food matrix testing was performed by two analysts on two instruments over a span of two days. One technician analyzed two samples and the other analyzed three samples. Linear regression was applied, plotting the determined concentration versus spiked concentration, and goodness of fit (R2) was calculated.

Results - Sample homogeneity of all the native matrices was confirmed by both AOAC and enzymatic method and is illustrated in Table 3 and 4. The evaluation of spiked matrices indicates an overall recovery of 99.6% for frozen tuna, 98.5% for fresh tuna, 93.7% for frozen mahi-mahi, 101.7% for canned tuna and 89.7% for pouched tuna. The overall recovery is well within the acceptable recovery range of 80 – 120%. The endogenous histamine content was included in the recovery calculations. The repeatability precision (% RSDr) was determined for all spiked levels including the native samples. The 0 – 72 ppm spike level shows an RSDr of less than 15% for all matrices. Results are listed in Table 3. All matrices exhibited strong linearity, with R2 values exceeding 0.9995 (Figure 3). Since the endogenous histamine concentration of the tuna samples used was high, LOD and LOQ experiments were not performed with these samples, as they would reflect artificially high values.

Matrix	Target Histamine Concentration	MaxSignal® Results				
		Mean (n=5)	S_r	RSDr (%)	Recovery (%)	Bias (ppm)
Fresh Tuna	0	5.9	0.1	1.7		5.9
	6	5.7	0.2	3.5	95.1	-0.3
	20	20.1	0.3	1.5	100.0	0.1
	50	50.1	1.1	2.1	101.0	0.1
	72	70.6	1.5	2.1	98.0	-1.4
					Mean 98.5	
Frozen Tuna	0	6.1	0.2	3.3		6.1
	6	6.4	0.2	3.1	107.0	0.4
	20	18.9	0.5	2.6	94.7	-1.0
	50	48.9	0.9	1.8	97.6	-1.1
	72	71.4	1.4	2.0	99.2	-0.6
					Mean 99.6	
Mahi-Mahi	0	3.9	0.1	2.5		3.9
	6	5.3	0.1	1.9	87.0	-0.8
	20	18.3	2.7	6.5	91.5	-1.6
	50	49.5	1.4	1.4	99.0	-1.1
	72	69.3	1.7	1.1	98.0	-1.6
					Mean 93.7	
Canned Tuna	0	4.2	0.2	4.8		4.2
	6	6.0	0.1	1.7	101.0	0.1
	20	20.1	0.5	7.0	100.0	0.2
	50	51.4	1.2	0.8	103.0	2.5
	72	74.6	1.8	1.5	103.0	2.6
					Mean 101.7	
Pouched Tuna	0	3.4	0.2	5.9		3.4
	6	4.9	0.3	6.1	82.0	-1.1
	20	18.1	0.8	4.4	91.0	-1.9

	50	47.0	1.4	3.0	94.0	-3.0
	72	66.0	2.5	3.8	92.0	-6.0
					Mean 89.8	

Table 3. Summary of matrix studies.

