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pH Dependence of the Formation of Malondialdehyde (MDA) in the Reaction of 2-Thiobarbituric Acid (2-TBA) with Local Yoghurt (Nunu)

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Abstract

The study investigates the pH dependence of malondialdehyde (MDA) formation in local yoghurt (Nunu) through its reaction with 2-thiobarbituric acid (2-TBA), using the Thiobarbituric Acid Reactive Substances (TBARS) assay. A total of 10 experimental setups were conducted across seven pH levels (5.6 to 9.0) and ten graded 2-TBA volumes (10 µL to 100 µL). Samples were allowed to equilibrate for up to 12 h before UV-Visible spectrophotometric measurements were taken at 532 nm. Descriptive statistics and comparative analyses were used to evaluate mean MDA concentrations across conditions. The results showed no clear linear relationship between pH and MDA levels; however, a consistent spike in MDA concentrations was observed at alkaline pH, notably at pH 8.6. The highest MDA concentration (1.0600 ± 0.0011 mg/L) was recorded at this pH, while the lowest was at pH 5.6 (0.241 ± 0.010 L), indicating enhanced lipid peroxidation or increased TBA-MDA complex stability in alkaline environments. This research highlights the complex pH sensitivity of lipid peroxidation in fermented dairy products and underscores the need to consider environmental factors such as pH when assessing oxidative stress markers like MDA. Future studies may benefit from refining the pH gradient intervals, extending incubation times, incorporating centrifugation steps, examining the effect of IHP (inositolhexakisphosphate) on the affinity of local yoghurt for 2-Thiobarbituric acid, and validating MDA levels through more specific techniques like HPLC. The findings hold implications for food quality monitoring, oxidative stress assessment, and health risk evaluations associated with lipid peroxidation products in traditional dairy foods.

Keywords: Malondialdehyde, pH dependence, Reaction, Local yoghurt, Thiobarbituric acid

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Introduction

In the view of nutrition and health, the interaction between bioactive compounds found in food and their effects on biological systems has become a focus of scientific exploration. In parallel, local yoghurt, colloquially referred to as "Nunu" in Nigeria, stands as a staple in the diet of many cultures. Nunu (local yoghurt), a traditional fermented milk product widely consumed in West Africa (especially Nigeria and Ghana), contains essential fatty acids (EFAs). Alpha-linolenic acid (ALA)—an omega-3 fatty acid found in milk, which is a precursor for EPA and DHA (though conversion is limited in the body), is an important compound for brain health and reducing inflammation [1]. It is produced from (commonly cow, goat, or sheep milk). These beneficial compounds contribute not only to physical health but also to the cultural significance of Nunu in communal meals and rituals. As such, it serves as a vital link between nutrition, tradition, and community identity in the regions where it is cherished. This interconnectedness highlights the importance of preserving traditional practices and understanding their nutritional value. By fostering a deeper appreciation for these foods, communities can enhance both their health and cultural heritage. Moreover, promoting awareness of these traditional foods can encourage sustainable agricultural practices and strengthen local economies. By valuing and supporting local food systems, communities can ensure that their culinary traditions continue to thrive for generations to come. This commitment not only benefits individual health but also fosters a sense of community and belonging. As people come together to celebrate and share their culinary heritage, they create bonds that enrich their social fabric and promote resilience in the face of modern challenges.

This communal approach to food can also lead to innovative solutions for environmental issues, as local producers often prioritise eco-friendly methods. Ultimately, embracing local foods paves the way for a more sustainable future while honouring the rich diversity of regional cuisines. This spontaneously fermented dairy product not only offers a nutrient-rich profile but also introduces probiotics that promote gut health. The outcome boasts a yoghurt-like tang (characterized by a pronounced acidic taste) and can be savoured on its own or alongside "Fura" [2]. Nunu is an excellent source of protein, rich in essential amino acids, and a good source of calcium, phosphorus, and vitamins A, B, C, E, and B complex. However, like other milk products, it is poor in ascorbic acid and iron [3].

Most of the organisms involved in the fermentation process are usually of three main groups: bacteria, yeast, and mould. Of these, Lactobacilli (*L. acidophilus* and *L. bulgaris*), Lactococci species (*L. cremoni* and *L. lactis*), Streptococcus thermophilus, Leuconostoc species, and Saccharomyces species seem to be the most prominent, each giving the product a characteristic flavor [4]. Local yoghurts' popularity as a source of protein, vitamins, and minerals, combined with their probiotic potential, underscores their significance in promoting dietary diversity and overall well-being [5].

Central to the study's focus is the compound 2-Thiobarbituric acid (2-TBA). Thiobarbituric acid is an organic and heterocyclic compound. It finds utility as a reagent in the assessment of malondialdehyde, known as the TBARS assay, a method for analyzing lipid peroxidation [6]. A notable limitation of this approach is the potential for TBARS formation after sampling [7, 8]. Additionally, other substances can also interact with TBA besides MDA [9], which can affect absorbance and consequently impact analysis outcomes. Furthermore, variations exist in the methodology employed, making result comparison challenging [10].

