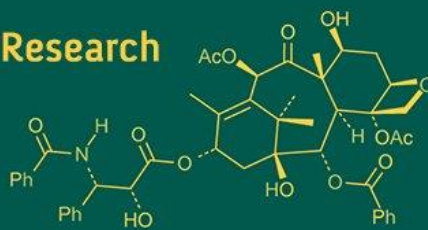


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Development and validation of spectrophotometric assays for rapid assessment of spoilage biomarkers in fresh chicken meat

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Abstract

This study developed and validated simple spectrophotometric methods for the evaluation of biochemical spoilage biomarkers in fresh chicken meat stored at 4 ± 1 °C for 7 days. Hypoxanthine, histamine and trimethylamine nitrogen (TMA-N) were selected as representative biomarkers based on their association with different spoilage-related biochemical pathways, including post-mortem nucleotide degradation, microbial decarboxylation of histidine and formation of volatile nitrogenous compounds. The analytical methods were optimized for application in the chicken meat matrix and validated for linearity, limit of detection, limit of quantification, recovery and precision. The developed assays showed reliable analytical performance, with linearity values of 0.9688, 0.9955 and 0.9996 for hypoxanthine, histamine and TMA-N, respectively. During refrigerated storage, all three biomarkers increased significantly, indicating progressive biochemical deterioration of chicken meat. Hypoxanthine increased from 1.13 to 3.95 µg/g, histamine from 20.13 to 73.03 mg/kg and TMA-N from 2.76 to 8.68 mg N/100 g over the storage period. The findings confirm that hypoxanthine, histamine and TMA-N can be effectively used as biochemical indicators of freshness loss and spoilage development in refrigerated chicken meat. The optimized methods provide a simple, rapid and cost-effective approach for routine assessment of meat quality without the requirement for advanced instrumentation.

Keywords: Meat spoilage, biomarkers, predictive microbiology, regression model

1. Introduction

Chicken meat is one of the most widely consumed animal-derived foods worldwide because of its high nutritional value, desirable sensory characteristics, easy digestibility and affordability [1]. Its demand is continuously increasing and global chicken meat production exceeded 151 million metric tons by 2025, indicating its major contribution to food security and the animal-source food sector [2]. However, despite its nutritional and economic importance, fresh chicken meat is highly perishable due to its high moisture content, nearly neutral pH and nutrient-rich composition, which provide favourable conditions for microbial growth and biochemical degradation [3].

Under refrigerated storage, the shelf life of fresh chicken meat is generally limited to about 5-7 days. Beyond this period, microbial proliferation and endogenous enzymatic activity lead to progressive deterioration in quality, which is commonly reflected by off-odours, slime formation, discoloration, texture changes and overall sensory rejection [4, 5]. Cold-chain interruptions, temperature abuse, repeated freeze-thaw exposure and improper handling during storage and distribution can further accelerate quality deterioration and compromise product authenticity, quality assurance and consumer safety [6].

During storage, spoilage of chicken meat is accompanied by the degradation of muscle constituents such as proteins, nucleotides and nitrogenous compounds. This results in the formation and accumulation of several spoilage-associated metabolites, including ammonia, hydrogen sulphide, total volatile basic nitrogen, trimethylamine nitrogen, biogenic amines and purine degradation products [7]. These compounds reflect the metabolic activity of spoilage microorganisms as well as post-mortem biochemical changes in meat.

Therefore, such metabolites can serve as useful biomarkers for monitoring freshness loss and spoilage progression in chicken meat.

Among spoilage-associated metabolites, hypoxanthine, histamine and trimethylamine nitrogen were selected because they represent different biochemical pathways of meat deterioration. Hypoxanthine primarily reflects post-mortem ATP degradation, histamine indicates microbial decarboxylation of histidine, whereas TMA-N is associated with the formation of volatile nitrogenous compounds and contributes to characteristic spoilage odours during advanced deterioration^[8-10].

