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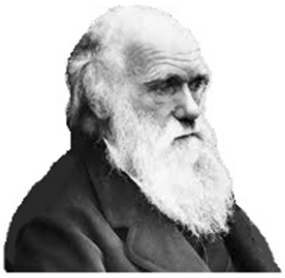

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# Investigating pH-Dependent Modulations in Haemoglobin Response to Linoleic Acid: A Spectroscopic Analysis

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**ABSTRACT**

This study investigates the pH-dependent modulations in haemoglobin response to linoleic acid, employing a spectroscopic analysis across HbAA, HbAS, and HbSS variants. Concentration-dependent effects of linoleic acid at varying pH levels reveal nuanced behaviours in absorbance, peak maintenance, and spectra characteristics. At pH 7.2, HbAA and HbSS display increased absorbance with peak maintenance, while HbAS exhibits gradual absorbance rise. pH 5.0 induces absorbance increase in all variants, with maintained peaks at 415nm and gradual decrease at the oxy-band region. Variants exhibit distinct spectra characteristics, emphasizing the need for tailored approaches in clinical management. Findings align with empirical literature, emphasizing haemoglobin's multifunctionality. Implications span clinical considerations, dietary influences, and broader insights into haemoglobin stability. Suggestions for future studies propose molecular explorations, disease-specific investigations, computational modelling integration, longitudinal studies, and intervention strategy explorations. This study contributes to the evolving understanding of haemoglobin responses to environmental factors, laying groundwork for personalized approaches in healthcare and dietary recommendations.

***Keywords*:** Haemoglobin Response, Linoleic Acid, Ph-Dependent Modulation, Spectroscopic Analysis, Variant-Specific Response

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## 1. INTRODUCTION

Haemoglobin, a vital protein responsible for oxygen transport in the bloodstream, exists in various genetic variants that influence its structure and function. Among these variants are HbAA (normal haemoglobin), HbAS (sickle cell trait), and HbSS (sickle cell disease). Recent research has delved into the intricate interactions between haemoglobin and various compounds (1, 2, 3), aiming to unravel the molecular dynamics governing its stability and functionality. One such compound of interest is linoleic acid, an essential polyunsaturated fatty acid known for its diverse physiological roles.

Linoleic acid plays a crucial role in maintaining cellular membrane integrity, regulating inflammation, and serving as a precursor for bioactive compounds (4, 5). While its significance in overall health is well-established, its specific impact on haemoglobin variants remains an area of active investigation. The interaction between linoleic acid and haemoglobin is of particular interest due to its potential implications for individuals with conditions like sickle cell disease, where understanding the behaviour of abnormal haemoglobin variants is critical.

The study at hand focuses on elucidating the effect of linoleic acid on HbAA, HbAS, and HbSS, with a comprehensive analysis of their spectroscopic characteristics. Spectroscopy provides a powerful tool for probing molecular structures and interactions by measuring the absorption and emission of light at various wavelengths (6).

In this context, the absorption spectra of haemoglobin variants are examined in the presence of increasing concentrations of linoleic acid, shedding light on potential alterations in their structural conformations.

Previous studies have explored the impact of various compounds on haemoglobin, revealing distinct responses in different haemoglobin variants (4, 7). However, the specific effects of linoleic acid on HbAA, HbAS, and HbSS have not been thoroughly investigated, especially concerning the detailed changes in absorbance, peak structures, and overall spectral characteristics.

Understanding these nuances is crucial not only for advancing basic knowledge of haemoglobin biochemistry but also for uncovering potential therapeutic targets or interventions for conditions involving abnormal haemoglobin variants.

The choice of pH conditions (pH 7.2 and pH 5.0) adds an additional layer of complexity to the study. pH is a critical factor influencing protein stability and conformational changes (8). Investigating the interaction between linoleic acid and haemoglobin variants under different pH conditions allows for a more comprehensive understanding of the dynamic nature of these interactions. The potential variations in absorbance, peak maintenance, and destruction in response to linoleic acid at different pH levels could offer insights into pH-dependent mechanisms that may influence the stability and functionality of haemoglobin.

