Single Laboratory Validation for the Determination of Six Biogenic Amines in Canned Tuna with Liquid-Liquid Extraction and Liquid Chromatography- Tandem Mass Spectrometry

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Abstract

FDA regulates histamine in seafood by sensory, chemical, and ELISA testing. Sensory analysis for histamine requires a unique and a highly specialized skill set with extensive and rigorous trainings. The ELISA assay tests only for histamine and no other compounds, whereas the HPLC method with fluorometric detection can detect other bioamines but is laborious and requires a derivatization step.

The Pacific Southwest Food and Feed Laboratory (PSFFL) developed an efficient and sensitive method for the detection, without derivatization of six biogenic amines (tyramine, putrescine, cadaverine, histamine, 2-phenylethylamine, and tryptamine) in canned tuna using LC-MS/MS. The utilization of ball-beating disruption and extraction with methanol, water and heptafluorobutyric acid followed by LC-MS/MS analysis allowed the rapid detection of the amines without derivatization.

Three different brands of canned tuna were spiked at three levels (0.25, 0.5 and 1.0 µg/g) in triplicates with each of the six amines; and the results are within the acceptable method validation criteria. The results demonstrate that the LC-MS/MS method is fit for use in the detection of biogenic amines in canned tuna, therefore giving regulators and the food industry a reliable method to accurately monitor the level of amines in seafood.

Key Words

Biogenic amine, Histamine, Tuna, LC-MS/MS, Ion-paring Chromatography

Introduction

Biogenic amines are formed by the decarboxylation of amino acids or by amination and transamination of aldehydes and ketones during normal metabolic processes in living cells. Fresh fish have low levels of biogenic amines. The levels of these amines increase as the post-mortem decomposition progresses [1,2]. Therefore, the biogenic amines can be used as an indicator of freshness or spoilage. In recent years, liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques have been used for the analysis of biogenic amines with or without a derivatization step. The LC-MS/MS methods without derivatization make the analysis more accurate, sensitive and efficient [5-7]. The FDA guidance set 50 ppm histamine as a critical level for sensory method that analyzes single fish as unit. However, for an instrument method, the fish collected for analysis may be composited for analysis if the action point is reduced accordingly. For example, a sample of 60 fish may be composited into 12 units of 5 fish each, provided the action point is reduced from 50 ppm to 10 ppm for each unit.

Current LC-MS/MS methodologies for analyzing biogenic amines in seafood can avoid the derivatization step. Developed by Naoki Ochi et al in 2019 [5], the method utilized ion pair solid phase to extract targeted analytes and volatile ion pair reversed phase liquid chromatography with tandem mass spectrometry to separate and quantify the individual biogenic amines. This method can maintain accuracy, sensitivity, and efficiency [5-7] and detect levels that were matched to the regulatory requirements. In addition, using an extraction method previously developed at PSFFL [3, 4] as a starting point, the modified extraction method was further optimized without having to rely on derivatization. A liquid-liquid extraction and LC-MS/MS method was developed and validated at PSFFL to test for the six biogenic amines: histamine, cadaverine, putrescine, tyramine, phenylethylamine & tryptamine in canned tuna with a target LOQ of 0.5 ppm for all six compounds.

To avoid the high incurred level of biogenic amines that affect the calculations of low-level spike recovery, a preliminary screening was performed for six brands of canned tuna. Three brands of canned tuna with low incurred levels were chosen for the validation study.

Method blanks, method blank spikes, matrix samples and matrix spikes were processed via the extraction protocol. Ultra-Centrifugal Filter Units were used as final filtration step to remove biomolecules with large size.

Once the method for six biogenic amine compounds was optimized, the calibration standards were prepared in solvent with serial dilution of all six biogenic amines.

Materials and Methods

Reagents and Consumables:

a) Water (Nanopure)
b) Acetonitrile (Fisher Scientific, LC/MS Grade)
c) Methanol (Fisher Scientific, LC/MS Grade)
d) Nonafluoropentanoic acid (ACROS Organics, CAS 2706-90-3)
e) Heptafluorobutyric acid (HFBA) (ACROS Organics, CAS 375-22-4)

f) Millipore Sigma Amicon Ultra Centrifugal Filter Units (Fisher Scientific)
g) Zorbax C8 1.8 µm column, 4.6 X 50 mm (Agilent)
h) Steel Ball (SPEX)
i) 50 mL Falcon Centrifuge Tube (Fisher Scientific)

Standards:

Table 1. Source of standards

Item		CCV		ICV	
	Standard	Vendor	Purity	Vendor	Purity
1	Histamine 2HCl	United States Pharmacopeia	100%	Sigma	≥99%
2	Cadaverine 2HCl	Sigma Aldrich	99.3%	TCI	99.0%
3	Putrescine 2 HCl	Research Products Int'l	99.23%	Alfa Aesar	99.6%
4	Tyramine HCl	Sigma Aldrich	100%	Alfa Aesar	98%
5	2-Phenylethyl-amine HCl	TCI	98.0%	Sigma Aldrich	100%
6	Tryptamine HCl	Aldrich	≥99.0%	Aldrich	≥98.5%

Internal standards: Histamine-α,α,β,β-d4 2HCl, 99.0 % (CDN Isotopes)
1,4-Butane-d_8-diamine 2HCl, 99.6 % (CDN Isotopes)

