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Development of a new High Performance Liquid Chromatography-Fluorescence Detection (HPLC-FL) method for the determination of biogenic amines in frozen fish sold in markets

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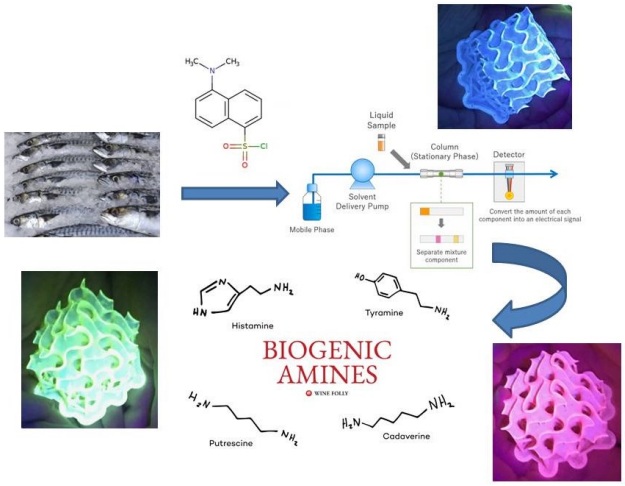
Graphical abstract



Abstract

It is now possible to measure the biogenic amines histamine, tryptamine, putrescine, cadaverine, spermidine, spermine, and tyramine in an easy, accurate, and selective manner. Dansyl chloride was used to purify the analytes, and high-performance liquid chromatography with detection of fluorescence was utilized for separating them. This is a useful method for identifying and measuring biogenic amines in fish samples in a selective manner. The C18 (250 mm × 4.6 mm × 5 µm) column was used for the separation by chromatography. A mobile phase comprising acetonitrile and phosphate buffer (pH=4.0) (60:40, v/v) was used at an average flow rate of 0.9 mL/min. The technique relied on utilizing fluorescence detection to determine the derivative (λ_{ex}=230 nm, λ_{em}= 350 nm) The suggested method's linearity was examined between 0.01 and 50 µg/mL (r^2=0.9996). In compliance with ICH criteria, the method was verified with respect to accuracy, precision, repeatability, specifity, robustness, and detection and quantification limitations. The suggested technique has been effective in tracking the production of biogenic amines in specimens of fish that can be purchased frozen in marketplaces. It was discovered that the recommended approach worked well for routinely analyzing fish sample biogenic amine analyses.

Keywords: Biogenic amines, HPLC, Fluorescence detection, Dansyl chloride, Fish samples

1. Introduction

The nitrogen-containing chemical molecules known as biogenic amines (BAs) are produced when certain fermented foods and beverages, like wine, beer, and some fruit juices, are consumed in excess by humans (Silla 1996). These items of food and drink include cheese, sausage, fermented vegetables, salami, and seafood. Human exposure to BAs is significant because of the possibility of toxicity. The quantity of BAs in food products is thought to serve as a quality indicator (Ancın-Azpiliceata *et al.* 2008). Foods with low concentrations of BAs can be tolerated since intestinal amine oxidase facilitates the detoxifying process. However, at high BA concentrations, amine oxidase's detoxifying process malfunctions, which can lead to major health issues (Spano *et al.* 2010). For each BA separately, the acceptable amounts that could be present in food products as well as the dangerous values were specified; however, data is lacking for a few of them. Additionally, each individual may have distinct hazardous limits as a result of food intolerance, allergy characteristics, genetics affecting the function of their enzymatic systems, and use of certain medications that restrict the action of their enzymes (Herrero *et al.* 2016; Ngapo *et al.* 2017).