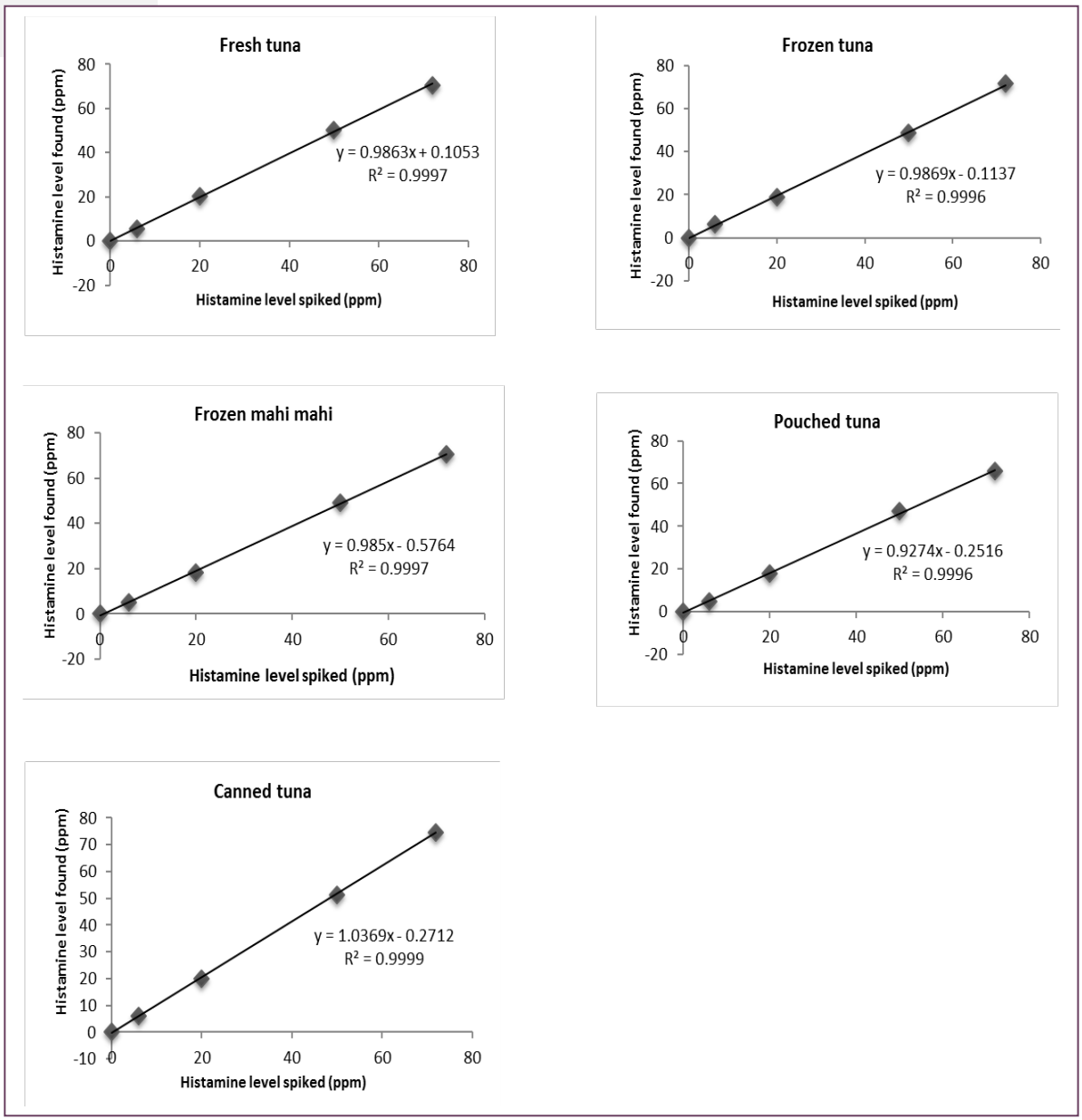

Figure 3. Matrix studies showing regression analysis of spiked versus found histamine concentrations.

IV. Detection Limits

For determination of LOD and LOQ, tuna and mahi-mahi samples with the lowest possible background histamine contamination were pre-screened and selected. No additional treatment was done to reduce the sample background.

Method - Ten replicates of the sample were measured according to the procedure described in Materials and Methods. The mean and Sr were calculated. The detection limit is expressed by using the equation,

$$\frac{X_0 + 3.3(s_b)}{1 - 1.65_m}$$

where Xo = mean, Sb is the intercept, and m is the slope. LOQ is expressed as three times the LOD, and was validated by spiking ten replicates of each matrix at or near the estimated LOQ. The spiked and recovered concentrations of histamine LOQ are listed in Table 4b. Repeatability precision was calculated.