Equipment:
Sciex 4500 QTrap mass spectrometer equipped with Exion LC system including pump, autosampler and column compartment (Sciex LLC)
Analytical balance (Mettler-Toledo XS105)
Blender (Robot Coupe RSI 2y-1)
Grinder (SPEX 2010)
Centrifuge (Eppendorf 5804 and/or Thermo Fisher Multifuge X3R)

Mobile Phases:
Mobile Phase A: 5 mM Nonafluoropentanoic acid (NFPA) in water
Mobile Phase B: 1:1 (vol) Acetonitrile / H_20 with 5 mM NFPA

Standards Preparation
Prepare the calibrants of biogenic amine mixture in solvent as shown in the following tables:

Table 2. Preparation of Stock Standards

1000 µg/mL **Stock Standard Solutions**	Weigh approximately 10 mg of each standard (adjust for salt) individually into a 10 mL volumetric flask, then dilute to volume with 75%MeOH and 25% water with 0.2% HFBA.
1000 µg/mL **Stock Internal Standard Solution**	Stock of internal standards are prepared same as the stock standards' solution at the same concentrations

Table 3. Preparation of Intermediate Standards and Spiking Solution

Step	**Starting Mixed Standards**	**Standard Vol** (mL)	**Final Vol** (mL)	**Final Concentration (**µg/mL**)**
1	1000 µg/mL std Stock	1	10.0	100
2	100 µg/mL std from Step 1	1	10.0	10
3	10 µg/mL std from Step 2	2.5	10.0	2.5

Table 4. Preparation of Intermediate Solvent Standards

Step	**Mixed Standard**	**Standard Vol** (µL)	**Solvent Vol** (µL)	**Final Concentration** (µg/mL)
1	10 µg/mL intermediate std	512	488	5.12
2	5.12 µg/mL std from Step 1	400	400	2.56
3	2.56 µg/mL std from Step 2	400	400	1.28
4	1.28 µg/mL std from Step 3	400	400	0.64
5	0.64 µg/mL std from Step 4	400	400	0.32
6	0.32 µg/mL std from Step 5	400	400	0.16
7	0.16 µg/mL std from Step 6	400	400	0.08

Table 5. Preparation Intermediate Solvent Standards of Internal Standard

Step	**Starting Mixed Standards**	**Standard Vol** (mL)	**Final Vol** (mL)	**Final Concentration (**µg/mL**)**
1	1000 µg/mL Stock solution	1	10.0	100
2	100 µg/mL from step 1	1	10.0	10
3	10 µg/mL from Step 2	0.5	5.0	1.0

Table 6. Preparation of Calibration Standards

Starting Mixed Standards (µg/mL)	Vol of Mixed Standards (µL)	Vol of 1 µg/mL Internal Std (µL)	Final Concentration (µg/mL)
0 (solvent)	300	300	0
0.08	300	300	0.040
0.160	300	300	0.080
0.320	300	300	0.160
0.640	300	300	0.320
1.28	300	300	0.640
2.56	300	300	1.280
5.12	300	300	2.560

Preparation of ISTD Spiked Extraction Solvent: 0.5 ppm Histamine-D4 + Putrescine D-8 in 75%(v/v) Methanol + 25 % Water (v/v) with 0.2% HFBA.

Table 7. Preparation of ISTD Spiked Extraction Solvent

Step	Mixed Standard	Standard Vol (mL)	Final Vol (L)	Concentration µg/mL (ppm)
1	1000 µg/mL Stock	2	4.0	0.5

LC/MS Parameters

The proposed method uses ABSciex 4500 QTrap LC-MS/MS with Agilent Zorbax C8 1.8 µm column to monitor two fragment ions of each biogenic amine. Also, the method gradient program is 11 minutes modified from the 16 minutes run which is published [4]. Acquity UPLC BEH C18 1.7 µm 2.1X100mm was evaluated. See Table 8 for other column parameters. Table 9 shows the fragment ions; two Q1/Q3 pairs are monitored. It meets recommendations of CVM Guidance #118.

Table 8. LC and MS Conditions

Column	Agilent Zorbax SB C8, 1.8µm 50X4.6 mm
Mobile Phase A (MPA)	5 mM Nonafluoropentanoic acid (NFPA) in water
Mobile Phase B (MPB)	Acetonitrile / H20 1:1 with 5 mM NFPA
Flow Rate	0.5 mL/min
LC Elution	0 - 1.0min: 95%A/5%B 1.0 to 7.5 min: ramp to 100%B 7.5-8.8 min: 100%B 8.8 to 11 min: 5%B (A=MPA and B=MPB) 5 min extra wash according to validation feedback
Other LC Parameters	Injection volume: 2 µL Column temperature: 40°C Autosampler temperature: 15°C

Table 9. MRM List for Ion Monitoring

Compound \ Ions	**Q1**	**Q3**	
Tyramine	138	121	51
Putrescine	89	72	55
Putrescine D_8	97	80	65
Cadaverine	103	86	69
Histamine	112	95	68
Histamine D_4	116	99	85
Phenylethylamine	122	105	51
Tryptamine	161	117	144
Source Parameters	Curtain gas: 30 IS: 4000 V TEMP: 400°C GS1: 50 GS2: 50 MRM Positive ESI		

Sample and Spiked Sample Preparation

Flush a Robot Coupe blender with hot tap water from the faucet for 3 minutes. Open a canned tuna and drain the liquid from the canned tuna. Place sample into the Robot Coupe blender and blend until homogeneous. Scoop the entire mixture with a spoon and place in the center of the blender and re-blend again. Repeat the previous scoop step to get a final paste-like homogeneous mixture.

