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Biophysical study of Folic Acid delivery by Pregnant's Serum proteins in The First and Third Trimester of Pregnancy

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Dedication

I dedicate this thesis to all of my beloved family and friends who have supported me throughout my life and allowed me to achieve my goals.

To my father Issam and my mother Hayfaa, thank you for being my source of inspiration, strength, and encouragement and for giving me your unlimited love and support. To my sister Sara, thank you for being a source of happiness and love. To my brothers, Qusai and Odai, thank you for your support and encouragement.

To my best friend, Sawsan! Thank you for teaching me to chase my dreams wherever they may be, for being the reason for positive development in my personality and every positive change in my mind. Thank you for the patience, support, and strength that you always give me. Thank you for all the beautiful times filled with laughter and true love. Thank you for every happy second. It lifts my spirit and it means the world to me that I mean thing to you. You believed in me before I believed in myself.

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To my friend Mary, thank you for your overwhelming love. To my friend Malak, thank you for your encouragement, support and love. To my friend Doaa, thank you for being a truly friend and for all the love you always show me. To my friend Bara'a, thank You for the attention, positive energy and great love that you always give me.

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To all my friends, thank you for your kind words, kind souls, and kind perspective of me. To every person in my life that wished the best for me. Thank you and I love you all.

Ghadeer I. Qawasmeh

Declaration

I certify that this thesis submitted for the degree of master, is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed: 

Ghadeer Issam "Abed Alfattah" Alqawasmeh

Date: 22 / 8 / 2022

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Abstract

Human serum albumin (HSA) is the most abundant protein in blood plasma. It is a biologically relevant protein that binds a variety of drugs and other small molecules. It plays a crucial role in the transportation and distribution of drugs, including Folic Acid in the human body. Folic Acid (FA) is a water-soluble bioactive food constituent from the vitamin B-family complex (B9). Folic Acid is recognized as an important component of the care of women of child-bearing age as it plays a crucial role in the prevention of birth defects. The strongest justification for Folic Acid supplementation during pregnancy comes from the association between adequate intake of Folic Acid and reduced risk of having a child with neural tube defects.

In this study, the structural changes and the molecular mechanism of the interaction of Folic Acid with human serum albumin (HSA) taken from pregnant women during early stage (First Trimester) and the late stage (Third Trimester) of pregnancy was investigated in a single molecular level (single functional group). The native blood protein concentration was almost constant in all samples in both First and Third Trimester periods with various Folic Acid contents.

FTIR total Absorption Spectroscopy, second derivative, Fourier self-deconvolution and curve-fitting methods were employed to analyze the Folic Acid binding sites, binding mechanisms and the induced structural effects on pregnant blood protein (HSA) by different Folic Acid concentrations in Pregnant's blood.

The molecular mechanism of types of interaction of Folic Acid with native serum albumin of pregnant women in the First and Third Trimester was extensively investigated in all major structural regions of protein using FTIR spectroscopy over a range of 4000–400 cm^{-1}. The interaction mechanism was demonstrated by a significant decrease in the total absorption intensities of the Amide A, ν_{as} CH_3, ν_{as} CH_2, Amide (I, II, III) in both Trimesters. The intensity difference (delta) of the maximum Absorption of the lowest and highest concentration of Folic Acid in FA-HSA complexes ($\Delta I = I_{5ng/ml} - I_{11ng/ml}$) were calculated in both Trimesters. For the Amide A and Amide B band (3500-3000 cm^{-1}) in the First and Third Trimesters were 0.327 and 0.090, respectively. In C-H Band (3000-2800 cm^{-1}), the intensity difference of ν_{as} CH_3 in the First and Third

trimester were 0.278 and 0.029, respectively, and of ν_{as} CH_2 in the First and Third Trimester were 0.286 and 0.029, respectively. Additionally, in Amide (I, II, III) bands, the intensity difference in the First Trimester were 0.299, 0.279 and 0.120, respectively. While in the Third Trimester were 0.083, 0.080 and 0.026, respectively. This indicates that the interaction between Folic Acid and serum albumin in the early stages (First Trimester) is greater than in the late stages (Third Trimester) of pregnancy.

Based on the overall all results obtained from the FTIR structural analysis, we could confirm that Folic Acid binds native serum albumin of Pregnant's via hydrophobic, hydrophilic and H-bonding interactions in both trimesters. Hence, at the molecular level, this gives evidence regarding major induced alterations occurring in the native serum albumin of Pregnant's women as a result of Folic Acid interaction in the early stages of pregnancy.