Based on the chemical structure (Figure 1), three categories of BAs can be distinguished. Among these are the aliphatic BAs putrescine and cadaverine, the aromatic BAs tyramine and phenylethylamine, and the heterocyclic BAs histamine and tryptamine. The amount of amine groups is the basis for another classification, which includes poly amines (spermidine and spermine), diamines (histamine, putrescine, and cadaverine), and mono amines (tyramine and phenylethylamine) (Tırıs *et al.* 2023). Bacterial decarboxylation of amino acids in food products produces beta-amylases (BAs), the amount of which varies depending on a number of factors during the

manufacturing process, including raw material hygiene, microbial makeup, fermentation conditions, and fermentation time. Critical criteria to regulate the rise in the content of bisphenol A (BAs) in food goods packaging include additives, hydrostatic pressure, irradiation, pasteurization, smoking, starting culture, and temperature (Ramos *et al.* 2014; Kiss *et al.* 2006).

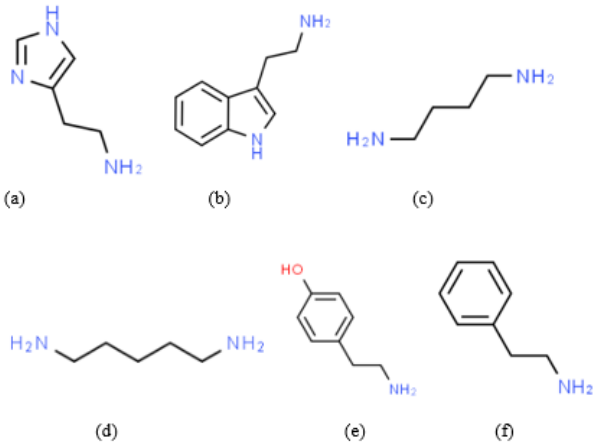


Figure 1. Chemical structures of some biogenic amines (a: histamine, b:tryptamine, c:putrescine, d:tryptamine, e:tyramine, f:phenylethylamine)

Food products' levels of BAs are significant indicators of their quality and healthfulness. Urine and human plasma concentrations are important markers of BA exposure as well. As a result, sensitive, straightforward, quick, and affordable analytical techniques are constantly needed. When we look at the literature, there are many methods for the determination of biogenic amines by liquid chromatographic methods (LC-MS/MS, LC-UV and LC-FL) (Byun and Mah, 2012; Romero-Gonzalez *et al.* 2012; Pawul-Gruba *et al.* 2014). Mass spectrometry is a unique analytical technique, but it is expensive when integrated with liquid chromatography. In addition, biogenic amines analyzed using UV and FL detectors, although cheap and simple methods, have disadvantages due to low sensitivity, long sample preparation procedures and complex chromatographic methods (gradient elution). The method we developed stands out with its features such as simple mobile phase, isocratic elution, simple sample preparation procedures, high sensitivity, high recovery and high reproducibility.

In this work, biogenic amines (histamine, tryptamine, putrescine, cadaverine, spermidine, spermine, and tyramine) in ten distinct frozen fish samples that were sold in markets were identified using a novel HPLC-FL approach that was developed and verified. The approach that has been devised is highly beneficial in identifying biogenic amines present in frozen fish-based goods.

2. Materials and methods

2.1. Chemicals and reagents

Sigma Aldrich (St. Louis, USA) provided the histamine, tryptamine, putrescine, cadaverine, spermidine, spermine, tyramine, and NBD-Cl. Merck (Darmstadt, Germany) provided the acetonitrile, monobasic and dibasic dihydrogen phosphate, hydrochloric acid (HPLC quality), and hexane (analytical quality). Human (Japan) ultrawater purification system was used for purifying the water.

2.2. Solutions

Biogenic amines (0.1 mg/mL) were produced as an initial solution and adjusted with water to yield standards ranging from 0.01 to 50 µg/mL. In 50 milliliters of water, 2.0209 grams of sodium phosphate dibasic and 0.3394 grams of sodium phosphate monobasic solution were combined to create phosphate buffer. A solution of 0.1 M hydrochloric acid was used to bring the pH level down to 4, and water was added to fill the container to a capacity of 100 mL. Fresh NBD-Cl solution was prepared at a dose of 5 mg mL-1 in methanol. The remainder of the solutions remained stable for at least two weeks when kept in storage at 4 ºC.

