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METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF HISTAMINE IN FERMENTED FOOD PRODUCTS USING FIRST DERIVATIVE UV-VISIBLE SPECTROPHOTOMETRY

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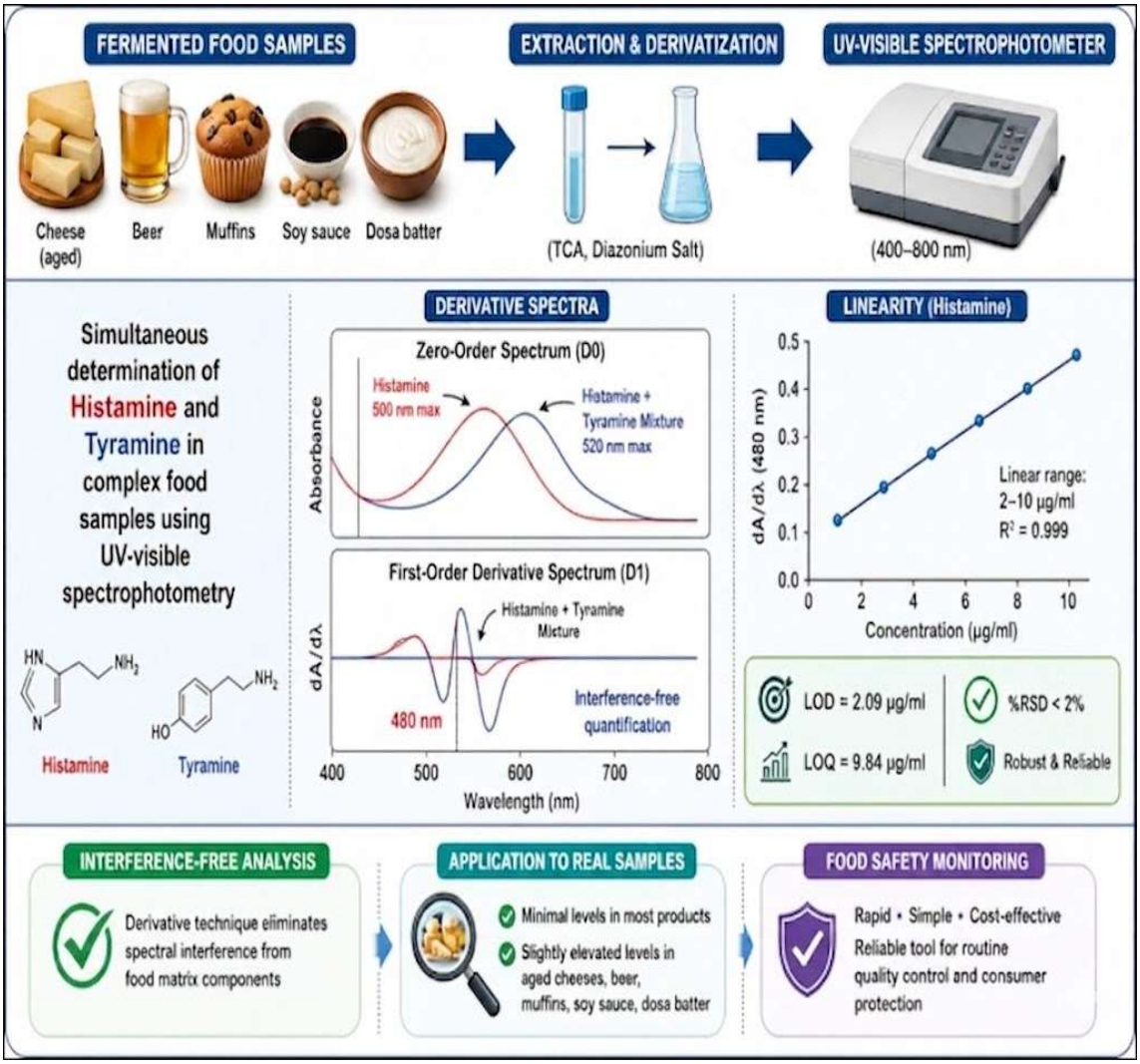
ABSTRACT