Several advanced analytical techniques, including liquid chromatography, mass spectrometry, electrophoretic methods and electrochemical sensing systems, have been used for the identification and quantification of spoilage biomarkers in meat and other food products. Although these techniques offer high sensitivity and selectivity, their routine application is limited by the requirement for costly instruments, trained personnel, complex sample preparation and longer analysis time^[11, 12]. These limitations restrict their use in basic laboratories, small-scale meat processing units and field-level applications where rapid and cost-effective assessment is required.

Hence, there is a need to develop simple, rapid, economical and easily performable analytical methods for detecting spoilage-associated biomarkers in chicken meat. Thus, spectrophotometric methods offer a practical alternative, as they are relatively simple, less expensive, less labour-intensive and suitable for routine laboratory analysis. Therefore, the present study aimed to identify and evaluate hypoxanthine, histamine and trimethylamine nitrogen as potential spoilage biomarkers in fresh chicken meat using simple spectrophotometric methods.

2. Materials and Methods

2.1 Sample collection and experimental design

Fresh broiler chicken meat (*Gallus gallus domesticus*, Cobb strain) was obtained from slaughtered birds sourced either from the Division of Post-Harvest Technology, ICAR-Central Avian Research Institute (CARI), Bareilly, or from hygienic retail outlets in Bareilly, Uttar Pradesh, India. Birds were 5-6 weeks old at the time of slaughter. The meat samples were aseptically handled, trimmed of visible fat and connective tissue, and aerobically packaged in sterile, colourless low-density polyethylene (LDPE) bags (50 μ m thickness). Samples were transported under chilled conditions in insulated ice boxes and subjected to post-mortem ageing at 4 $\pm$ 1 $^{\circ}$ C for 15-16 h upon arrival. Thereafter, samples were stored at 4 $\pm$ 1 $^{\circ}$ C for up to 7 days or until the onset of spoilage, with analyses performed at daily intervals to monitor spoilage progression. The experimental design consisted of three independent sample sets with duplicate analysis ($n = 6$) at each sampling interval.

2.2 Chemicals and reagents

All chemicals and reagents used were of analytical grade. Biomarker standards, including hypoxanthine, histamine and trimethylamine hydrochloride, along with enzymes such as xanthine oxidase and horseradish peroxidase, were procured from Sigma-Aldrich (USA). Other reagents, including perchloric acid, trichloroacetic acid, potassium

hydroxide, sodium nitrite, sulfanilic acid and picric acid, were obtained from HiMedia Laboratories (India) and Central Drug House (CDH), India.

2.3 Determination of spoilage biomarkers

Three biomarkers, namely hypoxanthine, histamine and trimethylamine nitrogen (TMA-N), were selected based on their association with different biochemical pathways. Analytical methods were adopted from literature with suitable modifications and optimized for application in the chicken meat matrix to minimize interference and ensure reliable quantification.

2.3.1 Method development and optimization of biomarker assays

The hypoxanthine assay was optimized under the present experimental conditions, while histamine and trimethylamine assays were adapted from established methods with minor modifications to suit the meat matrix.

The hypoxanthine assay was developed by evaluating key analytical parameters. Solubility was assessed in various solvent systems including diluted formic acid (2:1, v/v), phosphate buffer, ethanol, distilled water and dimethyl sulfoxide (DMSO), followed by evaluation of hypoxanthine over a concentration range of 1-100 mM at varying volumes (1-10 μ L). Enzymatic conditions were optimized by evaluating xanthine oxidase at two concentrations (0.15 and 15 U/mL) and incubation times (5 and 10 min). The chromogenic system comprising 3, 3', 5, 5'-tetramethylbenzidine (TMB; 0.2 and 10 mM) and horseradish peroxidase (HRP; 5 U/mL at 1 and 2 μ L) was also evaluated. The enzymatic reaction was carried out in phosphate buffer (0.1 M, pH 7.6), while color development was performed in sodium citrate buffer (20 mM, pH 5.0). Matrix-based extraction was evaluated using diluted formic acid (2:1, v/v) and perchloric acid (0.6 M), wherein spiked meat samples were extracted and analyzed at varying extract volumes (1-10 μ L) under identical experimental conditions.