This molecule is commonly employed in assays to quantify malondialdehyde (MDA) levels, a well-established marker of lipid peroxidation and oxidative stress. Despite the passage of five decades, the thiobarbituric acid-reactive substances (TBARS) assay remains the most widely utilized technique for both plant and animal samples [11]. The genesis of MDA originates from the degradation of polyunsaturated lipids catalyzed by reactive oxygen species (ROS) [12]. MDA is an outcome of prostaglandin synthesis [13]. Existing as a colourless liquid in its enol form, MDA displays substantial reactivity, making it a valuable biomarker for oxidative stress assessment in biomedical domains [14, 15]. It is a three-carbon dialdehyde formed as a by-product of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism [15]. Under more acidic conditions (pH < 4), beta hydroxyacrolein (HO—CH=CH—CHO) becomes prevalent. Its presence is discernible as both monomers and higher-order oligomers, a characteristic that makes it a fundamental gauge of lipid peroxidation.

While recognized as a marker of lipid peroxidation, MDA transcends its role as a mere indicator. It is a highly reactive and potentially hazardous compound known to interact with proteins and DNA, exerting atherogenic and conceivably mutagenic effects. Additionally, MDA is frequently found in significant quantities in spoiled food [16]. The peroxidation of membrane polyunsaturated fatty acids leads to MDA production, indicating cellular damage due to oxidative stress [17]. MDA stands out as one of the most widely used biomarkers, shedding light on the overall extent of lipid peroxidation [18]. Its application extends to being the preeminent biomarker for oxidative stress in a spectrum of health conditions, encompassing cancer, psychiatry, chronic obstructive pulmonary disease, asthma, and cardiovascular diseases, among others [19].

MDA formation can be instigated both nonenzymatically by Reactive Oxygen Species (ROS) and enzymatically by lipoxygenase activity [20]. Quantifying primary lipid hydroperoxide products presents challenges due to their instability and reactivity. Consequently, the estimation of lipid peroxidation often centers on measuring concentrations of secondary oxidation products derived from these initial hydroperoxides, primarily aldehydes like MDA [21]. MDA is a volatile, low-molecular-weight ($C_3H_4O_2$; formula weight = 72.07), short-chain, 1,3-dicarbonyl compound and a moderately weak acid (pKa = 4.46).

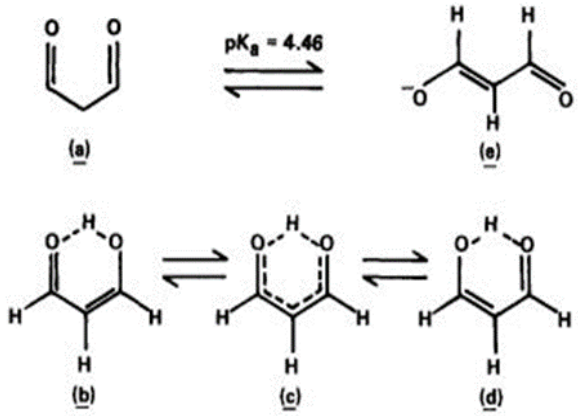

Figure 1: Structural chemistry of malondialdehyde [22]

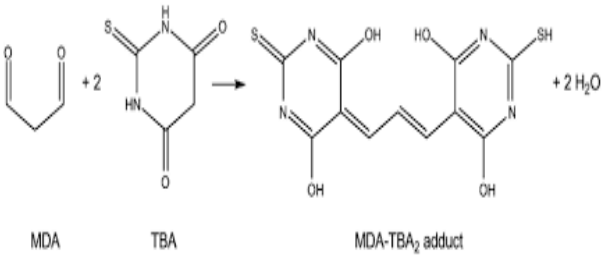

Figure 2: MDA-TBA$_2$ adduct formation

Haemoglobin undergoes a slow and reversible reaction with oxygen and CO_2 within the human body, facilitating the transportation of oxygen to all bodily tissues and the elimination of CO_2. Well-established regulators of haemoglobin's oxygen affinity include the erythrocyte enzyme 2,3-bisphosphoglycerate (BPG), which uses a regulatory mechanism involving competitive interactions with haemoglobin. This competition influences the release of oxygen to the body. Malondialdehyde's significance is often marked by its documented interaction with DNA, resulting in the formation of adducts with deoxyguanosine and deoxyadenosine. These adducts are recognized for their dual role as both mutagenic and carcinogenic agents, augmenting their importance as markers for oxidative stress. Notably, a predominant DNA adduct, termed pyrimidopurinone M1G, is a resultof these interactions. Given the increasing risk of cancer in modern times, it

becomes increasingly imperative to systematically monitor all forms of food, beverages, and chemicals that could potentially pose a threat due to their association with DNA adducts and related health outcomes [23]. In the context of this study, we endeavour to replicate a physiological environment external to the body by applying analogous principles and environmental variables. Based on this context, however, the study aimed to investigate the pH dependence of malondialdehyde (MDA) formation in local yoghurt (Nunu) through its reaction with 2-thiobarbituric acid (2-TBA), using the Thiobarbituric Acid Reactive Substances (TBARS) assay.

Materials and Methods

Research design

This study employed an experimental design to investigate the generation of malondialdehyde (MDA) in fermented milk (Nunu) using the TBARS assay. The experiment involved analysing the Nunu sample under seven different pH ranges and at ten different volumes of 2-TBA, with each sample analysed in triplicate, resulting in a total of 10 test samples. The same Nunu sample was then further analysed to assess its effect on the affinity for 2-TBA across the same seven pH ranges and with varying volumes of TBA, totalling 10 samples. Table 3shows the analysis measured MDA concentrations. The experiment was structured to test varying pH levels and treatment conditions using spectrophotometric analysis to quantify MDA levels [24].