Biogenic amines such as histamine and tyramine are naturally occurring compounds in various foods, particularly fermented products, and can pose significant health risks when present in excess. This study presents a simple, sensitive, and interference-free UV-visible spectrophotometric method employing first-order derivative analysis for the simultaneous determination of histamine in fermented food matrices. The wavelength of histamine 480nm enabled selective quantification, even in mixed samples. The technique showed good linearity of histamine (2-10µg/ml), with low limits of detection 2.09µg/ml and quantification 9.84 µg/ml. Precision studies yielded %RSD values below 2% and robustness tests confirmed reliability under minor operational changes. Application to commercial samples revealed minimal histamine levels in most products, with slightly elevated concentrations in aged cheeses, beer, muffins, soy sauce, and dosa batter. This validated approach offers an efficient tool for food safety monitoring and quality control, ensuring consumer protection against BA-related health hazards. The developed method was further validated according to standard

analytical validation guidelines, including parameters such as accuracy, specificity, repeatability, and intermediate precision. Recovery studies demonstrated satisfactory accuracy with percentage recoveries within the acceptable range, confirming the reliability of the proposed method for routine analysis. The derivative spectrophotometric technique minimized spectral interference from other food components, allowing precise determination of histamine even in complex fermented food matrices. Sample preparation involved simple extraction procedures, making the method rapid and cost-effective compared to advanced chromatographic techniques. Overall, the proposed analytical approach provides a practical alternative for laboratories involved in food safety evaluation and regulatory monitoring of biogenic amines in fermented food products.

Keywords: Histamine, First derivative, fermented food, tyramine, UV-Visible spectrophotometry

GRAPHICAL ABSTRACT



INTRODUCTION

The amino acid histidine is microbially decarboxylated to produce histamine, a naturally occurring biogenic amine **[1]**. It plays a crucial role as a mediator in several physiological processes, including gastric acid secretion, inflammation, and immunological responses. Histamine accumulation represents a significant food safety concern, particularly in fish species such as tuna, mackerel, and sardines, as well as in fermented products including cheese, sausages, wine, and soy-based foods, where it is primarily associated with microbial activity during spoilage or fermentation **[2]**. Excessive histamine intake can lead to adverse health effects such as flushing, headaches, pruritus, and gastrointestinal disturbances; in severe cases, it may result in scombroid poisoning **[3, 4, 5]**. Histamine concentrations in the range of 50–200 mg/kg may elicit moderate toxic responses in sensitive individuals, whereas levels below 50 mg/kg are generally considered safe **[6]**. Concentrations exceeding 200 mg/kg are regarded as hazardous and may pose serious health risks. Therefore, continuous monitoring of histamine levels is essential to ensure food quality and consumer safety **[7, 8, 9]**.

The literature review indicates that histamine has been determined using various analytical techniques, including high-performance liquid chromatography (HPLC) **[10-14]** thin-layer chromatography (TLC), liquid chromatography–mass spectrometry (LC–MS) **[15-18]**, gas chromatography–mass spectrometry (GC–MS) **[20, 21]**, hyphenated HPLC–GC methods **[22]**, gas chromatography (GC) **[23]**, and nuclear magnetic resonance (NMR) spectroscopy **[24]**. Although these methods are highly sensitive and reliable, they often involve complex sample preparation, derivatization procedures, and the requirement for sophisticated instrumentation. Notably, none of the reviewed studies report the development or validation of a first-derivative UV spectrophotometric method for the estimation of histamine in fermented food matrices. This highlights a significant analytical gap, emphasizing the need for a simple, rapid, and cost-effective spectrophotometric approach suitable for routine analysis **[25-47]**.

Therefore, the present study aims to develop and validate a novel first-derivative UV spectrophotometric method for the estimation of histamine in fermented food products. This method is intended to serve as an accessible alternative to conventional chromatographic techniques, facilitating efficient monitoring of biogenic amines for food safety evaluation and quality assurance.

MATERIALS AND METHODS

UV-Vis spectrophotometer Shimadzu UV-1900i with a laboratory solution The approach was developed and validated using UV Vis. A 1-cm quartz cell was used for spectrophotometric analysis between 200 and 400 nm. Weighing was done using the Infra Digi RS 232 analytical balance. Histamine and tyramine (99.78%) pure was obtained from oxford lab Fine Chem LLP, Milli q water was obtaine from Milli Q water purification system.