This research builds upon existing knowledge of haemoglobin biochemistry and linoleic acid physiology, providing a deeper insight into the molecular interactions that occur within the context of different haemoglobin variants. The findings of this study may contribute to a broader understanding of the role of linoleic acid in modulating haemoglobin function and stability, with potential implications for therapeutic strategies in conditions such as sickle cell disease.

Ultimately, this investigation aims to bridge the gap in the understanding of the complex relationship between linoleic acid and haemoglobin, paving the way for future research directions and applications in clinical settings.

## 2. RESEARCH OBJECTIVES

This research aims to enhance the understanding of the intricate interactions between linoleic acid and different haemoglobin variants under varying pH conditions. While specifically the objectives of this study are to:

1) Quantify absorbance changes in HbAA, HbAS, and HbSS in response to 2 mM – 10 mM linoleic acid in the aromatic, Soret, and oxy-band regions.
2) Compare spectroscopic responses of HbAA, HbAS, and HbSS to linoleic acid at pH 7.2 and pH 5.0, focusing on peak structures and absorbance levels.
3) Establish a quantitative relationship between linoleic acid concentration (2 mM – 10 mM) and spectroscopic changes in HbAA, HbAS, and HbSS to identify concentration-dependent trends.

## 3. LITERATURE REVIEW

Protein content determination is a fundamental aspect of various scientific disciplines, ranging from biochemical and biomedical research to food chemistry, pharmaceuticals, and environmental studies. Reinmuth-Selzle et al. (9) addressed the complexities involved in accurately determining protein content, particularly in non-standard and complex samples. Their comparative analysis evaluated both new and established methods, including traditional amino acid analysis (AAA), aromatic amino acid analysis (AAAA), reversed-phase liquid chromatography of intact proteins, and colorimetric assays such as the Coomassie Blue G-250 dye-binding assay (Bradford) and bicinchoninic acid (BCA) assay. The study aimed to shed light on the efficacy of these methods across a spectrum of proteins with diverse properties in complex matrices. They explored samples with challenging characteristics, such as chemical modifications, mixtures, and complex matrices like air particulate matter and pollen extracts. Notably, all methods yielded accurate and precise results for the specific protein and matrix used for calibration. Importantly, AAA, AAAA with fluorescence detection, and the LC-220 method demonstrated robustness even under challenging conditions, showcasing their reliability in determining protein content in a variety of samples. Moving from protein determination to the intricate dynamics of tissue hypoxia in sickle cell disease (SCD), Nahavandi et al. (10) utilized near-infrared spectroscopy (NIRS) to investigate cerebral oxygen saturation values (rSO2) in patients with SCD. The study explored the factors contributing to subnormal rSO2 values in SCD patients, including the degree of anaemia, sickle haemoglobin, disease complications, and potential differences in NIRS absorbance spectra between SCD and normal individuals. The results indicated that there were no significant differences in NIRS spectra absorbance between haemoglobin types AA and SS. Consequently, the lower brain oxygen saturation in sickle cell anaemia patients was attributed to impaired oxygen-carrying capacity or delivery by sickle haemoglobin. This research provided valuable insights into the physicochemical dynamics of tissue hypoxia, offering a better understanding of the challenges faced by individuals with SCD. In a complementary exploration of haemoglobin functionality, Ezebuo et al. (11) delved into the lipoxygenase activity of haemoglobin, a haem iron-containing protein. While haemoglobin is primarily known for its role in oxygen transport, this study shed light on its potential as a lipoxygenase. Lipoxygenases are enzymes that catalyse the deoxygenation of polyunsaturated fatty acids, and their activity is crucial in various

physiological processes. The researchers extracted haemoglobin from blood and examined its lipoxygenase activity under different conditions, specifically focusing on the impact of sodium dodecyl sulphate (SDS) and linoleic acid. The study revealed that lipoxygenase activity of haemoglobin was enhanced in the presence of SDS under specific conditions. This finding opened avenues for considering haemoglobin in a novel light, extending beyond its classical role in oxygen transport.