2.3.2 Determination of hypoxanthine

Hypoxanthine was quantified using a modified enzymatic colorimetric method^[13, 14] adapted for meat matrix analysis. A primary stock (1 M) was prepared by dissolving 136 mg hypoxanthine in 1 mL diluted formic acid (2:1, v/v), to obtain working standards of 0.106-13.6 μ g/g. Matrix-matched calibration standards were prepared by spiking these concentrations into 1 g minced chicken meat. Meat samples were extracted with 10 mL of 0.6 M perchloric acid, centrifuged at 10,000 rpm for 15 min at 4 $^{\circ}$ C using a refrigerated centrifuge (Model Z 446 K, Hermle Labortechnik GmbH, Germany), neutralized to pH 6.8-7.0 using 1 M KOH, and re-centrifuged (1200 rpm, 5 min) to obtain a clear extract. For analysis, 10 μ L extract was reacted with 75 μ L phosphate buffer (0.1 M, pH 7.6) and 15 μ L xanthine oxidase (15 U/mL) and incubated for 5 min. Color was developed by adding sodium citrate buffer (20 mM, pH 5.0), HRP (2 μ L; 5 U/ μ L), and 3, 3', 5, 5'-tetramethylbenzidine (TMB) (10 μ L; 10 mM). Hypoxanthine oxidation generated H₂O₂, which, in the presence of HRP, oxidized TMB to a blue chromogen measured at 630 nm using a microplate reader (Synergy HTX, BioTek Instruments, USA). Concentrations were determined using a matrix-matched calibration curve.

2.3.3 Determination of histamine

Histamine was determined using a modified spectrophotometric method based on the Pauly reaction [15, 16]. A stock solution (1 mg/mL) was prepared in deionized water and diluted to obtain working standards (10-100 µg/mL). For sample preparation, 1 g of meat was homogenized using an Ultra-Turrax tissue homogenizer (Model T-25, IKA Labortechnik, Germany) with 5 mL ice-cold 0.4 M perchloric acid, followed by centrifugation at 12,000 rpm for 15 min at 4 °C (Model Z 446 K, Hermle Labortechnik GmbH, Germany). The supernatant was neutralized to pH 6.8-7.0 using 2 M KOH and re-centrifuged (10,000 rpm, 10 min) to obtain a clear extract. The assay involved diazotization of sulfanilic acid with sodium nitrite, followed by coupling with histamine to form a colored azo compound. Briefly, 1 mL of 1% sulfanilic acid was mixed with 0.5 mL of 5% sodium nitrite and allowed to stand for 3 min. Subsequently, 1 mL of standard or sample extract and 1 mL of 10% sodium carbonate were added for color development. The mixture was incubated at room temperature for 15 min and absorbance was measured at 510 nm. Histamine concentration was determined using a calibration curve and expressed as mg/kg using the following equation:

$$\text{Histamine (mg/kg)} = (C \times V \times \text{DF})/W$$

where, C is the histamine concentration obtained from the calibration curve (mg/mL), V is the total extract volume (mL), DF is the dilution factor and W is the sample weight (kg).

2.3.4 Determination of trimethylamine nitrogen (TMA-N)

Trimethylamine nitrogen (TMA-N) was determined using a modified picrate-based colorimetric method [17-19]. A primary stock solution (50 µg/mL) of trimethylamine hydrochloride was prepared in hydrochloric acid and diluted with double distilled water and working standards (1-25 µg TMA-N/mL) were prepared in 10% trichloroacetic acid (TCA) to match the extraction matrix. For sample preparation, 1 g minced meat was homogenized with 10 mL of 10% TCA using an Ultra-Turrax tissue homogenizer (Model T-25, IKA Labortechnik, Germany) and centrifuged at 10,000 rpm for 15 min at 4 °C (Model Z 446 K, Hermle Labortechnik GmbH, Germany) to obtain a clear supernatant. For analysis, 1 mL extract was treated with 250 µL of 20% formaldehyde, followed by addition of 2.5 mL toluene and 1 mL of 10% potassium carbonate (K₂CO₃), vortexed and allowed to separate into phases. The upper organic layer was collected, dried over anhydrous sodium sulfate and reacted with 1.25 mL of 0.02% picric acid to form a yellow TMA-N picrate complex. Absorbance was measured at 410 nm and the concentration obtained from the calibration curve (µg TMA-N/mL) was expressed as mg N/100 g of meat.