Materials

Equipment used includes conical flasks, a UV-Visible spectrophotometer, micropipettes, pH meters, while reagents include disodium hydrogen phosphate, boric acid, sodium hydroxide, 2-TBA, deionised water, etc.

Sample collection

Nunu

A quantity of 136 mL of Nunu (local yoghurt) was sourced from Fulani women near Nasarawa State University, Keffi, and used as consumed locally, without any modification.

Preparation of reagents

Preparation of phosphate buffer

Phosphate buffer (PB) with ionic strength of 0.05 mol/dm^3 was prepared using 0.4 M $NaH_2PO_4.2H_2O$, (62.4 g $NaH_2PO_4.2H_2O$/ dm^3), 0.4 M of $Na_2HPO_4.12H_2O$ (143.2 g $Na_2HPO_4.12H_2O$/ dm^3), 3M NaCl and 0.4 M of NaOH (16 g NaOH/) in 1 dm^3 of distilled water and its pH was adjusted to (5.6-7.8) by addition of small volumes of solutions of NaH_2PO_4 and Na_2HPO_4 as needed. The quantities of the various reagents required for the preparation of the buffers at pH 5.6 - 7.8 as shown in Table 1 [25].

Table 1: Preparation of phosphate buffers with ionic strength of 0.05 M

pH	0.4 M NaOH (ml/dm^3)	0.4 M NaH_2PO_4 (dm^3)	3 M NaCl (dm^3)
5.6	1.00	25	13.0
6.2	4.28	25	12.2
6.8	11.80	25	10.2
7.6	21.40	25	8.1

Table 2: Preparation of borate buffer with ionic strength of 0.05 M

pH	0.3 M NaOH (ml/dm^3)	0.4 M H_3BO_3 (dm^3)	3 M NaCl (dm^3)
8.0	20.0	25	13.0
8.6	60.0	25	12.2
9.0	107.0	25	10.2

Preparation of borate buffer

Borate buffer (BB) with ionic strength 0.05 mol/dm^3 (pH 8.0 - 9.0) was prepared using 0.3 M of NaOH (10 g of NaOH /dm^3), 0.3 M of H_3BO_3 (18.55 g of H_3BO_3), 0.2M NaOH (8 g of NaOH /dm^3), 0.2 M H_3BO_3 (12.37 g of H_3BO_3 /dm^3), and 3 M NaCl in 1 dm^3 of distilled water and pH was adjusted to 8.0 - 9.0 by the addition of small volumes of solutions of NaH_2PO_4 and Na_2HPO_4 as needed. The quantities of the various reagents required for the preparation of the buffers at pH of 8.0 - 9.0 are shown in Table 2 [25].

Technique for data analysis and model specification

Descriptive statistics

Standard Deviation (SD), Standard Error (SE), and Coefficient of Variation (CV %) were calculated to summarise the absorbance and MDA concentration values obtained from the UV-Visible spectrophotometer readings.

Comparative analysis

Data across different pH levels (5.6–9.0) and treatment conditions were compared to determine trends and differences in MDA formation.

Justification of the methods

The methods employed in this study were carefully selected to ensure the accurate quantification of malondialdehyde (MDA), a well-established biomarker of lipid peroxidation, under varying pH conditions. The use of the Thiobarbituric Acid Reactive Substances (TBARS) assay was justified by its sensitivity, simplicity, and suitability for detecting MDA in complex biological and food matrices like Nunu.

Experimental procedure

A 1 cm^3 of the Nunu sample was introduced into 5 cm^3 of phosphate buffer solutions at varying pH levels (5.6, 6.2, 6.8, 7.6) and borate buffer solutions at pH values of (8.0, 8.6, and 9.0). These buffers were used to simulate physiological pH conditions and create an environment conducive to accurately measuring malondialdehyde (MDA) levels. The resulting mixtures were allowed to stand for 15 min [25].

Next, small, measured volumes of 2-thiobarbituric acid (2-TBA), ranging from 10 µL to 100 µL, were added to each mixture. These mixtures were then left to equilibrate for a period of 10 to 12 h. This procedure was repeated in triplicate for each pH condition. After equilibration, the samples were decanted to obtain the supernatant, and the absorbance was measured using a UV-Vis spectrophotometer at a maximum absorbance wavelength of 532 nm. The concentration of malondialdehyde (MDA) in each case was calculated using the relevant mathematical equation based on Beer-Lambert's Law, with absorbance (A_{532}) used to calculate MDA concentration using a modified formula (equation 1) [26]

$$c = \frac{A_{532} \times V + v_1}{11.5 \times 10^{-4} \times lv} \qquad 1$$

In this modification, the parameters are defined as follows;

C = concentration of Malondialdehyde (MDA) (mol/dm^3); A_{532} = maximum absorbance of MDA (nm); V = volume of buffer (ml); v_1 = volume of sample (*Nunu*) (ml); l = path length of cuvette (cm); ε = extinction coefficient at that wavelength (*Nunu*) (11.5 × 10^{-4} mol/dm^3).

Results and Discussion

Data showing the mean concentrations in mg/dm^3 and the corresponding pH are presented in Tables 3 – 12 and Figs 3- 12, showing MDA formation with 2-TBA at 10 – 100 µl. In this study, the analysis was conducted on a sample of locally made yoghurt (*Nunu*) to determine the presence of malondialdehyde (MDA) with 2-thiobarbituric acid (2-TBA) at varying pH levels ranging from 5.6 to 9.0. The TBARs method was employed for the analysis.