Spike tuna each sample as described in Table 10 as shown below.

Table 10. Fortification in 4 grams of Tuna Samples

Level of Spike (ppm)	Vol of 2.50 PPM Mixed Standard (µL)
0.25	400
0.50	800
1.0	1600

Extraction

Weigh 4 g $\pm$ 0.1 of sample in a 50 mL Falcon centrifuge tube, add 20 mL 75%/25% methanol/water with 0.2% HFBA containing 0.5 µg/mL mixed ISTDs, add a steel ball. Place on a Geno Grinder at 900 strokes/min for 10 minutes. Next, centrifuge for 5 min at 4000 rpm (2057 x*g*) and pipette 2 mL supernatant solution into an Amicon ultra-4 filter cartridge. Centrifuge for 20 min at 8000 rpm (8228 x*g*).

Pipette 0.5 mL of the filtered extract into a sample vial for instrument analysis. Each 1 mL of the extract contains 0.2 grams of sample (4 g/ 20 mL). The dilution factor is 5.

Calculations

Calibration of the aromatic biogenic amine standards (tyramine, 2-phenylethylamine and tryptamine) are performed by using the peak areas of the targeted aromatic biogenic amine peaks, without using internal standard.

$$Y = a + b X$$

where a is the y-intercept, and b is the slope of the curve; x is calibrant level (µg/mL), and y is a peak area.

Calibration of the aliphatic biogenic amine standards (putrescine, cadaverine, and histamine) are performed by using the relative peak area of the targeted biogenic amine peaks to that of the isotopically labeled internal standards. Histamine D4 was used for histamine and cadaverine, and Putrescine-D8 was used for putrescine as their internal standards. Putrescine-D8 was also fine used for putrescine as their internal standards.

$$Y = a + b X$$

where a is the y-intercept, and b is the slope of the curve; x is the ratio of calibrant level (µg/mL) and internal standard level (µg/mL), and y is a relative peak area.

Calculate targeted biogenic amines in concentration in extract in the units of µg/mL (µg biogenic amine/mL extract) in unknown sample as shown below:

$$X = (y - a)/b$$

where a is a y-intercept and b is slope of the curve, and y is a peak area or relative peak area. For the calibrations forced to zero, a=0.

Conversion of extract concentration units in µg/mL to matrix concentration ppm (µg/g)

In sample, the concentration of biogenic amines should be expressed as ppm that meant "how many micro grams of an amine in a gram of tuna sample". However, the solution density of sample extraction or calibration standard was unknown, the amine concentration in solution was expressed as µg/mL. The conversion as shown below:

Biogenic Amine concentration in matrix (ppm) = Extract Concentration (µg/mL) * 5

Calculate spike recoveries as shown below:

Spike Recovery (%) =
(Spiked matrix conc in ppm – Incurred Concentration in matrix blank in ppm) * 100/Spike Conc in ppm

Calculate ICV recovery (%) as shown below:

Recovery (%) =
(Calculated Conc of ICV standard in µg/mL) * 100/ (Theoretical ICV Conc in µg/mL)

Calculate CCV recovery (%) as shown below:

Recovery (%) =
(Calculated Conc in µg/mL of reinjected 0.640 µg/mL calibrant) * 100/ (Calculated Conc in µg/mL of the original 0.640 µg/mL calibrant)

Ion ratio confirmation (IRC)

IRC = (Peak area of confirmation ion) * 100 / (Peak area of quantitation ion)

Results and Discussion

According to Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 2nd Edition, Level Two validation, the validation protocol included the following factors:

1) 3 brands canned tuna as 3 matrix sources

2) Each matrix had 2 replicate analyses on different days
3) Each replicate batch had 3 spike levels and 2 methods blanks
4) Each replicate batch had 2 or 3 matrix blanks analyzed. For the incurred biogenic amines, the average levels were calculated for subtraction from the sample spikes.
5) Spike-recovery between 70-120%.
6) 7 replicate low level spiked sample were extracted and analyzed. The method detection limit (MDL) was calculated using EPA 40CFR136, Appendix B. This MDL was accepted as LOD
7) The difference of the ion ratio between samples and standards was within 10%
8) Nonafluoropentanoic acid (NFPA) is a high polar compound, it could be flushed out by 200 mM Ammonium Acetate solution. It is volatile reagent that does not stay in the vacuum chamber of the mass spectrometry. Comparing the cost and benefit, it is acceptable.