METHODOLOGY

Preparation of Standard and Working Solutions

Standard stock solutions of histamine and tyramine (1000 µg/mL) were prepared by dissolving 0.1 g of each analyte in 10 mL of distilled water using volumetric flasks. An intermediate working solution (100 µg/mL) was prepared by a 1:10 dilution of the stock. For the final working solution (10µg/mL), 1 mL of the intermediate solution was transferred to a 10 mL volumetric flask. To this, 5 mL of sodium carbonate and 2 mL of p-phenyl diazonium sulfonate reagent were added to facilitate the diazotization reaction, and the volume was adjusted with distilled water.

Sample Extraction and Processing

Sixteen diverse food matrices—including fermented dairy (curd, cheese, fermented milk), grain-based products (dosa batter, bread, muffins), and beverages (beer, soy sauce)—were analyzed. A 5 g portion of each sample was homogenized in 20 mL of 5% TCA and centrifuged at 3000 rpm for 10 min. The resulting supernatant was filtered through Whatman No. 1 paper and diluted to 50 mL with 5% TCA.

For further purification, 1 mL of the filtrate was combined with 2 mL of 5% TCA, 0.5 g of a salt mixture, (6.25g anhydrous sodium sulfate and 1g trisodium phosphate monohydrate) and 2 mL of methanol. After centrifugation at 3000 rpm for 15 min, the supernatant was evaporated to dryness. The residues were reconstituted in distilled water and derivatized using the optimized sodium carbonate/p-phenyl diazonium sulfonate system.

Spectrophotometric Conditions and Derivative Transformation

Absorption spectra were acquired in the UV-Vis range (400–800 nm) against a reagent blank. Zero-order spectra (D0) were transformed into first-order derivative spectra (D1) using a scaling factor of 10 and a $\Delta\lambda$ 2 nm. The UV spectrum of histamine and mixture of histamine and tyramine in deionized water has shown lamda max at 500nm and 520nm for zero order and 480 for first order respectively.

Method Validation

The analytical procedure was validated according to ICH Q2(R2) guidelines (Qarah & El-Maaiden, 2023) **[28-31]**.

Linearity and Range

Linearity was evaluated over a concentration range of 2–10 µg/mL for both pure histamine and histamine-tyramine mixtures. First-derivative absorbance values were recorded in triplicate, and a calibration curve was constructed by plotting the derivative response against concentration.

Precision and Repeatability

Method precision was determined through six replicates of 10 µg/mL histamine standards.

Repeatability (Intra-day)

Assessed by six consecutive injections under identical conditions.

Intermediate Precision (Inter-day)

Evaluated by analyzing six replicates on two different days within a one-week period.

Robustness

Robustness was tested by introducing deliberate minor variations in the detection wavelength ±2 nm at 478 nm and 482 nm. The relative standard deviation (RSD) of the absorbance values was calculated from five replicate injections at each condition.

The limits of detection and quantification for histamine were determined using the following equations, which utilized the standard deviation of the calibration curve:

DL = SD × 3.3 / slope

QL = SD × 10 / slope

RESULTS AND DISCUSSION

Optimization: The primary goal of the study was to create a straightforward, effective, sensitive, and precise analytical technique for measuring histamine without interference from tyramine, if it was present. The simplest and most popular way for creating analytical calibration is the zero-crossing technique. It is difficult to measure histamine in the presence of histamine using conventional UV spectrophotometry because a zero-order or D0 absorption of histamine showed substantial overlap with tyramine in deionized water **(Figure 1 and 2)**. First-order derivative UV spectrophotometry was successfully used to solve this issue. Following first-order derivatization of the spectra of the histamine and tyramine combination, sharp bands with larger amplitudes were found, permitting more precise identification estimate of histamine. At the zero-crossing of histamine at 480 nm (D1480), the first-order derivative amplitude of histamine was measured using a scaling factor of 10 and an interval of $\Delta\lambda = 2$. These particular wavelengths were selected since it was found that they were the best at removing interference when measuring the amount of histamine in the combination.

Method Validation

Linearity

The results were found to be linear for histamine over the range of 2.00–10.00 µg/, in accordance with Beer and Lambert's law. The linearity of the constructed calibration

curve was validated by the high correlation coefficient values. the analytical results from the standard mixture of histamine and tyramine and the calibration curves of histamine. UV spectra of histamine's first-order derivative overlay, histamine's calibration curve, and the standard mixture's linearity curve. For histamine, the linear regression equation and regression coefficient were determined to be y = 0.0018x – 0.0016 and R2 = 0.09993, while for the standard mixture, the results were y = 0.0036x – 0.0016 and R2 = 0.09993.