2.3. Derivatization process

The efficiency of the reaction product was examined and adjusted for the reaction circumstances of biogenic amines with NBD-Cl. While the other values remained unchanged, each parameter was altered independently. In order to stop the derivatization reaction, the ideal reaction time, temperature, pH, buffer type, amounts of acetonitrile-phosphate buffer, mole ratio of NBD-Cl/biogenic amines, concentration of HCL, and amount employed for acidification were all calculated.

2.4. Derivatization and sample preparation

For two minutes, 5% TCA was used to homogenize the 0.1 g of samples. After centrifuging at 3000 g for 10 minutes at 4 ºC, the supernatant was retrieved. 5% TCA was used in an equivalent amount to remove the residue. After combining the two supernatants, the volume was adjusted with 5% TCA to 25 mL. For 30 minutes at 60 ºC, the extract that had been pH-9.5 adjusted reacted with 10 mg/mL DNS-Cl to derivatize. To get rid of any remaining DNS-Cl, the reaction mixture was combined with 25% NH4OH and allowed to sit at room temperature for 30 minutes. After combining the solution vigorously for 30 seconds using a vortex mixer, 20 µL of the derivatized sample was added to the HPLC apparatus.

2.5. Instrumentation

The Shimadzu (Japan) LC 20 liquid chromatograph, which was equipped with a CTO 10 AC column oven, a SIL AH-HT autosampler component, an SPD-20A HT FL detector that was calibrated at 230 nm for excitation and 350 nm for emission, and an LC-20AT pump, was utilized for the HPLC studies. We used a GL Sciences (Japan) C18 (ODS) column with dimensions of 4.6 mm I.D., 250 mm length, and 5 µm particle size to accomplish isocratic chromatographic separation at 30 ºC. Acetonitrile-phosphate buffer (pH 4.0) was used as the phase of mobility, and its flow rate was 0.9 mL/min.

3. Results and discussion

3.1. Preparation of the calibration curve

The preparation of the calibration curve involved analyzing standard solutions containing biogenic amines at different concentrations ranging from 0.01 to 50 µg/mL. The techniques linear concentration ranges (each concentration was examined in a total of five imitates)

were identified using linear least-squares regression analysis. The calibration curve's equation, y = 89.544x + 35.641, was computed, where x denotes the biogenic amine concentration in 0.01–50 µg/mL and y represents the peak regions.

3.2. Chromatographic conditions

Chromatographic separation under isocratic elution was performed using a C18 Column (4.6×250mm×5µm), with the column temperature maintained at 30 ºC throughout the study. With a flow rate of 0.9 mL min-1, a mobile phase consisting of a mixture of acetonitrile (Mobile phase A) and phosphate buffer (pH adjusted to 4.0 with NaOH) (Mobile phase B) was utilized. Following an excitation wavelength of 230 nm, a 350 nm emission wavelength was used for fluorimetric detection. Chromatograms characteristic of this kind are shown in Figure 2. In the previous study, the retention time for histamine was approximataly 25 min (Arrieta and Prats-Moya, 2012). In our method, the retention time for histamine was 2.6 min. This highlights our method as a quality parameter.