Chromatogram and Calibration Curve

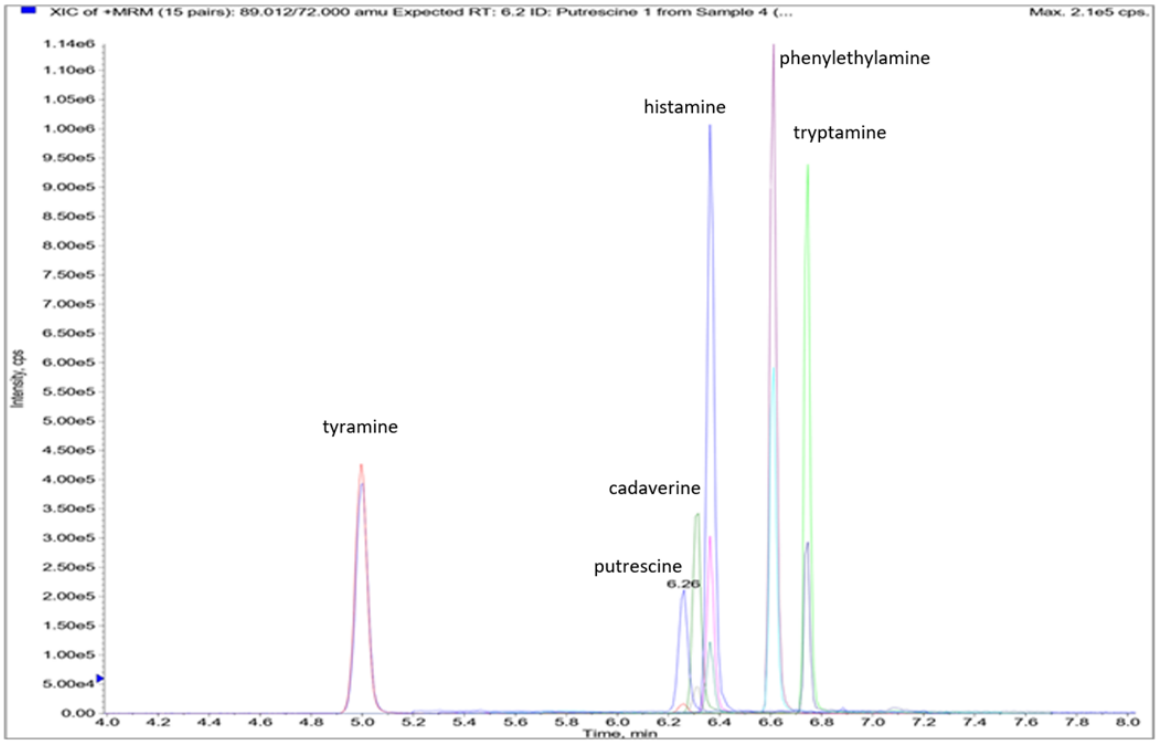

Fig. 1 Typical chromatograms of biogenic amines.