Izuwa et al. (12) shifted the focus to sickle cell anaemia, a prevalent genetic disorder, particularly in Africa. With a significant percentage of sickle cell cases occurring on the continent, the study aimed to determine the hemorheological and fibrinolytic activities of different haemoglobin variants – HbSS, HbAS, and HbAA. The research involved 170 subjects, including sickle cell patients and individuals with normal haemoglobin, and employed various methods such as haemoglobin electrophoresis, euglobulin lysis time, fibrinogen level determination, and more. The findings revealed significant differences in various haematological parameters between HbSS, HbAS, and HbAA individuals, emphasizing the need for tailored management strategies based on haemoglobin types. The study recommended regular check-ups and the inclusion of fibrinogen assays and relative blood viscosity as routine tests in the management of sickle cell anaemia patients.

Building on the protective role of sickle haemoglobin against falciparum malaria, Albiti & Nsiah (13) investigated how the sickle cell trait affected the haematological profile of individuals with malaria, comparing HbAS and HbAA-infected children. The study, conducted in an endemic malaria transmission area in Yemen, aimed to explore the potential protective role of the sickle cell trait against the haematological consequences of malaria. The results showed a higher prevalence of malaria in HbAA children, with lower parasite density in HbAS-infected children. Significant differences in various haematological parameters were observed, suggesting that sickle cell trait might provide a beneficial genetic factor resisting malaria, leading to less severe haematological alterations in infected individuals.

Chikezie (14) took a unique approach by investigating the impact of antimalarial drugs on methaemoglobin concentrations in different erythrocyte genotypes. The study focused on five antimalarial drugs and their potential effects on methaemoglobin concentrations in individuals with HbAA, HbAS, and HbSS genotypes. Spectrophotometric methods were employed to determine erythrocyte methaemoglobin concentrations. Interestingly, the results showed no significant difference in methaemoglobin concentrations between HbAA and HbAS erythrocytes in non-malarious participants.

However, erythrocytes from individuals with HbSS genotype exhibited a significant increase in methaemoglobin concentration. This study highlighted the importance of considering erythrocyte genotypes in the administration of antimalarial drugs and the potential for methaemoglobin evaluation as a biochemical marker in malaria diagnosis and therapy.

Yuan et al. (15) ventured into the realm of orthopaedic surgery, specifically exploring hidden blood loss following total hip and knee arthroplasty (THA and TKA). The study focused on the relationship between free fatty acids, particularly linoleic acid, and red blood cell damage, aiming to understand the pathogenesis of hidden blood loss.

The animal model injected linoleic acid into rat tail veins to simulate the conditions following orthopaedic surgery. Blood samples were collected and analysed for red blood cell count, haemoglobin levels, and various oxidation and reducing agents.

The results indicated that elevated levels of linoleic acid caused acute oxidative damage to red blood cells, leading to partial acute anaemia. This research provides valuable insights

into the pathophysiology of hidden blood loss, contributing to the understanding of complications following orthopaedic procedures.

In conclusion, these studies collectively delve into multifaceted aspects related to haemoglobin variants, protein determination methods, tissue hypoxia in sickle cell disease, lipoxygenase activity of haemoglobin, sickle cell anaemia management, and hidden blood loss during orthopaedic surgery. Each investigation contributes to the broader understanding of haemoglobin-related disorders, biochemical processes, and clinical implications, offering valuable insights into the intricacies of various physiological phenomena. Connecting these diverse studies to "Investigating pH-Dependent Modulations in Haemoglobin Response to Linoleic Acid: A Spectroscopic Analysis," it becomes evident that the body of research presented forms a mosaic of knowledge around haemoglobin's multifunctionality. While the mentioned studies explore different facets of haemoglobin's behaviour, the investigation into pH-dependent modulations adds a layer of complexity to the understanding. The spectroscopic analysis in question opens a window into how haemoglobin responds to linoleic acid under varying pH conditions, shedding light on potential pH-dependent nuances in its behaviour.