Results - The LOD for frozen tuna, fresh tuna, mahi-mahi, canned tuna, and pouched tuna was determined as 0.87 ppm, 2.07 ppm, 2.15 ppm, 2.99 ppm, and 2.11 ppm. Theoretical LOQ values based on the equation LOQ = 3 X LOD are 2.61 ppm, 6.21 ppm, 6.45 ppm, 8.96 ppm and 6.62 ppm in the respective matrices as listed above, therefore ten replicates of each matrix were spiked at 2.5 ppm, 6.0 ppm, 6.5 ppm, 9.0 ppm, and 7.0 ppm, respectively. The LOQ for frozen tuna, fresh tuna, mahi-mahi, canned tuna, and pouched tuna was determined as 2.63 ppm, 6.56 ppm, 5.93 ppm,

8.99 ppm and 7.39 ppm, respectively, with recoveries ranging from 80 – 120%. The RSDr values were less than 10%. The results of the LOD and LOQ studies are listed in Table 4a and 4b.

Sample ID	Frozen Tuna (ppm)	Fresh Tuna (ppm)	Mahi-Mahi (ppm)	Canned Tuna (ppm)	Pouched Tuna (ppm)
1	0.87	2.01	2.04	3.02	1.85
2	0.84	1.92	1.94	2.89	2.38
3	0.96	2.11	1.97	2.32	2.32
4	0.73	2.01	1.94	2.60	2.44
5	0.84	1.92	2.32	3.22	2.04
6	0.75	2.05	2.00	2.95	2.27
7	1.02	2.01	2.13	3.14	2.12
8	0.70	2.17	2.26	3.52	1.91
9	0.73	1.92	2.07	2.95	2.60
10	0.78	2.27	2.45	2.86	1.75
Mean	0.82	2.04	2.11	2.95	2.17
Sb	0.068	0.056	0.0593	0.0593	0.0593

m	0.10	0.095	0.0949	0.0949	0.0949
S_r	0.11	0.11	0.17	0.33	0.28
RSD(%)	12.89	5.61	8.27	11.12	12.94
LOD	0.87	2.07	2.15	2.99	2.21
LOQ	2.61	6.21	6.45	8.96	6.62

Table 4a. Results of LOD studies.

Sample ID	Frozen Tuna (ppm)	Fresh Tuna (ppm)	Mahi-Mahi (ppm)	Canned Tuna (ppm)	Pouched Tuna (ppm)
1	2.68	6.44	5.73	8.51	7.77
2	2.83	6.28	6.15	8.63	7.01
3	2.42	6.28	5.73	9.23	7.61
4	2.65	6.03	6.59	8.25	7.45
5	2.62	6.13	6.08	9.71	7.52
6	2.92	6.41	6.05	8.94	7.07
7	2.59	8.23	6.65	8.19	7.83
8	2.68	7.04	5.48	9.46	7.17
9	2.56	6.31	5.42	9.49	7.23
10	2.39	6.41	5.45	9.52	7.26
Mean	2.63	6.56	5.93	8.99	7.39
Spiked	2.5	6.0	6.5	9.0	7.0
S_r	0.16	0.27	0.45	0.56	0.29
RSD(%)	6.13	4.31	7.55	6.27	3.89

Table 4b. Results of LOQ studies.

V. Ruggedness

Minor, reasonable changes were introduced to evaluate the ability of the method to remain unaffected.

Method - The following conditions were investigated for ruggedness testing:

1. Short time frames where the kit is left at room temperature
    a. 4 hours, 1 hour, and not left at RT
2. Heating time during sample preparation – 10 min (according to protocol) to 5 min and 15 min
3. Enzymatic incubation time – 5 min (according to protocol) to 3 min and 10 min

The factorial design of the experiment is described in Table 5. Twenty replicates of frozen tuna (ten spiked to 25 ppm and ten un-spiked) were tested per condition. Combinations from 1 to 8 represent deviations from the assay method, and combination

9 represents the baseline assay method. The results from combination 1-8 were compared to the baseline.

Results - The method remained robust throughout the experimental deviations, with recoveries ranging from 80 – 120% (Table 6).

Combination	Time frame where the kit was left at RT (Hours)	Sample Preparation Heating (Min)	Enzymatic Incubation Time (Min)
1	4	5	3
2	4	5	10
3	4	15	3
4	4	15	10
5	1	5	3
6	1	5	10
7	1	15	3
8	1	15	10
9 (Baseline)	Not left at RT	10	5

Table 5. Factorial design of ruggedness study.