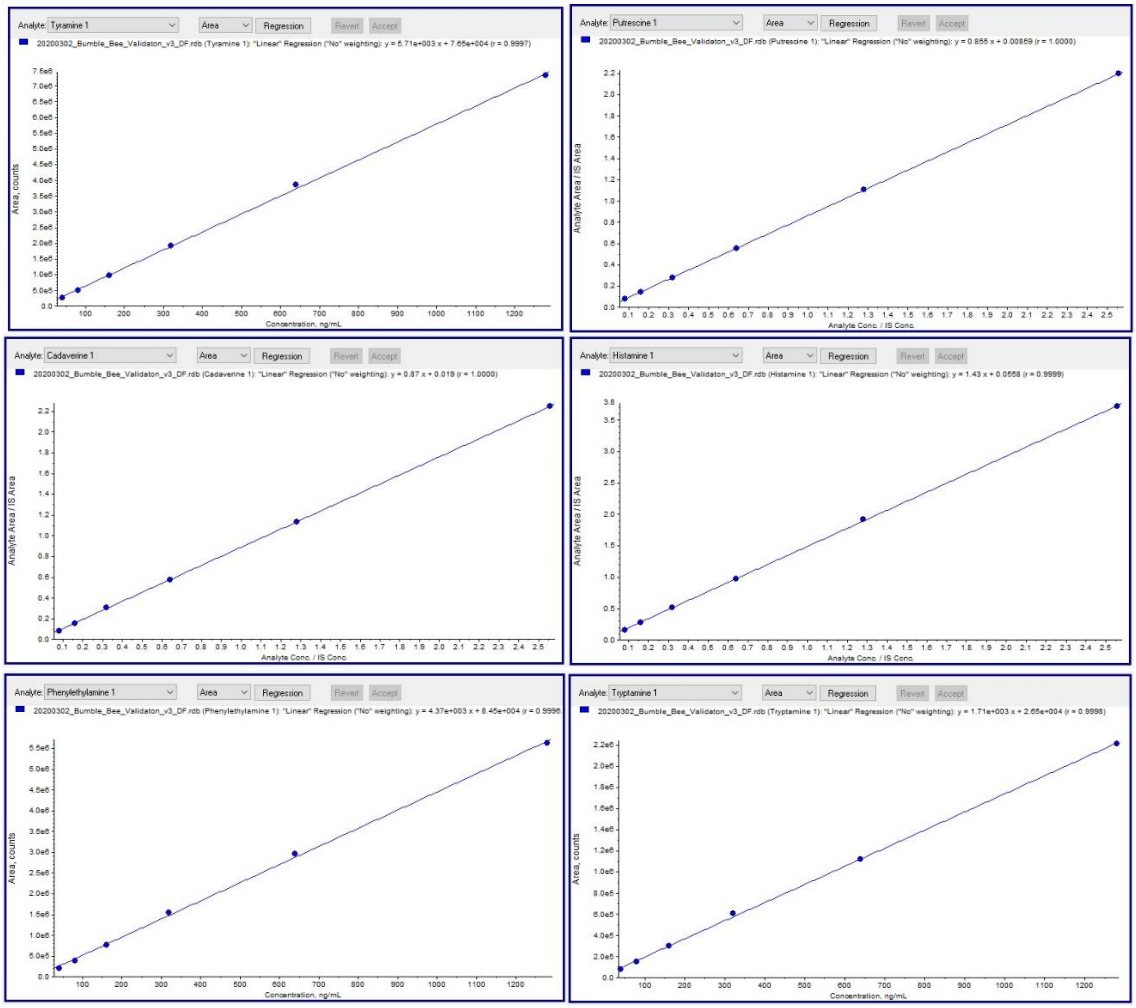

Fig. 2 Typical calibration lines of biogenic amines in solvent

Linearity

Six calibration levels of standards mix were used for the calibration curve. The linear regression showed that the correlation coefficient R^2 was above 0.995 for each compound in each batch of validation. The dynamic linear range was from 0.040 µg/mL to 1.280 µg/mL. Since the samples were extracted from 4g to the final volume of 20 mL, the quantitation of the biogenic amines in the sample could go up to 6.4 µg/g, or 6.4 ppm without further dilution. Cadaverine and histamine use Histamine D_4 as internal standard. Putrescine uses Putrescine D_8 as internal standard. The aromatic amines, Tyramine, Phenylethylamine and Tryptamine do not require the use of an internal standard. The three aromatic biogenic amines were not well correlated with two presented internal standards either. Table 11 was the results from 6 tests by three analysts in 6 days.

Table 11. Correlation coefficient of 6 biogenic amines, linear fitting

Batch #	**Valida-tion 1** Brand A	**Valida-tion 2** Brand B	**Valida-tion 3** Brand C	**Valida-tion 4** Brand A	**Valida-tion 5** Brand B	**Valida-tion 6** Brand C
Analytes	**R^2**	**R^2**	**R^2**	**R^2**	**R^2**	**R^2**
Tyramine	0.9999	0.9999	1.0000	0.9993	0.9998	0.9953
Putrescine	0.9999	0.9991	1.0000	0.9986	0.9987	0.9997
Cadaverine	1.0000	0.9999	1.0000	0.9998	0.9990	0.9998
Histamine	0.9998	0.9994	0.9998	0.9997	0.9999	0.9999
Phenylethyl-amine	0.9998	0.9995	0.9971	0.9974	0.9997	0.9980
Tryptamine	0.9998	0.9990	0.9999	0.9975	0.9998	0.9955

LOD and LOQ

The limit of detection and the limit of quantitation were calculated according to the guidelines. Seven replicates of 0.250 µg/g standard mix with internal standards spiked tuna, Brand C, were extracted and analyzed. The standard deviations of each compound were calculated. Using 40CFR 136 Appendix B,

$$MDLS = t(n-1, 1-\alpha = 0.99)Ss$$

Where:
MDLs = the method detection limit based on spiked samples
t(n-1, 1−α= 0.99) = the Student's t-value appropriate for a single-tailed 99th percentile t statistic and a standard deviation estimate with n-1 degrees of freedom. When n=7, t = 3.14
Ss = sample standard deviation of the replicate spiked sample analyses.

Table 12. Calculated concentration of biogenic amines from 7 replicates of tuna extractions. (µg/g)

Item	Extraction\ Analyte	Tyramine	Putrescine	Cadaverine	Histamine	Phenyl ethylamine	Tryp tamine
1	**Extraction 1**	0.262	0.253	0.263	0.221	0.196	0.189
2	**Extraction 2**	0.264	0.260	0.226	0.218	0.195	0.200
3	**Extraction 3**	0.255	0.246	0.255	0.248	0.198	0.193
4	**Extraction 4**	0.261	0.234	0.241	0.226	0.195	0.189
5	**Extraction 5**	0.258	0.243	0.240	0.242	0.184	0.194
6	**Extraction 6**	0.264	0.266	0.219	0.206	0.198	0.206
7	**Extraction 7**	0.269	0.219	0.227	0.207	0.187	0.184
8	**Average**	0.262	0.245	0.238	0.224	0.193	0.194
9	**SD**	0.0045	0.016	0.016	0.016	0.0053	0.0074
10	**LOD** (= 3.14*SD)	0.014	0.050	0.051	0.051	0.017	0.023
11	**LOQ** (= LOD*3)	0.043	0.150	0.153	0.153	0.051	0.069

Recovery:

Spike-recovery tests were performed with three brands of canned tuna by three chemists. Each brand had a duplicate test in different days. Three spike levels, 0.25, 0.50 and 1.00 µg/g (or ppm) were defined as low, medium and high levels. The percentage recovery was the calculated concentration divided by spiked concentration of each compound from each extraction. The RSD was the standard deviation of three replicates of each compound from extraction divided by the average calculated concentration. The recovery is between 70%-120%. The RSD in batch was 0.3%-9.0%. The average recovery of all six batches 81%-98.8%. The RSD was 7.7%-12.8%.

