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Hemoglobin adducts as biomarkers of psychoactive substance abuse

Beatriz Oliveira

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HEMOGLOBIN ADDUCTS AS BIOMARKERS OF PSYCHOACTIVE SUBSTANCE ABUSE

Master's Thesis in Legal Medicine, submitted to the Abel Salazar Institute of Biomedical Sciences at the University of Porto.

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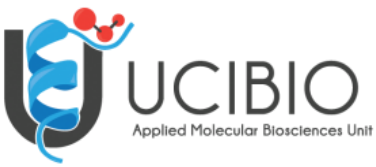

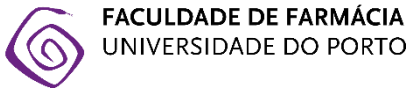

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Resumo

A hemoglobina é uma proteína essencial responsável pelo transporte de oxigénio no organismo, e a formação de adutos covalentes entre a hemoglobina e metabolitos do etanol, como o acetaldeído, está relacionada com a exposição ao álcool e o seu consumo prolongado. A deteção destes adutos, realizada principalmente por técnicas como a cromatografia líquida de alta eficiência e espectrometria de massa, enfrenta limitações devido à complexidade das amostras, custos elevados e dificuldades em obter informação estrutural detalhada. Neste contexto, surge a necessidade de abordagens alternativas mais informativas e acessíveis.

O objetivo desta dissertação foi avaliar o potencial da ressonância magnética nuclear de protão, utilizando a técnica de diferença de transferência de saturação [do inglês *Saturation Transfer Difference Nuclear Magnetic Resonance* (STD-NMR)], para a deteção de adutos hemoglobina-acetaldeído. Esta técnica constitui uma metodologia avançada para a investigação de interações ligando-proteína, permitindo a identificação seletiva e a caracterização estrutural de ligandos em matrizes complexas. Deste modo, foram incubadas soluções de hemoglobina humana com acetaldeído em concentrações entre 16 µM e 20 mM. A concentração de hemoglobina foi determinada por espectroscopia na região do ultravioleta-visível e a formação dos adutos foi monitorizada por NMR convencional e STD-NMR.

Os resultados revelaram o aparecimento de um novo pico a 1,47 ppm (referenciado ao padrão interno DSS) na análise de NMR, compatível com a presença de acetaldeído ligado à hemoglobina. Adicionalmente, a análise por STD-NMR demonstrou um aumento da área deste sinal em resposta ao acréscimo da concentração de acetaldeído, validando a capacidade da técnica para identificar especificamente a formação do aduto. Verificou-se que a resposta do sinal a 1,47 ppm é linear na gama de 80 µM a 6 mM, com um coeficiente de correlação R^2 de 0,9944, indicando uma relação direta entre a concentração de acetaldeído e a formação do aduto. A 20 mM observou-se a evidência de saturação dos sítios de ligação disponíveis na hemoglobina, sinalizando o limite da capacidade de interação nessa faixa de concentrações.

Estes resultados sugerem que a técnica de STD-NMR constitui uma alternativa promissora para a análise estrutural de adutos hemoglobina-acetaldeído e para o desenvolvimento de biomarcadores de exposição ao álcool. A possibilidade de detetar e caracterizar modificações induzidas pelo acetaldeído por via não destrutiva e com potencial de especificidade estrutural abre novas perspetivas na monitorização clínica e investigação toxicológica. Ainda assim, futuros estudos serão essenciais para validar a sensibilidade e aplicabilidade desta abordagem em matrizes biológicas mais complexas e contextos populacionais.

Abstract

Hemoglobin is an essential protein responsible for oxygen transport in the body, and the formation of covalent adducts between hemoglobin and ethanol metabolites, such as acetaldehyde, is associated with alcohol exposure and prolonged consumption. The detection of these adducts, primarily performed by techniques such as high-performance liquid chromatography and mass spectrometry, faces limitations due to sample complexity, high costs, and difficulties in obtaining detailed structural information. In this context, there is a need for alternative, more informative, and accessible approaches.

The objective of this dissertation was to evaluate the potential of proton nuclear magnetic resonance (NMR), using the Saturation Transfer Difference Nuclear Magnetic Resonance (STD-NMR) technique, for the detection of hemoglobin-acetaldehyde adducts. This technique represents an advanced methodology for investigating ligand-protein interactions, allowing selective identification and structural characterization of ligands within complex matrices. To this end, solutions of human hemoglobin were incubated with acetaldehyde at concentrations ranging from 16 µM to 20 mM. Hemoglobin concentration was determined by ultraviolet-visible (UV-vis) spectroscopy, and adduct formation was monitored by conventional NMR and STD-NMR.

The results revealed the appearance of a novel peak at 1.47 ppm (referenced to the internal standard DSS) in the NMR spectra, consistent with acetaldehyde bound to hemoglobin. Additionally, STD-NMR analysis demonstrated an increase in the area of this signal corresponding to increasing acetaldehyde concentration, validating the technique's ability to specifically identify adduct formation. The signal response at 1.47 ppm exhibited linearity between 80 µM and 6 mM, with a correlation coefficient (R^2) of 0.9944, indicating a direct relationship between acetaldehyde concentration and adduct formation. At 20 mM, evidence of saturation of available binding sites on hemoglobin was observed, marking the limits of interaction capacity within this concentration range.

These findings suggest that STD-NMR constitutes a promising alternative for the structural analysis of hemoglobin-acetaldehyde adducts and for the development of biomarkers of alcohol exposure. The ability to detect and characterize acetaldehyde-induced modifications non-destructively and with potential structural specificity opens new perspectives for clinical monitoring and toxicological research. Nonetheless, further studies will be essential to validate the sensitivity and applicability of this approach in more complex biological matrices and population contexts.

Abbreviations

1D - One-dimensional

2D - Two-dimensional

2,3-BPG - 2,3-bisphosphoglycerate

E - Extinction coefficient

A - Absorvance

ADH - Alcohol dehydrogenase

ALDH - Aldehyde dehydrogenase

Acetil-CoA - Acetyl coenzyme A

C - Concentration

Cl^- - Chloride ion

CO_2 - Carbon dioxide

DeoxyHb - Deoxyhemoglobin

DNA - Deoxyribonucleic acid

DSS - 2,2-dimethyl-2-silapentane-5-sulfonic acid

ELISA - Enzyme-linked immunosorbent assay

EtG - Ethyl glucuronide

Fe^{2+} - Ferrous iron

Fe^{3+} - Ferric iron

GC-FID - Gas Chromatography with Flame Ionization Detection

GS-MS - Gas Chromatography–Mass Spectrometry

Hb - Hemoglobin

HbA - Hemoglobin A

HbA_1c -Glycated hemoglobin

HbF - Fetal hemoglobin

HPLC - High-performance liquid chromatography

HSQC - Heteronuclear single quantum coherence

Kd - Dissociation constant

KNF - Koshland-Némethy-Filmer model

L - Molar ratio

MetHb - Methemoglobin

MWC - Monod-Wyman-Changeux model

$NaBH_4$ - Sodium borohydride

NMR - Nuclear magnetic resonance

NO - Nitric oxide

O_2- Oxygen

OxyHb - Oxyhemoglobin

pO_2 - Oxygen partial pressure

R state - Relaxed state

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

STD-NMR - Saturation transfer difference nuclear magnetic resonance

T state - Tense state

TOCSY - Total correlation spectroscopy

UPLC-MS/MS - Ultra-performance liquid chromatography coupled with tandem mass spectrometry

UV–Vis - Ultraviolet-visible spectroscopy

List of figures

List of Tables

Index

1. Introduction

Ethanol, the psychoactive and toxic compound present in alcoholic beverages, is a substance capable of inducing dependence and producing significant physiological and behavioral effects. From an epidemiological perspective, alcohol consumption represents a major public health concern worldwide. In Portugal, the per capita alcohol consumption is estimated at 11.9 liters per year, the highest among the countries of the Organisation for Economic Co-operation and Development (OECD), significantly exceeding the OECD average of 8.5 liters per capita (Health at a Glance 2025, 2025). Globally, approximately 2.6 million deaths were attributed to alcohol consumption in 2019, with a notable predominance among males, with 2 million deaths against 600,00 deaths among women (World Health Organization, 2024). It is further estimated that nearly 400 million people live with alcohol-use disorders, of whom 209 million meet criteria for alcohol dependence (World Health Organization, 2024).

Even low levels of alcohol consumption are associated with increased health risks. Alcohol use is causally linked to over 200 diseases, including various forms of cancer, cardiovascular diseases, and liver cirrhosis (World Health Organization,2024). Moreover, approximately 13% of deaths among individuals aged 20-39 years are attributed to alcohol-related causes. Beyond the direct health impact, alcohol use contributes to traffic and railway accidents, interpersonal violence, social problems, and congenital defects. (World Health Organization, 2024)

Current diagnostics focus on recent exposure via blood ethanol measurement, a gold standard using gas chromatography with flame ionization detection (GC-FID) or gas chromatography–mass spectrometry (GC-MS) with headspace sampling (Jones, 1996, 2010). Another method are breathalyzers, a rapid method suitable for emergency and legal contexts based on electrochemical or infrared, to quantify exhaled ethanol, correlating closely with blood levels (Jones, 1996). Urine tests for metabolites like ethyl glucuronide (EtG) and ethyl sulfate via gas chromatography (GS), high-performance liquid chromatography (HPLC), immunoassays, or Ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) (Helander & Beck, 2005; Wurst et al., 2015). Complementary assessments may also be conducted, including blood counts, electrolytes, liver enzymes (alanine aminotransferase / aspartate aminotransferase / gamma-glutamyl transferase), renal function, blood gases, glucose levels, and imaging. These are typically performed using automated clinical analyzers that employ

spectrophotometric, colorimetric, or potentiometric methods (Andresen-Streichert et al., 2018).

These methods detect recent or acute intake but fail to capture chronic patterns, highlighting the need for biomarkers that persist post-abstinence, enable precise diagnosis, relapse monitoring, and personalized treatment to curb alcohol's societal impact (Litten et al., 2010; K. Peterson, 2004). Hemoglobin emerges as a promising chronic biomarker, persisting throughout erythrocyte lifespan to reflect prolonged exposure; its abundance, easy access, and lack of rapid repair mechanisms position it ideally for public health, therapy validation, and harm reduction.

1.1. Hemoglobin: molecular structure, biochemical properties, and physiological relevance

Hemoglobin was one of the first proteins to have its three-dimensional structure determined by X-ray in the 1960s (Perutz et al., 1960). It is the most abundant protein in human red blood cells and plays a central role in physiology by reversibly binding oxygen and transporting it from the lungs to tissues throughout the body. As illustrated in Figure 1, hemoglobin exhibits cooperative oxygen binding and release, evidenced by the O_2 equilibrium curve (Gell, 2018). The oxygen dissociation curve shows the link between oxygen partial pressure (pO_2) and hemoglobin's oxygen saturation. It forms a sigmoidal shape, indicating hemoglobin's cooperative binding, where one oxygen molecule binding increases the affinity for others. This allows efficient oxygen uptake in the lungs, where pO_2 is high, and release in tissues with lower pO_2 (Sil, 2024). The position and shape of the oxygen dissociation curve are influenced by physiological factors such as pH, temperature, carbon dioxide, and 2,3-bisphosphoglycerate levels, allowing hemoglobin to fine-tune oxygen delivery according to tissue demands (Khee & Leow, 2007). In addition to its role in oxygen transport, hemoglobin also contributes to blood buffering capacity and helps preserve the vasoactive mediator nitric oxide (Rubana & Aulik, 1989).

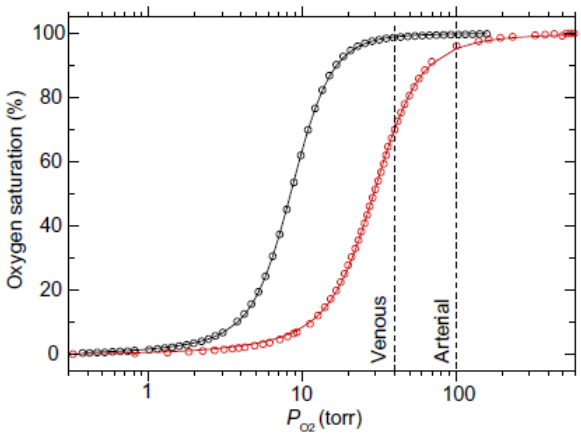


Figure 1. Hemoglobin saturation curve. Adapted from (Gell, 2018).

Several hemoglobin variants emerge at different developmental stages of the human body. The predominant type is hemoglobin A (HbA, α2β2), constituting approximately 97% of adult hemoglobin in healthy individuals. Hemoglobin A2 (HbA2, α2δ2) represents about 2.5%, while fetal hemoglobin (HbF, α2γ2) accounts for roughly 0.5% (Barbara J. Bain, 2006). Fetal hemoglobin has a greater affinity for oxygen, aiding in the transfer of O_2 from mother to fetus, yet its levels typically drop after birth. Chromatographic techniques have identified minor fractions such as HbA1a, HbA1b, and HbA1c, with glycated hemoglobin (HbA1c) being the most recognised variant (Carter, 2007). HbA1c forms through the non-enzymatic binding of glucose to the N-terminal valine of HbA's β-globin chains (American Diabetes Association [ADA],2011; Yadav & Kumar Mandal, 2023).

The biological functions of HbA are linked to its three-dimensional structure and its associated properties. HbA's hierarchical structure includes primary, secondary, tertiary, and quaternary levels, each contributing to this molecule's stability, specificity, and functional dynamics (Bunn & Forget, 1986; Perutz, 1970).

1.1.1. Conformational architecture: Primary, secondary, tertiary, and quaternary structure

The primary structure of HbA is composed of two α-chains (α1 and α2), each containing 141 amino acids, and two β-chains (β1 and β2) with 146 amino acids (Bunn & Forget, 1986). As illustrated in Figure 2, each polypeptide is defined by its unique linear sequence of amino acids, which determines its chemical properties and governs its folding and interaction potential (Bunn & Forget, 1986; Gell, 2018).

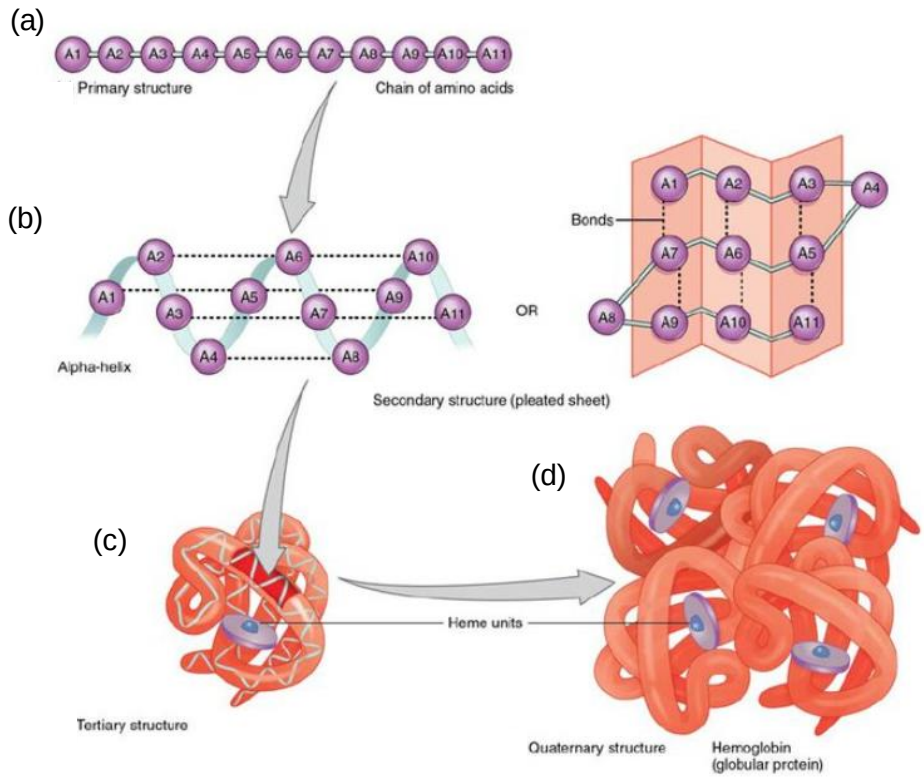


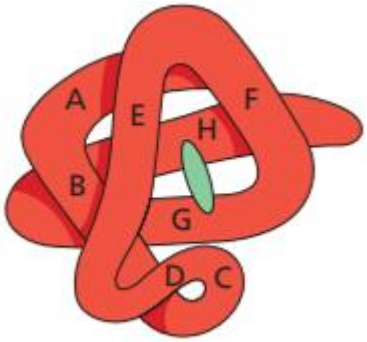
Figure 2. Hemoglobin Conformational Architecture, including the (a) primary structure as a sequence of amino acids,(b) secondary, (c) tertiary, and (d) quaternary structures. Adapted from (Moitra, 2015).

The secondary structure involves the formation of alpha helices within each polypeptide chain (Figure 2), which are stabilized by hydrogen bonds between the carbonyl and amine groups. Helices 7 and 8, respectively, from the α and β subunits, labelled A-H (Figure 3), are connected by non-helical segments (Gell, 2018). Each subunit has a binding pocket for heme formed by the E and F helices (Perutz, 1970). Heme is essential for oxygen transport; it is a functional prosthetic component of human hemoglobin, necessary for oxygen binding. It comprises a protoporphyrin IX ring linked to a central ferrous iron ion (Fe2+). The Fe is also covalently anchored to hemoglobin at the heme proximal pocket by an imidazole of a histidine residue located on the F helix (known as the proximal histidine or His F8). This configuration allows the binding of oxygen or other gases to the ferrous ion, where the imidazole of a histidine residue at the distal pocket (His E7) stabilises the bound through hydrogen-bond interactions (Gell, 2018; Nelson & Cox, 2001; Perutz, 1970).

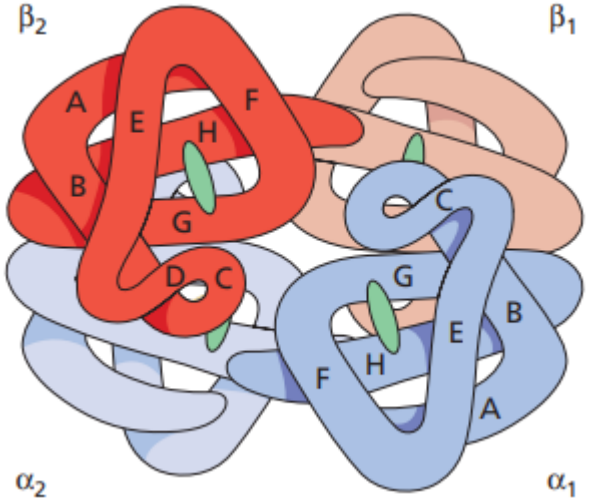
The folding of each polypeptide chain into a three-dimensional conformation is known as its tertiary structure, shown in Figure 2. This folding involves a hydrophobic pocket where the heme group resides is stabilised by hydrogen bonds, salt bridges, hydrophobic interactions, and sometimes disulfide bonds (Ahmed et al., 2020).