	Spike Level (ppm) Mean (n = 10)		Mean Recovery %
Combination #	0	25	
1	1.21	23.2	88.1
2	4.06	27.2	93.0
3	2.17	24.9	93.0

4	5.07	27.2	88.5
5	1.31	24.0	91.0
6	3.54	25.4	87.4
7	3.10	24.0	84.0
8	5.06	27.5	90.0
9 (baseline)	2.40	26.0	95.0

Table 6. Results of ruggedness testing data.

VI. Lot-to-Lot Consistency

Lot-to-lot variability was assessed by testing samples of frozen tuna among three lots of MaxSignal® Histamine Assay kits (lot A, lot B, lot C) to compare statistical differences in detection.

Method - The study was carried out with blank spiking solution and 25 ppm frozen tuna spiking solution. Five blank and five 25 ppm spiked replicates were tested per condition. The blank and 25 ppm sample replicates used for testing accelerated stability were used in this study. A test kit variation study was also performed to examine the variation between three test kits of a single lot of MaxSignal® Histamine Assay Kit (lot B).

Results - Results generated from each lot of reagents were comparable and no significant statistical difference was found, as shown in Table 7 and Table 8.

Sample Identity			Product Consistency	
Lot ID	Spike Status	Mean (ppm)	STD (ppm)	RSD_r (%)
A	Unspiked	3.8	0.2	5.3
	25 ppm	27.4	0.5	2.1
B	Unspiked	3.2	0.3	9.4
	25 ppm	27.7	0.6	1.2
C	Unspiked	2.7	0.3	11.1
	25 ppm	25.7	0.4	1.7

Table 7. Table showing results of lot-to-lot consistency study.

Sample Identity			Product Consistency	
Lot ID	Spike Status	Mean (ppm)	STD (ppm)	RSD_r (%)
A	Unspiked	3.1	0.3	9.7
	25 ppm	28.8	0.5	1.9
B	Unspiked	3.2	0.4	12.5
	25 ppm	29.6	0.2	0.8
C	Unspiked	3.5	0.2	5.7
	25 ppm	26.5	0.2	0.9

Table 8. Results summary of test kit variation.

VII. Stability

To validate a 1 year claimed shelf life of the kit at 4°C, an accelerated stability study was performed based on an Arrhenius model[5]: assuming Ea= 20 Kcal, 1 year at 5°C = 32 days at 25°C.

Method - Five replicates each of un-spiked and 25 ppm histamine spiked samples were tested with kits for which starting days of incubation were staggered at 25°C for incubation periods of 1, 16 and 32 days. Staggering of the kits was implemented to provide for a single evaluation date. Three kits from lot B were used in this study. Real time stability was also carried out simultaneously using reagents of the same lot.

Results - Data indicates good stability throughout the testing period (Table 9).

		Mean (ppm)		Sr (ppm)		RSDr (%)	
	Spike level (ppm)	0	25	0	25	0	25
Accelerated Stability	Day 1	3.5	28.7	0.3	0.6	8.6	2.2
	Day 15	3.2	28.5	0.1	0.3	3.3	1.1
	Day 32	3.5	28.8	0.1	0.4	5.2	1.5
Real-time Stability	1 Month	3.7	28.0	0.2	0.1	8.0	2.8
	6 Months	2.5	27.3	0.2	0.1	8.0	2.6
	1 Year	2.5	26.4	0.2	1.1	7.0	4.8

Table 9. Accelerated and real time stability test results.

VIII. Reference Method Comparison

The AOAC 977.13 OMA method was used for establishing background histamine content in frozen/fresh tuna, canned tuna, pouched tuna, and mahi-mahi samples used in the study. The ratios of AOAC/MaxSignal® method suggested good correlation between the methods as seen in Table 10.