2.4 Validation of biomarker assays

The analytical performance of the developed biomarker assays was evaluated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ) and accuracy, to determine the suitability of the optimized methods for

quantitative estimation of spoilage biomarkers in chicken meat matrix.

2.4.1 Linearity, limit of detection (LOD) and limit of quantification (LOQ) of biomarker assays

Linearity, LOD and LOQ of the assays for hypoxanthine (Hx), histamine and trimethylamine nitrogen (TMA-N) were evaluated in accordance with International Council for Harmonisation [20]. Matrix-matched calibration for hypoxanthine was performed over a concentration range of 0.106-13.6 µg/g, while histamine and TMA-N were analyzed over ranges of 10-100 µg/mL and 1-25 µg TMA-N/mL, respectively. Calibration curves were constructed by plotting absorbance against concentration and linear regression analysis was performed using Microsoft Excel (version 2019), with linearity assessed based on the coefficient of determination (R²). The LOD and LOQ were estimated from the standard deviation of the intercept (σ) and slope (S) of the calibration curve as:

$$\text{LOD} = 3.3 (\sigma/S) \text{ and } \text{LOQ} = 10 (\sigma/S)$$

2.4.2 Assessment of accuracy of biomarker assays

Accuracy of the developed biomarker assays was evaluated through recovery studies in minced chicken meat samples following ICH Q2 (R1) guidelines [20] with slight modifications. Initially, the natural concentrations of hypoxanthine, histamine and trimethylamine nitrogen (TMA-N) were determined using the optimized methods and corresponding calibration curves. The same samples were then spiked (n = 6) at low, medium and high concentration levels, selected based on FDA limits for histamine [21] and literature-reported ranges for TMA-N and hypoxanthine [22, 23]. After extraction under optimized conditions, the samples were reanalyzed using the respective methods. Recovery (%) was calculated by considering the native concentration and the added amount, and results were expressed as Mean ± SE to assess method accuracy.

2.5 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 29.0.2; IBM Corp., USA). All experiments were performed in triplicate with duplicate samples (n = 6) and results were expressed as mean ± standard error (SE). Data were analyzed using one-way ANOVA followed by Tukey's HSD (Honestly Significant Difference) test as the post hoc test at a significance level of p < 0.05.

3. Results

3.1 Optimization of biomarker assays

The hypoxanthine assay was optimized based on previously reported XOD-HRP-TMB spectrophotometric methods [14] and the optimized assay conditions are summarized in Table 1, wherein hypoxanthine exhibited complete solubility in formic acid: water (2:1, v/v), enabling stable stock and working solution preparation. A concentration-dependent increase in absorbance was observed within the range of 1-100 mM, with 10 mM (1 µL) providing a consistent and reliable response. Enzymatic conditions were optimized using xanthine oxidase (15 U/mL) with an incubation time of 5 min, ensuring rapid and stable reaction performance. The chromogenic system comprising TMB and HRP showed optimal performance at 10 mM TMB (10 µL) and

HRP (5 U/mL, 2 μ L), producing a stable and measurable color response. The reaction proceeded efficiently in phosphate buffer (0.1 M, pH 7.6), while sodium citrate buffer (20 mM, pH 5.0) supported consistent color development under optimized conditions. For application in

the meat matrix, extraction using 0.6 M perchloric acid was found to be more effective than diluted formic acid, yielding better recovery of hypoxanthine from spiked samples, with an optimized extract volume of 10 μ L providing consistent assay response.