The assembly of the four subunits into a functional tetramer refers to the quaternary structure of the protein (Figure 2). The four subunits interact through non-covalent forces,

assembling into a compact globular protein complex. This quaternary structure is critical for cooperative oxygen binding. When oxygen binds to the heme site, conformational changes are transmitted across the subunit interfaces, increasing the oxygen affinity for binding, a phenomenon known as allosteric cooperativity (Bunn & Forget, 1986; Gell, 2018; Perutz, 1970).



Tertiary structure of the β chain



Quaternary structure of the hemoglobin A molecule

Figure 3. Diagram illustrating the tertiary structure of a hemoglobin monomer, which includes an alpha or beta globin chain with a heme group, along with the quaternary structure of hemoglobin. Adapted from (Barbara J. Bain, 2006).

1.1.2. Hemoglobin's allosteric regulation and cooperativity

Allosteric proteins are those in which the binding of a substrate, product, or effector at a site distinct from the active site, known as the allosteric site, induces a conformational change that modulates the protein's function, thereby regulating its activity (Monod et al., 1965).
The transition from T state (tense state) to R state (relaxed state) is a hallmark of HbA´s allosteric regulation, where the binding to one heme site increases the affinity of the remaining sites. This phenomenon, known as cooperativity, is fundamental to the ability of hemoglobin to fulfil its functions and deliver oxygen efficiently throughout the body. Perutz was the first to introduce the ideas of deoxy- and oxy-hemoglobin (Perutz, 1970; Perutz et al., 1960). The T-structure, or deoxy-structure, has a lower ligand affinity compared to the R-structure, which is also known as the oxy-structure, represented in Figure 4. The oxygen binding is cooperative, which means the binding of the first oxygen molecule increases the affinity of the Hb molecule for additional oxygen molecules. These authors also postulate that inter- and intra-subunit salt bridges stabilize the Hb molecule in the T structure (Perutz, 1970; Perutz et al., 1960).

To explain the cooperative binding of oxygen to hemoglobin, several allosteric models have been proposed, the most prominent being the Monod-Wyman-Changeux (MWC) model and Koshland-Nemethy-Filmer (KNF) model (Koshland et al., 1965; Monod et al., 1965). The MWC model proposes that the transition between the R and T states occurs without intermediates. This implies that ligand affinity of neighbouring subunits within the same quaternary structure; instead, all subunits undergo the conformational change simultaneously. A key feature of this model is that the four subunits must simultaneously switch between states (Koshland et al., 1965; Monod et al., 1965).

On the other hand, the KNF model proposes that hemoglobin initially exists in a single conformation in the absence of ligand, and that each subsequent ligand binding event induces a conformational change in the bound subunit. This change is then sequentially transmitted to neighbouring subunits, generating a series of intermediate states between the T and R conformations (Koshland et al., 1965).

A combination of these two models emerged from Max Perutz, who proposed a stereochemical mechanism incorporating both models. This new combined model suggests that ligand binding to each subunit of the tetramer induces tertiary conformational changes, which are transmitted to other subunits through direct communication between the α1 and β2 subunits. This leads to a sequential increase in affinity for the ligand at other heme sites, shifting the allosteric equilibrium from the T to the R state (Perutz, 1972; Perutz et al., 1998).

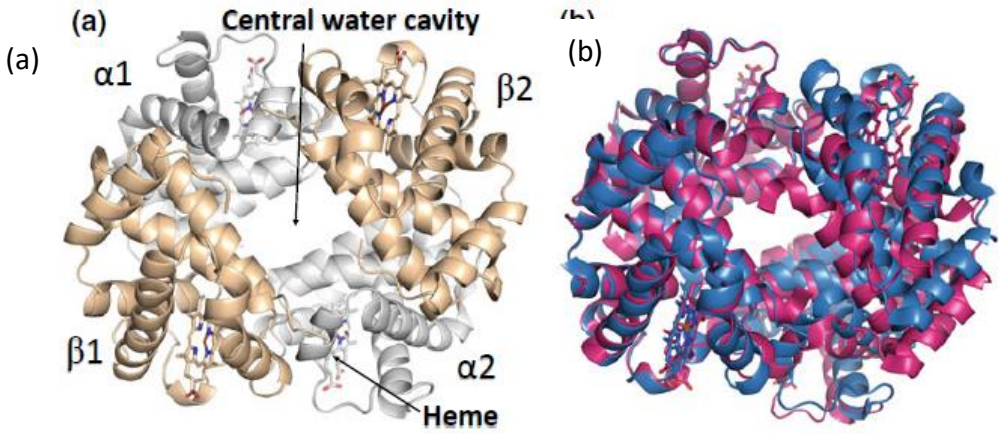


Figure 4. Crystal structure of hemoglobin, (a) quaternary structure of hemoglobin, representing the two a-chains in grey and the two b-chains in tan, with heme group identified by an arrow; (b) Oxygenated (R state) Hemoglobin (magenta) overlapped on the deoxygenated (T state) hemoglobin (blue). Adapted from (Hoeger et al., 2020)

In addition to oxygen, hemoglobin's affinity is influenced by allosteric modulators such as 2,3-biphosphoglycerate (2,3-BPG), protons (H^+), carbon dioxide (CO_2), chloride (Cl^-), nitric oxide (NO), or synthetic effectors. These substances alter Hb-O_2 affinity and shift the

equilibrium between the R and T states, either by stabilising one state or the other (Ho & Perussi, 1994; Perutz, 1970).

2,3-BPG is one of the modulators that interferes with oxygen affinity to hemoglobin. It binds to the central cavity of deoxyhemoglobin, interacting with βHis2, βLys82, βHis143, and βHis146, thereby linking the two β-subunits together and stabilizing the T state, while promoting oxygen release in tissues (R. Benesch & Benesch, 1967).

CO2 binds to the N-terminal amino groups of globin chains, forming carbamino compounds, which also stabilize the T state (Ferguson & Roughton, 1934).

NO binds to either the heme groups of hemoglobin or to cysteine residues, forming nitrosylhemoglobin or S-nitrosohemoglobin (SNO-Hb), respectively (Allen et al., 2009).

Changes in proton concentration also contribute to stabilizing either the T or R state of hemoglobin through the Bohr effect. The Bohr effect refers to the change in oxygen affinity of hemoglobin with changes in pH. This happens because the pK values of certain ionisable groups are modified in the hemoglobin molecule through oxygenation. At pH values of 6 or higher, the oxy-form of hemoglobin becomes more negatively charged than the deoxy form due to oxygen-linked ionisable groups (Ahmed et al., 2020).

When the pH is < 6, the Bohr effect occurs, hemoglobin takes up protons when binding oxygen, and oxygen affinity decreases. When the pH is ≥ 6, the alkaline Bohr effect occurs, and the opposite happens, which involves the release of protons, and hemoglobin shifts to the oxygenated state. The most likely amino acids involved in this effect are the imidazole groups of histidine residues and N-terminal amino groups, as their pK values can change significantly during oxygenation, leading to the alkaline Bohr effect (Dong & Caughey, 1994; Perutz, 1970).

1.2. Interaction of hemoglobin with small molecules

Hemoglobin adducts can serve as biomarkers for detecting exposure to various substances, as many hemoglobin adducts are already known. These adducts are stable, and their elimination relies on the lifespan of erythrocytes (120 days in humans). Therefore, they indicate prolonged exposure and are reliable markers of long-term exposure (Arnold et al., 2013; Sabbioni & Day, 2022). Hemoglobin is also present in high concentrations in the blood, ranging from 13.5 to 17.5 g/dL in men and 12.0 to 16.0 g/dL in women, making it easy to collect and analyse (Carolina et al., 2023). It is preferred over DNA for several reasons, such as its availability, the variety of identification methods, and its well-defined lifespan due to the absence of repair mechanisms (Ogawa et al., 2006).

Hemoglobin interacts with various external small molecules, which have broad implications in toxicology and clinical (Puscas et al., 2018; Zagrean-Tuza et al., 2024). For example,

glycated hemoglobin (HbA1c) is of clinical importance, as it results from a non-enzymatic reaction between glucose and the N-terminal valine of the β-globin chain (Koenig et al., 1976). This glycation results in a stable hemoglobin derivative that reflects long-term blood sugar control over approximately 8 to 12 weeks, corresponding to the lifespan of erythrocytes (Association, 2010). As stated by the American Diabetes Association (2010), measuring HbA1c is the gold standard for diagnosing and managing diabetes mellitus (Association, 2010). In this process, circulating glucose forms stable covalent adducts with valine residues in hemoglobin, enabling the estimation of average blood glucose levels over the past two to three months.

Beyond HbA1c, hemoglobin has been observed to form covalent adducts with reactive xenobiotics (Puscas et al., 2018; Zagrean-Tuza et al., 2024). For example, exposure to aromatic amines, antioxidants, anti-sickling agents, anti-inflammatories, alkylating agents, and environmental carcinogens has been shown to create hemoglobin adducts at nucleophilic amino acid residues, such as N-terminal valine or lysine (Sabbioni & Jones, 2002; Zagrean-Tuza et al., 2024). These adducts can be used as biomarkers of exposure, benefiting from the erythrocyte's lifespan that reflects cumulative exposure over several months. Moreover, these interactions can occur through covalent or non-covalent mechanisms, involving coordination bonds to the heme iron ion, hydrophobic interactions, or electrostatic contacts with amino acid residues within the protein structure (Zagrean-Tuza et al., 2024).

Non-covalent interactions involve the binding of molecules like glutathione (GSH) at the interface between hemoglobin's alpha and beta chains, with affinities around 2 μM for oxyhemoglobin and 17 μM for deoxyhemoglobin. These interactions influence vital hemoglobin functions, such as oxygen binding, and impact its structural stability and antioxidant properties (Zagrean-Tuza et al., 2024).

It is also well-established that certain drugs and chemicals can cause changes in hemoglobin. For example, oxidizing agents like dapsone and benzocaine have been found to increase methemoglobin levels by converting Fe^{2+} into Fe^{3+}, which reduces oxygen transport and results in methemoglobinemia (Rehman, 2001).

Similar to glycated hemoglobin and other small molecules that form adducts with hemoglobin, this protein can also serve as a biomarker by binding ethanol metabolites, such as acetaldehyde, and the resulting adducts may therefore indicate chronic alcohol consumption, making the adducts of ethanol metabolites a potential biomarker of alcohol intake.

Detection and quantification of hemoglobin-acetaldehyde adducts have been carried out using various techniques, such as Liquid Chromatography coupled with Mass Spectrometry (LC-MS), Nuclear Magnetic Resonance (NMR), and High-Performance Liquid

Chromatography (HPLC), which provide high specificity and sensitivity (Chen et al., 1995; Toennes et al., 2010). Additionally, many studies have employed immunoassays; however, these approaches often lack specificity, since the identification of distinct acetaldehyde-protein adducts for antibody development remains challenging (Itälä et al., 1995; Latvala et al., 2001a; Lin et al., 1990; Lin, Shahidi, Kelly, et al., 1993; Niemelä et al., 1990).

1.2.1 Adducts of acetaldehyde with hemoglobin

Acetaldehyde, the primary product of ethanol metabolism, is a small, water-soluble molecule commonly derived from the consumption of alcoholic beverages, and it exerts a range of physiological and pathological effects in the human body. Ethanol is rapidly absorbed through the gastrointestinal tract and is detoxified primarily in the liver through a series of oxidative metabolic reactions.

The three principal stages of ethanol metabolism, represented in Figure 5, are as follows: firstly, the reversible oxidation of ethanol to acetaldehyde; secondly, the non-reversible metabolism of toxic acetaldehyde to acetate; and thirdly, the subsequent breakdown of acetate to water and carbon dioxide (Zakhari, 2006).

The initial phase of ethanol oxidation metabolism is catalysed by two enzymes: alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). In the initial phase, ethanol is oxidized by ADH into acetaldehyde, a highly reactive and toxic intermediate. Subsequently, acetaldehyde is subject to further oxidation by ALDH, resulting in the formation of acetic acid. This acid is subsequently converted into acetyl-CoA, thus entering the citric acid cycle (Contreras-Zentella et al., 2022; Zakhari, 2006).

In addition to the primary oxidative pathway, ethanol can also be metabolized by cytochrome P450 (CYP2E1) and through catalase, particularly in cases of chronic alcohol exposure or elevated ethanol concentrations. These alternative pathways have been demonstrated to contribute to an increase in the production of reactive oxygen species (ROS), which in turn leads to a state of oxidative stress, lipid peroxidation, and subsequent cellular damage. The subsequent step is the metabolism of acetaldehyde to acetate by mitochondrial aldehyde dehydrogenase (ALDH). The resulting acetate is unstable and can spontaneously decompose into water and carbon dioxide (Jiang et al., 2020; Zakhari, 2006). When the mechanisms become overwhelmed, an accumulation of acetaldehyde occurs, leading to toxic effects.

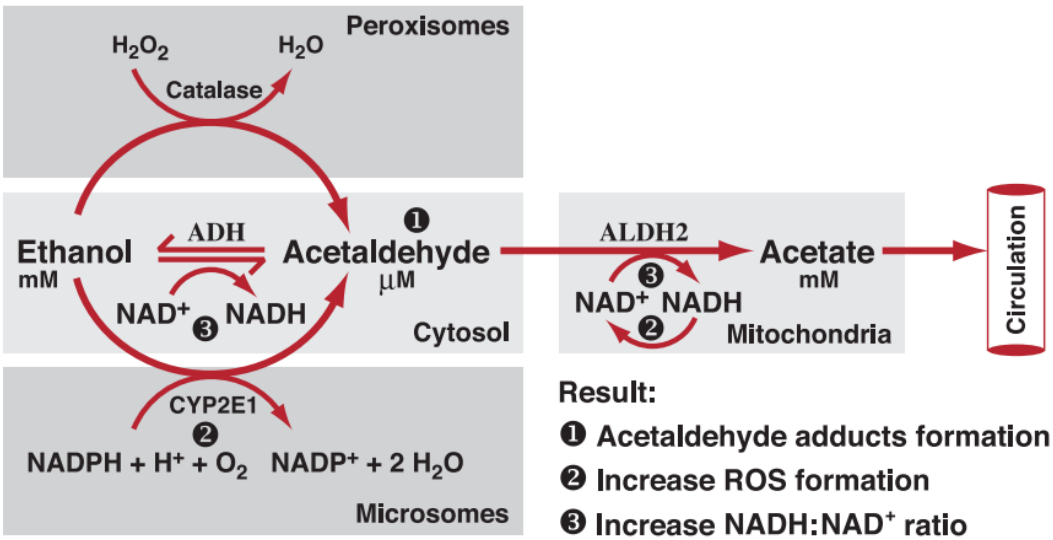


Figure 5. Illustration of oxidative pathways of ethanol metabolism. Adapted from (Zakhari & director, 2006).

Acetaldehyde is particularly relevant due to its cytotoxic, genotoxic, and carcinogenic properties. As the first metabolite of ethanol, it is responsible for a variety of adverse effects of ethanol, such as covalently binding to proteins, DNA, and lipids, damaging their structure and function, and potentially leading to immune responses (Latvala et al., 2001). Concerning the acetaldehyde concentration in alcoholics, some values have been described. According to Eriksson (1980) (Eriksson, 1980), it is usually found in the bloodstream of people consuming alcohol at a concentration of 1- 50 µM. At the same time, other authors have described it as higher, such as 200 µM (or 550 µM in severe cases) by Brecher & Adamu (2002) (Brecher & Adamu, 2002), below 100 µM according to Lindros et al. (1980)(Lindros et al., 1980), or even between 0 and 500 µM (Hazelett et al., 1993).

First reported by Stevens et al., 1981(Stevens et al., 1981), acetaldehyde was shown to form adducts with hemoglobin, as evidenced by an increase in fast-eluting species on cation exchange chromatography. This discovery led to the concept of “fast-moving” hemoglobin, referring to hemoglobin variants that migrate more rapidly than normal hemoglobin during chromatographic analysis (Gross et al., 1992).

Several studies have attempted to confirm hemoglobin-acetaldehyde adduct formation. Stevens et al. (1981) postulate that valine, lysine, and tyrosine residues of globin are sites of reaction, which was confirmed by Lumeng and Durant (1985) (Lumeng & Durant, 1985; Stevens et al., 1981). San George and Hoberman studies revealed seven acetaldehyde-modified fractions, with the sites of attachment being the free amino groups of valine residues in the α and β chains of hemoglobin, with the β chains being preferred, and the site of attachment being the imidazoline structure (San George & Hoberman, 1986).

Additionally, Braun et al. (1997) found that stable adducts to lysine sidechains are created consistent with Schiff base structure (Braun et al., 1997).
These findings support the possibility of selecting hemoglobin-acetaldehyde adducts as a promising biomarker for alcohol consumption, as it reflects a cumulative exposure to alcohol.

1.3. State of the Art in the identification and characterization of hemoglobin-acetaldehyde adducts

A systematic literature search focused on the determination of hemoglobin-acetaldehyde adducts was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Page et al., 2021). Two online databases were used, namely PubMed and Web of Science. To optimize the search, the following advanced search string was used: “hemoglobin” AND “Adducts” AND “Acetaldehyde.” This string was used because the primary goal of this study is related to acetaldehyde adduct formation to hemoglobin. The articles were screened based on their titles, abstracts, and keywords. Articles were considered eligible if they addressed topics related to acetaldehyde-hemoglobin adducts detection and structural understanding and were excluded if the topics referred to before did not match this study’s aim.
The screening process utilized specific inclusion criteria, focusing on studies investigating hemoglobin adduct formation, detection methods such as NMR or chromatography, and computational modelling of ligand interactions. In this phase, unavailable studies (n=7), written in languages other than English or Portuguese, or classified as reviews (n=8), comments, or unrelated to the topic, were excluded. Motivations for exclusion (indicated as “not relevant”, n=21) included studies on illness variants of hemoglobin, reactions of molecules other than acetaldehyde with hemoglobin, acetaldehyde adducts in inflammatory mediators, studies focused on obesity and diabetes, adducts coexisting with liver disease, formation of acetaldehyde-Hemoglobin adducts during treatments, adducts in pregnant women, and evolving hepatic fibrosis Consequently, 26 studies were selected for an in-depth review and further analysis. This procedure, adhering to the PRISMA flow diagram, is illustrated in Figure 1, which outlines each step from initial identification to final inclusion, ensuring transparency, reproducibility, and minimizing bias in the literature review.