Table 3: UV–Visible analysis of MDA with 2-TBA at 10 µL

pH	Mean Conc. (mg/L×10^{-4})	S.D(×10^{-6})	S.E(×10^{-6})	%CV
5.6	0.466	0.213	0.121	0.461
6.2	0.581	0.352	0.210	0.612
6.8	0.357	0.016	0.010	0.352
7.6	0.370	0.041	0.020	0.111
8.0	0.591	0.123	0.072	0.214
8.6	0.652	0.053	0.022	0.075
9.0	0.544	0.052	0.260	0.082

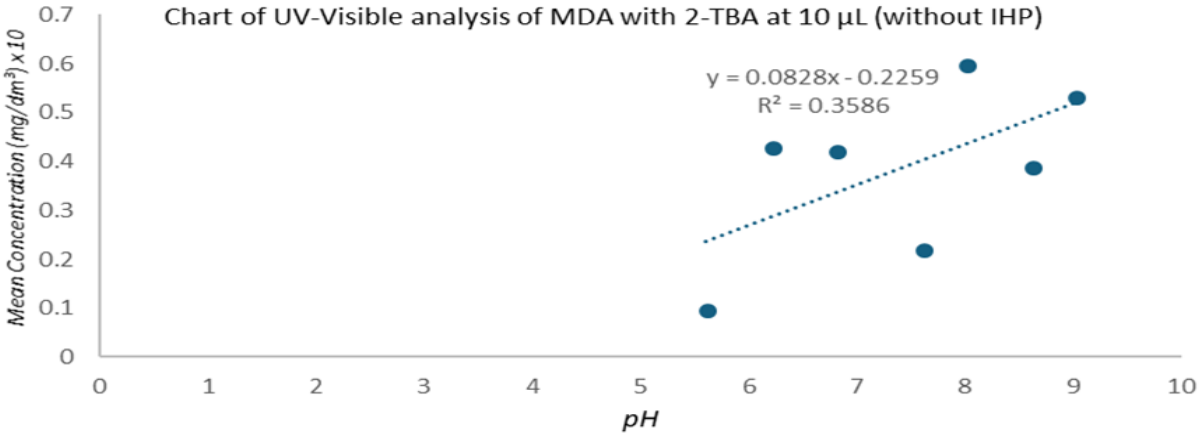

Figure 3: Chart of UV-Visible analysis of MDA with 2-TBA at 10 µL

This chart shows the measured absorbance values (at 532 nm) corresponding to malondialdehyde (MDA) concentrations formed in local yoghurt (Nunu) samples across varying pH conditions. The sample was treated with 10 µL of 2-thiobarbituric acid.

Table 4: UV–Visible analysis of MDA with 2-TBA at 20 µL

pH	Mean Conc. (mg/L×10^{-4})	S.D(×10^{-6})	S.E(×10^{-6})	%CV
5.6	0.352	0.060	0.034	0.173
6.2	0.566	0.262	0.150	0.472
6.8	0.510	0.241	0.114	0.470
7.6	0.370	0.045	0.021	0.110
8.0	0.656	0.111	0.070	0.186
8.6	0.680	0.070	0.042	0.104
9.0	0.583	0.052	0.031	0.082

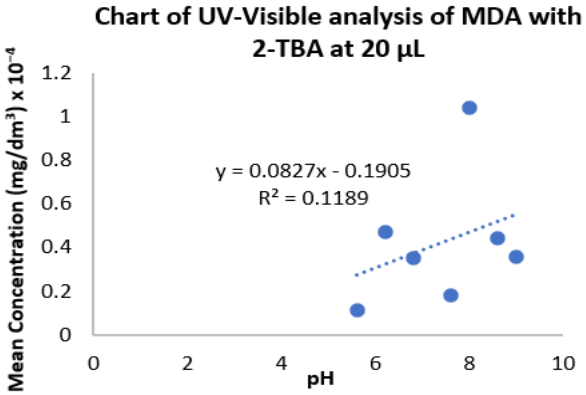

Figure 4: Chart of UV-Visible analysis of MDA with 2-TBA at 20 µL

This chart shows the measured absorbance values (at 532 nm) corresponding to malondialdehyde (MDA) concentrations formed in local yogurt (Nunu) samples across varying pH conditions. The sample was treated with 20 µL of 2-thiobarbituric acid.

Table 5: UV–visible analysis of MDA with 2-TBA at 30 µL

pH	Mean Conc. (mg/L×10^{-4})	S.D(×10^{-6})	S.E(×10^{-6})	%CV
5.6	0.301	0.012	0.010	0.030
6.2	0.532	0.081	0.051	0.166
6.8	0.372	0.102	0.064	0.272
7.6	0.341	0.043	0.023	0.101
8.0	0.611	0.121	0.101	0.278
8.6	0.683	0.107	0.061	0.156
9.0	0.600	0.037	0.027	0.040

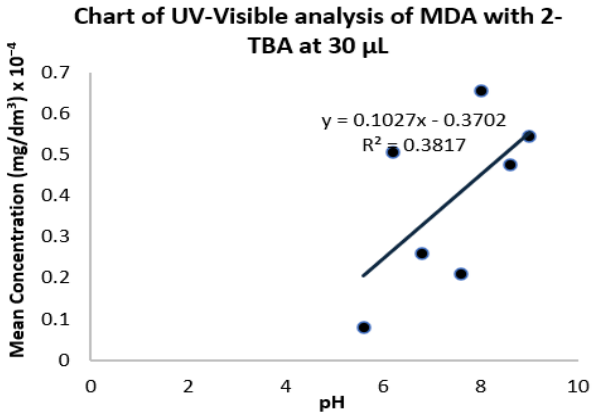

Figure 5: Chart of UV-visible analysis of MDA with 2-TBA at 30 µL

This chart shows the measured absorbance values (at 532 nm) corresponding to malondialdehyde (MDA) concentrations formed in local yoghurt (Nunu) samples across varying pH conditions. The sample was treated with 30 µL of 2-thiobarbituric acid.