Besides the structural changes FTIR measurements, determination of the conformational changes of the native protein secondary structure induced by Folic Acid in the Amide I region (1700–1600 cm^{-1}) have been made using Fourier self-deconvolution methods. From these advanced analyses, it has been found that Folic Acid biding to native serum albumin induce conformational and microenvironmental changes on protein secondary structure by major alternation in the α-helix (reduced) and β-sheet (increased) structures in First and Third Trimesters. While, the occurrence of major structural alterations of blood protein for the Pregnant women was happened clearly in the early gestation ages (First Trimester) than later ages (Third Trimester). Therefore, taking Folic Acid as supplement by Pregnant's women during the First Trimester of pregnancy can efficiently contribute to the public health action to decrease severe, disabling and potentially lethal Neural Tube Defects (birth defects).

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Acronyms

A	**Absorbance**
E	**Energy**
ECL	**ElectroChemiLuminescence**
EM	**Electromagnetic**
FA	**Folic Acid**
FTIR	**Fourier Transform Infrared**
HSA	**Human Serum Albumin**
IR	**Infrared**
K	**Force constant**
MIR	**Mid-Infrared**
NTDs	**Neural Tube Defects**
pH	**Potential of Hydrogen**
Q	**Speed of Rotation**
RBC	**Red Blood Cells**
RCF	**Relative Centrifugal Force**
R	**Distance**
T	**Transmittance**
UV	**Ultraviolet**
WBC	**white Blood Cells**
$A(\bar{\nu})$	**Spectral Power Intensity**
ν	**Frequency**
c	**Speed of Light**
λ	**Wavelength**
h	**Plack's Constant**
V(r)	**Potential Energy**
ν_i	**Fundamental Frequency of the Particular Mode**
n_i	**Vibrational Quantum Number**
E_{vib}	**Vibrational Energy states**
x_i	**Anharmonicity Constant**
$\bar{\vartheta}$	**Natural Frequency of Vibration of a bond**
μ	**Reduced Mass of the System**
m	**Mass**
$\bar{\nu}$	**Wavenumber**
$I(\bar{\nu})$	**Intensity of the Radiation**
ν (__)	**Stretching Vibrations**
δ (__)	**Scissoring Vibrations**
COOH	**Carboxyl Group**
NH_2	**Amino Group**

Chapter 1: Introduction

1.1 Problem Statement

For several decades, the interaction between drugs and bio-macromolecules has had great interest among researchers. Among those bio-macromolecules was serum albumin, which is the major soluble protein constituent of the circulatory system and has several physiological functions [1, 2]. The main function of serum albumin is that can act as a transporter protein for many exogenous compounds. The binding of drugs with tissues and blood proteins can significantly influence the pharmacokinetics of drugs, and therefore, the toxicological action and pharmacodynamics of drugs [3]. The interactions of drugs and protein may form a stable drug-protein complex, which can have an important effect on the distribution, free concentration and metabolism of the drug in the bloodstream. Thus, can be considered the drug–albumin complex as a model for gaining a fundamental understanding of the drug-protein interactions. Therefore, studies of the binding of a drug with protein present a facilitated interpretation of the metabolism and transporting process, and explain the relationship between the functions and structure of a protein [4].

Folic Acid (FA) is a vitamin B9, which can be found in many foods and multivitamin supplements. Medical and biochemical studies have proven that Folic Acid as a coenzyme is very important to the biosynthesis of purines, pyrimidines, nucleic acid, and protein, and to the formation and division of cells [5]. A shortage of Folic Acid causes the decline of physiological function and some diseases such as gastrointestinal dysfunction megaloblastic anemia, mental retardation, and defects in newborns [6, 7].

Particularly, Folic Acid is important for women who could become pregnant. Numerous research studies have revealed that Folic Acid plays a crucial role in reducing the risk of many birth defects including anencephaly, spina bifida and some heart defects [8]. For many embryos, a shortage of Folate during pregnancy causes a failure of the primitive neural tube to close and differentiate normally and results in neural tube birth defects (NTD). Many studies have confirmed the importance of enough intake of folate during the weeks just before and after conception. Folic Acid can reduce many

birth defects of the brain and spinal cord by more than 70%. Additionally, it has been shown that diets and blood levels of women pregnant who have a fetus with a neural tube defect are low for several micronutrients, especially Folate [9-13].

However, the function of the Folic Acid-binding blood proteins and the molecular mechanism of the interaction between Folic Acid and the carrier protein, i.e human serum albumin on a single molecular level are not addressed yet. Our research study will result in a full understanding of the molecular mechanism of Folic Acid function as an inhibitor or reducer of many of the birth defects of the brain and the spinal cord using native blood protein from pregnant women in the First and Third Trimesters of pregnancy.