Precision

Method repeatability and intermediate precision were used to evaluate the developed method's precision. For tyramine, the relative standard deviation values for method repeatability were 0.09772. The absorbance was measured and the RSD was calculated for two days in a row with intermediate precision. For day 2, the RSD values were 0.0499, 0.0499. The precision test was passed since the final RSD was found to be less than 2% **Table 1**.

Robustness

Evaluated the robustness of the wavelength variation (Absorbance at 478nm and 482nm) method. **Table 2** contained the results. Since there was less than 2.0% difference between the initial and sample results, the method passed the robustness test.

Limit of detection and limit of quantification

Low DL and QL values were found in the evaluation of the developed UV method's DL and QL. For histamine, the corresponding DL and QL values 2.09µg/ml and quantification 9.84 µg/ml.

Histamine content in tested fermented food products

A total of 16 samples consisting of marketed curd (5 samples), cheese (4 samples), dosa batter (1 sample), bread (2 sample), fermented milk (1 sample), soya sauce (1 sample), beer (1 sample) and muffins (1 sample) were analysed by developed UV/Visible method. These samples underwent testing for five days in a row. the fermented food sample's histamine content (mg/100g) was below the USFDA-recommended acceptable limit. Because it was a market sample, the handling, storage, and presence of bacteria that produce histamine affected the amount of histamine produced in the fish sample.

Interpretation of results

The histamine content of sixteen fermented food products was analyzed for five consecutive days using the first-derivative UV spectroscopic method. The study aimed to assess the influence of fermentation duration on histamine formation, since histamine—like tyramine—is a biogenic amine generated through microbial

decarboxylation of histidine during fermentation.

During the first two days (Day 1 and Day 2), no detectable levels of histamine (NF: Not Found) were observed in any of the samples. Detectable levels of histamine appeared only from Day 3 onward, and even then, in limited samples, mainly the cheese-based products and a few other fermented foods.

This delayed appearance indicates that histamine formation begins only after prolonged fermentation or storage, when microbial decarboxylase activity reaches measurable levels.

Curd samples (1–3), Bread, Cake bread, Cheese balls, Fermented milk, and Lassi

Histamine was not detected (NF) throughout the five-day study period. These products, although fermented, possibly underwent mild fermentation with low histidine decarboxylase activity, or contained microorganisms that do not favour histamine production. The absence of histamine could also result from lower free histidine substrate or short fermentation duration.

Cheese samples (1–4)

Histamine was first detected on Day 3 in all cheese samples, remaining constant through Day 5. Cheese sample 1, 3, and 4 showed 0.026 mg/100 g. Cheese sample 2 recorded a slightly higher value of 0.027 mg/100 g. The presence of histamine only in cheese samples is consistent with literature, as cheese ripening involves proteolysis and microbial decarboxylation processes that liberate histidine and convert it into histamine. The steady concentration from Day 3 to Day 5 indicates that histamine formation reached a plateau, possibly due to limited substrate availability or reduced microbial activity in later stages.

Beer, Muffins, Soy sauce, and Dosa batter

Each of these samples exhibited a low but consistent histamine concentration of 0.026mg/100 g from Day 3 to Day 5. These low values may result from tracemicrobia activity during storage or residual amine formation from raw materials. Despite being fermented or aged products, histamine levels remained negligible compared to typical safety thresholds.

Table 1: Results of Linearity of histamine standard and standard mixture

S. No.	Concentration (µg/ml)	Absorbance of Std Histamine	Absorbance of Std Mixture
1	2	0.0021	0.0021
2	4	0.0053	0.0054
3	6	0.0091	0.009
4	8	0.0128	0.0128
5	10	0.0162	0.0162

Table 2: Results of precision

Concentration of Histamine (µg/ml)	Method Repeatability	Intermediate Precision	
	Absorbance	Day 1	Day 2
10µg/ml	0.017	0.017	0.017
10µg/ml	0.015	0.015	0.015
10µg/ml	0.016	0.016	0.016
10µg/ml	0.013	0.017	0.017
10µg/ml	0.017	0.016	0.016
10µg/ml	0.014	0.017	0.017
MEAN	0.015333	0.016	0.0163
SD	0.001491	0.00081	0.00081
%RSD	0.09722	0.0499	0.0499