Table 13. Results of spike recovery tests

COMPOUND	Tyramine			Putrescine			Cadaverine			Remark
SPIKE LEVEL	0.25 µg/g	0.5 µg/g	1.0 µg/g	0.25 µg/g	0.5 µg/g	1.0 µg/g	0.25 µg/g	0.5 µg/g	1.0 µg/g	
% Recovery 1	83.6	79.6	72.8	92.4	91.4	94.3	86.0	90.6	93.6	
% Recovery 2	95.6	79.2	74.5	91.2	92.6	95.3	88.0	97.4	95.0	Valida-
% Recovery 3	92.8	81.2	74.3	105.6	95.8	87.3	89.2	95.8	95.5	tion 1
Average	**90.7**	**80.0**	**73.9**	**96.4**	**93.3**	**92.3**	**87.7**	**94.6**	**94.7**	Brand A
RSD %	**6.9**	**1.3**	**1.3**	**8.3**	**2.4**	**4.7**	**1.8**	**3.8**	**1.0**	
% Recovery 1	93.9	93.1	90.1	99.8	103.1	101.7	103.0	113.5	106.7	
% Recovery 2	96.3	89.9	88.9	91.4	110.3	106.7	93.4	107.9	116.7	Valida-
% Recovery 3	98.7	95.5	91.7	95.4	103.1	98.7	96.6	98.7	114.7	tion 2
Average	**96.3**	**92.9**	**90.2**	**95.5**	**105.5**	**102.3**	**97.7**	**106.7**	**112.7**	Brand B
RSD %	**2.5**	**3.0**	**1.6**	**4.4**	**3.9**	**3.9**	**5.0**	**7.0**	**4.7**	
% Recovery 1	114.4	91.4	77.5	107.3	99.5	101.4	91.2	86.4	95.5	
% Recovery 2	110.4	86.8	76.3	100.5	85.9	91.4	101.6	96.2	91.5	Valida-
% Recovery 3	106.4	88.2	78.0	110.5	91.1	95.4	96.0	99.2	93.5	tion 3
Average	**110.4**	**88.8**	**77.3**	**106.1**	**92.1**	**96.1**	**96.3**	**93.9**	**93.5**	Brand C
RSD %	**3.6**	**2.7**	**1.1**	**4.8**	**7.4**	**5.2**	**5.4**	**7.1**	**2.1**	
% Recovery 1	72.8	72.6	70.7	114.0	98.6	113.4	80.7	100.4	113.2	
% Recovery 2	70.4	75.2	70.8	114.4	114.8	118.4	79.5	105.4	116.2	Valida-
% Recovery 3	72.8	72.0	70.4	114.8	110.8	114.4	94.3	117.0	105.2	tion 4
Average	**72.0**	**73.3**	**70.6**	**114.4**	**108.1**	**115.4**	**84.8**	**107.6**	**111.5**	Brand A
RSD %	**1.9**	**2.3**	**0.3**	**0.3**	**7.8**	**2.3**	**9.7**	**7.9**	**5.1**	
% Recovery 1	83.5	74.1	98.0	83.0	79.7	88.7	91.0	72.5	78.2	
% Recovery 2	80.7	74.5	98.0	87.8	75.9	88.4	85.0	72.1	80.6	Valida-
% Recovery 3	83.9	72.5	99.0	91.4	74.1	95.8	91.8	71.5	78.2	tion 5
Average	**82.7**	**73.7**	**98.3**	**87.4**	**76.6**	**90.9**	**89.3**	**72.1**	**79.0**	Brand B
RSD %	**2.1**	**1.4**	**0.6**	**4.8**	**3.7**	**4.6**	**4.2**	**0.7**	**1.8**	
% Recovery 1	95.6	83.2	76.0	105.9	96.1	92.3	96.8	91.4	91.0	
% Recovery 2	94.8	85.2	77.4	90.3	96.3	94.3	86.0	93.4	90.0	Valida-
% Recovery 3	95.6	85.4	76.0	91.5	106.5	100.3	95.2	95.0	86.0	tion 6
Average	**95.3**	**84.6**	**76.5**	**95.9**	**99.7**	**95.6**	**92.7**	**93.3**	**89.0**	Brand C
RSD %	**0.5**	**1.4**	**1.1**	**9.1**	**6.0**	**4.4**	**6.3**	**1.9**	**3.0**	
Grand AVG	91.2	82.2	81.1	99.3	95.9	98.8	91.4	94.7	96.7	
RSD %	12.6	7.7	10.1	10.1	11.7	9.2	6.8	13.0	12.7	

Table 13. (continue)