1.2 Hypothesis

Our system mainly consisted of native serum albumin; which is the major soluble protein in the blood circulatory system and used as a carrier for Folic Acid at physiological pH.

The interactions of Folic Acid with pregnant's serum protein may lead to the formation of a stable Folic Acid-serum albumin complex, which has a significant impact on the distribution, free concentration, and metabolism of Folic Acid in the bloodstream. In this research project, the molecular mechanism of the Folic Acid complexes with serum albumin will be investigated to understand how the pharmacodynamic and pharmacokinetic properties are affected by the drug binding to the secondary structure of pregnant’s albumin using Fourier Transform Infrared Spectroscopy (FTIR) as shown in **Figure 1.1**. FTIR spectroscopy was used to determine the chemical structure of serum albumin collected from pregnant women in the First and Third Trimesters which taken Folic Acid as a supplement and compared between them.

Figure 1.1: Schematic Diagram of our model system including major steps to prepare the serum albumin samples from pregnant women which taken Folic Acid as a supplement for measurement and analysis using FTIR spectroscopy.

1.3 Significance of Work

The metabolism of several biologically active compounds in the body whether natural products or drugs is correlated with their binding with serum albumin. Also, the drug distribution is controlled by serum albumin, because most drugs travel in the blood and reach the target tissue by binding to human serum albumin (HSA) [14]. Thus, the study of the interaction of Folic Acid with albumin is of fundamental and imperative importance.

A recent study by P. Bourassa and his group, suggests that the serum albumins can act as carrier proteins for Folic Acid and delivered to target molecules [15]. Fourier Transform Infrared Spectroscopy, Ultraviolet-visible and fluorescence spectroscopic methods, and molecular modelling were used to study the interaction of Folic Acid with serum albumin which includes the analysis of binding sites, the binding constant, and effect of Folic Acid on the stability and conformations of HSA. The structural analysis explained that Folic Acid binds HSA via both hydrophilic and hydrophobic contacts with overall binding constants of $K_{FA\text{-}HSA}$ equal 8.1 ($\pm$0.5)$\times 10^4$ M^{-1} [15]. Molecular modelling showed the contribution of several amino acids in Folic Acid–albumin

complexes stabilized by a hydrogen bonding network. Folic Acid complexation altered protein secondary structure by major reduction of α-helix from 59% (free HSA) to 35% (Folic Acid-complex) with an increase in random coil, turn and β-sheet structures indicating protein unfolding [15].

The binding of Folic Acid to human serum albumin also was studied using ATR-FTIR, fluorescence spectroscopy, cyclic voltammetry, and electrochemical impedance spectroscopy. The small distance found between albumin Trp 214 and Folic Acid and the decrease in the average lifetime of serum albumin after the addition of Folic Acid pointed to that there is an energy transfer between the two molecules. The entropic and enthalpic terms showed that the complex formation is an exothermic process and the reaction is entropically driven [16].

Moreover, the interaction of Folic Acid with serum albumin was studied by both cyclic voltammetry and electrochemical impedance spectroscopy; the variation of electrochemical parameters showed that the Folic Acid binds to the site of serum albumin and the binding constant was ten times higher than the value obtained when the interaction takes place between the free molecules in the solution [16].

1.4 Objective

The main goal of this study was to investigate the molecular mechanism, drug loading and the binding efficiency of the Folic Acid with serum albumin, which was taken from pregnant women in the First and Third Trimester of pregnancy.

In this study, the Fourier Transform Infrared Spectroscopy was used to understand the role of serum albumin as a carrier protein for Folic Acid in the aqueous solution under physiological conditions that can be used in different therapies.

Our results may be used in future studies to improve drug delivery systems or cellular uptake of drugs such as Folic Acid and food components conjugated to albumin nanoparticles or Nano-capsules.

Chapter 2: Literature Review and Theoretical Background

2.1 Biology Approach

2.1.1 Blood Components

Blood is a vital body liquid, a combination of cellular components, colloids, and crystalloids make up whole blood [17]. The Blood is circulated through the heart, arteries, capillaries, and veins. Also, it carries oxygen and nutrients to the body cells and removes carbon dioxide and waste products. A person's body contains different amounts of blood based on their age, sex, general health, and even where a person lives. The volume of blood in the human body has been estimated to be approximately 7% of the body weight [18].

The main Components of the Blood are plasma, red blood cells (RBC), white blood cells (WBC), and platelets. The Blood of humans contains about 55% of plasma, 45% of RBC, 0.1% of WBC and about 0.17% of platelets as show in **Figure 2.1** [18].