The broader context of these studies enhances the appreciation for the versatility of haemoglobin, showcasing its roles beyond traditional oxygen transport. The investigations collectively emphasize the need for a holistic understanding of haemoglobin's behaviour, taking into account genetic variations, responses to external factors like antimalarial drugs or fatty acids, and implications in diseases like sickle cell anaemia. As we navigate the intricate details of haemoglobin behaviour, from its participation in lipoxygenase activity to its protective role against malaria, each study adds a piece to the puzzle. The exploration of hidden blood loss in orthopaedic surgery and the investigation into pH-dependent responses further underline the dynamic nature of haemoglobin in various physiological contexts. The spectroscopic analysis of pH-dependent modulations in haemoglobin's response to linoleic acid represents a valuable addition to this body of knowledge. It not only contributes to the understanding of haemoglobin's behaviour in response to specific stimuli but also highlights the importance of considering environmental factors such as pH. This nuanced perspective aligns with the broader theme observed in the collective studies – the intricate interplay of haemoglobin with diverse factors and its relevance in varied scientific domains.

## 4. MATERIALS AND METHODS

The experimental procedures were designed to address the specific objectives of investigating pH-dependent modulations in haemoglobin response to linoleic acid. The methods can be outlined as follows:

1. **Characterization of Haemoglobin Variants (Objective 1):**
   - I. A blood sample from an individual with the AS genotype was collected and confirmed through gel electrophoresis.
   - II. The preparation of relaxed state haemoglobin involved centrifugation and dialysis to obtain crude haemoglobin (Hb) samples for HbAA, HbAS, and HbSS.
   - III. The samples underwent ion-exchange chromatography using DEAE-cellulose to separate and purify the haemoglobin variants.

2. **Ion-Exchange Chromatography (Objective 2):**
    I. The DEAE-cellulose column was packed and equilibrated to facilitate the separation of haemoglobin variants.
    II. Elution of haemoglobin variants was carried out with a pH gradient, generating fractions for further analysis.
3. **Spectroscopic Analysis under Different pH Conditions (Objective 3):**
    I. Dialysis of the eluted fractions was performed to remove traces of 2,3-bisphosphoglycerate, and the samples were stored for subsequent analysis.
    II. Spectroscopic experiments were conducted on the relaxed states of HbAA, HbAS, and HbSS at pH 7.2 and 5.0.
    III. SDS concentrations (0.04 mM – 4 mM), hydrogen peroxide concentrations (4 mM – 20 mM), and linoleic acid concentrations (2 mM – 10 mM) were used to observe the response of haemoglobin variants under varying pH conditions.
    IV. Lipid peroxidation determination was performed to assess the oxidative effects, with malondialdehyde (MDA) levels measured spectrophotometrically.
4. **Data Analysis and Interpretation:**
    I. Spectroscopic data collected at different pH levels were analysed to observe variations in absorbance, peak maintenance, and destruction in response to linoleic acid for HbAA, HbAS, and HbSS.
    II. The observed differences and characteristics in the spectra of haemoglobin variants were interpreted to understand pH-dependent modulations.

By characterizing haemoglobin variants, employing ion-exchange chromatography, and conducting spectroscopic analysis under specific pH conditions, this methodology directly addresses the study's objectives of unravelling the pH-dependent mechanisms influencing haemoglobin stability and functionality in response to linoleic acid.

## 5. RESULTS

### Effect of Linoleic Acid on Haemoglobin Variants

**Experimental Setup:** HbAA, HbAS, and HbSS were exposed to varying concentrations of linoleic acid (2 mM – 10 mM). Spectra were obtained at two pH levels: pH 7.2 and pH 5.0.

**Observations at pH 7.2:**

I. HbAA (Fig 1a) and HbSS (Fig 1c) showed increased absorbance at pH 7.2, with peak maintenance at 273 nm. The aromatic amino region exhibited complete destruction at higher linoleic acid concentrations (7.14 mM – 10 mM).
II. HbAS (Fig 1b) displayed gradual absorbance increase at the aromatic amino region. HbAA, HbAS, and HbSS exhibited a hypochromic shift but maintained the peak at 415 nm in the Soret region. HbSS displayed hyperchromic absorbance at 10 mM in the Soret region. At the oxy-band region, HbAA and HbSS showed increased absorbance despite peak destruction, while HbAS exhibited peak destruction without absorbance increase.