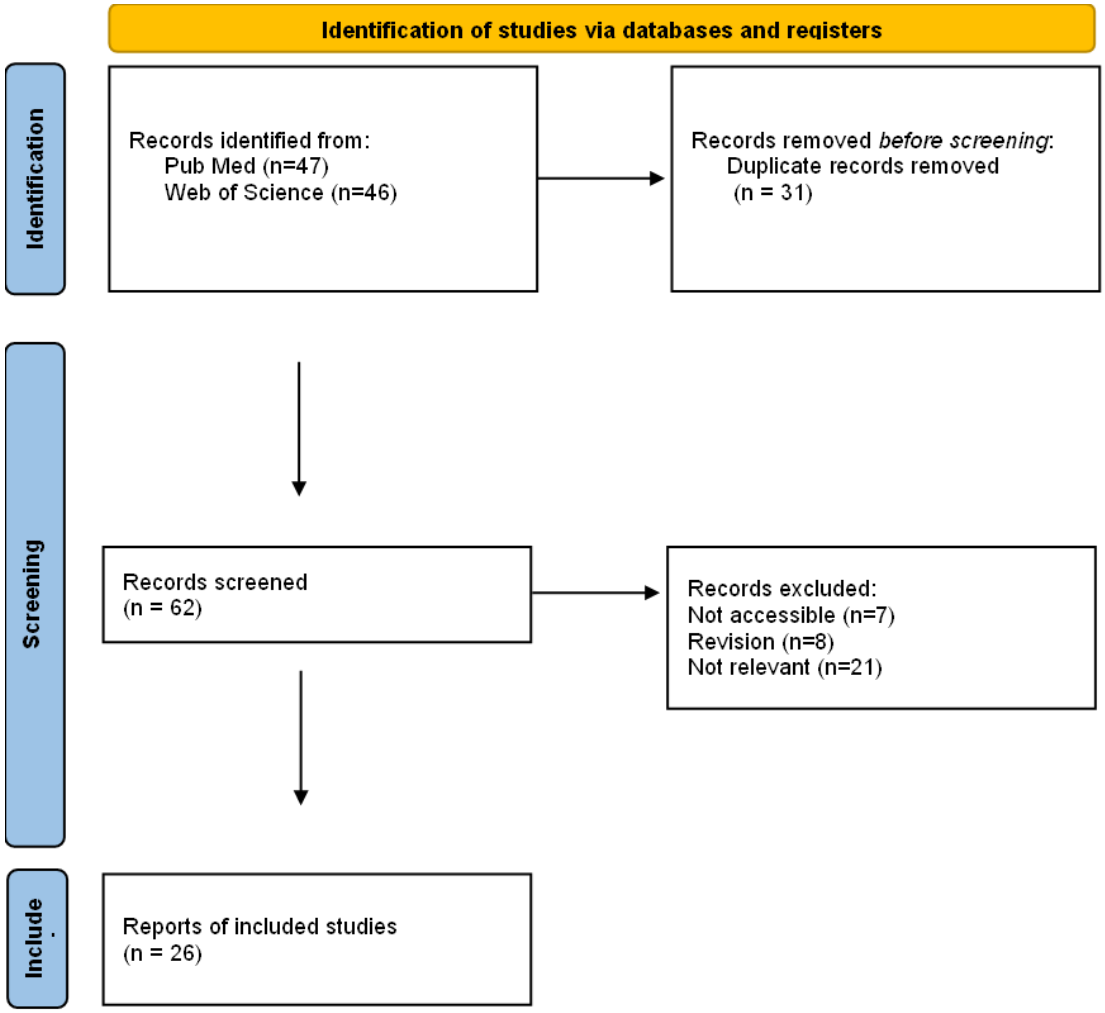


Figure 6. PRISMA Flow diagram illustrating the process of study identification, screening, eligibility, assessment, and inclusion, for studies related to hemoglobin acetaldehyde adducts. Two databases were used, PubMed and Web of Science, and in search keywords "acetaldehyde", "hemoglobin", and "adducts" were implemented. Duplicated records were removed (n=31), leaving 62 records, of which revisions (n=8), not relevant (n=21), and not accessible articles(n=7) were removed. A total of 26 articles were included.

This review summarizes 26 articles on acetaldehyde adducts with hemoglobin, exploring their formation, characterization, and potential as biomarkers for alcohol consumption. The goal is to understand the chemical reactions involved, including binding sites, stability, and reactivity, and to develop reliable detection methods for monitoring alcohol intake under various conditions. The references of the selected articles are listed in Table 1, and the acetaldehyde and hemoglobin concentrations, objectives, experimental design, and main results of each article are summarized in the Appendix (Table A1).

Table 1. Articles included in the literature review, including the Article title, year of publication, and respective reference. (Continued on the next page)

Article title	Year	Reference
Acetaldehyde adducts with hemoglobin	1981	(Stevens et al., 1981)
Modification of hemoglobin by acetaldehyde: A time course Study by High Pressure Liquid Chromatography	1984	(Nguyen & Peterson, 1984a)
The Effect of Acetaldehyde Concentrations on the relative Rates of Formation of Acetaldehyde-Modified Hemoglobins	1984	(Nguyen & Peterson, 1984b)
Acetaldehyde-hemoglobin adducts: an unreliable marker of alcohol abuse	1984	(Homaidan et al., 1984)
Regulation of the formation of stable adducts between acetaldehyde and blood proteins	1985	(Lumeng & Durant, 1985)
Reaction of acetaldehyde with hemoglobin	1986	(San George & Hoberman, 1986)
Differential modification of hemoglobin Chains by acetaldehyde	1986	(Nguyen & Peterson, 1986)
Investigation of an Acetaldehyde-Hemoglobin Adduct in Alcoholics	1988	(Ten L. Stockham, 1988)
Hemoglobin-Acetaldehyde Adducts in human Volunteers Following Acute Ethanol Ingestion	1990	(Niemelä et al., 1990)
Protein-Acetaldehyde Adducts in Serum of Alcoholic Patients	1990	(Lin et al., 1990)
Detection of a New Acetaldehyde-Induced Hemoglobin Fraction HbA1ach by Cation Exchange Liquid Chromatography	1990	(Sillanaukee & Koivula, 1990)
The Identification and Partial Characterization of Acetaldehyde Adducts of Hemoglobin Occurring in Vivo: A Possible Marker of Alcohol Consumpsion	1992	(Gross et al., 1992)
The Formation of Stable Acetaldehyde-Hemoglobin Adducts in a Red Blood Cell Model	1992	(Gapstur, Demaster, et al., 1992)
Measurement of Hemoglobin-Acetaldehyde Adduct in Alcoholic Patients	1993	(Lin, Shahidi, Kelly, et al., 1993)
Production of Antibodies That Recognize the Heterogeneity of Immunoreactive Sites in Human Hemoglobin Chemically Modified by Acetaldehyde	1993	(Lin et al., 1993)
Improved Separation of Acetaldehyde-Induced Hemoglobin	1993	(Hazelett et al., 1993)
Evidence for the formation of multiple types of acetaldehyde-haemoglobin adducts	1994	(Gross et al., 1994)

Table 1 (continued). Articles included in the literature review, including the Article title, year of publication, and respective reference.

Article title	Year	Reference
Separation of hemoglobin acetaldehyde adducts by high-performance liquid chromatography-cation exchange chromatography	1995	(Itälä et al., 1995)
Validated Fluorimetric HPLC Analysis of Acetaldehyde in Hemoglobin Fractions Separated by Cation Exchange Chromatography: Three New Peaks Associated with Acetaldehyde	1995	(Chen et al., 1995)
Structural Analysis of Peptide-Acetaldehyde Adducts by Mass Spectrometry and Production of Antibodies Directed Against Nonreduced Protein-Acetaldehyde Adducts	1995	(Lin et al., 1995)
A structural assignment for a stable acetaldehyde-lysine adduct	1995	(Brauni et al., 1995)
Structural characterisation of acetaldehyde adducts formed by a synthetic peptide mimicking the N-terminus of the hemoglobin β-chain under reducing and nonreducing conditions	1996	(Sillanaukee, 1996)
Stable acetaldehyde adducts: Structural characterization of acetaldehyde adducts of human hemoglobin N-terminal β-globin chain peptides	1997	(Braun et al., 1997)
Acetaldehyde Adducts in Blood and Bone Marrow of Patients with Ethanol-Induced Erythrocyte Abnormalities	2001	(Latvala et al., 2001)
A new CE-ESI-MS method for the detection of stable hemoglobin acetaldehyde adducts, potential biomarkers of alcohol abuse	2009	(De Benedetto & Fanigiliulo, 2009)
Application of LC-TOF MS to analysis of hemoglobin acetaldehyde adducts in alcoholic detoxification patients	2010	(Toennes et al., 2010)

The studies included in the literature review, spanning 1981 to 2010, are predominantly older, with relatively few recent publications, as summarized in Table 1.

The first study regarding acetaldehyde-hemoglobin adducts was documented by Stevens et al. in 1981, these authors discovered that acetaldehyde could form nonenzymatically stable adducts with hemoglobin (Stevens et al., 1981). The stability of these adducts to dialysis has been described as generally good (15-25%), and their formation is dependent on the concentration of acetaldehyde and the number of exposures to acetaldehyde. It has been established that valine, lysine, and tyrosine residues of globin can be pointed as reaction sites, primarily with the ε-amino group of lysine and the α-amino group of valine, through an initial Schiff's base reaction (Stevens et al., 1981), San George and Hoberman

(1986), using 13C NMR, showed that the sites of attachment of the aldehyde were the free amino groups of the N-terminal valine residues of the α and β chains of hemoglobin, with derivation of the β chain being preferred (San George & Hoberman, 1986). Furthermore, seven acetaldehyde-modified fractions were detected, with acetaldehyde reacting with the amino termini of hemoglobin to form stable cyclic imidazolidinone derivatives.

Most studies use controlled incubation of hemoglobin or blood samples with acetaldehyde at concentrations from nanomolar to millimolar, simulating physiological or high alcohol intake states (Ten L. Stockham, 1988). Incubation times range from minutes to days, sometimes under reducing or non-reducing conditions to test stability (Lin et al., 1995; Nguyen & Peterson, 1984a, 1984b; Sillanaukee, 1996). The formation of these adducts depends on factors such as acetaldehyde levels, pH, hemoglobin oxygenation, and the presence of reactants or antioxidants like ascorbic acid. For instance, deoxygenated hemoglobin and low oxygen tension tend to react more quickly. The stability of these adducts varies with incubation conditions and reducing agents; some remain detectable after dialysis, while others may dissociate over time (De Benedetto & Fanigiliulo, 2009; Nguyen & Peterson, 1984b; Stevens et al., 1981).

Analytical techniques such as HPLC (Nguyen & Peterson, 1984b, 1986b; Stevens et al., 1981), ^{13}C NMR (Brauni et al., 1995; San George & Hoberman, 1986; Sillanaukee, 1996), Raman spectroscopy (Braun et al., 1997), Enzyme-linked immunosorbent assay (ELISA) (Lin et al., 1990, 1993; Niemelä et al., 1990), Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Lin et al., 1993), and cation exchange chromatography (Chen et al., 1995; Homaidan et al., 1984; Ten L. Stockham, 1988) were used to identify and analyze adducts in different settings. Methods like cation-exchange chromatography and peptide mapping after trypsin digestion help pinpoint modification sites and quantify adduct formation in various conditions (Chen et al., 1995; Itälä et al., 1995).

UV-Vis spectroscopy, widely used in many studies, is an analytical technique based on the absorption of ultraviolet (190-400 nm) and visible (400-700 nm) light by molecules. The absorbed energy promotes electrons from the ground state to an excited state, enabling their detection and quantification. This straightforward technique facilitates rapid analysis without compromising the integrity of the sample, rendering it appropriate for both qualitative and quantitative evaluations (Werle et al., 2025). Notably, hemoglobin displays unique absorbance bands, which include the Soret bands (380-440 nm), methemoglobin-specific bands (metHb, 490-510 nm and 600-660 nm), and oxyhemoglobin-specific bands (oxyHb, 510-560 nm and 560-600 nm), represented in Figure 7 (Werle et al., 2025). The Soret peak is attributed to the central iron atom in protoporphyrin. Beer-Lambert's Law, which relates absorbance to concentration, allows for the measurement of hemoglobin levels when the molar absorptivity (ε) is known, thereby enabling accurate determination of hemoglobin

concentration. (R. E. Benesch et al., 1973; Dhmiri et al., 2022; van Assendelft & Zijlstra, 1975). This technique is also helpful for assessing the oxidative state of hemoglobin. Changes in hemoglobin's oxidative states result in different conformations, affecting its ligand properties. Oxyhemoglobin (OxyHb) is formed by the reversible binding of oxygen to deoxyhemoglobin (deoxyHb), which can spontaneously convert to methemoglobin (Werle et al., 2025). Although a reproducible and straightforward method, careful calibration, precise blank subtraction, and attention to sample purity are essential to ensure the integrity of the results.

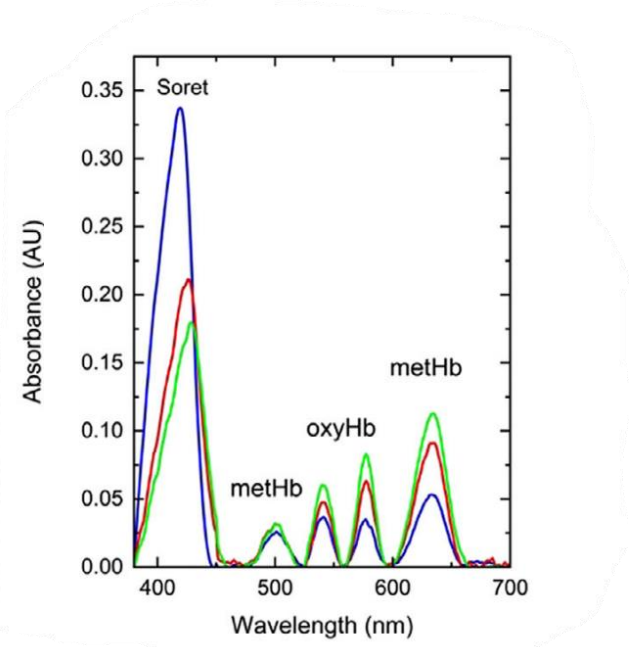


Figure 7: Peak analysis process of the UV–Vis spectra of normal Hb (red). Baseline absorbance at 700 nm was subtracted, and the valley-to-valley peak integration method was employed. Two metHb peaks and two oxyHb peaks were revealed. Adapted from (Werle et al., 2025)

Some authors employ Nuclear Magnetic Resonance spectroscopy, a particularly efficacious technique for the analysis of carbon-containing molecules, including organic compounds. This is because spins respond to their surroundings and can couple through chemical bonds and space (Lukin & Ho, 2003). As NMR utilizes the magnetic properties of nuclei placed in an applied magnetic field, nuclei with non-zero spin (^{1}H, ^{13}C) can absorb and re-emit electromagnetic radiation at characteristic radio frequencies that depend on the local chemical environment. This enables the extraction of detailed information about molecular structure, electronic distribution, and interatomic connectivity. The properties of this technique make it valuable for structural identification, quantifying compounds, studying molecular dynamics, monitoring chemical reactions in real time, and investigating molecular interactions, such as protein-ligand binding (Ho & Perussi, 1994; Lukin & Ho, 2003).

NMR is characterized by five parameters: the chemical shift (δ), the resonance intensity or area (proportional to concentration), the multiplet structure (linked to the spin- spin coupling constant, J), the spin- lattice relaxation time (T_1), and the spin- spin relaxation time (T_2) (Ho & Perussi, 1994; Lukin & Ho, 2003). Together, the five parameters are essential for analysing NMR spectra. Combining these methods allows for a thorough understanding of molecular structure, composition, and dynamics, cementing NMR's role as a key tool in chemistry, biochemistry, and material science (Ho, 1992; Ho & Perussi, 1994; Lukin & Ho, 2003). The chemical shift (δ) is the most critical parameter, indicating the resonance frequency of a nucleus relative to a standard, typically tetramethylsilane (TMS) for proton and carbon NMR, measured in parts per million (ppm). It reflects the electron cloud's shielding effect around the nucleus, causing variations in resonance frequencies based on the chemical environment. This shift is highly sensitive to electronic and structural factors, aiding in identifying functional groups and elucidating molecular structure.

Relative to a hemoglobin NMR spectrum, represented in Figure 7, peaks are well established, with those between +6 and +10 ppm from DSS corresponding to aromatic and amide protons, while those between 0 and +4 ppm are attributed to aliphatic protons. Additionally, resonances spanning from 0 down to -20 ppm originate from ring-current effects and hyperfine-shifted protons located on or near the heme groups. Peaks at 12.9 and 12.1 ppm from DSS are assigned to protons involved in H-bonds across the α1β1-subunit interface. (Ho, 1992; Lukin & Ho, 2003)

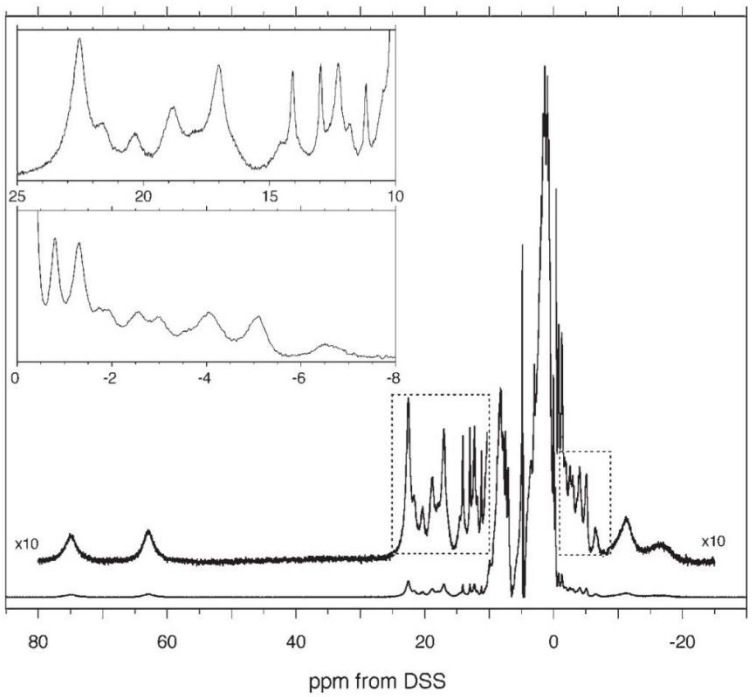


Figure 8. The 300-MHz proton NMR spectrum of deoxyHbA in 0.1 M phosphate at pH 7.1 in H_2O is presented, recorded at 29°C. Water suppression was done using the jump-and-return pulse sequence, producing a spectrum where signals upfield and downfield of the water resonance appear with opposite signs. For clarity, the negative signals have been inverted. Adapted from ***(Lukin & Ho, 2003)****.*

Molecular docking is another technique used by many authors. It is a computational method that forecasts the binding conformations and free energies of small-molecule ligands interacting with a macromolecular target. This technique is widely used to elucidate biomolecular interactions and their underlying mechanisms. By utilizing scoring functions, it evaluates thousands of possible ligand conformations and orientations in the receptor binding area, ranking them based on expected binding energy or affinity. The primary goal is to simulate how a potential ligand may attach to the macromolecule (Kitchen et al., 2004; Morris & Lim-Wilby, 2008).

This method has been employed in various studies examining the interaction of a small ligand with a macromolecule, and the results obtained validated the effectiveness of this technique in predicting the bound conformations of the two molecules (He et al., 2014; Kamaljeet et al., 2017; Puscas et al., 2018; Zagrean-Tuza et al., 2024).

Relative to the main results found in the literature review, many studies indicate that acetaldehyde mainly reacts with amino groups on valine, lysine, and tyrosine residues of hemoglobin, forming stable surface adducts (Stevens et al., 1981). The nature of these adducts has been found to be Schiff base, but there are also results indicating acetaldehyde reacts with the amino termini of hemoglobin to form a stable cyclic imidazolidinone derivative (Braun et al., 1997; Brauni et al., 1995; Homaidan et al., 1984; Lin et al., 1995; Nguyen & Peterson, 1984; Sillanaukee, 1996a). The presence of these adducts correlates with acetaldehyde levels and repeated alcohol exposure. Besides, the adduct's stability is influenced by conditions and reducing agents such as sodium borohydride ($NaBH_4$) (Itälä et al., 1995; Nguyen & Peterson, 1984a). Both laboratory and in vivo studies show that these adducts are more common in heavy drinkers and alcoholics than in non-drinkers or social drinkers (Gross et al., 1992; Itälä et al., 1995; Lin et al., 1990).