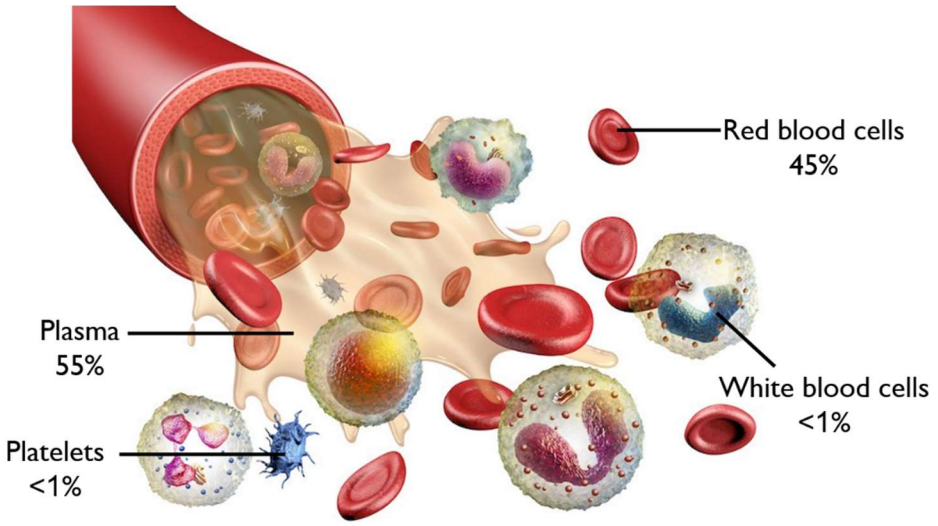


Figure 2.1: Blood components Blood [19].

Blood and blood components carry out a variety of essential body activities [20]. The red blood cells supply oxygen to several tissues of the body and carry waste products including carbon dioxide. White blood cells fight the diseases and can produce

antibodies to strengthen the body's immunity. While the plasma; is a liquid part of the blood, it contained about 92% water, 7% vital proteins such as albumin, gamma globulin, anti-hemophilic factor, and other clotting factors, and 1% sugars, mineral salts, fats, vitamins, and hormones. The platelets are produced in the bone marrow and their main function is to prevent bleeding where it plays an important role in blood clotting [18].

2.1.2 Protein and Protein Structure

Proteins consider the very basis of life. It is the most abundant organic chemical substance in living systems and considers a major target for several medications in our body [21]. Proteins might be regulatory, structural, protective or contractile; they are involved in numerous physiological processes, including clotting, immunological response, molecular transport, osmotic balance, and tissue leakage [21, 22]. Proteins carry out their functions through three-dimensional tertiary and quaternary interactions between the substrates such as RNA and DNA [23].

Understanding protein function requires knowledge of the three-dimensional structure of proteins. Proteins are biological polymers. The monomeric units of proteins are amino acids, which are arranged in a linear sequence and joined by covalent bonds [22]. Proteins are made from twenty distinct amino acids, each of which has a carboxylic acid group (COOH), an amino group (NH_2), and one of the twenty functional (R) groups. Amongst the amino acid glycine's straightforward hydrogen atom and tryptophan's complex structure, the R group, or side-chain, differs between amino acids [23]. The basic structure of an amino acid was shown in **Figure 2.2**.

Proteins can be identified by the number and the sequence of amino acids units they contain [24]. Changing one amino acid or removing it from the protein sequence can be detrimental to its structure [23]. A polymer can be formed by connecting the amino group on one amino acid with the carboxylic acid group on another amino acid to form

an amide bond (peptide bond). When an amide bond links two amino acid together, the molecule is called a dipeptide, while when many amino acids are linked together, the molecule is called a polypeptide. The proteins formed by a few polypeptides. For a polypeptide to be considered a function protein, it must be able to fold into three-dimensional structure [24].

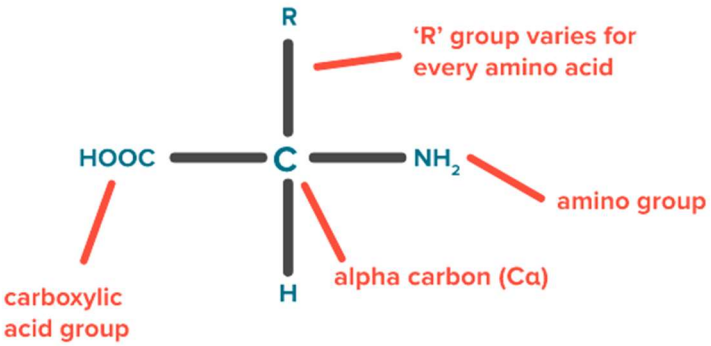


Figure 2.2: General structure of an amino acid [25].