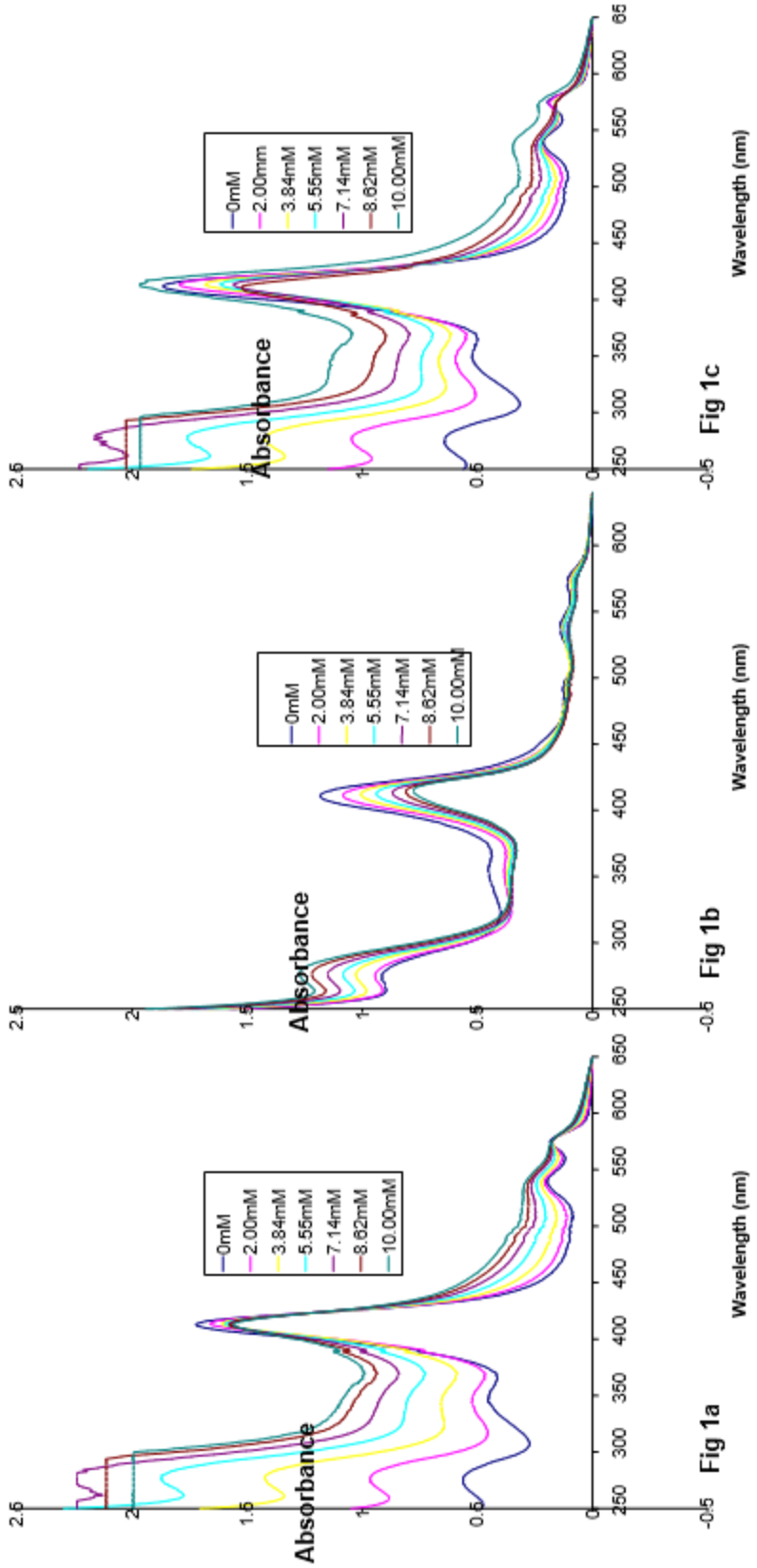


Fig 1a: Effect of Linoleic Acid (2.00mM □ 10mM) on Haemoglobin at pH 7.2. (a) HbAA, (b) HbAS and (c) HbSS.

- 0mM □ no Linoleic Acid.