Sample Type	MaxSignal®	AOAC 977.13	Ratio
Frozen Tuna	6.1	5.4	0.9
Mahi-Mahi	3.9	3.1	0.8
Canned Tuna	4.2	3.4	0.8
Fresh Tuna	5.9	5	0.8
Pouched Tuna	3.4	3.6	1.1

Table 10. Correlation between AOAC 977.13 and MaxSignal® methods.

Independent Validation Study

I. Matrix Study

Sample Preparation - Two boxes containing a total of 37, blind- coded 50-mL conical tubes of 4.0 g portions of frozen spiked tuna were received by the Silliker Food Science Center, Illinois. Temperature upon receipt was -30°C. Five samples containing known histamine concentrations were evaluated, covering the analytical range of the method (6-72 ppm). The matrix study determined the bias, recovery, repeatability precision, LOD, and LOQ of the MaxSignal® Histamine Enzymatic Assay. The MaxSignal® Histamine Enzymatic Assay was validated for frozen tuna. Naturally contaminated matrices were not available, therefore artificially spiked samples were used.

Method - The matrix study was performed by two analysts using one instrument. Five samples per concentration were analyzed using the MaxSignal® Histamine Enzymatic Assay and 2 samples were analyzed using the OMA 977.13 method. From each blind- coded sample, a 4.0 g test portion was weighed, extracted, and analyzed according to the MaxSignal® Histamine Enzymatic Assay protocol (refer to Table 11 for details).

Matrix Sample Set	Histamine Level (ppm)	MaxSignal® Histamine Ezymatic Assay Replicates	AOAC 977.13 Replicates
1	0	5	NA
2	6	5	NA
3	20	5	NA
4	50	5	NA
5	72	5	NA

6	0	NA	1
7	50	NA	1
8	5	10	NA

Table 11. Matrix study details for MaxSignal® Histamine Enzymatic Assay.

Results - Results of bias, recovery, and repeatability precision determination of histamine in five concentrations for frozen tuna are presented in Table 12. The evaluation of spiked matrix indicates an overall recovery of 94%. The repeatability precision (RSDr) of all five replicates at the spiked concentration and native samples were determined. The RSDr at 6 ppm was < 5%, at 20 ppm was < 2%, at 50 ppm was < 3%, and at 72 ppm was 2%. The native sample exhibited a higher RSDr at 21%, which can be attributed to the fact that the mean concentration was below the LOQ. This is further confirmed by the OMA 977.13 method, where endogenous histamine content was found to be less than 1 ppm (Table 13). Figure 4 shows the regression analysis of the target vs. actual histamine concentration results, where the correlation coefficient (R2) was 0.9994.

Matrix	Target Histamine Concentration	MaxSignal® Results				
		Mean (n=5)	S_r	RSDr (%)	Recovery (%)	Bias (ppm)
Frozen Tuna	0	1.18	0.25	21.00		1.18
	6	5.64	0.26	4.60	93.95	-0.36
	20	18.34	0.34	1.83	91.69	-1.66
	50	47.97	1.06	2.22	95.95	-2.03
	72	68.8	1.43	2.07	95.56	-3.20

Table 12. Independent lab validation results summary of the MaxSignal® Histamine Enzymatic Assay.

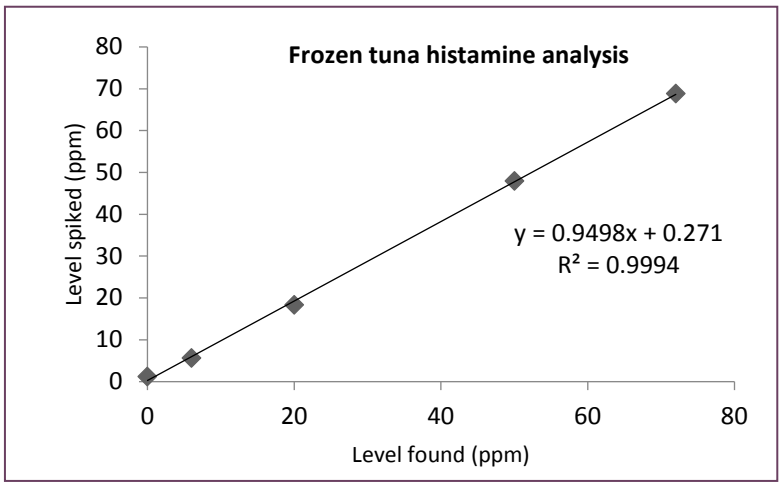

Figure 4. Independent lab regression analysis of spiked versus found histamine concentrations in frozen tuna.