Recent research shows that these adducts form quickly and can be detected shortly after exposure. However, they tend to dissociate over time, making them useful markers for recent or ongoing alcohol use (Toennes et al., 2010). Advanced detection techniques like ELISA and chromatography improve diagnostic accuracy. ELISA assays with antibodies against acetaldehyde-modified hemoglobin show good sensitivity, with some reaching over 75% accuracy in identifying alcoholics (Lin et al., 1993; Niemelä et al., 1990). The development of sensitive chromatography and mass spectrometry further enhances the detection of low-level adducts, allowing non-invasive, rapid, and precise assessment of alcohol consumption.

Overall, evidence supports the potential of acetaldehyde-related hemoglobin modifications as reliable markers of alcohol intake. These modifications reflect both recent and cumulative drinking, aiding in monitoring and managing alcohol-related health issues. Developing

standardized, sensitive detection methods could significantly improve clinical assessment of drinking patterns and support better intervention strategies.

Despite considerable progress in understanding acetaldehyde–hemoglobin adducts, several critical gaps remain. Although it is known that acetaldehyde mainly targets nucleophilic residues like valine, lysine, and tyrosine via Schiff base chemistry, the detailed atomic structures and the distribution of cyclic and non-cyclic adducts under physiological conditions are not fully characterized (San George & Hoberman, 1986; Stevens et al., 1981).

A major methodological challenge is the limited sensitivity and specificity of current detection techniques. Traditional methods such as HPLC, ELISA, and even some advanced mass spectrometry protocols often fail to detect the low levels of adducts present during moderate exposure or early clinical stages, partly due to acetaldehyde's high reactivity, short half-life, and erythrocyte turnover (Ten L. Stockham, 1988).

Adding to this complexity is the limited focus on how the oxidative state of hemoglobin and other physiological factors influence adduct formation. Few systematic studies have explored how redox conditions, co-factors such as pyridoxal 5'-phosphate, and oxygen levels affect adduct development, despite their known roles in hemoglobin chemistry. (Lumeng & Durant, 1985). The variability in redox status in clinical samples adds uncertainty to biomonitoring data interpretation (Itälä et al., 1995).

Although the use of acetaldehyde–hemoglobin adducts as biomarkers for alcohol intake and related diseases is conceptually promising, population-based quantitative studies have yielded inconsistent results. Elevated adduct levels correlate with chronic alcohol consumption. However, findings are affected by small sample sizes, genetic differences affecting acetaldehyde metabolism (e.g., ALDH2 variants), and inconsistent analytical methods. (Latvala et al., 2001; Lin et al., 1993)

Another significant gap is the specificity of these adducts as markers of ethanol metabolism. In vivo, erythrocytes are exposed to various endogenous and external aldehydes, especially malondialdehyde from lipid peroxidation. The formation of mixed adducts like malondialdehyde–acetaldehyde further complicates attributing specific adducts solely to alcohol metabolism, reducing their specificity.

Most existing data are from in vitro studies, leaving the in vivo dynamics of adduct stability and clearance largely unexplored. Details about the mechanisms and kinetics of adduct formation and removal, within the context of competing proteins and different in vivo compartments, are poorly understood.

Finally, although potential genotoxic and immunogenic effects have been proposed, with possible impacts on erythrocyte function and immune regulation, robust data on how these

adducts affect erythrocyte lifespan or oxygen transport are limited. The clinical relevance, including causality or correlation with disease, remains a subject of ongoing debate.

In summary, the main limitations of current research are the inadequate sensitivity and specificity of detection methods, an incomplete understanding of the underlying mechanisms and structures, a lack of large-scale clinical validation, and the challenge of distinguishing adducts formed from ethanol versus other aldehydes. Overcoming these gaps will require advanced analytical techniques, standardization of methods, and an integrated systems biology approach (Sabbioni & Jones, 2002).

In the absence of precedent in the field of acetaldehyde-hemoglobin, it was deemed appropriate to utilize the STD-NMR spectroscopy technique. STD-NMR is a promising technique for higher sensitivity; its use in clinical settings is still rare, and standardized, high-throughput detection protocols are needed (Sabbioni & Jones, 2002; Viegas et al., 2011).

1.4. Potential of saturation-transfer difference NMR (STD-NMR) for ligand screening studies

STD-NMR has been used to characterize and identify ligand-receptor complexes. Additionally, it has become a preferred method for analyzing weak protein-ligand interactions, as it is based on the principle that for weakly binding ligands, there is an exchange between the bound and free ligand. This technique detects interactions with dissociation constants (Kd) in the micromolar to millimolar range (10^{-6}–10^{-3} M) (Mayer & Meyer, 2001). Besides, its efficiency results from saturation transfer occurring optimally only when the ligand rapidly exchanges between these states, allowing magnetization from the saturated receptor to be transferred and detected in the ligand signal (Viegas et al., 2011).

As illustrated in Figure 9, the STD experiment involves subtracting a spectrum in which the protein was selectively saturated (the on-resonance spectrum, obtained by irradiating a region of the spectrum that contains only the resonances of the receptor/protein) from one recorded without protein saturation (off-resonance), with signal intensities I_{SAT} and I_0, respectively (Viegas et al., 2011). In the difference spectrum ($I_{STD}=I_0-I_{SAT}$), only the ligands that received saturation transfer from the protein will remain. The ligand's occupancy of the binding site affects the signal intensity, which in turn relates to its binding affinity. While this technique does not directly yield dissociation constants, one can estimate the Kd value through ligand titration experiments and by evaluating the STD amplification factor ($\mathrm{ASTD} = \frac{I0-ISAT}{I0} \times \frac{L}{P}\ \mathrm{molar\ ratio} = \frac{ISTD}{I0} \times \frac{L}{P}\ \mathrm{molar\ ratio}$, where I_0 and I_{SAT} are the intensity of the signals in the reference and saturated spectra, respectively) in relation to ligand concentration (Angulo & Nieto, 2011; Viegas et al., 2011).

This makes STD-NMR an effective tool for qualitative detection of ligand binding and for semi-quantitative analysis of binding strength under conditions where saturation and protein concentration are well controlled. Its efficiency and simplicity explain its popularity in fragment-based drug discovery and biomolecular screening (Angulo & Nieto, 2011; Viegas et al., 2011).

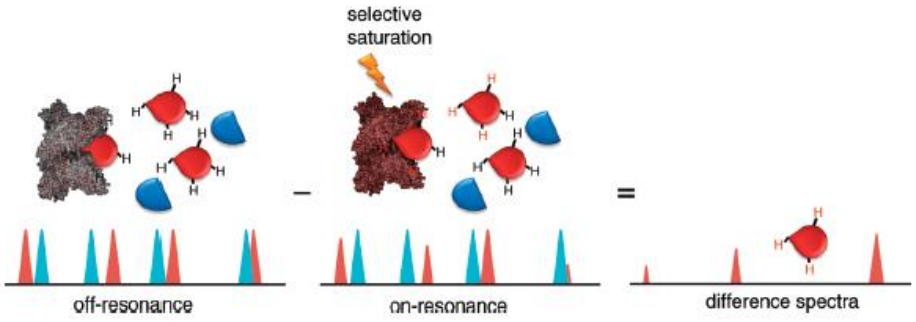


Figure 9. STD NMR technique: ligands (red) exchange between free and receptor-bound states, while non-binding molecules (blue) stay constant. Receptor resonance saturation transfers magnetization to bound ligands. When the ligand dissociates, the magnetization remains, appearing in the free ligand signal. The difference spectrum, obtained by subtracting on- and off-resonance spectra, highlights ligand protons in contact with the receptor. Adapted from (Viegas et al., 2011).

2. Aims of this thesis

The aim of this thesis was to evaluate a methodology based on STD-NMR for the detection and structural characterization of hemoglobin-acetaldehyde adducts. This work specifically aimed to investigate the feasibility of applying STD-NMR, for the first time, to selectively identify and analyze the molecular interactions between acetaldehyde and hemoglobin under controlled experimental conditions. Additionally, the thesis sought to assess the performance and sensitivity of STD-NMR across a relevant concentration range of acetaldehyde, providing insight into detection limits and the saturation behavior of hemoglobin binding sites. Furthermore, the study aimed to explore the potential of these adducts as reliable biomarkers of chronic alcohol exposure, thereby establishing a foundation for future mechanistic and biomarker research focused on hemoglobin modifications induced by xenobiotic metabolites.

3. Materials and Methods

3.1. Reagents

Acetaldehyde (CAS-No 75-07-0, ≥99.0%) and human hemoglobin (CAS No 9008-02-0) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Deuterium oxide (D_2O, CAS 7789-20-0, 99.96% D) for NMR analyses was obtained from Eurisotop (Saint-Aubin, France). Sodium phosphate monobasic (NaH_2PO_4, CAS-No 7558-80-7, ≥99%) was bought from Scharlau (Barcelona, Spain), and sodium phosphate dibasic (Na_2HPO_4, CAS-No 7558-79-4, ≥99.0%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (CAS 7732-18-5) was produced by a Milli-Q purification system (Merck Millipore, Darmstadt, Germany) and was employed for all solution preparations. All chemicals were handled and stored according to the manufacturer's recommendations.

3.2. Preparation of buffer, hemoglobin, and acetaldehyde

The phosphate buffer was prepared using sodium phosphate dibasic (Na_2HPO_4) and sodium phosphate monobasic (NaH_2PO_4) with ultrapure water, with a final concentration of 0.1 M, and pH adjusted to 7.4.

A solution of 0.2 mM hemoglobin was prepared. The suspension particles were then removed by centrifuging the solution at 2,570 g for 5 minutes at 12 °C. The upper portion was then extracted, and the hemoglobin was stored at 4 °C until needed. Subsequently, a volume of 25 mL oxidized hemoglobin was subjected to a reduction process using Sephadex G-25 resin columns and sodium dithionite, following the supplier's procedure. It was important to prevent the column from drying out during the process. First, 1 mL of buffer containing 50 mg of sodium dithionite, serving as a reducing agent, was added. An additional 500 µL of buffer was then added, followed by 2.5 mL of the hemoglobin solution. Then, 4 mL of buffer solution was added (2 mL at a time), and the of purified hemoglobin was collected. The procedure was repeated until the passage of the total volume of hemoglobin. Afterwards, 8 mL of 0.1M NaOH was added to the column to ensure thorough cleaning, and the final drop was retained before storing the column.

Figure 10. Molecule separation using a Sephadex G-25 column according to their relative size, where small molecules (like sodium dithionite) are efficiently separated from high-molecular-weight molecules of interest, hemoglobin in this case.

A stock solution of 1 mL of 2.5 M acetaldehyde was prepared in 0.1 M phosphate buffer, pH 7.4. From that solution, dilutions were performed to achieve final concentrations of acetaldehyde of 16 µM, 80 µM, 200 µM, 400 µM, 2 mM, 6 mM, 10 mM, and 20 mM, 40 mM.

3.3. Incubation of hemoglobin with acetaldehyde

Hemoglobin was incubated with different concentrations of acetaldehyde, resulting in final concentrations of 16 µM, 80 µM, 200 µM, 400 µM, 2 mM, 6 mM, 10 mM, 20 mM, and 40 mM acetaldehyde. The Eppendorf tubes were sealed with parafilm, thoroughly vortexed, and incubated at 4 °C for 30 minutes, followed by ^{1}H NMR analysis.

3.4. Analytical methods

3.4.1. UV-vis Spectroscopy

The hemoglobin solution was diluted 1:5 and analyzed by UV-VIS spectroscopy using a Shimadzu UV-1800 UV-Visible Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Analysis was made using quartz cells to reduce interference. First, the full spectrum was acquired from 800 nm to 250 nm, to check the oxidation state of hemoglobin. For data processing, we used Spectragryph (version 1.2.16), applying baseline correction. The final hemoglobin concentration was obtained by reading the absorbance at 541 nm and 576 nm and calculated according to the Beer-Lambert law (Concentration (C) = Absorbance(A) / (Extinction coefficient(ε) × path length (L)), where ε = 13.8 mM^{-1} and L= 1 cm) and multiplied by 5 times according to the dilution (Dhmiri et al., 2022; Meng & Alayash, 2017).

3.4.2. RMN spectroscopy: Sample preparation, acquisition, and processing parameters

To prepare the NMR samples, we pipetted 630 µL of the hemoglobin-acetaldehyde mixture, hemoglobin, or acetaldehyde into a new Eppendorf and added 70 µL of buffer solution 0.1M, pH 7.4 with 100% deuterated water and DSS 10 µM, resulting in a total volume of 700µL. DSS was used to reference chemical shifts. After that, 600 µL were transferred to 5 mm NMR tubes and analyzed.

To determine the optimal temperature for our procedure, we tested different values. Specifically, temperatures of 290K (17°C) and 310K (37°C) were evaluated. The results showed that the signals for hemoglobin and acetaldehyde were stronger at 310K, which was therefore selected as the optimal temperature.

All NMR experiments were performed on a Bruker Avance III HD 600 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a cryoprobe at 310 K. Both one-dimensional (1D) and two-dimensional (2D) NMR experiments were acquired to enable a comprehensive structural and dynamic analysis of the hemoglobin-acetaldehyde adducts. The following pulse sequences were employed: *zgesgp* for standard proton pulse sequence with water suppression, *stddifefsgp* for STD with shaped pulse train for saturation, *dipsi2gpph19* for ^{1}H-^{1}H Total Correlation Spectroscopy (TOCSY), and *hsqcedetgpsisp2.2* for ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC).

For the 1D water suppression experiment (*zgesgp*), the spectral width (SW) was set to 119047.6 Hz to fully encompass the relevant proton signals, and the acquisition time (AQ) was set to 0.14 s. The number of scans (NS) was adjusted to 256 to achieve an adequate signal-to-noise ratio, and the relaxation delay (d1) was set to 2 s to allow full relaxation between transients.

The *stddifefsgp* sequence was implemented for STD experiments using a shaped pulse train for selective saturation of the receptor signals. The spectral width (SW) was set to 20.0 ppm, with an acquisition time (AQ) of 1.36 s. Data were collected with 64 scans per increment, and a relaxation delay (d1) of 1.5 s. The on-resonance frequency was set to 4335.74 Hz, and the off-resonance frequency was 8000.00 Hz. The saturation time was selected by testing different saturation times (0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 s), with a relaxation delay of 1.25s more than the saturation time, to obtain the STD buildup curves. After interpretation of the STD amplification factor as a function of saturation time, the saturation time (d20) of all experiments was set to 0.25 s.

For the 2D TOCSY experiment, using the *dipsi2gpph19* pulse program, the mixing time (d9) was adjusted to 80 ms to allow efficient spin-lock transfer among scalar-coupled spins. Spectral widths were set to 7211.5 Hz in F2 and 7211.5 Hz in F1, and the number of data

points (TD) was chosen as 2048 in F2 and 256 in F1. The number of scans (NS) per increment was 48, and the relaxation delay (d1) was 3 s.

The 2D ^{1}H-^{13}C HSQC experiment employed the *hsqcedetgpsisp2.2* pulse sequence, with a spectral width (SW) of 7211.5 Hz for ^{1}H (F2) and 30182.7 Hz for ^{13}C (F1). The number of data points (TD) was 2048 in F2 and 256 in F1. Both the number of scans (NS) per increment and the relaxation delay (d1) were set to 48 and 2 s.

All spectra were processed in TopSpin 4.5.0 and referenced to the internal standard DSS. I_{STD} and I_0 spectra were integrated, and integrals were calibrated accordingly to the reference spectrum and correspond to $(I_0\text{-}I_{SAT})/I_0$. This process was repeated for all saturation times. To compare the relative STD effects STD amplification factor was calculated according to the $\mathrm{ASTD} = \frac{\mathrm{I0}-\mathrm{ISAT}}{\mathrm{I0}} \times \frac{\mathrm{L}}{\mathrm{P}}\ \mathrm{molar\ ratio} = \frac{\mathrm{ISTD}}{\mathrm{I0}} \times \frac{\mathrm{L}}{\mathrm{P}}\ \mathrm{molar\ ratio}$, where I_0 and I_{SAT} are the intensity of the signals in the reference and saturated spectra, respectively. This process was repeated for all saturation times, and a graph of A_{STD} versus saturation time (s) was created. Similarly, the procedure was carried out for all concentrations, resulting in a graph of A_{STD} versus concentration (µM).

4. Results

4.1. UV-Vis spectroscopy of hemoglobin: Oxidation state assessment and quantification

UV-vis spectroscopy revealed that the commercial hemoglobin was in the methemoglobin and oxyhemoglobin state, indicating that it was oxidized, as indicated by the prominent peaks at 500 nm and 635 nm as seen in Figure 11. After elution through Sephadex G-25 resin columns, as previously described in the materials and methods section, these peaks disappeared, leaving only the characteristic peaks of oxyhemoglobin at 541 nm and 576 nm. Consequently, the conversion of hemoglobin to the oxyhemoglobin state is particularly relevant in this context. The Beer–Lambert law ($C = A/(\varepsilon \times L)$, where $\varepsilon = 13.8\ mm^{-1}$ was used to determine hemoglobin concentration at two wavelengths (541 nm and 576 nm) (Meng & Alayash, 2017). The concentration was calculated separately for each wavelength, and the mean value was taken, resulting in an estimated hemoglobin concentration of approximately 300 µM.

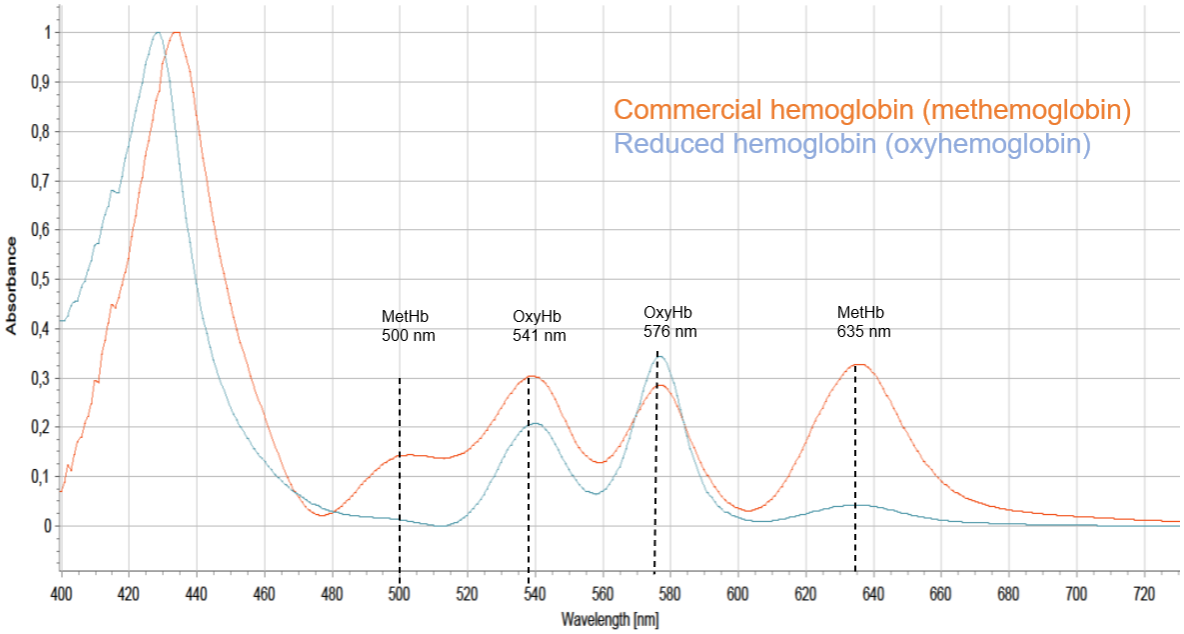


Figure 11. UV-vis spectra of hemoglobin before (red) and after (blue) passing through Sephadex columns. Baseline correction was employed using Spectragryph. There are two visible metHb peaks (500 nm and 635 nm) and two oxyHb peaks (541 nm and 576 nm).