Table 3: Robustness of the proposed analytical method

S. No.	Concentration (µg/ml)	478nm	480nm	482nm
1	10	0.014	0.017	0.013
2	10	0.012	0.016	0.011
3	10	0.012	0.017	0.013
4	10	0.015	0.015	0.011
5	10	0.011	0.016	0.012
6	10	0.013	0.017	0.013
MEAN		0.012833	0.014333	0.012167
SD		0.001344	0.000745	0.000898
%RSD		0.104705	0.052002	0.073769

Table 4: Histamine content in fermented food products (mg/100g)

Samples	Day-1	Day-2	Day-3	Day-4	Day-5
1.Curd sample 1	-	-	-	-	-
2. Cheese balls	-	-	-	-	-
3. Bread	-	-	-	-	-
4. Cake bread	-	-	-	-	-
5. Curd sample 2	-	-	-	-	-
6. Curd sample 3	-	-	-	-	-
7. Yakult	-	-	-	-	-
8. Lassi	-	-	0.026mg	0.026mg	0.026mg
9. Cheese sample 1	-	-	0.027mg	0.027mg	0.027mg
10. Cheese sample 2	-	-	0.026mg	0.026mg	0.026mg
11. Cheese sample 3	-	-	0.026mg	0.026mg	0.026mg
12. Cheese sample	-	-	0.026mg	0.026mg	0.026mg
13. Beer	-	-	0.026mg	0.026mg	0.026mg
14. Muffins	-	-	0.026mg	0.026mg	0.026mg
15. Soy sauce	-	-	0.026mg	0.026mg	0.026mg
16. Dosa batter	-	-	0.026mg	0.026mg	0.026mg