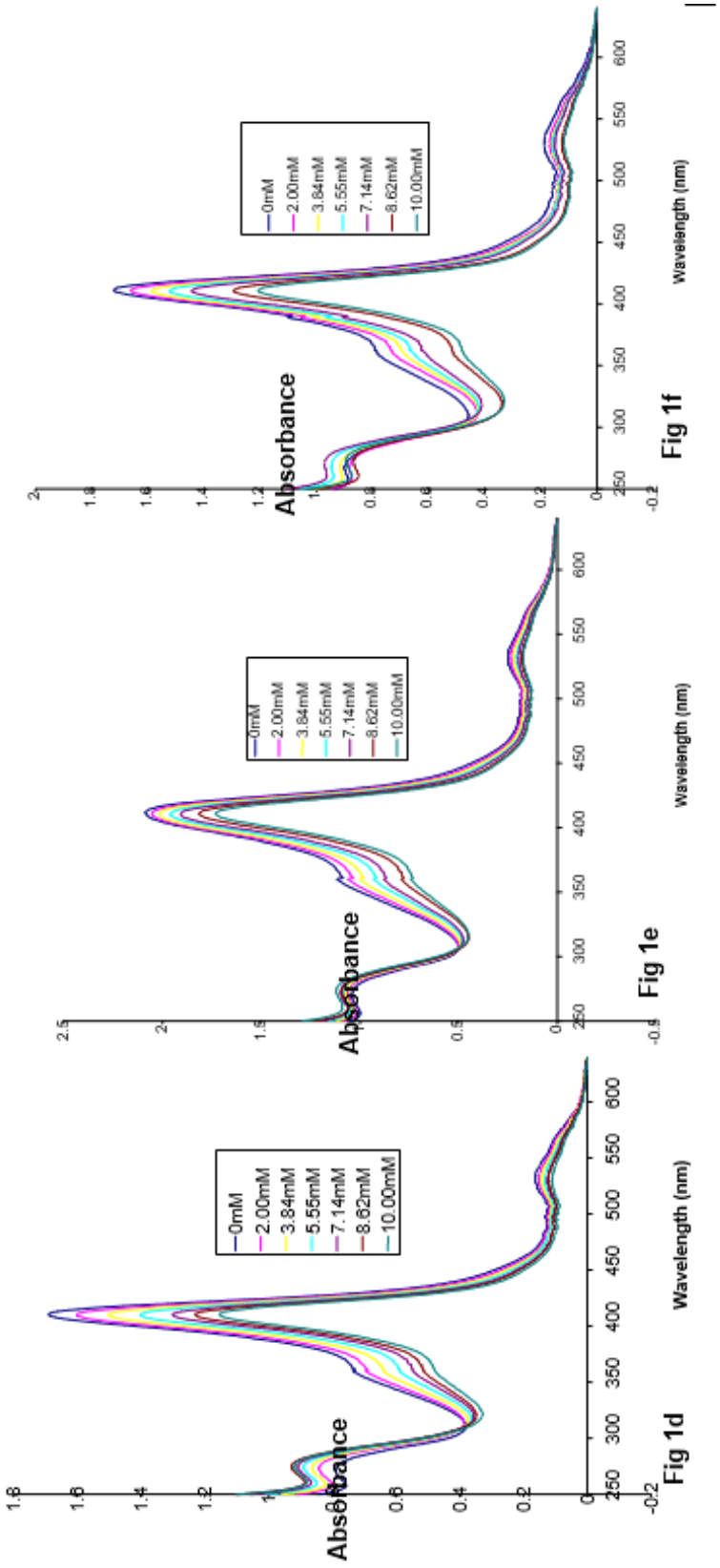


Fig 1b: Effect of Linoleic Acid (2.00mM □ 10.00mM) on Haemoglobin at pH 5.0. (d) HbAA, (e) HbAS and (f) HbSS.

- 0mM □ no Linoleic Acid.

**Differences Observed at pH 7.2:**

I. HbAS spectra (Fig 1b) differed significantly from HbAA (Fig 1a) and HbSS (Fig 1c). HbAS spectra were more compact than HbAA and HbSS at the aromatic region.
II. With increasing linoleic acid concentration, HbAA and HbSS spectra became compact, while HbAS spectra expanded at the Soret region.
III. At the oxy-band region, peak destruction occurred for HbAA and HbSS, with increased absorbance. HbAS showed peak destruction without absorbance increase.