4.2. 1D and 2D NMR characterization of hemoglobin and acetaldehyde

As previously described in the materials and methods section, the ^{1}H NMR spectrum of the buffer was processed to demonstrate that no impurities were present in the buffer solution. This spectrum revealed peaks, including the internal standard DSS (peaks marked with 1) and water (peak 2), as shown in Figure 12.

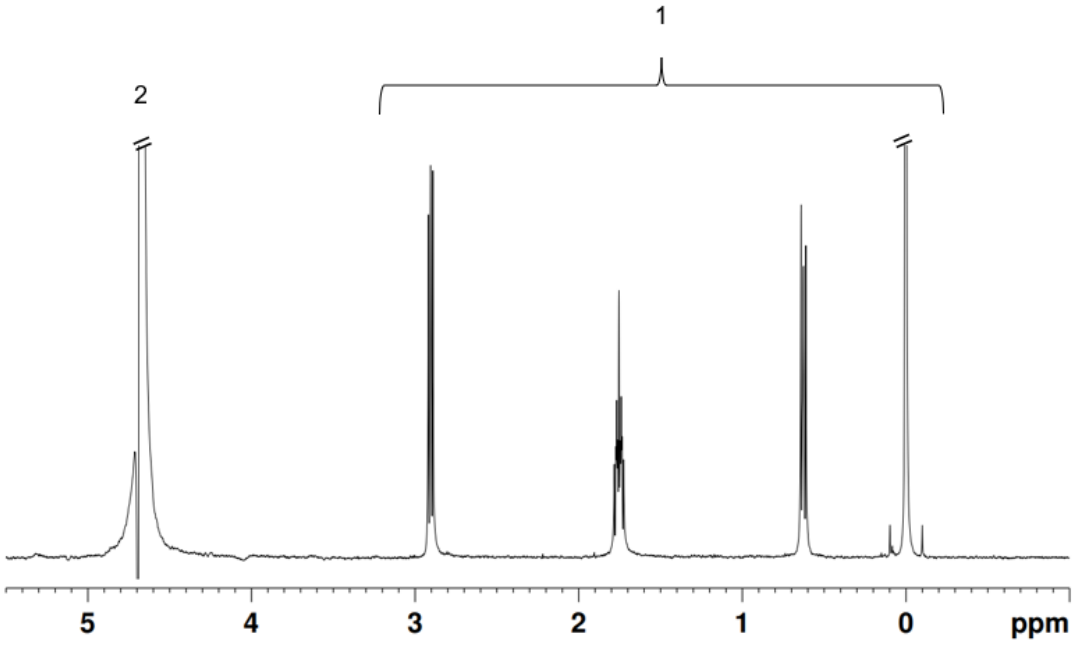


Figure 12. ^{1}H NMR spectrum of phosphate buffer 0.1M, pH 7.4 with DSS in 10% D_2O, where peaks numbered with 1 correspond to DSS at 0 ppm, 0.63 ppm, 1.75 ppm, and 2.91 ppm; and peak numbered with 2 is water (H_20) at 4.68 ppm.

The ^{1}H NMR spectrum of 10 mM acetaldehyde in phosphate buffer exhibited the peaks identified previously in Figure 12, along with four additional signals. Two peaks correspond to free acetaldehyde (2.23 ppm and 9.47 ppm, both labelled as 3), while the other two were assigned to acetaldehyde hydrate (1.31 ppm and 5.25 ppm, both labelled as 2). These assignments were made according to Peterson & Waterhouse (2016), who reported the presence of different acetaldehyde species in aqueous medium, with the hydrate being the predominant form *(A. L. Peterson & Waterhouse, 2016)*. The purpose of these analyses was to verify the purity of both the buffer and acetaldehyde solution, ensuring the absence of potential contaminants.

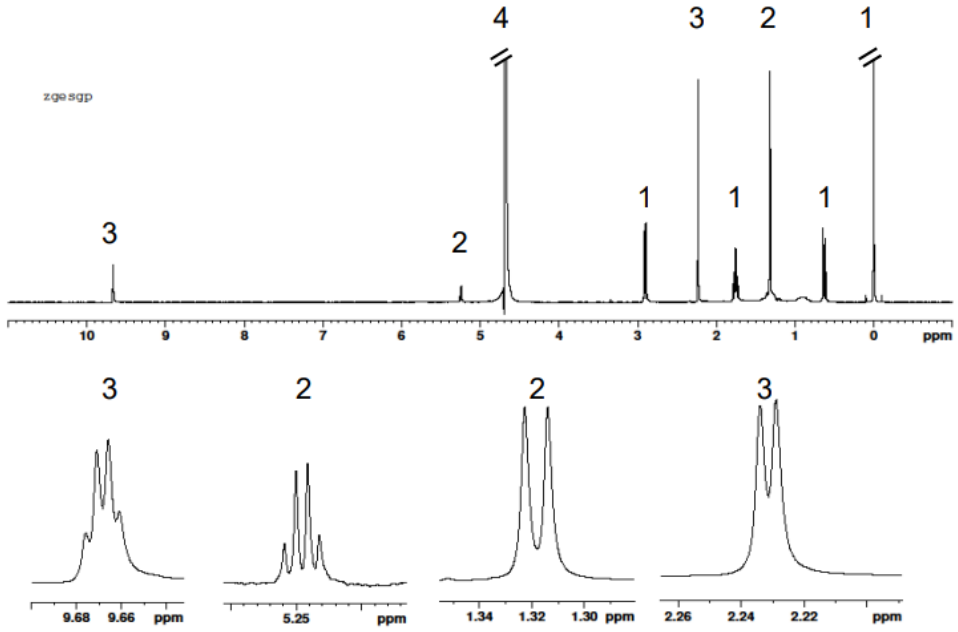


Figure 13. ^{1}H NMR spectrum of 10mM acetaldehyde in phosphate buffer 0.1M, pH 7.4, and DSS in 10% D_2O. Where peaks (1) are DSS at 0 ppm, 0.62 ppm, 1.75 ppm, and 2.90 ppm; (2) acetaldehyde hydrate ($CH_3CH(OH)_2$) at 1.31 ppm and 5.25 ppm; (3) free acetaldehyde (CH_3CHO) at 2.23 ppm and 9.47 ppm; and (4) water at 4.68 ppm. Peaks 2 and 3, corresponding to acetaldehyde hydrate and free acetaldehyde, respectively, are expanded below for better visualization.

As shown in Figure 14a, we can observe the hemoglobin ^{1}H NMR spectrum, and in Figure 14b, the hemoglobin with acetaldehyde 10 mM ^{1}H NMR spectrum. A comparison between the two spectra reveals the appearance of a new broad signal at 1.47 ppm close to the DSS reference peak. This additional resonance, highlighted in the expanded view of the spectrum, is attributed to acetaldehyde bound to hemoglobin.

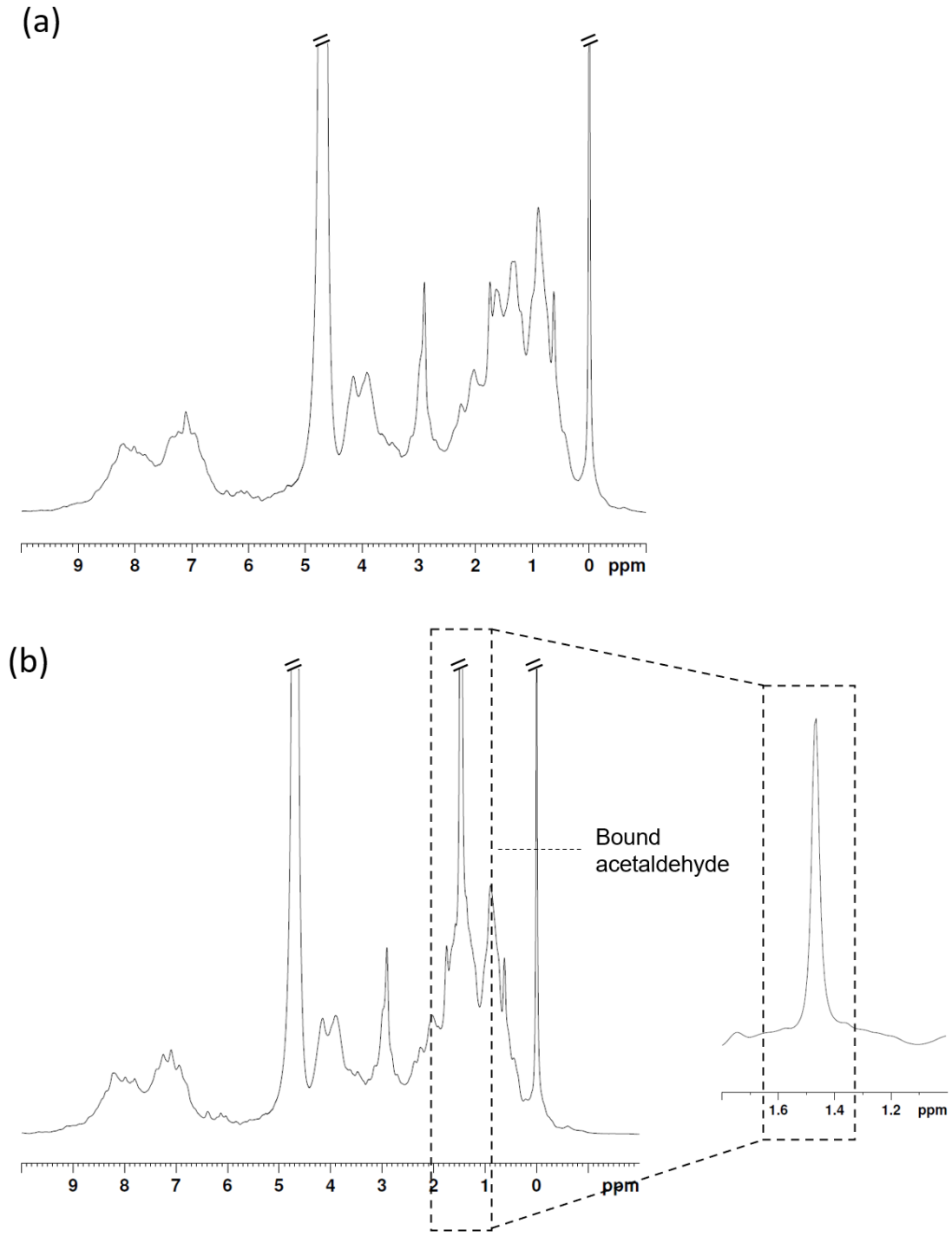


Figure 14. (a)1D ^{1}H spectrum of hemoglobin phosphate buffer 0.1M, pH 7.4, and DSS in 10% D_2O; (b)1D ^{1}H spectrum of hemoglobin with 10mM acetaldehyde in phosphate buffer 0.1M, pH 7.4, and DSS in 10% D_2O.

Figures 15a show the 2D ^{1}H-^{1}H TOCSY of hemoglobin; the experimental conditions revealed predominantly DSS signals, with only one signal pertaining to hemoglobin. The hemoglobin signal was detected at 0.90 ppm from DSS, representing the methyl (CH_3) group of aliphatic protons (leucine, isoleucine, valine).

The spectrum in Figure 15b displays the signals of acetaldehyde hydrate at 5.25 ppm and 1.31 ppm, and free acetaldehyde at 9.70 ppm and 2.23 ppm. The methyl group of acetaldehyde (CH_3) corresponds to 2.23 ppm, and the aldehyde (CHO) group to 9.70 ppm. A clear correlation is also evident between the signals of the methyl (CH_3, 2.23 ppm) and the aldehyde proton (CHO, 9.7 ppm) characteristic of the free form of acetaldehyde. A similar occurrence is observed in the acetaldehyde hydrate peaks, where a correlation is identified between the methyl group (CH_3, 1.31 ppm) and the $CH(OH)_2$ group (5.25 ppm).

In Figure 15d, it is possible to observe the same hemoglobin signal revealed in Figure 15a and another one at 1.47 ppm, corresponding to the signal of bound acetaldehyde to hemoglobin.

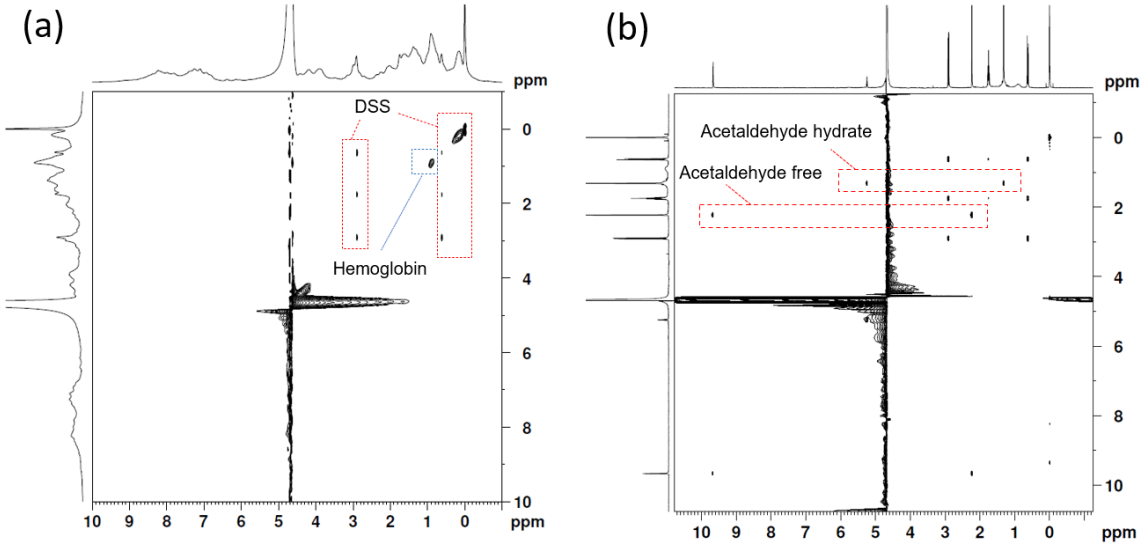


Figure 15. (a)2D (^{1}H-^{1}H) TOCSY spectrum of hemoglobin in 0.1M phosphate buffer, pH 7.4 and DSS with 10% D_2O; (b)2D (^{1}H-^{1}H) TOCSY spectrum of acetaldehyde 10mM in 0.1M phosphate buffer, pH 7.4 with DSS and 10% D_2O.
(Continued on the next page)

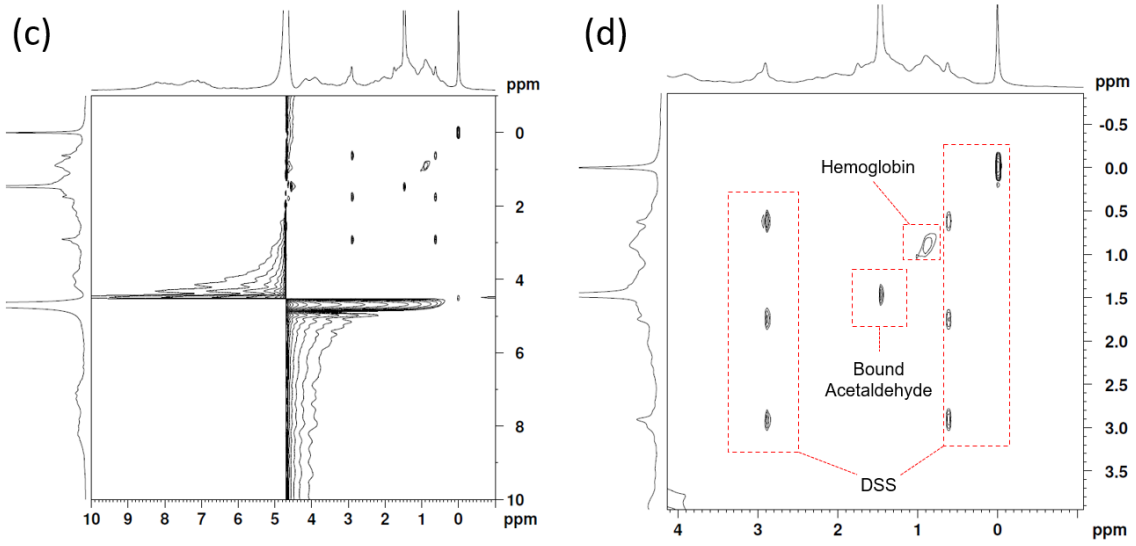


Figure 15 (continued). (c) 2D (^{1}H-^{1}H) TOCSY spectrum of hemoglobin with acetaldehyde 10mM in 0,1M phosphate buffer, pH 7.4 with DSS and 10% D_2O; (d) expansion between F1 (4 ppm to1 ppm) and F2 (4 ppm to -1 ppm) of 2D (^{1}H-^{1}H) TOCSY spectrum of hemoglobin with acetaldehyde 10mM in 0.1M phosphate buffer, pH 7.4 with DSS and 10% D_2O.

The HSQC experiments of hemoglobin revealed two sets of correlations that match the resonance pattern of the DSS used as an internal reference, along with four associated hemoglobin peaks as seen in Figure 16b. In the 2D ^{1}H-^{13}C HSQC spectrum of 10 mM acetaldehyde, signals of free acetaldehyde and acetaldehyde hydrate are also visible (Figure 16d). In the hemoglobin sample incubated with 10 mM acetaldehyde, a new signal at 1.47 ppm was revealed (Figure 16f), indicating bound acetaldehyde. All correlations of the signals represented are summarized in Table 2, representing the chemical shifts (**δ**) of ^{1}H and ^{13}C, observed for hemoglobin, acetaldehyde hydrate, free acetaldehyde, and bound acetaldehyde, highlighting the chemical environments that enable discrimination between the different molecular species.