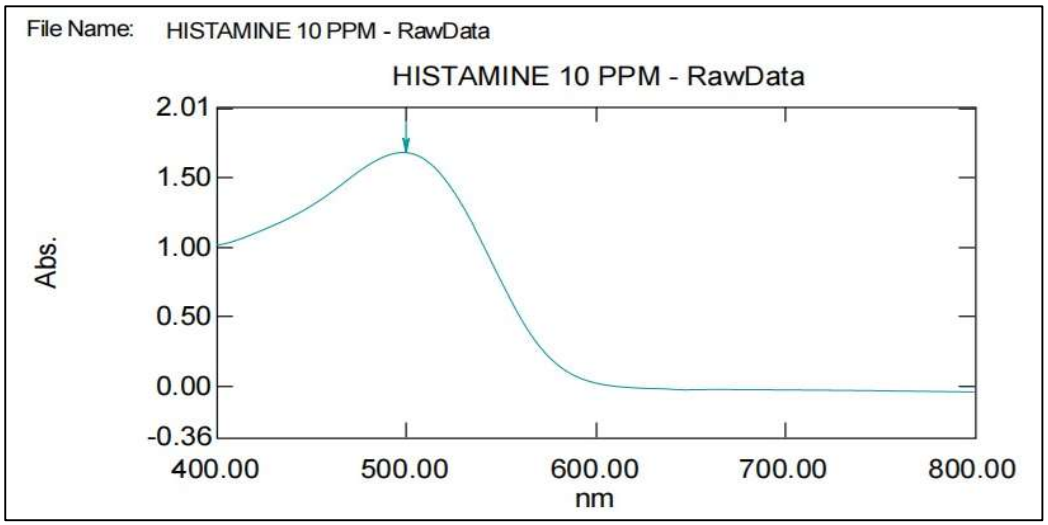


Figure 1: Zero order spectrum of histamine

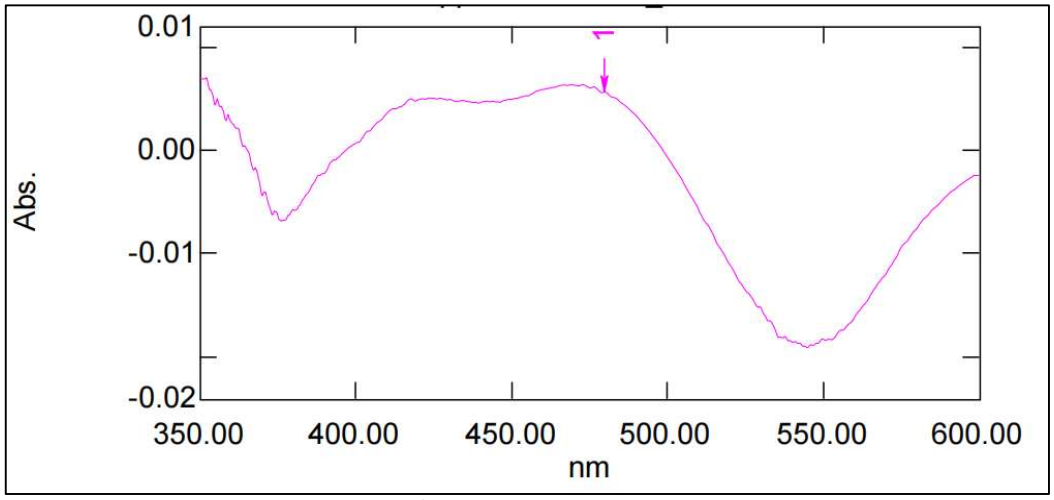


Figure 2: 1st Derivative spectrum of histamine

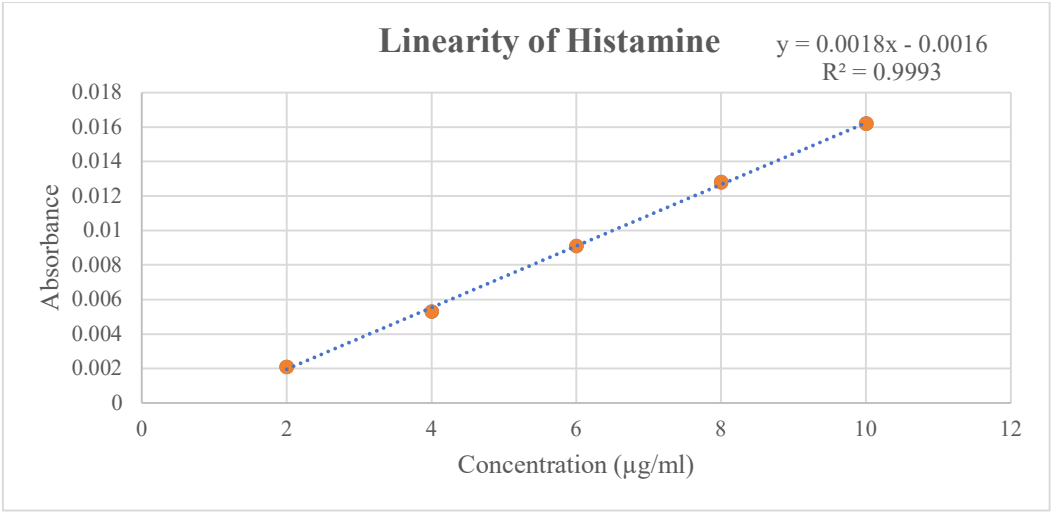


Figure 3: Linearity of Standard Histamine

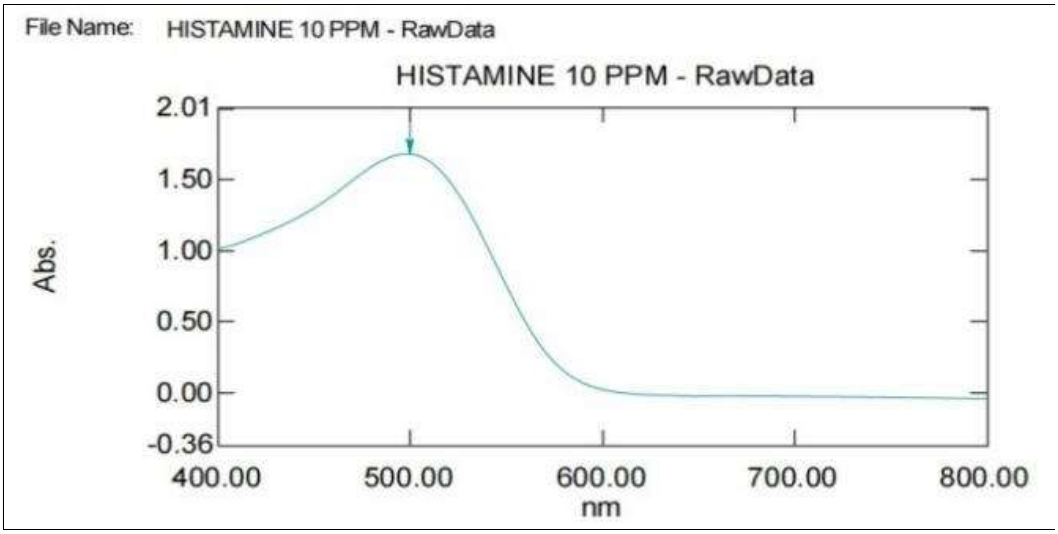


Figure 4: Zero order spectra of histamine (10µg/ml)