2.1.2.1 Structure Levels of Protein

Understanding the four levels of protein structure (primary, secondary, tertiary, and quaternary) **(Figure 2.3)** is necessary to understand how the protein assumes its final shape or conformation [26]. The four levels of protein structure become highly structured and effective biological machines as a result of several ionic and molecular interactions within the protein [23]. The first level of protein structure called the primary structure. The primary sequence is the linear order of the amino acids are linked together in the polypeptide chain [22]. It is sometimes called the "covalent structure" of proteins because most of the covalent bonding within Proteins defines the primary structure [27]. The primary structure of each protein leads to the unique folding pattern that is characteristic of that specific protein [22].

The next level is called the secondary structure of the protein. A secondary structure is a regular structure formed via hydrogen bonding mainly within the peptide backbone [23]. Also, it describes the local conformation of the amino acids in the protein chain [28]. α-helix and β-pleated sheet considered the most common types of secondary

structures **(Figure 2.4)** [23]. These two structures are held in shape by hydrogen bonds. The hydrogen bonds form between the oxygen atom in the carbonyl group (C=O) in one amino acid and another amino acid that is four amino acids farther along the chain [26].

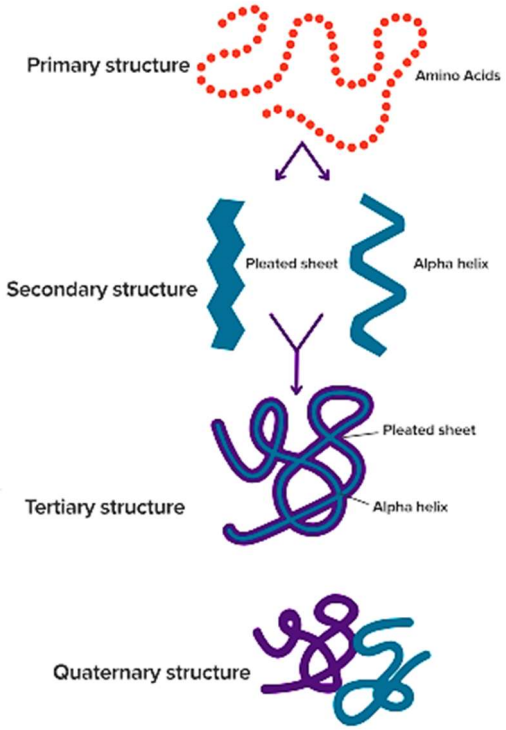


Figure 2.3: The four levels of protein structure [29].

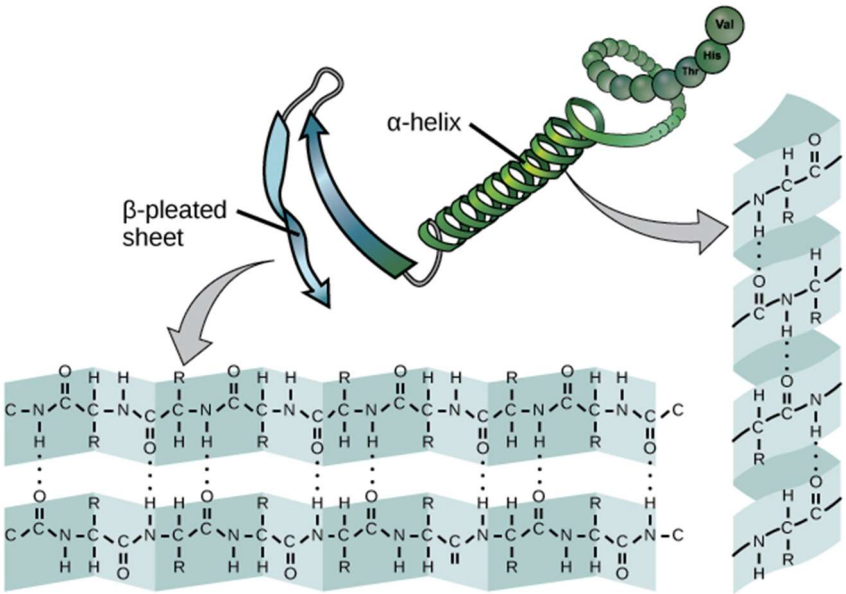


Figure 2.4: Protein's secondary structure, α-helix and β-pleated sheet [29].