COMPOUND	Histamine			Phenylethylamine			Tryptamine			Remark
SPIKE LEVEL	0.25 µg/g	0.5 µg/g	1.0 µg/g	0.25 µg/g	0.5 µg/g	1.0 µg/g	0.25 µg/g	0.5 µg/g	1.0 µg/g	
% Recovery 1	86.8	88.2	87.8	79.6	75.8	74.6	83.2	78.2	73.9	
% Recovery 2	85.6	93.8	90.8	82.4	77.0	74.6	86.4	78.0	73.3	Validation 1
% Recovery 3	78.4	94.8	90.8	84.8	80.2	71.4	84.0	80.6	71.6	
Average	**83.6**	**92.3**	**89.8**	**82.3**	**77.7**	**73.5**	**84.5**	**78.9**	**72.9**	Brand A
RSD %	**5.4**	**3.9**	**1.9**	**3.2**	**2.9**	**2.5**	**2.0**	**1.8**	**1.6**	
% Recovery 1	91.6	94.6	89.3	100.8	99.0	96.5	106.8	103.6	96.6	
% Recovery 2	84.8	91.6	92.3	100.4	94.8	94.9	105.6	97.6	97.6	Validation 2
% Recovery 3	85.2	84.6	90.3	105.6	105.4	99.1	105.6	99.6	93.7	
Average	**87.2**	**90.3**	**90.6**	**102.3**	**99.8**	**96.8**	**106.0**	**100.3**	**96.0**	Brand B
RSD %	**4.4**	**5.7**	**1.7**	**2.8**	**5.4**	**2.2**	**0.7**	**3.0**	**2.1**	
% Recovery 1	92.0	94.0	96.0	94.0	80.6	74.9	79.6	75.4	74.3	
% Recovery 2	92.0	82.0	94.0	91.6	81.8	74.2	76.4	76.6	72.5	Validation 3
% Recovery 3	104.0	94.0	91.0	86.8	79.4	74.0	75.2	73.4	73.4	
Average	**96.0**	**90.0**	**93.7**	**90.8**	**80.6**	**74.4**	**77.1**	**75.1**	**73.4**	Brand C
RSD %	**7.2**	**7.7**	**2.7**	**4.0**	**1.5**	**0.6**	**3.0**	**2.2**	**1.2**	
% Recovery 1	115.8	115.3	117.7	79.2	75.8	76.0	84.0	77.8	77.7	
% Recovery 2	115.0	117.3	119.7	77.6	78.2	74.3	80.4	82.0	76.3	Validation 4
% Recovery 3	117.8	117.3	117.7	79.6	76.2	73.5	81.6	78.8	76.0	
Average	**116.2**	**116.6**	**118.3**	**78.8**	**76.7**	**74.6**	**82.0**	**79.6**	**76.7**	Brand A
RSD %	**1.2**	**1.0**	**1.0**	**1.3**	**1.7**	**1.7**	**2.2**	**2.8**	**1.2**	
% Recovery 1	90.0	79.4	86.6	76.0	75.4	98.6	88.6	80.1	98.3	
% Recovery 2	86.0	74.0	91.6	74.0	70.4	94.7	89.4	75.1	92.6	Validation 5
% Recovery 3	100.8	75.4	89.6	84.4	71.0	98.5	97.0	76.3	98.3	
Average	**92.3**	**76.3**	**89.3**	**78.1**	**72.3**	**97.3**	**91.6**	**77.2**	**96.4**	Brand B
RSD %	**8.3**	**3.7**	**2.8**	**7.1**	**3.8**	**2.3**	**5.1**	**3.4**	**3.4**	
% Recovery 1	86.5	95.7	88.8	95.6	90.2	84.1	91.2	88.0	82.9	
% Recovery 2	88.5	91.7	92.8	99.6	90.0	84.5	92.0	86.0	83.9	Validation 6
% Recovery 3	100.5	97.7	90.8	96.4	89.4	83.2	86.8	86.4	80.9	
Average	**91.9**	**95.0**	**90.8**	**97.2**	**89.9**	**83.9**	**90.0**	**86.8**	**82.6**	Brand C
RSD %	**8.2**	**3.2**	**2.2**	**2.2**	**0.5**	**0.8**	**3.1**	**1.2**	**1.9**	
Grand AVG	94.5	93.4	95.4	88.3	82.8	83.4	88.5	83.0	83.0	
RSD %	11.8	12.8	10.8	9.9	9.8	10.6	9.7	9.0	10.3	

Ion ratio confirmation

Two fragment ions signal from each biogenic amine were collected as chromatograms. The acceptance range was that the peak area ratio should be within 10% difference between samples and calibration standards. The validation results showed that the ratios from each compound in each spiked sample met this requirement. Table 14 showed an example in a validation batch. Three biogenic amines were confirmed in the non-spikes sample that showed low incurred levels. All six biogenic amines spiked in the sample were confirmed by the corresponded ion area ratios.

Table 14. Ion area ratio from spiked tuna. "+": area ratio within acceptable range; "-": area ratio out of acceptable range; "N": no peak

Validation #	V1			V2			V3			V4			V5			V6		
Method Blank																		
Tyramine	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N
Putrescine	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N
Cadaverine	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N
Histamine	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N
Phenylethyl-amine	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N
Tryptamine	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N
Matrix Blank																		
Tyramine	+	+	N	-	-	N	-	-	N	+	+	N	-	-	N	+	+	N
Putrescine	+	+	N	+	+	N	+	+	N	+	+	N	+	+	N	+	+	N
Cadaverine	+	+	N	+	+	N	+	+	N	+	+	N	+	+	N	+	+	N
Histamine	+	+	N	+	+	N	+	+	N	+	+	N	+	+	N	+	+	N
Phenylethylamine	-	-	N	-	-	N	-	-	N	-	-	N	-	-	N	+	+	N
Tryptamine	-	-	N	-	-	N	-	-	N	-	-	N	-	-	N	-	-	N
0.25 ppm spiked																		
Tyramine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Putrescine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cadaverine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Histamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylethylamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tryptamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.50 ppm spiked																		
Tyramine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Putrescine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cadaverine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Histamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylethylamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tryptamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1.00 ppm spiked																		
Tyramine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Putrescine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cadaverine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Histamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylethylamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tryptamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Ruggedness

The retention time (RT) of chromatography peaks was evaluated as the robustness of the LC-MS/MS method. In six validation batches, there were system suitability, calibration standards, ICV, CCV and replicates of spike-recovery samples' injections. The average retention time of each compound's peaks from each injection was calculated. The maximum (MAX) and minimum (MIN) retention each batch were listed to show the shift, then, found out the MAX and MIN in all batches (highlighted in Table 2.4) to calculate the percentage shift by using the difference between MAX or MIN and average RT divided by the average RT. The RT shift was less than 0.2 min, or less than 0.2%. A summary showed in Table 15. The results of spike-recovery, linearity, retention time and detection limits from six validation batches in different days by 3 chemists were well correlated, see Tables 11-15.