Table 1: Optimized assay conditions for spoilage biomarkers in chicken meat

Hx concentration	1 mM	10 mM	20 mM	50 mM	100 mM
	0.092 $\pm$ 0.005 ^c	1.576 $\pm$ 0.099 ^a	1.130 $\pm$ 0.054 ^{ab}	1.660 $\pm$ 0.278 ^a	0.406 $\pm$ 0.257 ^{bc}
XOD	0.15U/ml	15U/ml			
	0.0014 $\pm$ 0.0003 ^b	0.0423 $\pm$ 0.0012 ^a			
TMB	0.2mM	10Mm			
	0.0059 $\pm$ 0.0031 ^b	0.8390 $\pm$ 0.0092 ^a			
HRP	1 μ l	2 μ l			
	0.0333 $\pm$ 0.003 ^b	0.0461 $\pm$ 0.0026 ^a			
Incubation time	5 min	10 min			
	0.0435 $\pm$ 0.0026 ^a	0.0213 $\pm$ 0.0029 ^b			
Extraction Buffer	FA (1 μ l)	FA (10 μ l)	PCA (1 μ l)	PCA (10 μ l)	
	0.004 $\pm$ 0.001 ^b	-0.032 $\pm$ 0.001 ^b	0.013 $\pm$ 0.001 ^b	0.117 $\pm$ 0.030 ^a	

Hx: Hypoxanthine; XOD: xanthine oxidase; HRP: horseradish peroxidase; TMB: 3, 3', 5, 5'-tetramethylbenzidine; PCA: perchloric acid; FA: formic acid

Histamine extraction using 0.4 M perchloric acid resulted in consistent recovery from the meat matrix, with stable color development at 510 nm after 15 min and a proportional increase in absorbance with increasing concentration, confirming assay reliability [15]. Similarly, TMA-N extraction using 10% trichloroacetic acid enabled efficient recovery and reproducible response, where the picrate-based reaction produced a stable yellow chromophore measurable at 410 nm, with absorbance increasing proportionally with TMA concentration [17].

Overall, the optimized spectrophotometric assays are simple, cost-effective and easy to perform, demonstrating rapid color development within a short incubation period while requiring minimal reagent and enzyme consumption and eliminating the need for complex instrumentation,

thereby making them suitable for routine analysis.

3.2 Analytical performance of developed biomarker assays

The calibration characteristics and analytical performance of the developed biomarker assays are presented in Fig. 1 and Table 2, demonstrating reliable quantitative behavior across all biomarkers. The standard curves (Fig. 1a-c) revealed a strong linear relationship between concentration and absorbance (OD), confirming the suitability of the developed assays for quantitative analysis, while the corresponding regression equations further support method reliability. In addition, the distinct colorimetric response observed in the microplate (Fig. 1d) highlights the visual detection capability of the assay.

Table 2: Recovery evaluation and analytical performance of biomarker assays

Biomarker	Linearity (R ²)	LOD	LOQ	Spiked concentration	Measured (Mean $\pm$ SE)	Recovery (%) (Mean $\pm$ SE)	RSD%
Hypoxanthine (μ g/g)	0.9688	0.044 μ g/g	0.134 μ g/g	3.4	3.370 $\pm$ 0.109	99.119 $\pm$ 3.207	5.603
				6.8	6.559 $\pm$ 0.223	96.459 $\pm$ 3.278	5.887
				13.6	12.439 $\pm$ 0.092	91.460 $\pm$ 0.674	1.276
					Average	95.679 $\pm$ 2.245%	
Histamine (mg/kg)	0.9955	0.29 mg/kg	0.88 mg/kg	20	22.566 $\pm$ 0.137	112.832 $\pm$ 0.685	1.051
				40	38.460 $\pm$ 0.237	96.150 $\pm$ 0.593	1.068
				60	56.489 $\pm$ 0.119	94.148 $\pm$ 0.198	0.364
					Average	101.043 $\pm$ 5.922%	
Trimethylamine nitrogen (mgN/100 g)	0.9996	0.028 mgN/100 g	0.085 mgN/100 g	5	4.892 $\pm$ 0.015	97.843 $\pm$ 0.296	0.524
				10	9.964 $\pm$ 0.332	99.640 $\pm$ 3.315	5.63
				15	14.957 $\pm$ 0.023	99.712 $\pm$ 0.151	0.262
					Average	99.065 $\pm$ 0.611	

Mean $\pm$ SE (n = 6), LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation.