Table 6: UV–visible analysis of MDA with 2-TBA at 40 µL

pH	Mean Conc. (mg/L×10^{-4})	S.D(×10^{-6})	S.E(×10^{-6})	%CV
5.6	0.281	0.097	0.212	0.050
6.2	0.551	0.182	0.312	0.580
6.8	0.471	0.081	0.131	0.284
7.6	0.322	0.011	0.022	0.051
8.0	0.772	0.134	0.088	0.282
8.6	0.826	0.011	0.011	0.015
9.0	0.610	0.010	0.000	0.017

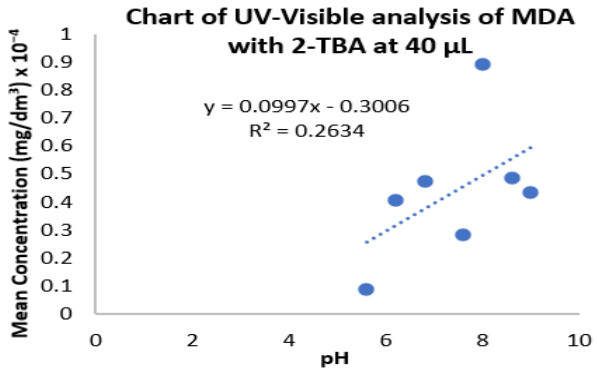

Figure 6: Chart of UV-visible analysis of MDA with 2-TBA at 40 µL

This chart shows the measured absorbance values (at 532 nm) corresponding to malondialdehyde (MDA) concentrations formed in local yoghurt (Nunu) samples across varying pH conditions. The sample was treated with 40 µL of 2-thiobarbituric acid.

Table 7: UV–visible analysis of MDA with 2-TBA at 50 µL

pH	Mean Conc. (mg/L×10^{-4})	S.D(×10^{-6})	S.E(×10^{-6})	%CV
5.6	0.291	0.010	0.012	0.042
6.2	0.370	0.021	0.011	0.042
6.8	0.391	0.013	0.012	0.030
7.6	0.303	0.002	0.014	0.052
8.0	0.772	0.172	0.010	0.221
8.6	0.845	0.100	0.062	0.112
9.0	0.601	0.023	0.013	0.041

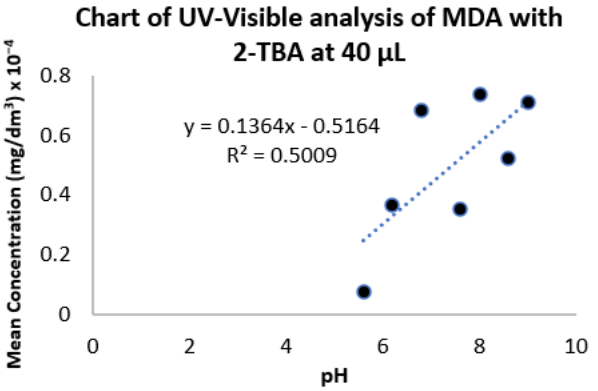

Figure 7 Chart of UV-visible analysis of MDA with 2-TBA at 50 µL

This chart shows the measured absorbance values (at 532 nm) corresponding to malondialdehyde (MDA) concentrations formed in local yogurt (Nunu) samples across varying pH conditions. The sample was treated with 50 µL of 2-thiobarbituric acid.

Table 8: UV–visible analysis of MDA with 2-TBA at 60 µL

pH	Mean Conc. (mg/L×10^{-4})	S.D(×10^{-6})	S.E(×10^{-6})	%CV
5.6	0.241	0.010	0.000	0.020
6.2	0.474	0.240	0.140	0.522
6.8	0.340	0.025	0.014	0.064
7.6	0.326	0.011	0.012	0.034
8.0	0.851	0.210	0.120	0.251
8.6	0.980	0.093	0.051	0.095
9.0	0.652	0.023	0.001	0.021

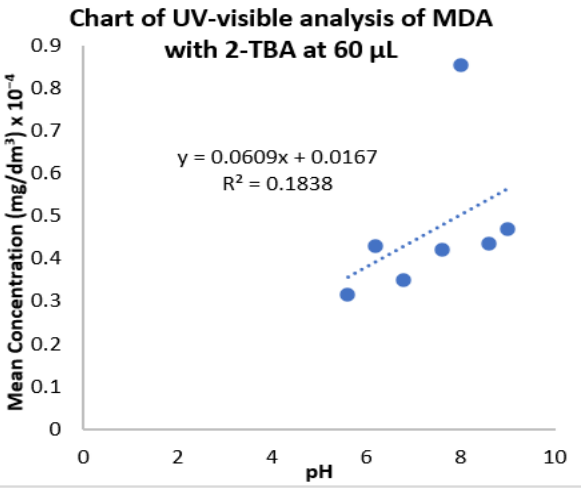

Figure 8: Chart of UV-visible analysis of MDA with 2-TBA at 60 µL

This chart shows the measured absorbance values (at 532 nm) corresponding to malondialdehyde (MDA) concentrations formed in local yoghurt (Nunu) samples across varying pH conditions. The sample was treated with 60 µL of 2-thiobarbituric acid.