In an α-helix the polypeptide backbone is coiled, where the hydrogen bonding occurs between the carbonyl (C=O) of one amino acid and the amino H (N-H) of an amino acid that is four down the chain [24]. This pattern of bonding pulls the polypeptide chain into a helical structure that resembles a curled ribbon, with each turn of the helix containing 3.6 amino acids. The R groups of the amino acids stick outward from the α-helix, where they are free to interact [30].

In the β-pleated sheet, the hydrogen bonding between atoms on the backbone of the polypeptide chain forms the "pleats". The R groups attach to the carbons and extend above and below the folds of the pleat. The polypeptide chains that form the β-structure are called β-strands [26]. The β-strands in the β-sheet can interact in two ways; parallel in which all amino acids in the aligned β-strands run in the same direction, or antiparallel in which the amino acids in successive strands run in opposite directions [31].

when the secondary structures are spatially further apart along the polypeptide chain, the polypeptide chains start to interact with their respective side chains, creating a more complex level of folding, a tertiary structure. Tertiary structure is the complete three-dimensional shape of the entire protein and is a unique and defining feature of that protein. The tertiary structure is primarily due to interactions between the R groups of the amino acids that make up the protein [29]. These interactions include; covalent interactions between cysteine groups, non-covalent electrostatic interactions between polar groups, and Van der Waal interactions between non-polar R groups [23]. Also significant to tertiary structure are hydrophobic interactions, in which amino acids with nonpolar, hydrophobic R groups cluster together on the inside of the protein, leaving hydrophilic amino acids on the outside to interact with surrounding water molecules [29]. Interaction between cysteine side chains forms disulfide linkages in the presence of oxygen, the only covalent bond forming during protein folding. These all interactions determine the final three-dimensional shape of the protein. When a protein loses its three-dimensional shape, it is usually no longer be functional [26].

Quaternary structure describes how several polypeptide chains come together to form a single functional protein [32]. The interactions between the chains are not different from those in tertiary structure, but are distinguished only by being inter-chain rather than intra-chain [23].

2.1.2.2 Human Serum Albumin

Several transport proteins are found in the human blood plasma. The most abundant one is human serum albumin (HSA), 66.5 kDa, makes up ~50% of the total plasma protein [33] and naturally synthesized in the liver and released into the bloodstream afterward [16].

HSA is a multi-functional plasma protein plays many critical roles within the body. The main function of HSA is to maintain the osmotic pressure in the blood [34]. Additionally, it regulates blood pH, transports solutes like hormones or medications, mediates lipid metabolism, eliminates toxins, and acts as an anti-inflammatory and an antioxidant [35-37]. Moreover, it has the ability to bind a range of medicines as well as insoluble substances like fatty acids [38].

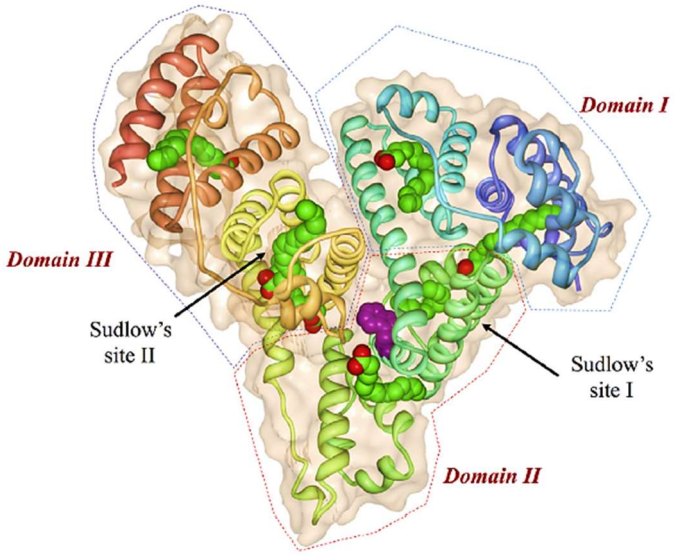


Figure 2.5: The Human serum albumin structure [39].

The distribution of the binding sites across the molecule's three-dimensional structure determines the binding characteristics of albumin. Crystallographic analyses showed that the serum albumin **(Figure 2.5)** is a single polypeptide chain of 585 amino acids with a largely-helical (67%) triple-domain structure that assemble to form a heart-shaped molecule [40, 41]. HSA consisted of three structurally similar domains; domain I, II and III **(shown in Figure 2.5)**. Each domain has two subdomains (A and B), and 17 disulphide bridges stabilize it [42].