The analytical performance of the developed biomarker assays was consistent with previously reported biosensor and spectrophotometric methods. For hypoxanthine, a μ PAD-based biosensor reported by [24] indicated strong linearity (R² = 0.9957) along with an LOD of 1.8 mg/L (~18 mg/kg in meat), LOQ of 6.1 mg/L, recovery of ~95.8% and precision (RSD $\approx$ 4.75%), confirming the applicability of colorimetric biosensing approaches for practical quantification. Similarly, a strip-based colorimetric method

for histamine reported by [25] showed excellent linearity over a wide concentration range of 70-2240 mg/kg, with an R² value of 0.992 and a detection limit of 70 mg/kg. The method also showed good analytical performance, with recovery values ranging from 89.29% to 112.17% and RSD values between 1.72% and 3.25%, indicating acceptable accuracy and precision. However, in complex biological matrices, some degree of matrix-related variability may occur in colorimetric assays due to co-extracted endogenous

compounds that alter chromogenic reactions, resulting in increased absorbance and possible overestimation. In the case of trimethylamine nitrogen, an advanced spectrophotometric method based on HS-SDME coupled with microvolume UV-vis reported by [26] demonstrated

excellent linearity ($R^2 = 0.9981$) at very low concentration levels, with a highly sensitive detection limit of 6×10^{-4} mg N/100 g, recovery of 97.1-103.2%, and precision (RSD < 5%), where the enhanced sensitivity is attributed to the preconcentration step.

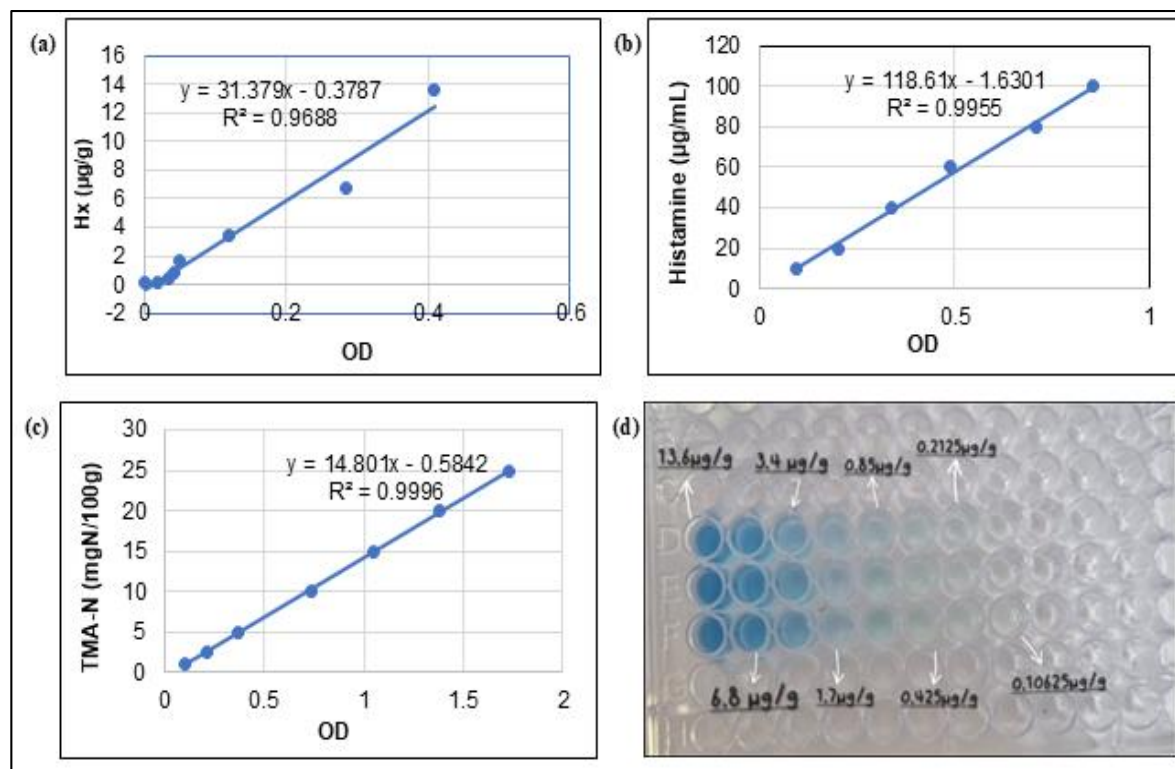


Fig 1: Calibration and assay development of biochemical spoilage biomarkers: (a) hypoxanthine standard curve (b) histamine standard curve (c) trimethylamine nitrogen (TMA-N) standard curve (d) representative colorimetric response of hypoxanthine standards in a microplate