Table 9: UV–visible analysis of MDA with 2-TBA at 70 µL

pH	Mean Conc. (mg/L×10^{-4})	S.D(×10^{-6})	S.E(×10^{-6})	%CV
5.6	0.370	0.023	0.014	0.051
6.2	0.432	0.094	0.050	0.211
6.8	0.470	0.124	0.072	0.241
7.6	0.311	0.012	0.000	0.022
8.0	0.991	0.212	0.120	0.212
8.6	1.010	0.220	0.131	0.220
9.0	0.652	0.351	0.020	0.056

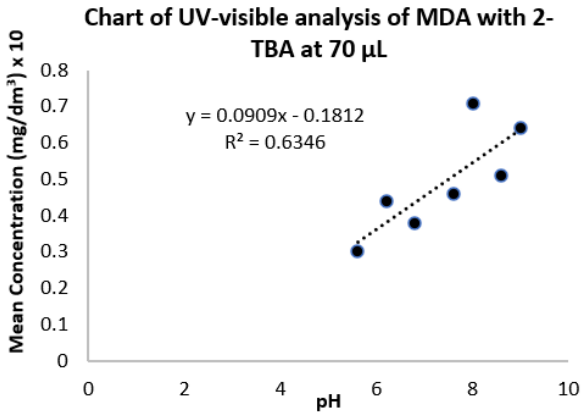

Figure 9: Chart of UV-visible analysis of MDA with 2-TBA at 70 µL

This chart shows the measured absorbance values (at 532 nm) corresponding to malondialdehyde (MDA) concentrations formed in local yogurt (Nunu) samples across varying pH conditions. The sample was treated with 70 µL of 2-thiobarbituric acid.

Table 10: UV–visible analysis of MDA with 2-TBA at 80 µL

pH	Mean Conc. (mg/L×10^{-4})	S.D(×10^{-6})	S.E(×10^{-6})	%CV
5.6	0.381	0.433	0.033	0.121
6.2	0.352	0.011	0.010	0.033
6.8	0.530	0.024	0.141	0.501
7.6	0.340	0.023	0.010	0.052
8.0	0.962	0.170	0.102	0.177
8.6	1.060	0.011	0.010	0.011
9.0	0.671	0.021	0.021	0.030

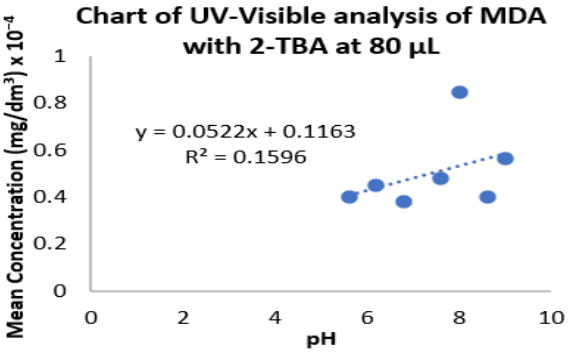

Figure 10: Chart of UV-visible analysis of MDA with 2-TBA at 80 µL

This chart shows the measured absorbance values (at 532 nm) corresponding to malondialdehyde (MDA) concentrations formed in local yoghurt (Nunu) samples across varying pH conditions. The sample was treated with 80 µL of 2-thiobarbituric acid.

Table 11: UV–visible analysis of MDA with 2-TBA at 90 µL

pH	Mean Conc. (mg/L×10^{-4})	S.D(×10^{-6})	S.E(×10^{-6})	%CV
5.6	0.390	0.111	0.067	0.282
6.2	0.361	0.061	0.032	0.163
6.8	0.474	0.111	0.061	0.236
7.6	0.384	0.032	0.025	0.074
8.0	0.963	0.134	0.084	0.142
8.6	1.032	0.045	0.021	0.041
9.0	0.640	0.011	0.010	0.002

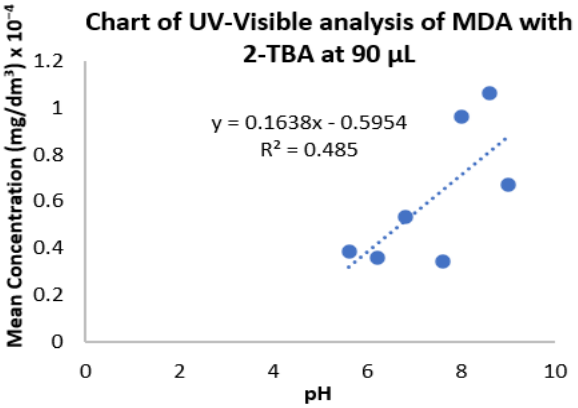

Figure 11: Chart of UV-visible analysis of MDA with 2-TBA at 90 µL

This chart shows the measured absorbance values (at 532 nm) corresponding to malondialdehyde (MDA) concentrations formed in local yoghurt (Nunu) samples across varying pH conditions. The sample was treated with 90 µL of 2-thiobarbituric acid.