High Histamine Sample Analysis

If the concentration of histamine is higher than 12.5 ppm, we reduced the sample amount from 4 grams to 1 gram. Then, diluted the sample extract 12 folds for LC-MS/MS analysis. The overall dilution was 1 gram sample to 240 mL extraction solution. Accordingly, the internal standard, Histamine-D4 was spiked to be 24 ppm in the sample to get 0.1 ppm in the final extract. This modification can analyze the samples with 12.5 to 600 ppm histamine. See Attachment 1, Memorandum of Analysis.

Table 15. Retention time shift range and percentage shift of RT

Batch #	Valida-tion 1		Valida-tion 2		Valida-tion 3		Valida-tion 4		Valida-tion 5		Valida-tion 6		Overall		
Analytes	max	min	max	min	max	min	max	min	max	min	max	min	avg	max%	min%
Tyramine	7.90	7.89	7.74	**7.73**	7.88	7.86	7.85	7.84	**7.90**	7.80	7.81	7.80	7.82	100.0	99.9
Putrescine	**8.28**	8.27	8.13	**8.12**	8.26	8.24	8.24	8.23	8.23	8.21	8.20	8.19	8.22	100.1	99.8
Cadaverine	**8.31**	8.30	8.16	**8.15**	8.29	8.28	8.28	8.26	8.26	8.25	8.24	8.23	8.25	100.1	99.9
Histamine	**8.34**	8.33	8.20	**8.18**	8.33	8.31	8.31	8.30	8.28	8.29	8.27	8.26	8.28	100.2	99.9
Phenylethy-lamine	**8.60**	8.58	8.44	**8.43**	8.58	8.56	8.56	8.55	8.54	8.53	8.52	8.51	8.53	100.1	99.9
Tryptamine	**8.71**	8.70	8.55	**8.54**	8.69	8.67	8.67	8.66	8.66	8.65	8.63	8.62	8.65	107.2	99.9

Conclusions

The validated method is highly sensitive, reliable and would be a great tool in regulatory sample analysis to accurately determine biogenic amines concentrations in canned tuna. The simplified sample extraction method combined with fast and robust LC-MS/MS application makes the method suitable for identification and quantitation of biogenic amines in canned tuna. The method has demonstrated it is fit for use in the analysis of Biogenic amines in canned tuna. The method has a promising application to other fish matrix through matrix extension validation study.

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References

[1] Fish and Fishery Products Hazards and Controls, Fourth Edition, March 2020, https://www.fda.gov/food/seafood-guidance-documents-regulatory-information/fish-and-fishery-products-hazards-and-controls, last accessed: May 21, 2021
[2] CPG Sec 540.525 Decomposition and Histamine Raw, Frozen Tuna and Mahi-Mahi; Canned Tuna; and Related Species; https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-540525-decomposition-and-histamine-raw-frozen-tuna-and-mahi-mahi-canned-tuna-and-related, last accessed: May 21, 2021
[3] Benjamin S. Liao, Jackie Sram, Teresa T. Cain, and Kenneth Halcrow, "Simultaneous Analyses of Histamine, Putrescine and Cadaverine for Scombrotoxic Fish by Cation Ion Chromatography", Laboratory Information Bulletin, (2006) No. 4375 1-14
[4] Benjamin S. Liao, Jackie Sram, Teresa T. Cain, and Kenneth Halcrow,"Aqueous Sulfuric Acid as the Mobile Phase in Cation Ion Chromatography for Determination of Histamine, Putrescine, and Cadaverine in Fish Samples", Journal of AOAC International, (2011) Vol 94, No. 2
[5] Naoki Ochi, "Simultaneous determination of eight underivatized biogenic amines in salted mackerel fillet by ion-pair solid-phase extraction and volatile ion-pair reversed-phase liquid chromatography-tandem mass spectrometry", *Journal of Chrom A*, 1601 (2019) 115-120
[6] Randy L. Self, Wen-Hsin Wu, and Heidi S. Marks, "Simultaneous Quantification of Eight Biogenic Amine Compounds in Tuna by Matrix Solid-Phase Dispersion followed by HPLC–Orbitrap Mass Spectrometry, *J. Agric. Food Chem.* (2011), 59, 11, 5906–5913
[7] Randy L. Self, Wen-Hsin Wu, "Determination of eight biogenic amines in selected seafood products by MSPD extraction followed by UHPLC-Orbitrap MS", *Journal of Food Composition and Analysis* (2012) 27(2):169–173
[8] Randy L. Self, Wen-Hsin Wu, and Heidi S. Marks, "Ultra-high Performance Hydrophilic Interaction Chromatography (UPLC-HILIC) Coupled with Orbitrap Mass Spectrometry for Quantitation of Eight Biogenic Amines", Laboratory Information Bulletin, (2011) No. 4470

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