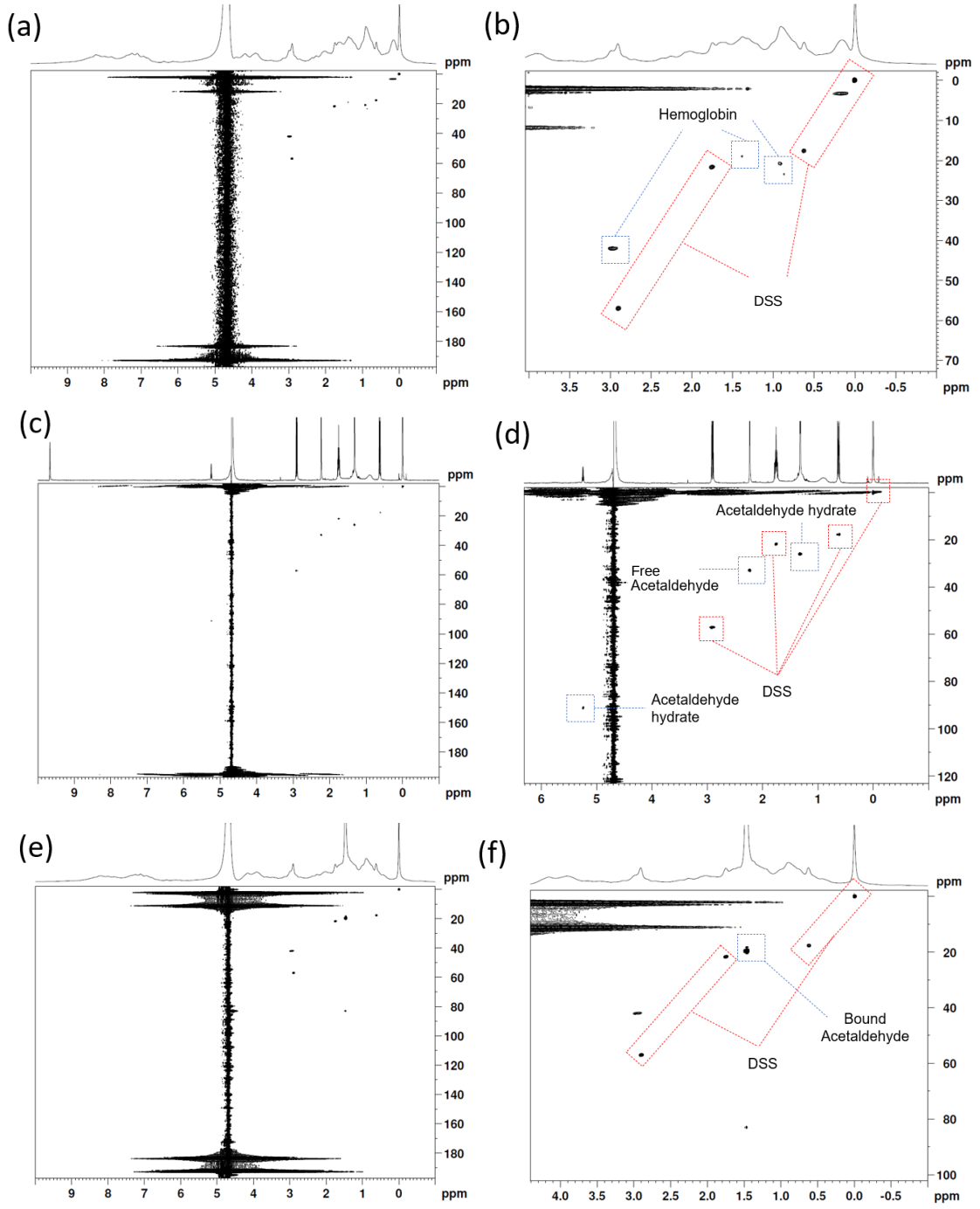


Figure 16. (a) 2D (^{1}H-^{13}C) HSQC spectrum of hemoglobin in 0,1M phosphate buffer, pH 7.4 with DSS and 10% D_2O; (b) amplification of 2D (^{1}H-^{13}C) HSQC spectrum of hemoglobin in 0.1M phosphate buffer, pH 7.4 with DSS and 10% D_2O; (c) 2D (^{1}H-^{13}C) HSQC spectrum of acetaldehyde 10 mM in 0.1M phosphate buffer, pH 7.4 with DSS and 10% D_2O; (d)amplification of 2D (^{1}H-^{13}C) HSQC spectrum of acetaldehyde 10 mM in 0.1M phosphate buffer, pH 7.4 with DSS and 10% D_2O; (e) 2D (^{1}H-^{13}C) HSQC spectrum of hemoglobin with acetaldehyde 10mM in 0.1M phosphate buffer, pH 7.4 with DSS and 10% D_2O; (f) amplification of 2D (^{1}H-^{13}C) HSQC spectrum of hemoglobin with acetaldehyde 10mM in 0.1M phosphate buffer, pH 7.4 with DSS and 10% D_2O.

Table 1. Assigned ^{1}H and ^{13}C chemical shift correlations obtained from 2D HSQC NMR spectra.

Molecule	**δ ^{1}H ppm**	**δ ^{13}C ppm**
(1) Hemoglobin	0.88	23.52
	0.93	20.73
	1.41	19.19
	2.99	41.98
(2) Acetaldehyde hydrate	1.31	26.05
	5.52	90.77
(3) Free acetaldehyde	2.23	32.74
(4) Bound acetaldehyde	1.47	19.74

4.3. STD-NMR characterization of hemoglobin–acetaldehyde adduct

4.3.1. STD amplification factor as a function of saturation time

In STD-NMR analysis, the reference spectra (I_0) and the saturated spectra (I_{SAT}) were acquired to obtain the final I_{STD} spectra. The difference between these spectra is shown in Figure 17. In the reference spectrum without saturation, I_0, (Figure 17a), the peak at 1.47 ppm is enhanced and appears as a doublet, being attributed to the signal of acetaldehyde bound to hemoglobin, while in the I_{STD} spectrum (Figure 17b), only a doublet at 1.47 ppm remains, corresponding to the signal of acetaldehyde bound.

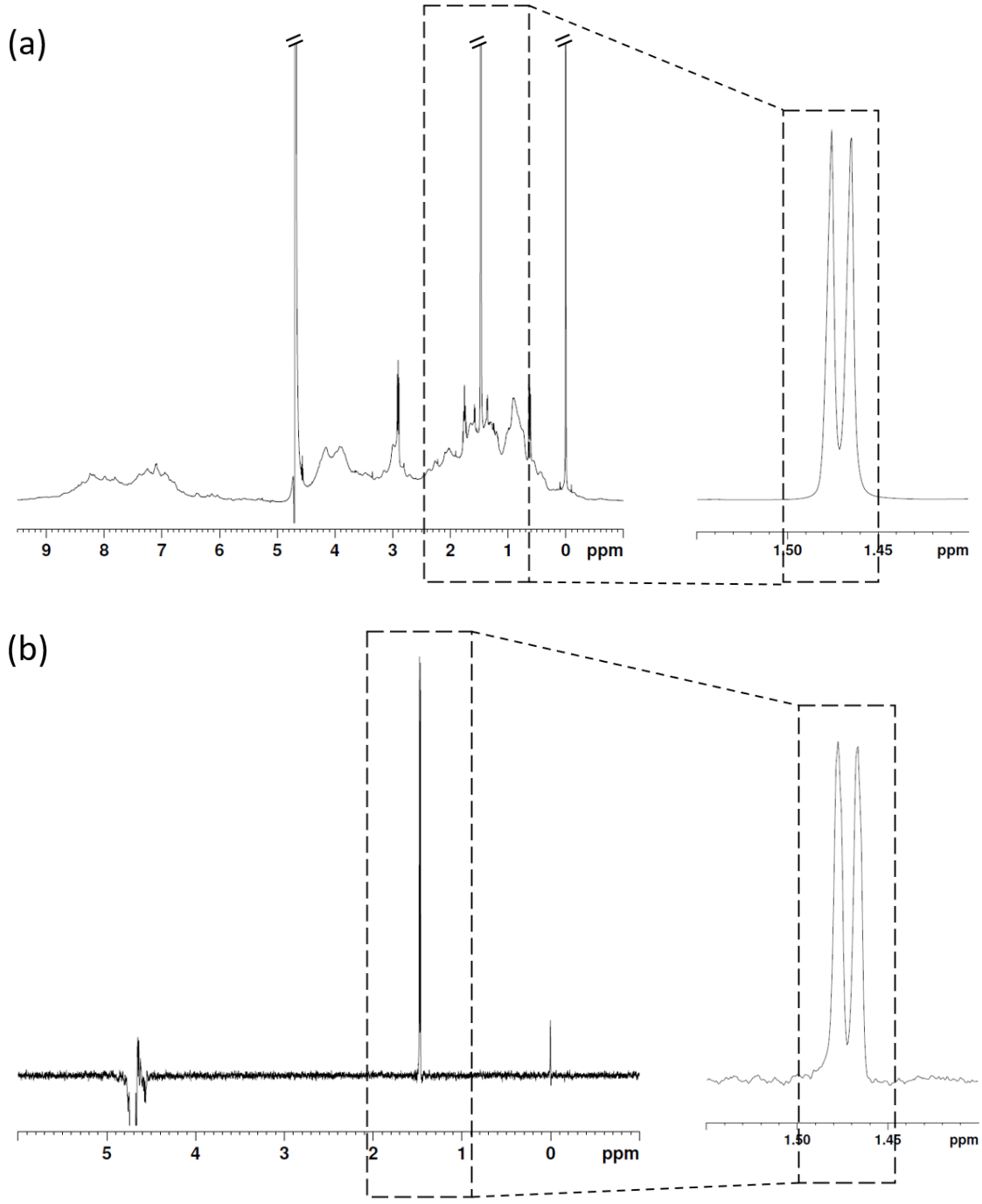


Figure 17. (1) I_0 spectrum shows the off-resonance signal of hemoglobin with 10 mM acetaldehyde, highlighting a doublet at 1.47 ppm. (2) I_{STD} spectrum with hemoglobin and 10 mM acetaldehyde also displays a doublet at 1.47 ppm.

I_{STD} spectra was processed and evaluated at each saturation time (0.25 s, 0.5 s, 0.75 s, 1 s, 1.5 s, 2 s, 2.5 s, 3 s, 4 s, 5 s) for hemoglobin incubated with acetaldehyde 20 mM (hemoglobin/acetaldehyde ratio 1:67) at the peak 1.47 ppm and hemoglobin incubated with acetaldehyde 40 mM (hemoglobin/acetaldehyde ratio 1:133) at peaks 1.47 ppm and 9.47 ppm. The evaluation of I_{STD} spectra was conducted to identify the optimal saturation time, meaning the point at which the peak intensity is highest. Visual examination of all spectra

and the STD-Amplification factor based on saturation time, as shown in Figure 18a, revealed that the signal intensity/peak area was higher at 0.25 s, and subsequent analyses were therefore conducted using 0.25 s. For hemoglobin with acetaldehyde at 20 mM and 40 mM, peak integration was only feasible up to a saturation time of 2.5 seconds.

In the case of hemoglobin with acetaldehyde at 20 mM, in peak 1.47 ppm, when plotting the I_{STD} values against saturation times, the graph showed a decreasing trend with increasing saturation time, as seen in Figure 18b.

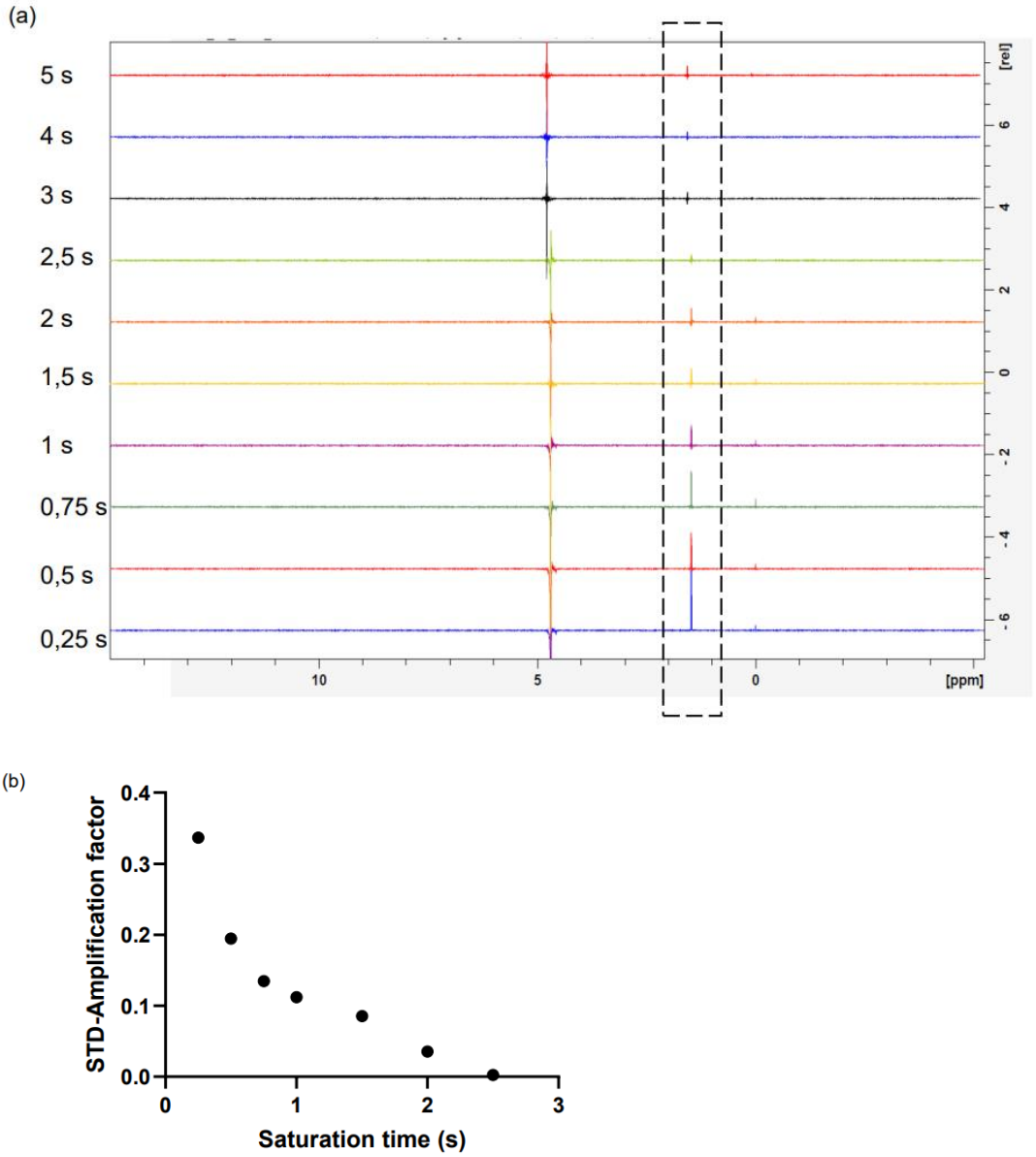


Figure 18. (a) STD-NMR spectrum showing the variation of the STD-amplification factor (A_{STD}) with saturation times (0.25 s, 0.5 s, 0.75 s, 1 s, 1.5 s, 2 s, 2.5 s, 3 s, 4 s, 5 s) using hemoglobin with 20 mM acetaldehyde at the peak at 1.47 ppm. (b) Plotting of STD-amplification factor according to saturation time using hemoglobin with 20 mM acetaldehyde at the peak at 1.47 ppm.

In the hemoglobin sample containing 40 mM acetaldehyde, the STD amplification factor values were plotted as a function of saturation time. The peak at 1.47 ppm displayed a dispersed trend, whereas the peak at 9.47 ppm exhibited a clear decreasing trend, as shown in Figures 19b and 19c.

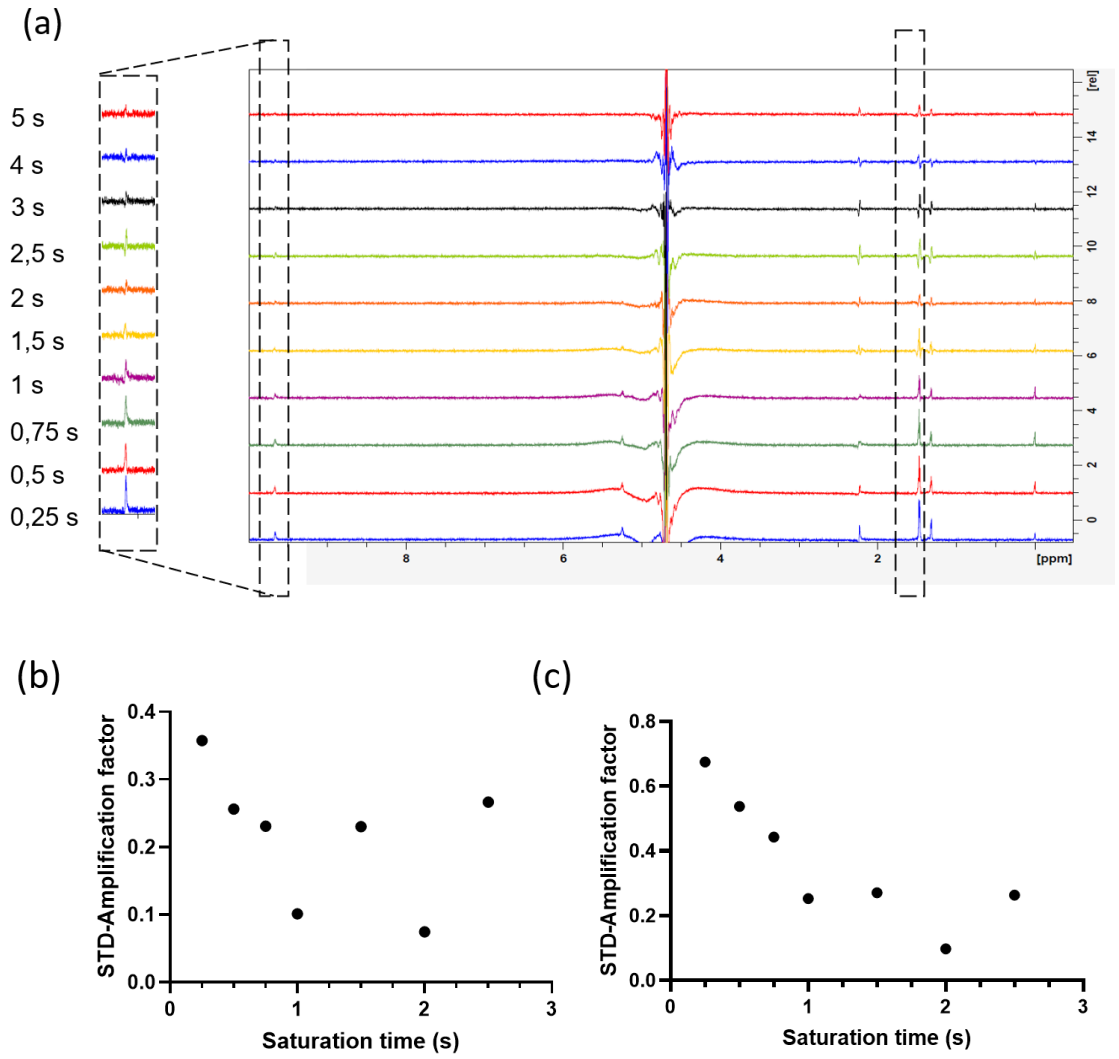


Figure 19 (a)STD-NMR spectra for the variation of the STD-amplification factor (A_{STD}) with the saturation time (0.25s, 0.5s, 0.75s, 1s, 1.5s, 2s, 2.5s, 3s, 4s, 5s) using hemoglobin with acetaldehyde 40 mM, integrating peaks 1.47 ppm and 9.70 ppm; (b) Plotting of STD-amplification factor according to saturation time using hemoglobin with 40 mm acetaldehyde at the peak at 1.47 ppm; (c) Plotting of STD-amplification factor according to saturation time using hemoglobin with 40 mm acetaldehyde at the peak at 9.70 ppm.