Table 12: UV–visible analysis of MDA with 2-TBA at 100 µL

pH	Mean Conc. (mg/L×10^{-4})	S.D(×10^{-6})	S.E(×10^{-6})	%CV
5.6	0.340	0.233	0.011	0.072
6.2	0.402	0.212	0.121	0.532
6.8	0.322	0.021	0.014	0.052
7.6	0.353	0.020	0.015	0.057
8.0	0.984	0.142	0.081	0.141
8.6	1.005	0.161	0.098	0.163
9.0	0.680	0.040	0.020	0.050

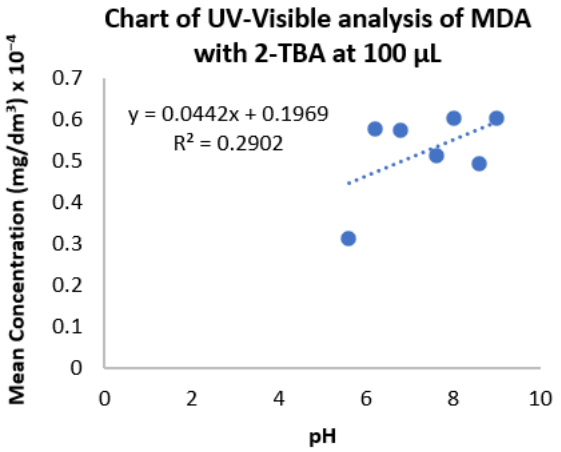

Figure 12: Chart of UV-Visible analysis of MDA with 2-TBA at 100 µL

This chart shows the measured absorbance values (at 532 nm) corresponding to malondialdehyde (MDA) concentrations formed in local yoghurt (Nunu) samples across varying pH conditions. The sample was treated with 100 µL of 2-thiobarbituric acid.

The results demonstrate a clear influence of pH on the concentration of malondialdehyde (MDA) measured using the thiobarbituric acid reactive substances (TBARS) assay. Across the different volumes of 2-thiobarbituric acid (2-TBA) investigated, MDA concentrations generally increased under mildly alkaline conditions, with pH 8.0 frequently producing the highest measured concentration. In contrast, pH 5.6 consistently produced the lowest MDA concentration. However, the response across the entire pH range was not strictly linear, indicating that the relationship between pH and the measured MDA concentration is complex.

For the experimental condition presented in Table 3, the highest MDA concentration was observed at pH 8.0 (0.652 ± 0.053 mg/L), whereas the lowest concentration occurred at pH 5.6 (0.357 ± 0.016 mg/L). A similar pattern was observed in Table 4, where MDA concentration increased from 0.352 ± 0.060 mg/L at pH 5.6 to a maximum of 0.680 ± 0.070 mg/L at pH 8.0. These observations indicate that the measured formation of the MDA–TBA chromogen was favoured under mildly alkaline conditions.

The same general behaviour was observed in Tables 5–7. In Table 5, the MDA concentration increased from 0.303 ± 0.012 mg/L at pH 5.6 to 0.683 ± 0.107 mg/L at pH 8.0. In Table 6, the corresponding values were 0.281 ± 0.097 and 0.826 ± 0.011 mg/L, respectively, while Table 7 recorded 0.291 ± 0.012 mg/L at pH 5.6 and 0.845 ± 0.100 mg/L at pH 8.0. Thus, despite some fluctuations at intermediate pH values, pH 5.6 and pH 8.0 consistently represented the lower and upper extremes, respectively, for several of the experimental conditions.

At higher 2-TBA volumes, the increase in measured MDA concentration became more pronounced. MDA concentrations of 0.980 ± 0.093, 1.010 ± 0.120, and 1.060 ± 0.011 mg/L were recorded at pH 8.0 under successive experimental conditions. Conversely, the corresponding minimum values at pH 5.6 were 0.241 ± 0.010, 0.370 ± 0.023, and 0.381 ± 0.433 mg/L, respectively. These results suggest that increasing the amount of 2-TBA enhanced the measured TBARS response, particularly under mildly alkaline conditions. Nevertheless, the relationship was not proportional throughout the entire pH range.

An important observation was made at the highest 2-TBA volumes. Rather than continuing to increase, the measured MDA concentrations decreased slightly at 90–100 µL of 2-TBA, with values of 1.032 ± 0.045 and 1.005 ± 0.161 mg/L, respectively. This decline suggests that an increase in 2-TBA concentration does not necessarily result in a corresponding increase in the measured MDA–TBA chromogen. At sufficiently high reagent concentrations, factors such as reagent excess, competing reactions, changes in chromogen stability, or spectrophotometric response may influence the apparent MDA concentration. Therefore, an optimum 2-TBA concentration may exist beyond which further increases in reagent volume provide little analytical benefit.

In contrast to the pronounced response observed around pH 8.0, the results obtained at pH 5.6 showed a relatively consistent increase in MDA concentration as the volume of 2-TBA increased from 10 to 100 µL. This observation suggests that the effect of 2-TBA volume was not completely independent of pH. Rather, the two variables appear to interact in determining the intensity of the measured TBARS response. The relatively steady increase at pH 5.6, compared with the more pronounced maximum under alkaline conditions, further demonstrates that pH can modify the response of the assay to changes in reagent concentration.

The results obtained using the fixed 2-TBA volumes further support this interpretation. For example, at a 20 µL 2-TBA volume, the MDA concentration varied considerably across the investigated pH range. The highest value was recorded at pH 8.0 (0.680 ± 0.070 mg/L), whereas the lowest was observed at pH 5.6 (0.352 ± 0.060 mg/L). Similar behaviour was observed

under the other experimental conditions, with the maximum MDA response generally occurring around pH 8.0–8.6. In one of the later experimental conditions, the maximum concentration shifted towards pH 8.6, reaching 1.005 ± 0.161 mg/L, while the lowest concentration was 0.340 ± 0.233 mg/L at pH 5.6.