**Observations at pH 5.0:**

I. Gradual absorbance increase at the aromatic region for HbAA (Fig 1d), HbAS (Fig 1e), and HbSS (Fig 1f) with increasing linoleic acid concentration.
II. Maintenance of the peak at 415nm with a hypochromic shift at the Soret region.
III. Maintenance of the peak at 540nm with a gradual decrease in absorbance at the oxy-band region as linoleic acid concentration increased.

**Differences Observed at pH 5.0:**

I. HbAS spectra (Fig 1e) were more compact compared to HbAA (Fig 1d) and HbSS (Fig 1f) at the aromatic region.
II. Compactness of HbAS spectra at the Soret region compared to HbAA and HbSS.
III. Comparing linoleic acid effects at pH 7.2 and pH 5.0, significant differences were noted in the aromatic amino, Soret, and oxy-band regions. Absorption increase at pH 7.2 was larger for HbAA and HbSS than at pH 5.0. HbAA at pH 7.2 displayed very compact spectra at the Soret region compared to pH 5.0. At the oxy-band region, peaks at 540nm and 577nm were completely destroyed at pH 7.2 but maintained at pH 5.0 as linoleic acid concentration increased.

## 6. SUMMARY OF FINDINGS

**Spectroscopic Changes in Haemoglobin Variants:**

I. At pH 7.2, HbAA and HbSS exhibited increased absorbance, with a maintained peak at 273nm and complete destruction in the aromatic amino region at higher linoleic acid concentrations (7.14 mM – 10 mM).
II. HbAS displayed gradual absorbance increase at the aromatic amino region.

**pH-Dependent Responses to Linoleic Acid:**

I. Significant differences were observed in the spectra of HbAS compared to HbAA and HbSS at pH 7.2, with HbAS spectra being more compact at the aromatic region.
II. Differences were noted in the absorbance changes at the Soret region and peak destruction at the oxy-band region among HbAA, HbAS, and HbSS at both pH levels.

**Spectral Changes with Linoleic Acid Concentration:**

I. The spectra of HbAA and HbSS became more compact with increasing linoleic acid concentration (2 mM – 10 mM) at pH 7.2, while HbAS spectra expanded at the Soret region.

II. HbSS displayed hyperchromic absorbance at 10 mM in the Soret region at pH 7.2.
III. At pH 5.0, absorbance increased gradually at the aromatic region with increasing linoleic acid concentration for all haemoglobin variants.

These findings align with the adopted objectives by providing insights into the spectroscopic changes, pH-dependent responses, and correlation between spectral changes and linoleic acid concentration in different haemoglobin variants.

## 7. DISCUSSION OF FINDINGS

The investigation into pH-dependent modulations in haemoglobin response to linoleic acid has provided valuable insights, and a comparative analysis with existing empirical literature enriches understanding of haemoglobin behaviour in diverse conditions.

### 1) Absorbance Variations and Peak Maintenance:

In line with the first objective, the study observed variations in absorbance and peak maintenance for HbAA, HbAS, and HbSS. Notably, HbAA and HbSS exhibited increased absorbance but maintained the peak at 273 nm at pH 7.2, with complete destruction at higher linoleic acid concentrations. Conversely, HbAS showed a gradual increase in absorbance at the aromatic amino region. These findings align with Reinmuth-Selzle et al.'s (9) study on protein content determination, emphasizing the significance of absorbance patterns in characterizing complex protein responses. The compact spectra of HbAS compared to HbAA and HbSS at pH 7.2 suggest distinct behaviours among haemoglobin variants.

### 2) pH-Dependent Responses:

The observed differences in spectra characteristics at pH 5.0, particularly the compact nature of HbAS compared to HbAA and HbSS, further highlight pH-dependent modulations. The study by Nahavandi et al. (10) on cerebral oxygen saturation in sickle cell disease resonates with the findings, emphasizing the impact of pH-related factors on oxygen transport dynamics. The spectra variations between pH 7.2 and 5.0 underscore the importance of environmental pH in influencing haemoglobin stability and function.