4.3.2. STD amplification factor as a function of acetaldehyde concentration

The I_{STD} spectra obtained at different acetaldehyde concentrations (16 µM, 80 µM, 200 µM, 400 µM, 2 mM, 6 mM, 10 mM, and 20 mM, 40 mM) revealed that the signal at 1.47 ppm was undetectable at 16 µM. At 80 µM, a weak but detectable signal emerged, which progressively increased with higher concentrations, as seen in Figure 20a.

After acquiring the I_{STD} spectra, the STD amplification factor was calculated. In addition to the observed increase in peaks in the I_{STD} spectra, plotting the I_{STD} amplification factor against concentration and performing linear regression in the range of 80 µM and 20 mM (Figure 20b), yielded a correlation coefficient (R^2) of 0.9505. When the analysis was restricted to the concentration range of 80 µM - 6 mM, the linearity improved significantly, with a correlation coefficient of 0.9944, as seen in Figure 20c. This result suggests that the available binding sites on the hemoglobin molecule begin to saturate above 6 mM. However, free acetaldehyde remains undetectable. Furthermore, a second-degree polynomial fit (Figure 20d) indicated a possible onset of a plateau phase at concentrations exceeding 20 mM.

(a)

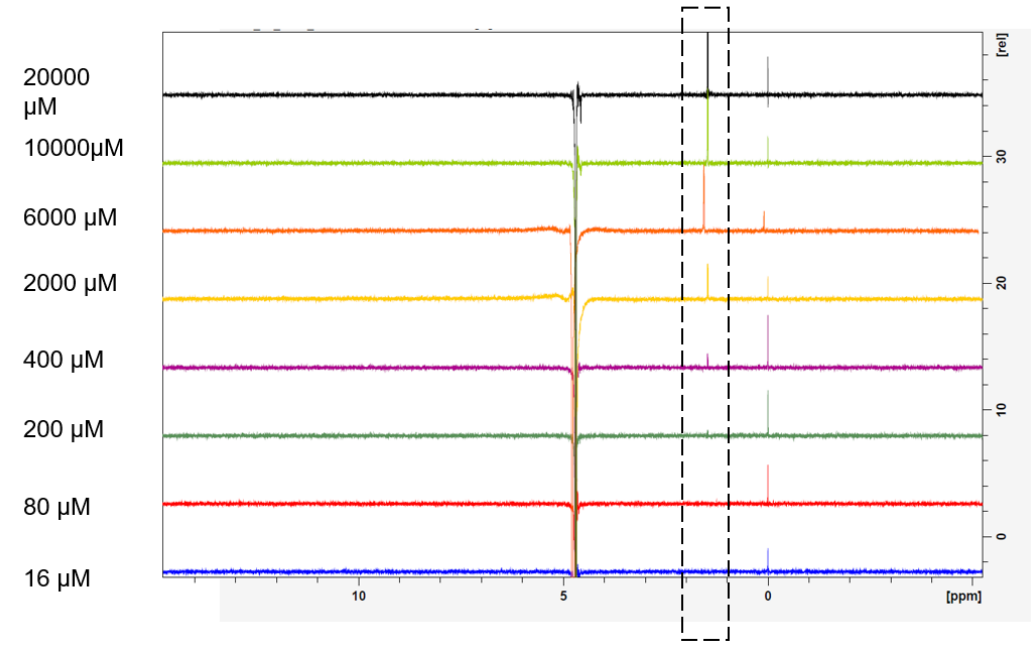


Figure 20. (a) STD-NMR spectra showing the variation of the STD-amplification factor (A_{STD}) with concentration, using the acetaldehyde peak at 1.47 ppm; (Continued on the next page)

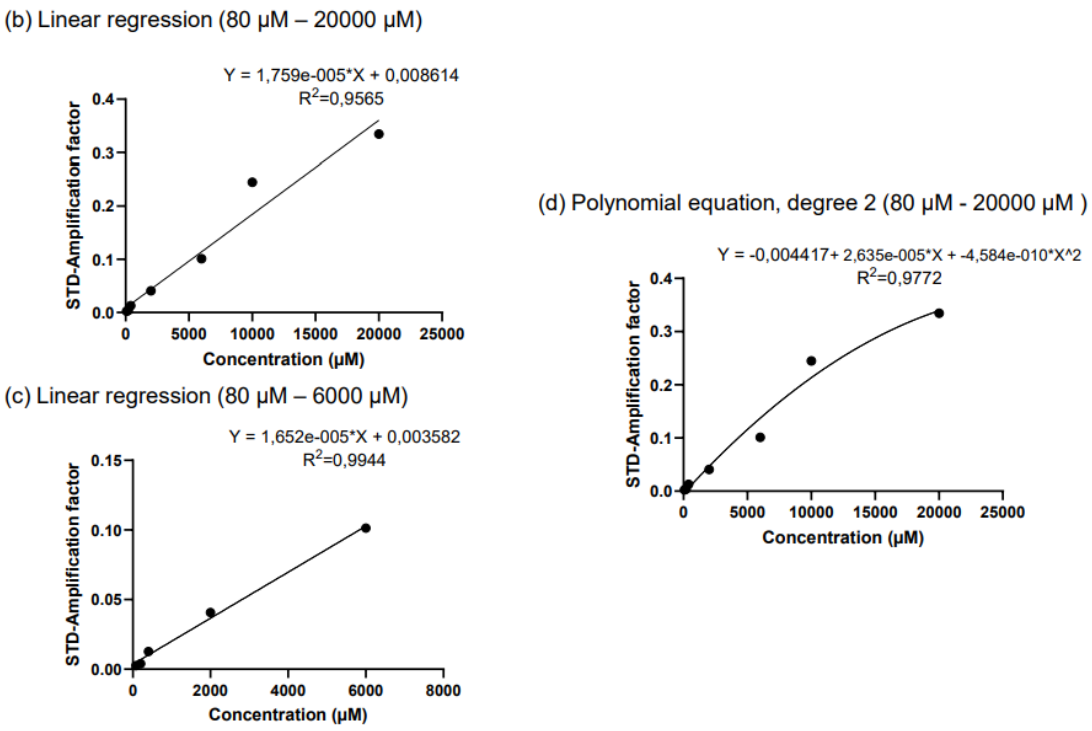


Figure 20 (continued). (b) Plot of the STD-amplification factor according to concentration ranging from 80 µm to 20 mM of acetaldehyde at the peak at 1.47 ppm, and fitting with a linear regression, with resulting values of A_{STD} of 0, 0.0026, 0.0040, 0.0127, 0.0407, 0.1013, 0.2447, and 0.03346 for saturation times of 0.25s;(b) Plot of the STD-amplification factor according to concentration ranging from 80 µm to 6 mM of acetaldehyde at the peak at 1.47 ppm, and fitting with a linear regression; (b) Plot of the STD-amplification factor according to concentration ranging from 80 µm to 20 mM of acetaldehyde at the peak at 1.47 ppm, fitting with a polynomial equation, degree 2.

5. Discussion

The formation of acetaldehyde–hemoglobin adducts has been extensively investigated due to their potential utility as biomarkers of alcohol consumption and their relevance to related disease processes. Hemoglobin's reactivity with small molecules like acetaldehyde is profoundly influenced by its oxidation state. Lumeng and Durant (1985) demonstrated that acetaldehyde exhibits reduced reactivity with oxyhemoglobin (oxyHb) and carbonylated oxyhemoglobin forms but has enhanced reactivity with deoxyhemoglobin (deoxyHb), where the heme iron remains in the ferrous (Fe^{2+}) state (Lumeng & Durant, 1985). In contrast, methemoglobin (metHb), which contains ferric (Fe^{3+}) heme iron, shows significantly lower reactivity due to the altered electronic state of the heme group. In these hemoglobin forms, the heme group remains in the ferrous (Fe^{2+}) state, which promotes rapid reactions with other molecules. In contrast, methemoglobin contains the ferric (Fe^{3+}) state of heme, leading to significantly reduced reactivity. In the present study, the commercial hemoglobin was

partially present as methemoglobin, with heme in the ferric state (Fe^{3+}), which cannot bind acetaldehyde.

In this study, commercial hemoglobin initially contained a significant fraction of metHb, limiting acetaldehyde binding. To address this, the hemoglobin solution was processed by size-exclusion chromatography using Sephadex G-25 columns and chemical reduction with sodium dithionite, restoring heme to the ferrous state and regenerating oxyhemoglobin capable of interacting with acetaldehyde (Cooper et al., 2008; Khan et al., 2008). This reducing process ensured that the hemoglobin used in subsequent assays was predominantly in the oxyHb form, consistent with its physiological reactive state.

Various analytical methods have been used for characterizing hemoglobin-acetaldehyde adducts; however, NMR spectroscopy was chosen for its robustness, high reproducibility, and detailed molecular-level information. NMR also preserves sample integrity, making it well-suited for dynamic interaction studies (Ho & Perussi, 1994; Lukin & Ho, 2003). One-dimensional ^{1}H and two-dimensional ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC experiments characterized hemoglobin and acetaldehyde individually and in mixed solutions to identify chemical shifts indicative of binding.

Unlike most prior studies, which incubated samples at 37°C (Lin et al., 1995a), this study was unable to detect acetaldehyde signals in the standard 1D ^{1}H NMR spectrum after incubation at physiological temperature, likely due to volatilization. Based on the literature, incubation at 4°C was applied to minimize acetaldehyde loss while maintaining non-reducing conditions, ensuring measurable ligand presence. In addition, two different temperatures were tested for NMR acquisition, namely 310 K (37°C) and 290 K (16.85°C), with higher signal intensities at 310 K highlighting enhanced sensitivity at physiological temperatures, relevant for biological interpretation.

A distinct peak arising at 1.47 ppm upon incubating hemoglobin with 10 mM acetaldehyde was assigned to the formed adduct, supported by spectral analysis. To probe these interactions with higher specificity, STD-NMR was employed for the first time with hemoglobin-acetaldehyde systems. STD-NMR selectively detects transient and weak ligand–protein interactions by transferring saturation from protein to ligand protons, a method proven effective for characterizing weak complexes (Angulo & Nieto, 2011; Viegas et al., 2011).

Optimizing saturation time revealed that STD signal intensity initially increased but decreased with prolonged saturation. Specifically, samples with 20 mM and 40 mM acetaldehyde showed STD signals at 1.47 ppm (20 and 40 mM) and at 9.70 ppm (40 mM only), the latter corresponding to free acetaldehyde. The absence of a free ligand signal at 20 mM supports nearly complete ligand binding. The decrease in STD signal intensity with prolonged saturation time may result from a combination of complex processes, including

relaxation-driven loss of magnetization (Jayalakshmi & Krishna, 2002; Mayer et al., 1999). Moreover, the presence of multiple binding modes, such as transient Schiff base intermediates, may introduce heterogeneity in the local molecular environments, further contributing to the attenuation and variability of the STD-NMR signal over time. Together, these factors reflect the dynamic and complex nature of the hemoglobin-acetaldehyde interaction observed in the study. At the highest concentrations (40 mM), the STD response became variable, possibly due to factors such as multiple binding sites with distinct affinities, ligand aggregation, or hemoglobin conformational heterogeneity (Viegas et al., 2011). These observations agree with previous reports emphasizing the influence of aggregation and conformational diversity on STD signals (Monaco et al., 2023; Viegas et al., 2011).

Concentration-dependent analysis showed no significant binding signal at 16 µM acetaldehyde, but clear STD responses appeared from 80 µM upwards, demonstrating the high sensitivity of STD-NMR compared to previous studies performed in the decade of 1980s, which reported negative results below 0.5-1 mM (Nguyen & Peterson, 1984; Ten L. Stockham, 1988). However, it is important to consider that analytical techniques, including mass spectrometry, have significantly advanced since the 1980s, greatly improving their sensitivity. Nevertheless, the present study highlights STD-NMR's capability to detect weak and transient interactions that remain challenging for conventional methods.

Fitting the STD amplification factor versus acetaldehyde concentration initially with a linear regression (80 µM–20 mM) showed an acceptable overall correlation but deviated at high concentrations, where saturation effects emerge. Subsequent fitting with a second-degree polynomial markedly improved the fit, supporting a model of specific, saturable binding consistent with protein-ligand interaction theories (Mayer et al., 1999; Monaco et al., 2023). Saturation of available hemoglobin binding sites above ~6 mM acetaldehyde is indicated, in agreement with biochemical evidence of finite binding capacities and conformational changes reported previously (Itälä et al., 1995; Lumeng & Durant, 1985). Overall, these findings establish that acetaldehyde binds in a concentration-dependent manner, to hemoglobin, forming adducts detectable by STD-NMR even at low micromolar concentrations (Itälä et al., 1995; Nguyen & Peterson, 1984a; Viegas et al., 2011). This validates STD-NMR as a valuable tool for probing protein modifications by ethanol metabolites with potential applications in biomarker discovery and toxicological studies.

6. Conclusion and Future Perspectives

This thesis successfully demonstrated the application of STD-NMR to detect and characterize hemoglobin–acetaldehyde adducts, representing the first use of this technique for such a system. The study confirmed that hemoglobin incubated with acetaldehyde forms specific adducts, as evidenced by a distinct NMR signal at 1.47 ppm. STD-NMR provided sensitive and selective detection of these interactions, revealing a concentration-dependent binding behavior starting from low micromolar levels (≥80 µM) and saturation of hemoglobin binding sites at higher acetaldehyde concentrations (~20 mM).

The dynamic nature of the ligand-protein interaction was reflected in the STD signal's dependence on saturation time, consistent with nuclear relaxation and exchange dynamics, and the potential presence of diverse binding modes such as Schiff base intermediates. These findings underscore the complexity of acetaldehyde binding to hemoglobin.

Future research combining NMR techniques with computational modeling can deepen our understanding of binding mechanisms and adduct formation, providing valuable molecular insights into alcohol metabolism. Furthermore, validation of these findings in vivo is essential. Investigation of acetaldehyde–hemoglobin adducts in clinical samples from alcohol-exposed individuals will clarify the physiological relevance and biomarker potential of these modifications in real biological contexts, where complex biochemical environments may influence adduct chemistry differently than controlled laboratory conditions.

In summary, this thesis lays a solid methodological foundation for using STD-NMR to detect and characterize hemoglobin-acetaldehyde adducts. While offering promising evidence of the specificity, dose-dependency, and saturable binding behavior of the adduct formation, it also underscores the need for further methodological refinement and clinical validation to establish these adducts as robust and routine biomarkers of alcohol consumption and its related health impacts.

7. Annex

Table A1: Summary of Articles included in literature review, 26 articles including the main objective, hemoglobin and acetaldehyde concentrations used in the study, Experimental design, techniques used, main results, and respective references. (Continued on the next pages)

Tabel A1(Continued): Summary of Articles included in literature review, 26 articles including the main objective, hemoglobin and acetaldehyde concentrations used in the study, Experimental design, techniques used, main results, and respective references.

Objective	Hemoglobin	Acetaldehyde	Experimental design	Techniques used	Results	References
Investigate the formation of acetaldehyde adducts with hemoglobin, identify the reaction sites, understand their stability, and explore potential clinical applications for monitoring alcohol consumption.	1-2 mM Erythrocytes, hemolysate and hemoglobin A were used	0.03, 0.3 and 3.0 mM	Effect of acetaldehyde concentration: hemolysate incubated at 37 °C for 2 hours alone or with different concentrations of Ac (0.03, 0.3, 3 mM). Adduct formation: Reaction mixtures of hemolysate incubated with 0.3 mM Ac at 22 °C	High performance-liquid chromatography Cation-exchange chromatography Radioactivity and mass spectrometry for adduct analysis	The presence of acetaldehyde-hemoglobin adducts was found to be 15-25% stable to dialysis and dependent on the acetaldehyde concentration and the number of exposures. Valine, lysine, and tyrosine residues of globin were identified as sites of reaction, mainly reacting with the ε-amino group of lysine and the α-amino group of valine, probably through an initial Schiff's base reaction.	(Stevens et al., 1981)

					Hemoglobins from alcoholics showed significantly elevated levels of hemoglobin-acetaldehyde adducts compared to controls The accumulation of adducts would appear to explain the fact that alcoholics in this study had elevated minor hemoglobins but below normal levels of hemoglobin A1c	
Assessing the formation and detection of hemoglobin-acetaldehyde adducts at physiological	1.8 µM Isolated from the blood of healthy volunteers measured by Drabkin method	0, 0.05, 0.1, 0.25, 0.5, 1, 5 mM	Acetaldehyde was added to hemoglobin at 4 °C, sealed, and incubated at 37 °C water bath	HPLC with a cation Exchange column for the separation of hemoglobin fractions	HPLC method detected significant differences between amounts of minor hemoglobin from diabetic patients and normal subjects, but no	(Nguyen & Peterson, 1984a)

acetaldehyde levels using HPLC and incorporating ^{14}C acetaldehyde, comparing separation methods and relating in vitro findings to in vivo evidence using HPLC.	intervals up to 24 hours.	difference was observed in minor hemoglobins from alcoholics and normal subjects. Hemoglobin incubation with acetaldehyde ranging from 0.05 to 5 mM led to the formation of detectable adducts, particularly in the HbA1a+b and HbA1c fractions. Significant formation of the HbA1a+b fraction was observed after 2 hours at concentrations ≥1 mM, while [^{14}C] acetaldehyde incorporation indicated rapid modification of hemoglobin. Acetaldehyde nonenzymatically forms

					stable adducts with hemoglobin	
Identify which minor hemoglobin induced by acetaldehyde could have clinical relevance, to compare the rates at which acetaldehyde-modified hemoglobin forms in different rapidly eluting HPLC fractions, and to assess how chemical reduction of hemoglobin affects the chromatographic behavior of these	1-2 mM hemolysate	0, 0.5, 5mM	Incubation at 37 °C for various time intervals (10, 30,60 and 120 minutes). Duplicate 0.5 mL aliquots were added to sodium phosphate buffer or sodium borohydride ($NaBH_4$) Cyanonosodium borohydride was used to reduce acetaldehyde-modified hemoglobins formed after 2-	HPLC	5 mM acetaldehyde increased the amounts of HbA1a+b and HbA1c fractions, but no increase was observed when incubated with 0,5 mM. The rate of formation of the HbA1c fraction was faster than that of HbA1a+b. Hemoglobin-acetaldehyde reduction with $NaBH_4$ resulted in decreased amounts of the HbA1a+b fractions but no changes in the HbA1c fractions. Stabilization of acetaldehyde-Schiff base	(Nguyen & Peterson, 1984b)

hemoglobin variants			hour incubation at 37 ºC.		adducts led to changes in chromatographic properties of hemoglobin.	
Evaluate the reliability of acetaldehyde-hemoglobin adducts as a marker of alcohol abuse by cation exchange chromatography or agar gel electrophoresis	2 to 3 mM	0, 3.7, 15, 30, 45, 0.9, 3.7, 15 mM	Incubation of hemoglobin at 37 °C for 2 to 24h with various concentrations of acetaldehyde according to the method of Steven et al., 1981	Cation exchange chromatography Agar gel electrophoresis NMR Gas Chromatographic-mass spectroscopic analysis	In vitro: Hemoglobin undergoes a concentration-dependent reaction with acetaldehyde to produce an increase in the “fast” hemoglobin fraction Clinical studies: An increase in the “fast hemoglobin” was not found by the chromatographic or agar gel methods Formation of adducts may result from the reaction of hemoglobin with aldehydic impurities or with acetaldehyde	(Homaidan et al., 1984)

					condensation products (aldol) or from acetaldehyde condensation/dehydration products (crotonaldehyde) Reaction of acetaldehyde with amino groups of proteins is a reversible reaction (Schiff base formation);	
Analyse how factors beyond acetaldehyde concentration influence the development of stable adducts between acetaldehyde and blood proteins	2.5 mg/mL Hemoglobin 20 mg/mL protein	5 µM	Anaerobic conditions at 37 °C, 2 hours, pH 7.4	HPLC Radioactivity	Factors like oxygen tension, pyridoxal 5'-phosphate and ascorbic acid influence the acetaldehyde binding to Hb Acetaldehyde reacted more slowly with oxyHbAo and carbonyl	(Lumeng & Durant, 1985)

					HbAo, and reacted more readily with deoxyHb Low concentrations of acetaldehyde can react with hemoglobin	
Elucidate how acetaldehyde binds to hemoglobin in stable adducts, as well as the stoichiometry of addition and the location of the attachment sites, to understand the addition reaction's mechanism	1 mM	0.2 to 5.0 mM	Incubation for 20 hours at 37 °C in 0.002 M Bis-tris, 0.15M NaCl, pH 7.3 ^{13}C NMR:100 mM isotopic aldehyde and 6mM peptide at 37 ºC for 72h	HPLC ^{13}C NMR	Acetaldehyde reacts with hemoglobin to form stable adducts The sites of attachment of aldehyde were the free amino groups of the N-terminal valine residues of the α and β chains of hemoglobin. Derivation of the β chains was preferred. Seven acetaldehyde-modified fractions were detected.	(San George & Hoberman, 1986)

					^{13}C NMR analysis of the peptide adducts formed with [1,2-^{13}C] acetaldehyde indicated that tetrahedral diastereomeric derivatives were produced. Acetaldehyde reacts with the amino termini of hemoglobin to form stable cyclic imidazolidinone derivatives.	
Examination of the modifications by acetaldehyde in hemoglobin´s α and β chains by cation exchange chromatography	1.38 mM	0, 0.1, 0.2, 0.5 mM	Hemolysate and acetaldehyde were added at 4 °C and incubated at 37 °C bath for 1hour	Cation exchange chromatography	A linear relationship was seen between increasing levels of [^{14}C] acetaldehyde and the radioactivity of the α and one of the β chains of the adducts.	(Nguyen & Peterson, 1986)

Acetaldehyde reacts differentially with the α and β-hemoglobin subunits and with the undissociated hemoglobin molecule. Reacts preferentially with the β-chain.