The absence of a strictly linear relationship between pH and MDA concentration is important when interpreting these findings. Although the results consistently indicate enhanced MDA responses under mildly alkaline conditions, the concentration did not increase progressively with every incremental increase in pH. Instead, the data showed fluctuations, followed by a pronounced increase around pH 8.0–8.6 and, in some cases, a subsequent decline. This behaviour suggests that the observed response may reflect not only the actual formation of MDA but also the chemical behaviour of MDA and the MDA–TBA chromogen under different pH conditions.

One possible explanation for the increased MDA concentration under mildly alkaline conditions is enhanced availability or formation of reactive aldehydic products from lipid oxidation. Changes in pH can influence lipid oxidation reactions and the chemical stability of oxidation products. However, the TBARS assay does not measure MDA exclusively; other substances capable of reacting with 2-TBA can contribute to the measured absorbance. Consequently, the increased absorbance observed at pH 8.0 should be interpreted as an enhanced TBARS response rather than definitive evidence that the actual MDA concentration in the sample increased to the same extent.

Another possible explanation is the influence of pH on the formation and stability of the MDA–TBA chromophore. The TBARS reaction depends on the chemical environment in which MDA reacts with 2-TBA to form a coloured adduct that can be quantified spectrophotometrically, which is in agreement with the research carried out by Janero [22]. Consequently, changes in pH may affect reaction kinetics, chromogen formation, chromogen stability, and the molar absorptivity of the coloured species. The relatively high concentrations observed around pH 8.0 therefore may reflect a combination of increased availability of reactive aldehydic products and favourable conditions for formation or stabilisation of the coloured MDA–TBA complex.

The results also demonstrate the importance of optimising the analytical conditions of the TBARS assay. The repeated observation of the highest MDA response around pH 8.0, together with the consistently low response at pH 5.6, indicates that pH is an important experimental variable that should be carefully controlled when comparing TBARS measurements. Similarly, the decline observed at 90–100 µL of 2-TBA indicates that simply increasing the reagent volume may not improve assay sensitivity indefinitely. An appropriate balance between pH and 2-TBA concentration is therefore necessary to obtain a reliable and reproducible analytical response.

Overall, the findings indicate that pH substantially influences the apparent concentration of MDA measured in the sample using the TBARS assay [27]. Across the experimental conditions, pH 5.6 generally produced the lowest MDA concentrations, whereas pH 8.0, and in some cases pH 8.6, produced the highest values. The response was not strictly linear across the investigated pH range, demonstrating that MDA measurement by TBARS is influenced by the chemical environment of the reaction. The results further suggest that the enhanced response under mildly alkaline conditions may arise from increased lipid oxidation and/or improved formation or stability of the MDA–TBA chromogen. The decrease observed at the highest 2-TBA volumes further indicates that an optimum reagent concentration may be required for maximum analytical response.

Conclusion

The results of this study reveal that malondialdehyde (MDA), as an indicator of lipid peroxidation, is not only a compound of biological significance but also one which can react with proteins and nucleic acids. The change in the levels of MDA at different pH values shows that pH is an important parameter affecting lipid oxidation and/or the stability and formation of the TBA-MDA adduct. Thus, the increase in MDA detected or formed in the alkaline medium demonstrates the sensitivity of oxidative processes and the measurements of TBARS to the physicochemical conditions of the medium of fermented dairy products. Therefore, pH should be controlled and reported in studies when MDA is used as an oxidative stress or lipid-peroxidation marker.

The results of the study also have biological importance since MDA formed from lipid peroxidation in red blood cells may react with cellular proteins, among which is haemoglobin (HbA). Although the current study was performed in vitro, its results give important information about the properties of MDA and the parameters that may affect the formation/detection of MDA in complex biological polymeric materials and food systems. Moreover, the role of inositolhexakisphosphate (IHP) in this system is a basis for further research of IHP in relation to oxidative processes and reactions with MDA.

Future research would benefit from using a wider pH range and greater resolution in order to perform a more thorough evaluation of the effect of pH on MDA formation and TBA-MDA complex stability. In particular, the neutral to alkaline region, which appears to demonstrate the most pronounced effect according to the presented results, requires further study. Including additional pH values in the range of pH 5.0 to 11.0 with smaller increments would help to define the optimal conditions and the points of transition.

Since the TBARS test is relatively unspecific and might be affected by other thiobarbituric acid reactive substances, future research should include more specific methods such as HPLC to evaluate MDA content. Using such analysis in parallel with spectrophotometry

will increase the reliability and analytical accuracy of the results obtained.

It is also worth investigating the kinetics of TBA-MDA complex formation as well as studying affinity for 2-TBA using longer incubation periods such as 6, 12, and 24 h. Centrifuging of the mixture prior to spectrophotometry may improve the clarity of the supernatant and reduce the effects of suspended particles, which could be important in the case of fermented dairy products.

These findings highlight the need for careful optimisation and standardisation of pH and 2-TBA volume when applying the TBARS assay for MDA determination in fermented milk products such as *nunu*. Finally, systematic studies of IHP should be performed to determine its effectiveness and potential mechanisms of action in oxidative processes under different experimental conditions. Studying factors such as IHP concentration, pH, incubation time, and interactions with MDA and other lipoperoxidation products would help clarify its potential applications in oxidative stress management and food science.

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