The protein's capability of binding aromatic and heterocyclic compounds largely depends on the existence of two main binding regions, known as Sudlow's sites I and II. The first one was located in the core of the IIA subdomain and the second in the IIIA subdomain, more exposed to the solvent than the former one [43]. Besides these two sites, there are another five sites, with a high affinity for fatty acids [41, 44].

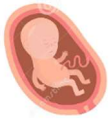
2.1.3 Pregnancy

Pregnancy is the description of the period when a woman has a growing fetus inside of her womb or uterus. Where the fetus is developed after the fertilization of an egg by a sperm and implantation in the uterus. Pregnancy lasts about 40 weeks from the start of the last menstrual period to childbirth [45]. Pregnancy has three trimesters, each of which is marked by specific fetal developments. As mentioned before, pregnancy is considered full-term at 40 weeks; approximately every 14 weeks is considered a unique stage of pregnancy. The fetus in each trimester meets specific developmental milestones **(see Table 2.1)** [46].

The First Trimester lasts from the occurrence of pregnancy until 13 weeks. It is considered the most important stage of the baby's development because the baby's organ systems and body structure develop during it. All major organs of the baby and external body structures have begun to form during this trimester. The beating of the baby's heart

started with a regular rhythm and the external sex organs define the sex of the baby. At the end of this period, the baby will be about 7.4 cm long and a 1.3 KG weight. This trimester is as crucial for the baby as for the woman. The next period of pregnancy is the Second Trimester (14 weeks to 26 weeks). In this stage, the mother will starts to feel is the baby's fluttering movements somewhere between 16 weeks and 20 weeks. During the Second Trimester, the baby can hear and swallow and its lungs are formed but do not work. At the end of this Trimester, the baby can store fat and weighs about 7.5 KG and will be about 36.6 cm long. It can consider a baby to be full-term and all of his organs to be functional at the end of the third trimester [47-49].

Table 2.1: Pregnancy Trimester stages [50].

	Trimester	Months	Weeks	Normal development (Weight, Length)
	First Trimester	1-3	1-13	1.3 KG, 7.4 cm
	Second Trimester	4-6	14-26	7.5 KG, 36.6 cm
	Third Trimester	7-9	27-40	12.0 KG, 51.2 cm

2.1.4 Folate and Folic Acid

Folate is a water-soluble member of the vitamin B-family compounds; the synthetic form of this vitamin is called Folic Acid, and it differs from Folate just slightly. Compared to naturally occurring Folate, Folic Acid has the benefit of being better absorbed by the body and having a significantly higher bioavailability [51]. The main Folate sources in foods are liver, sprouts, kidneys, various fruits such as oranges, and leafy green vegetables such as spinach [52].

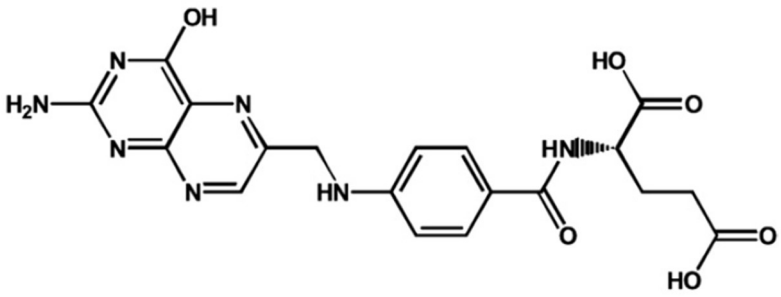


Figure 2.6: Chemical structure of Folic Acid [15].

Folates are a group of heterocyclic compounds with a 4-(pteridin-6-methylamino) benzoic acid skeleton, conjugated with one or more residues of l-glutamic acid [53]. The external supply of this compound is necessary to prevent nutritional deficiency; because human cells are unable to synthesize it [54]. The oxidized and most active form of this vitamin is Folic Acid; it is rarely found in food and is typically used in vitamin preparations and food fortification. Folic Acid are characterized by (4((pteridin-6-yl-methyl) amino) benzoic acid) **(Figure 2.6)**; it is the most stable form of folate [53].

2.1.4.1 Folic Acid Function

Folic Acid is required to maintain an organism's regular vital functions; its function is mainly as a methyl-group donor which is involved in many important biological processes [55]. Studies in biochemistry and medicine showed that the Folic Acid is a coenzyme that is crucial for the development and division of cells, as well as the manufacture of purines, pyrimidines, nucleic acids, and proteins [56]. Folic acid deficiency can causes various declines of physiological function and some diseases such as gastrointestinal dysfunction, mental retardation, and neurovascular abnormal newborn [7].