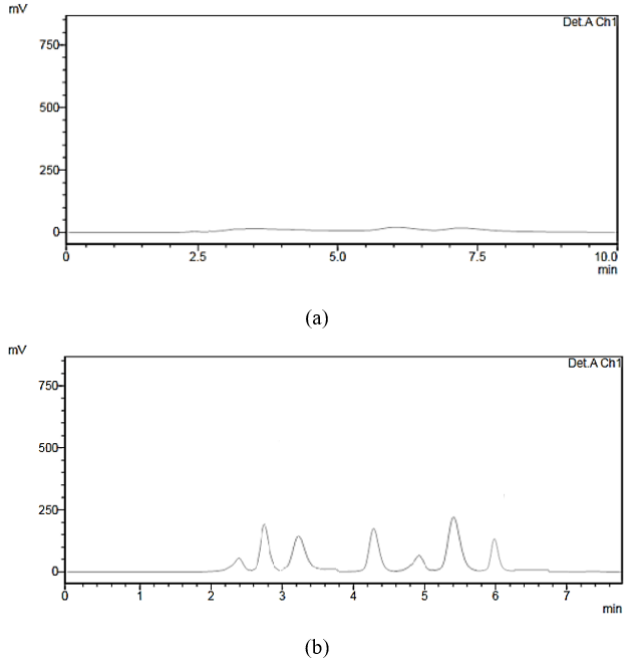


Figure 2. Aqueous medium (blank) and real sample (frozen fish) are shown in a and b.

3.3. Validation of the method

The International Conference on Harmonization (ICH) provided the following guidelines, which were followed during the method's validation (ICH 2005).

3.3.1. The limit of detection (LOD) and limit of quantitation (LOQ)

The formula used to calculate LOD and LOQ was LOD or LOQ=kSDa/b, where k=3 for LOD and 10 for LOQ, Sda being the intercept's standard deviation, and b being the slope. Table 1 provides an overview of the characteristics governing the analytical efficacy of the suggested approach. In the previous study, the lowest LOD value for biogenic amines was determined as 0.1 mg/mL (Tuberoso *et al.* 2017). However, in our current study, the LOD value was found to be 0.003 µg/mL. This highlights our method as a quality parameter.

Table 1. Analytical parameters of the method

Parameters	Method
Concentration range [a] (µg mL^{-1})	0.01-50
Regression equation[b]	y=89.544x+35.641
Intercept± SD	35.641 ± 1.213
Slope± SD	89.544 ± 2.886
Correlation coefficient (r^2)	0.9996
LOD (µg mL^{-1})	0.003
LOQ (µg mL^{-1})	0.01

[a]Average of six determinatons

[b]y=xC +b where C is the concentration in µg mL^{-1} and y is the peak area

3.3.2. Accuracy, precision and recovery

The determination of QC samples at three concentration levels was used to measure precision and accuracy. Fish specimens with three distinct concentrations (0.01, 5 and 50 µg/mL) were prepared, which are then categorized as low, medium, and high concentrations (n = 5). Recovery values were used to convey accuracy, and RSD values of the recovery results in five imitate trials were used to determine the precision of the recovery research. By extracting spiked fish samples and comparing the peak regions collected from the same volumes of water unextracted biogenic amine solutions, the absolute recovery of biogenic amines from fish samples was studied. Comparing the quantities injected to the spiked and derived from the calibration curve yielded a mean relative recovery of 99.36%.

For daily precision and accuracy, five duplicates of all concentrations of material were measured on one single day, and for hourly precision and accuracy, five distinct days were chosen. Both the daily and hourly assays had RSD values that were less than 3.91%. Table 2 summarizes all these findings and shows that satisfactory precision and accuracy were noted. In the previous study, intra-day and inter-day RSD values were found to be lower than 4.86% (Angula *et al.* 2020). In our study, this value was 3.91%. This is another indication of how sensitive out work is.

3.3.3. Robustness

Determining the QC samples at three concentration levels—as mentioned in the validation section above—was how robustness was evaluated (n=3). The variables that were altered by Ara in order to assess the method's resilience are the flow rate, temperature of the column oven, acetonitrile concentration, and the mobile phase's water-based contents. The relative amounts of the mobile phase (acetonitrile-buffer solution) were adjusted from 60:40 to 55:45 and 65:35. Additionally, the flow rate was modified from 0.9 to 0.7 and 1.0 mL/min, and the oven temperature was changed from 30 ºC to 25 ºC and 35 ºC. The outcomes were displayed in Table 3. Peak area and resolution were not significantly impacted by these modifications. The figures from our prior study (Serim *et al.* 2023) are not as good as the recent recovery percentage

values of 101.15, 103.19, and 100.15 which shows a significant progress.