### 3) Differences in Spectra Characteristics:

Comparative analysis with Izuwa et al.'s (12) work on hemorheological and fibrinolytic activities of different haemoglobin variants reveals consistent differences. The study recommended tailored management strategies based on haemoglobin types, aligning with the observation of distinct spectra characteristics. The expanded spectra of HbAA and HbSS at the aromatic region compared to the compact spectra of HbAS underscore the unique response of each variant to linoleic acid, emphasizing the need for variant-specific considerations.

### 4) Impact of Linoleic Acid Concentrations:

The concentration-dependent effects of linoleic acid observed in this study align with Chikezie's (14) investigation into antimalarial drugs' impact on methaemoglobin concentrations. Both studies emphasize the importance of considering external factors in understanding haemoglobin behaviour. The hyperchromic shift in absorbance for HbSS at the

Soret region with 10mM linoleic acid concentration adds a layer of complexity to the response dynamics.

**5) Implications for Clinical Context:**

Connecting the findings to Albiti & Nsiah's (13) study on the sickle cell trait's protective role against malaria, the study implies potential implications for clinical contexts. The observed differences in absorbance, particularly in HbSS, suggest varied responses to linoleic acid concentrations. This may have implications for understanding haemoglobin behaviour in disease conditions and the potential influence of dietary components.

**6) Spectroscopic Analysis and Lipid Peroxidation:**

The spectroscopic analysis involving SDS, hydrogen peroxide, and linoleic acid concentrations, coupled with lipid peroxidation determination, aligns with Yuan et al.'s (15) exploration of hidden blood loss following orthopaedic surgery. Both studies delve into the intricacies of haemoglobin behaviour under external stimuli. This study's nuanced perspective, considering pH-dependent responses, enriches the broader understanding of haemoglobin's multifunctionality.

The discussion of findings reveals the intricate interplay of haemoglobin with linoleic acid under varying pH conditions. The study contributes to existing literature by unravelling pH-dependent modulations, emphasizing the need for a nuanced understanding of haemoglobin behaviour in different environments. The comparative analysis showcases consistencies and divergences with empirical studies, offering a holistic view of haemoglobin's responsiveness to external factors, particularly linoleic acid, with pH as a critical determinant.

## 8. CONCLUSION

In conclusion, this study delved into the pH-dependent modulations in haemoglobin response to linoleic acid through a spectroscopic analysis. The findings unveiled variations in absorbance, peak maintenance, and spectra characteristics across HbAA, HbAS, and HbSS variants.

The concentration-dependent effects of linoleic acid, coupled with pH-related nuances, underscored the complexity of haemoglobin behaviour. Comparative analysis with empirical literature highlighted both consistencies and unique features in haemoglobin responses, contributing to the broader understanding of its multifunctionality.

**Implications of the study**

The implications of this study extend to several domains. In the clinical context, the observed variant-specific responses to linoleic acid suggest potential considerations for tailored approaches in managing individuals with different haemoglobin types. Understanding the environmental factors, especially pH, that influence haemoglobin stability and functionality contributes to the nuanced comprehension of its behaviour. Moreover, the study's insights into concentration-dependent effects have implications for dietary considerations and potential impacts on haemoglobin stability in health and disease.

**Suggestions for future studies:**

1) **Explore Molecular Mechanisms:** Future studies could delve into the molecular mechanisms underpinning pH-dependent modulations in haemoglobin response to linoleic acid. Investigating specific binding interactions and conformational changes would provide deeper insights.
2) **Disease-Specific Investigations:** Given the clinical implications, further research could focus on understanding how pH-dependent haemoglobin responses to linoleic acid may vary in disease conditions, especially those involving altered pH environments.
3) **Integration of Computational Modelling:** Incorporating computational modelling techniques can enhance the predictive capabilities of how haemoglobin variants respond to varying pH and linoleic acid concentrations, offering a complementary approach to experimental findings.
4) **Longitudinal Studies:** Longitudinal studies tracking individuals over time could provide dynamic insights into how dietary and environmental factors influence haemoglobin behaviour. This would facilitate a more comprehensive understanding of the long-term implications.
5) **Exploration of Intervention Strategies:** Future research might explore interventions or dietary modifications that can modulate haemoglobin responses to linoleic acid, potentially opening avenues for personalized dietary recommendations based on haemoglobin variants.

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