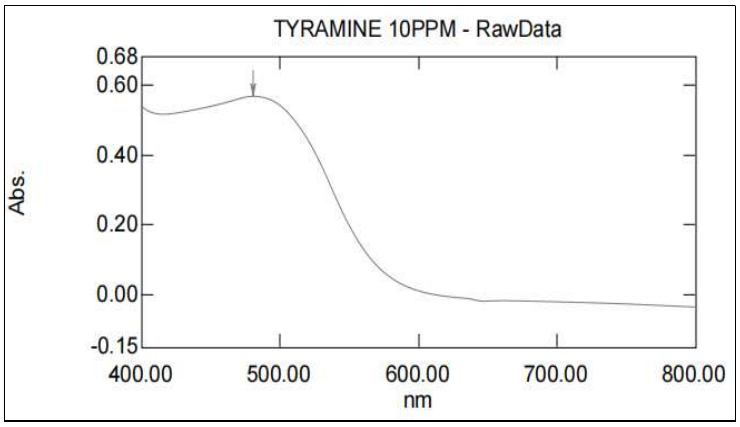


Figure 5: Zero order spectra of tyramine (10µg/ml)

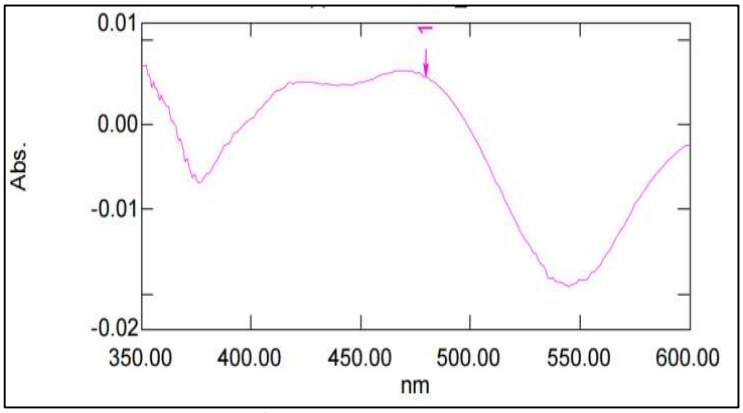


Figure 6: 1st Derivative spectrum of histamine

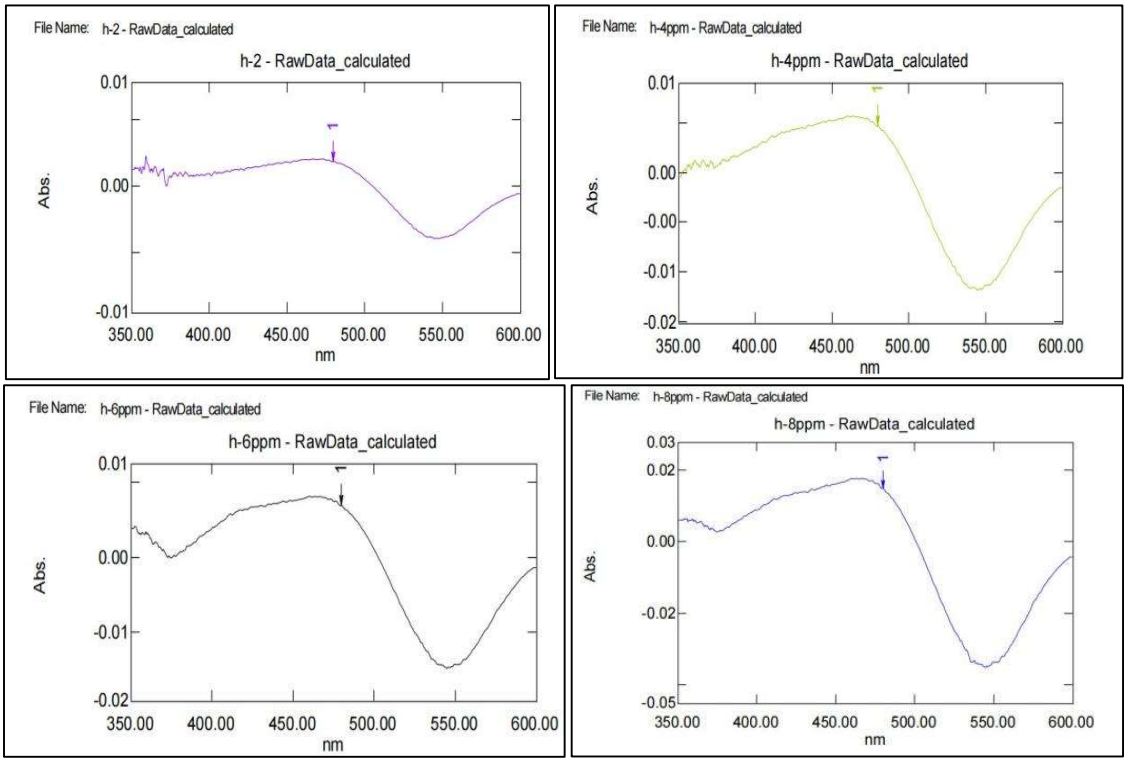


Figure 7: Linearity graphs of histamine

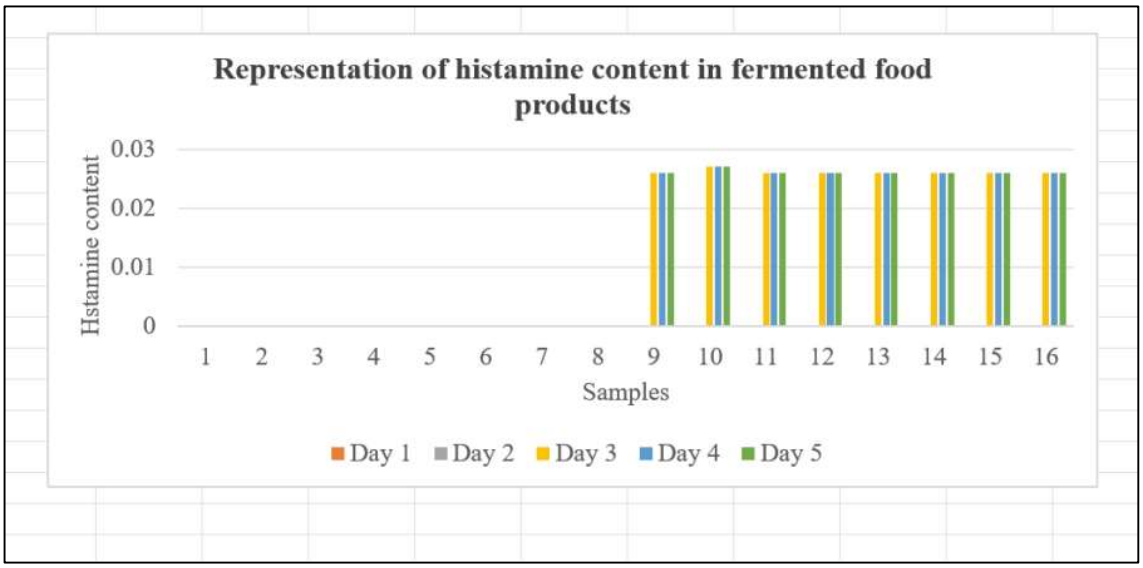


Figure 8: Representation of Histamine content in food products

CONCLUSION

This study presents a validated first-order derivative UV–visible spectrophotometric method for the selective and reliable determination of histamine in fermented food matrices. The method exhibited excellent analytical performance, with good linearity (2–10 µg/mL), low detectionn and quantification limits are 2.09µg/ml and quantification 9.84 µg/ml. and high precision (%RSD < 2%). Derivative spectroscopy effectively minimized spectral interference, enabling accurate quantification at 480 nm even in complex sample systems. Application to commercial products demonstrated that histamine formation is both time- and matrix-dependent, with detectable levels appearing only after extended fermentation. Aged cheese showed relatively higher histamine concentrations, whereas other products such as beer, soy sauce, muffins, and dosa batter contained only trace levels within safe limits. The method's simplicity, rapid analysis, and cost-effectiveness make it a practical alternative to chromatographic techniques. Overall, this approach is well-suited for routine food safety monitoring, supporting regulatory compliance and consumer protection against biogenic amine-related risks.

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Author contributions

Authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work, ensuring its accuracy and integrity.

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