Target Histamine Level (ppm)	OMA 977.13
0	< 1
50	44

Table 13. Independent lab determination of histamine in spiked and un-spiked tuna samples using OMA 977.13 method.

II. LOD and LOQ Determination

LOD and LOQ determination results are derived in the following manner: LOD was calculated by graphing Sr vs mean for candidate method results and using equation:

$$LOD = \frac{X_0 + 3.3(s_b)}{1 - 1.65_m}$$

The calculated LOD was 1.6 ppm with a resulting LOQ of 4.84 ppm (LOQ = 3 X LOD). Ten replicates for LOQ samples were pre-spiked at 5 ppm and received by the independent lab. An LOQ level of 5 ppm was obtained by internal analysis at Bioo Scientific, therefore ten replicates were spiked at 5 ppm. This was further verified and validated by the independent lab. The validated LOQ, determined by assaying the spiked LOQ samples, was 5.22 ppm (Table 14).

Sample ID	Adjusted level	Mean	S_r
1	5.35		
2	5.46		
3	5.13		
4	4.85		
5	5.05		
6	5.20		
7	5.06		
8	5.25		
9	5.45		
10	5.39		
Cumulative		5.22	0.20

Table 14. Independent lab LOQ determination results summary.

Discussion

Using a linear fit curve, the linearity of the assay ranging from 0 – 108 ppm for MaxSignal® Histamine Enzymatic Assay Kit was provided. Six standards are supplied with the kit, ranging from 0 – 72 ppm, and an additional 1000 ppm histamine spike is supplied for spiking samples to verify recoveries. Minimal cross-reactivity of < 1% was found for twelve of the fourteen biogenic amines tested. Negligible cross reactivity of 2% and 4% was found with Putrescine and Agmatine, respectively. The determined cross-reactivity is within acceptable levels, and there was no interference from the fourteen biogenic amines tested.

The overall recovery of histamine from all food matrices tested was within the acceptable range of 80 – 120%. The endogenous histamine contamination of un-spiked samples determined by MaxSignal® Histamine Enzymatic Assay Kit exhibited good correlation with the AOAC 977.13 method. The repeatability precision at the defect action level imposed by the FDA at 50 ppm is < 5%. LOD and LOQ determination based on the naturally histamine contaminated samples used in food matrix studies could have resulted in artificially high values. For this reason, the LOD and LOQ studies were conducted using samples with the lowest possible histamine contamination. Since no other methods were used to lower the histamine content of the samples, LOD and LOQ calculated could still be an overestimation. There were no observable lot- to-lot differences, and the 25 ppm samples tested with three different lots gave consistent results.

The claimed shelf-life of one year at 4°C was verified by both accelerated and real-time stability data, proving that the kit was very stable throughout the testing period. Robustness studies indicated no significant changes in the detection of histamine upon introduction of minor changes to the assay protocol. The independent lab studies revealed that the kit works with the same precision in minimally trained hands as well as expert examiners.

Conclusions

The efficient and solvent-free MaxSignal® Histamine Enzymatic method for the determination of histamine exhibited good recovery rates and good repeatability precision for the tested matrices of frozen tuna, fresh tuna, mahi-mahi, canned tuna and pouched tuna. The method is rugged, stable, and displays excellent product consistency between lots, with minimal cross-reactivity from potential interfering compounds. The performance of MaxSignal® Histamine kit was at least equivalent to the AOAC 977.13 reference method in the AOAC PTM validation study, and therefore has been accepted as AOAC Performance Tested Method℠ 051701.

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