Unreacted cysteine residues were more frequently detected at the higher acetaldehyde concentration, consistent with the formation of cysteine adducts that are labile to acid hydrolysis or the shielding of cysteine residues in acetaldehyde modified Hb against subunit separation by HMB treatment.

Isolation and characterization of acetaldehyde-hemoglobin adducts and their purification	Commercial Hemoglobin (HbA0) concentration was determined by the cyanmethemoglobin method of Chernoff	5 mM and 10 mM to assess adduct formation in vitro 100 µM (approximates circulating levels in chronic drinkers) to evaluate adduct formation at physiological concentrations	24, 48 and 72 hours	Adduct purification: Cation exchange chromatography and p-aminophenylboronic acid affinity chromatography Adducts characterization was performed using HPLC and mass spectrometry.	Acetaldehyde-Hemoglobin adducts were formed in vitro with concentrations of acetaldehyde such as 5 and 10 mM, but not with 100 µM (pharmacological concentration). Adduct formation might not be detectable by the methodology used	(Stockham & Blanke, 1988)
Detect and measure acetaldehyde-protein adducts	104 to 180 U/L	Ethanol 1.3 to 2.9 g/kg body wight,	Hemoglobin with various concentrations of acetaldehyde in	Enzyme-linked immunosorbent assay (ELISA) HPLC	Following acute ethanol ingestion, there is an increase in acetaldehyde conjugation to	(Niemelä et al., 1990)

from human blood following acute ethanol ingestion using immunological methods		administrated over 8 hours	the presence of 10 mM sodium cyanoborohydrid e for 24h under sterile conditions	Gas chromatography	hemoglobin that can be detected using sensitive immunological methods. Acetaldehyde-hemoglobin condensates stay elevated longer than alcohol is measurable.	
Evolve and utilize a sensitive enzyme-linked immunosorbent assay (ELISA) to detect protein-acetaldehyde adducts in human serum, aiming to identify a marker for alcohol abuse, by comparison of direct ELISA with sandwich ELISA	Not specified	250mM	Room temperature, 1 hour Added sodium borohydride and incubated for 3 hours	ELISA (direct and sandwich)	Sandwich ELISA was more sensitive than direct Serum protein-acetaldehyde reacted more strongly with anti-myoglobin-AA lgG than with anti-hemocyanin-AA lgG. Alcoholic patients had significantly higher levels of serum protein-AAs compared to non-drinking control subjects.	(Lin et al., 1990)

Development of a sensitive liquid chromatography method to analyse the acetaldehyde binding to hemoglobin	9.2 mg/L	0, 10, 50, 100, 250, 500, 1000, 5000 µmol/L	Incubation of hemolysate with acetaldehyde for 15 min at 37°C ^{14}C acetaldehyde was incubated for 10 min at 37°C with hemoglobin	Cation exchange, Liquid chromatography	When incubated with hemoglobin, acetaldehyde produced two new fractions (HbA1c and HbA1ach), which increased rapidly, but decreased slowly, never returning to the initial levels. Low acetaldehyde concentrations from 10 to 100 µmol/liter caused detectable changes in hemoglobin Incorporation with [^{14}C] acetaldehyde revealed that radioactivity was bound specially to HbA1ach, HbA1c and HbA0 fractions	(Sillanaukee & Koivula, 1990)

					Even the lowest concentration of acetaldehyde induced small but detectable chA0 fraction	
Identification and characterization of hemoglobin-acetaldehyde adducts.	2 mg/mL	Acetaldehyde: 3.6mM Radioactive ethanol: 12.8 mM	37°C, 5 hours, phosphate buffer 20 Mm, pH 7.4	HPLC Peptide mapping mass spectrometric Plasma desorption mass spectrometry (PDMS)	Heavy drinkers had significantly higher amounts of "fast" hemoglobin (structural variants of hemoglobin) compared to abstainers. Hemoglobin was found to be modified by acetaldehyde in the alpha and beta chain N-termini.	(Gross et al., 1992)
Develop an in vitro model using intact red blood cells to evaluate the effect of	Total hemoglobin was measured using Dranbkin's reagent	20, 50, 200 nmol/ml (uM) [14C] acetaldehyde	37°C for 3, 24 or 48 hours Aldehyde dehydrogenase	Reverse-phase HPLC Mass spectroscopy	The amount of stable hemoglobin-acetaldehyde adducts is proportional to the	(Gapstur, DeMaster, et al., 1992)

acetaldehyde concentration and incubation time on the formation and quantification of stable hemoglobin adducts, comparing treated and control cells.	was inhibited by cyamide-pretreatment	increasing acetaldehyde concentrations. Modifications have been identified at least on two sites: the N-terminal valine residues of both the α and β chains of hemoglobin. Stable adducts are formed in metabolising blood cells exposed to concentrations of acetaldehyde ranging from 6-70 µM Inhibition of red blood cells aldehyde dehydrogenase substantially increased adduct formation

Discover a sensitive and specific method to diagnose and manage alcoholic patients	Blood from alcoholics or nondrinkers	Nondrinkers or people who consumed less than 20 g of ethanol/week	Venous blood drawn with heparin as the anticoagulated and placed in ice Antibodies were raised against: Keyhole limpet hemocyanin (KLH); Peptide consists of eight amino acid residues (8-pep, V1 to K8); Segment of HbA β-chain consists of 11 amino acids rich in lysine or K (11-pep,G56-k663)	ELISA	The anti-KLH-AA IgG antibody showed 78% sensitivity in detecting alcoholic patients, while anti-11-pep-AA IgG exhibited 75% sensitivity, and anti-8-pep-AA IgG demonstrated 43% sensitivity. The site of Hb modified by acetaldehyde in vivo is mainly located in a surface-accessible region near the centre of the β-chain of HbA, where several lysine residues are clustered. Hb-AA has the potential to be a good marker for alcohol abuse	(Lin, Shahidi, Kelly, et al., 1993)

Improvement of the separation of Hb variants (HbA1-Ach, HBA1a+b, Hb pre-A1c, and HbA1d3) induced by acetaldehyde using a polyaspartic acid column	Hemoglobin concentration was determined by the absorbance of the supernatant (diluted 1:100) at 540 nm.	0-500 µM	Acetaldehyde was incubated with hemolysate at 37 °C for specified times Saline-washed red blood cells were treated with sodium acetate buffer to remove unstable Schiff bases	HPLC separation using a polyaspartic acid column	HbA1-ACH, HBA1a+b, Hb pre-A1c, and HbA1d3 (hemoglobin variants induced by acetaldehyde) were significantly increased by Acetaldehyde incubation, and the changes were only partially reversible with time.	(Hazelett et al., 1993)
Compare some physicochemical properties of acetaldehyde-modified human hemoglobin prepared under two different conditions.	5 µg/ml	250mM, 500mM	250 mM acetaldehyde for 1 hour, followed by reduction with 100 mM NaCNBH3 for 4 hours 500 mM acetaldehyde for	SDS-PAGE Agarose gel electrophoresis ELISA	ELISA tests confirmed that anti-KLH-AA IgG reacted strongly with Hgb-AA but showed negligible binding to Hgb-AA or unmodified Hgb, even at high concentrations.	(Lin et al., 1993)

			10 days, followed by reduction with one mM NaBH4 for 1 hour		These findings indicate that antibodies raised against protein-AA antigens produced under different conditions recognize different epitopes.	
Identify and characterize different types of stable acetaldehyde-hemoglobin adducts formed both in laboratory settings and in living organisms, evaluate their reactivity and stability, and determine their usefulness as	0,06 mM	14C acetaldehyde: 20, 50, and 200 mM	Incubated for 3, 24, and 48 hours Hemoglobin samples were digested with 5% (w/w) trypsin for 30 min at 4°C, and the digestion was stopped by addition of 10% trifluoroacetic acid	HPLC	Nine peptides with AcH modifications were identified, with six found even at the lowest AcH concentration (20 mM) Two peptides were identified as AcH modifications of the Hb α- and β-chain N-termini. Analysis of [^{14}C]AcH-peptide adducts revealed that at least two peptides exhibited different reactivities for AcH	(M D Gross 1, 1994)

specific biomarkers for various drinking behaviors.					compared to the N-termini of Hb, suggesting that multiple modifications may be useful in developing markers of alcohol consumption.	
HPLC as a clarifying method to separate the hemoglobin fraction, especially the acetaldehyde hemoglobin adducts, without interference from glucose or acetylsalicylic acid, with a good separation and in a short analysis time	Venous blood of volunteer social drinkers collected, mixture of HbA0, HbA2, HbF and HbS was purchased, HbA0 purchased	5; 10; 25; 50; 100; 250; 500; 1000 µM	Effect of concentration: 37 °C, 24h Time dependence of ac adduct formation: 1000 µM ac and 0.5 incubation volume analysed after 1, 2, 4, 8, 24, 48h	HPLC Poly cat A cation-exchange chromatography	New peak found to be formed after acetaldehyde incubation (HbA1ach3). This showed a dose and time dependence on acetaldehyde incubation HbA1ach3 is stable and an adduct of HbA0. Physiological acetaldehyde concentrations (5-250 µM) induced the formation of the new	(Itälä et al., 1995)

					peak and increased HbA1ach1, HbA1prec and HbA1d3. More substantial concentrations of ac (1000 µM) induced minor changes	
Investigate which hemoglobin fractions form adducts with acetaldehyde both in vivo and after in vitro addition of acetaldehyde, using an improved cation exchange system and a non-radioisotopic method for	2.2 mM	5mM Dose-response: 0, 5, 50, 500, 5000 pM	37 °C for 30 minutes and washed with H_2O to remove free acetaldehyde and labile adducts	Fluorometric HPLC Cation exchange chromatography Mass spectroscopy	Most acetaldehyde resides in the HbA1a+b fraction Reaction with acetaldehyde in vitro led to a significant increase in fast-eluting minor hemoglobin species on cation exchange chromatography, specifically in the HbA1a+b, HbA1-AcH, and HbA1c fractions Three new cation exchange	(Chen et al., 1995)

acetaldehyde quantification					Chromatographic peaks after reaction with acetaldehyde: HbA 1-ACH-3, HbA 1a-1 and HbA1a+1	
Elucidate the nature of chemical modifications of acetaldehyde adducts with hemoglobin using FAB/MS	Used three peptides to mimic hemoglobin chains (8-pep, 11-pep-gly, 11-pep-pro)	Non-reduced conditions: 1Mm Reduced conditions: 250mM	Reduced conditions (with NaCNBH3): 37 ºC for 1 hour Non-reduced conditions: 4 ºC for 7 days	FAB-MS (Fast atom bombardment mass spectrometry) ELISA	By FAB/MS, under reduced conditions 8-pep formed an imidazolidinone at the N-terminal valine, 11-pep-gly formed a Schiff's base at Lys4. Under reduced conditions, modifications involved mono- and diethylated derivates of lysine residues and the N-terminal amino group.	(Lin et al., 1995)

					Acetaldehyde modifies peptides in reduced and unreduced conditions Lysine residues, although located in a close cavity in a peptide, are not equally reactive in forming stable adducts Antibodies raised by nonreduced MAP-11-pep-AA immunogens can recognise both reduced and nonreduced epitopes, the adduct	
Characterize the formation of stable acetaldehyde-lysine adducts, specially Schiff base structures in	1 mM	0, 1.5, 5, 8 mM	Incubation of Hb with ac for 1 hour at 37 °C	Mass spectroscopy NMR Raman spectroscopy	A Schiff base adducts between pentalysine and acetaldehyde, stable to the preparative and analytical procedures, supporting the formation	(Brauni et al., 1995)

			hemoglobin and understand their chemical properties		of stable Schiff bases with lysine residues. acetaldehyde forms stable Schiff base adducts predominantly at lysine residues in the proteins. MS data revealed the formation of monoadducts with a mass increase of approximately 26 atomic mass units, consistent with Schiff base formation. NMR spectra showed signals at around 155-156 ppm, further supporting the presence of Schiff bases, while Raman spectra displayed

					characteristic peaks at approximately 1640 cm^{-1} indicative of imine (C=N) structures.	
Elucidate the structure of Hemoglobin β chain N-terminal residues modifications induced by acetaldehyde under different experimental conditions	Synthetic peptide that represents the N-terminal residues of the Hb β-chain (Val-His-Leu-Thr-Glu-Cys)	20 mM	1:1000, 1:10 and 1:1 molar ratios 1:10- adduct formation at different pH values (5, 7, 7.4, 7.8) Adduct formation was followed for 48h at pH 7, 7.4 and 7.8	Reverse-phase HPLC Fast-atom-bombardment MS (FAB-MS) Linked-scan (B/E)	Under non-reducing conditions at pH 7.4, two adducts were found, shown to be a Schiff base and a cyclic 2-methyl-imidazoline-4-one With reducing conditions at pH 7.4, an N-ethyl valine adduct was predominant, whereas at pH 9.0, rapid formation of diethylated adducts was observed. adduct stability was pH-dependent, with some structures dissociating over time	(Sillanaukee , 1996)

Characterise stable Schiff base acetaldehyde adducts of Val-His-Leu-Thr-Pro and Val-His-Leu-Thr-Pro-Val-Glu-Lys at both active sites	1 mM	1.5, 5 or 8 mM	37 °C for 1 hr and chilled on ice until purified	Mass spectroscopy 13C NMR Raman spectroscopy HPLC Electrospray ionization (ESI) mass spectroscopy	Stable acetaldehyde adducts to lysine sidechains occur under mild incubation (37 °C, 1 hr, 10 µm to 5 mM acetaldehyde with one mM peptide), and those adducts are consistent with a Schiff base structure on both the N-terminal peptides of the β-chain and the ε-amino groups of lysine Mass spectrometry revealed mass differences consistent with Schiff base structures for the adducts, with monoadducts observed for both pentapeptide and octapeptide samples	(Braun et al., 1997)

					NMR and Raman spectroscopy provided further evidence supporting the formation of Schiff bases, with no confirmatory evidence for imidazolidinone products under the study's conditions	
Examine the formation of acetaldehyde-derived protein modifications in erythrocytes and bone marrow precursors.	Not mentioned	Various concentrations of [^{14}C] acetaldehyde with $NaCNBH_3$ (5 µM-10 mM)	Incubated 3 hours at room temperature	HPLC ELISA	Acetaldehyde-derived epitopes were found to occur both on the cell membrane and inside the erythrocytes. Separation of the erythrocyte-proteins on HPLC showed the formation of fast hemoglobin fractions that reacted with antibodies	(Latvala et al., 2001)

					against acetaldehyde adducts. Acetaldehyde-erythrocyte adducts are formed in vivo with excessive alcohol consumption and this formation likely contributes to the observed erythrocyte abnormalities in alcoholic patients.	
Develop a new Capillary Electrophoresis-Electrospray Ionization-Mass Spectrometry (CE-ESI-MS) method for the detection of stable	2mg/mL	0.2-20 mM	Incubation times over 24 hours Hemoglobin incubated with different acetaldehyde concentrations at 37 °C in 20 mM	CE-ESI-MS (Capillary electrophoresis-electrospray ionisation- mass spectroscopy)	Identified modifications in: α and β N-amino terminal, condensation of ach molecules on the histidine residue in position 4 in α4, and asparagine residue in position 2 in β3. The last two, internal modifications, were	(De Benedetto & Fanigiliulo, 2009)

hemoglobin acetaldehyde adducts, which are potential biomarkers of alcohol abuse			Bis-Tris buffer (pH 7.4)		identified for the first time. The study demonstrated that the CE-ESI-MS method offers improved sensitivity	
Detect changes in adduct levels in alcohol detoxification patients using a novel, sensitive method combining liquid chromatography with time-of-flight mass spectrometry (LC–TOF MS), aiming to establish analytic procedures for	1 mL of 0.1 g/mL -1 water Hemoglobin extracted from erythrocytes	5 µM to 500 mM	Incubation for 24h at 37°C	Liquid chromatography coupled to time-of-flight mass spectrometry (LC-TOF)	Acetaldehyde has been found to react with hemoglobin 21 modified peptide fragments were identified after in vitro incubation of acetaldehyde with hemoglobin and subsequent trypsin digestion, only eight were found in human blood samples Elevated levels of adducts were only found in recent ethanol	(Toennes et al., 2010)

detecting these adducts and evaluate their potential as indicators of alcohol abuse			consumption and decreased during five days of abstinence. No differences were found between samples taken at 5, 10 or 15 days after admission, suggesting rapid degradation within five days of abstinence and it to be a potential biomarker for short-term ethanol ingestion.

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