Overall, the consistency of linearity, detection limits, recovery and precision with literature-reported values confirms the analytical robustness of the developed assays and supports their suitability for application in complex meat matrices.

3.3 Application of developed biomarker assays in monitoring spoilage of chicken meat during refrigerated storage

A significant ($p < 0.05$) increase in biochemical spoilage biomarkers was observed during storage (Table 3). Hypoxanthine increased from 1.13-3.95 $\mu\text{g/g}$, histamine from 20.13-73.03 mg/kg, and TMA-N from 2.76-8.68 mg N/100 g, indicating progressive biochemical deterioration of

the meat matrix. The enzymatic assay effectively captured the continuous rise in hypoxanthine, reflecting ongoing ATP degradation and supporting its role as an early-stage spoilage indicator. The observed range aligns with reported values in chicken meat [22]. Histamine showed a gradual increase due to microbial decarboxylation of histidine, with levels remaining below fish regulatory limits (100-200 mg/kg); [27], suggesting its relevance as a quality deterioration marker rather than a safety concern. Similar trends have been reported by Esposito *et al.* [28] TMA-N also increased significantly, indicating accumulation of volatile nitrogenous compounds associated with spoilage. Its levels approached reported spoilage thresholds (~ 10 mg N/100 g) [23], supporting its role as a late-stage indicator.

Table 3: Changes in spoilage biomarkers in chicken meat during refrigerated storage (4 ± 1 °C)

Parameter	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Hypoxanthine ($\mu\text{g/g}$)	1.13 ± 0.04^f	1.29 ± 0.05^f	1.75 ± 0.06^c	2.10 ± 0.01^d	2.57 ± 0.12^c	3.40 ± 0.08^b	3.95 ± 0.08^a
Histamine (mg/kg)	20.13 ± 0.84^s	40.35 ± 1.23^f	49.55 ± 0.67^c	58.35 ± 0.51^d	63.58 ± 0.44^c	68.00 ± 0.44^b	73.03 ± 0.44^a
TMA-N (mgN/100 g)	2.76 ± 0.28^d	2.80 ± 0.38^d	3.31 ± 0.65^d	4.37 ± 0.54^{cd}	5.93 ± 0.84^{bc}	7.76 ± 0.19^{ab}	8.68 ± 0.17^a

Mean $\pm$ SE (n = 6), bearing different superscripts (a-g) within a row differ significantly ($p < 0.05$), TMA-N: Trimethylamine nitrogen)

Overall, the results confirm that the optimized assays effectively track both early and advanced biochemical changes associated during refrigerated storage.

4. Conclusions

The present study established that hypoxanthine, histamine and trimethylamine nitrogen are effective biochemical

indicators for monitoring refrigerated spoilage progression in fresh chicken meat. The optimized spectrophotometric methods showed reliable analytical performance in the meat matrix, with acceptable linearity, detection limits, recovery and precision. During storage at 4 ± 1 °C, all three biomarkers increased significantly, confirming progressive biochemical deterioration of the meat. Hypoxanthine

increased from 1.13 to 3.95 µg/g, indicating early nucleotide degradation; histamine increased from 20.13 to 73.03 mg/kg, reflecting microbial decarboxylation activity; and TMA-N increased from 2.76 to 8.68 mg N/100 g, indicating accumulation of volatile nitrogenous compounds during advanced spoilage. Thus, the study confirms that these simple, rapid and cost-effective spectrophotometric assays can be used for routine assessment of freshness loss and spoilage development in refrigerated chicken meat.

5. Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The research work was funded by ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India.

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