Table 2. The results of the accuracy and precision studies

Existant concentration (µg mL^{-1})	Added concentration (µg mL^{-1})	Found concentration (µg mL^{-1}) (Mean±SD[1])	Recovery (%)	RSD of recovery	RSD of intraday variation	RSD of interday variation
	0.01	4.99±0.22	99.80	1.26	2.67	3.91
5	5	9.87±1.04	98.70	1.81	2.52	3.90
	50	54.78±2.74	99.60	1.33	2.62	3.83
Mean relative recovery = 99,36						

For each concentration n=3

Table 3. Results of the robustness

Condition	Value	Recovery %	RSD %
Flow rate (mL min^{-1})	0.8	101.15	1.46
	1.0	102.02	1.41
Mobile phase composition (methanol:aqueous phase)	55:45	103.19	3.43
	65:35	104.11	3.41
Column temperature	25	100.15	2.73
	35	100.63	2.03

n=3 for all QC sample levels

Table 4. Biogenic amines concentrations and method reproducibility

Sample number	Recovery	%RSD	HIS µg/mL	TRP µg/mL	PUT µg/mL	CAD µg/mL	SPD µg/mL	SPM µg/mL	TYR µg/mL
1	% 82.3	2.44	1.11	0.63	N.D	1.01	0.92	N.D	N.D
2	% 80.6	3.73	0.88	0.75	1.20	N.D.	N.D.	1.41	1.25
3	% 88.4	2.27	3.42	2.94	0.85	0.62	0.44	0.16	0.39
4	% 86.5	2.35	2.53	3.72	1.28	1.12	0.36	0.08	0.27
5	% 85.6	2.14	2.32	4.16	1.04	0.98	0.21	N.D.	0.12
6	% 87.5	2.97	2.88	3.14	0.65	0.52	0.30	0.04	0.21
7	% 79.4	3.11	0.80	0.48	N.D.	0.93	0.77	N.D.	N.D.
8	% 78.6	3.24	0.68	0.40	N.D.	0.85	0.70	0.02	N.D.
9	% 81.4	3.62	0.81	0.86	1.34	0.46	N.D.	1.24	1.05
10	%80.6	3.70	0.77	0.66	1.18	N.D.	0.22	0.85	1.35

HIS:histamine, TRP:tryptamine, PUT:putrescine, CAD:cadaverine, SPD:spermidine, SPM:spermine, TYR:tyramine, N.D.:not detected, RSD:relatedive standard deviation

3.3.4. Stability

Prior to assessment, four freeze-thaw cycles have been applied to spiked biogenic amines standards at 0.01, 5, and 50 µg/mL in order to examine the impact of freezing and thawing on biogenic amine concentrations. Additionally, the stability of the biogenic amines standard was assessed after being kept at ambient temperature for a full day and at -20 ºC for a period of two weeks. When kept at -20 ºC, stock solutions of biogenic amines remained stable for a minimum of a full month. The concentration of biogenic amines in fish samples did not drop the following twenty consecutive days.

3.4. Examination of actual samples (frozen fish)

Following technique and validation experiments, biogenic amine concentrations in frozen fish samples were calculated and are presented in Table 4.

4. Conclusion

A class of compounds known as BAs is created from amino acids by the processes of decarboxylation, amination, and transamination of aldegydes and ketones. They can be classified as heterocyclic, aromatic, or aliphatic compounds based on the precursor amino acids. Not only are they harmful to people, but some of them are necessary for optimal physiological function in humans. As a result of their low volatility and the ability to derivatize their functional groups for spectrophotometric detection, liquid chromatography (LC) techniques are more preferred over gas chromatography (GC).

In overall, fluorogenic characteristics and chromophore groups are absent from BAs. As a result, scientists frequently create methodologies for analysis that rely on derivatization processes. From the same angle, this research assessed the biogenic levels in frozen seafood available in markets and discovered that they were non-toxic.

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

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