2.1.4.2 Folic Acid and Pregnancy

Folic acid is acknowledged as a crucial element of the preconception care given to women of childbearing age ; it plays an important role in the prevention of birth defects [57]. The greatest argument for Folic Acid supplements for expectant mothers comes from the association between adequate Folic Acid intake and a lower chance of having a child with neural tube abnormalities (NTDs).Therefore, it is necessary for the fetus's appropriate neural tube development and closure, which happens between the second and third week of pregnancy [58].

Anencephaly and spina bifida are the most frequently occurring congenital birth disorders that damage the brain and spinal cord. Neural tube defects are a subclass of birth defects. Neural tube defects can drastically affect a baby's growth or even be deadly. A mother with low Folate may have a higher risk of miscarriage, placental abruption, preclampsia, and preterm delivery due to the link between low Folate and homocysteine levels. Furthermore, there has been some convincing evidence that Folate may reduce the risk of other birth defects as well [10, 12, 13, 59].

2.1.5 Recent Studies

Claudia G. Chilom, et al (2018), make investigated the interaction of Folic Acid with serum albumin using ATR-FTIR, fluorescence spectroscopic, cyclic voltammetry, and electrochemical impedance spectroscopy to understand the role of serum as a carrier protein of Folic Acid in aqueous solution. They studied the quenching of serum albumin in the presence of Folic Acid and determined the binding constant. Also, was shown that Folic Acid interacts with serum albumin and made changes in protein conformation by partially unfolding it. The small distance found between albumin Trp 214 and Folic Acid and the decrease in the average lifetime of serum albumin after the addition of Folic Acid pointed to that there is an energy transfer between the two molecules. The negative value of ΔG indicates the spontaneity of the complex formation. The entropic

and enthalpic terms showed that the complex formation is an exothermic process and the reaction is entropically driven.

Also, the interaction of immobilized serum albumin with free Folic Acid was proved by both cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The variation of electrochemical parameters for both CV and EIS proved that the Folic Acid binds to the site of serum albumin and the binding constant was ten times higher than the value obtained when the interaction takes place between the free molecules in the solution) [16].

P. Bourassa, et al (2017), analyzed the loading efficacy of Folic Acid with serum albumin and determined the effect of Folic Acid conjugation on albumin morphology. Structural analysis indicates that the Folic Acid binds with serum albumin via hydrophilic, hydrophobic, and H-bonding contacts. Molecular modeling showed stabilizing Folic Acid-albumin conjugates as a result of the presence of several H-bonding systems. Folic acid conjugation changes the protein conformation by major alternating in the α-helix and β-sheet. TEM images appear major albumin morphological changes, which induce albumin aggregation upon the interaction with Folic Acid. The results indicate that the albumin can deliver Folic Acid to the target molecules. Thus, the interaction of Folic acid with albumin induces major alterations in human serum albumin conformations, and this carrier protein is capable of Folic Acid delivery in vitro [60].

Zannat MR, et al (2016), make a study to evaluate and assess the level of serum albumin in the First and Third Trimester of pregnancy. The decrease in the level of the serum albumin that occurs in the First and Third Trimester of pregnancy might be related to increased maternal and fetal mortality and morbidity. Serum aluminum was evaluated by the Bromocresol green (BCG) method in the First and Third Trimesters of pregnant women and in non-pregnant women. Statistical analysis of data was made using an unpaired student's t-test. The results showed that the serum albumin levels decreased significantly both in First and Third Trimester and maximum decrease seen

in Third Trimester. From this study, it is concluded that the decreasing albumin level during the First Trimester of pregnancy is mainly due to hemodilution, while in the Third Trimester of pregnancy its due to progressive hemodilution, increasing capillary permeability, dietary protein deficiency, and increased loss of albumin through urine attribute hypoalbuminaemia [61].

Miriam G, et al (2016), presented an overview of the works of literature relating to the use of the Folic Acid in the peri-conceptional period and the prevention of birth defects, especially neural tube defects (NTDs). Moreover, the study presented evidence for the protective function of Folic Acid in the prevention of neural tube defects, when Folic Acid is taken prior to conception and throughout the First Trimester of pregnancy. Also, confirmed that maximizing the intake of Folic Acid in the peri-conceptional period can concretely contribute to the public health action to decrease severe, disabling and potentially lethal NTDs [57].

Thaler C. J. (2014), this study emphasizes that Folate metabolism affecting on ovarian function, implantation, embryogenesis, and the entire process of pregnancy. In addition to its confirmed effect on the incidence of neural tube defects was found associated between reduced Folic Acid levels and increased homocysteine concentrations on the one hand, and recurrent spontaneous abortions and other complications of pregnancy on the other. For the infertility patients who undergo IVF/ICSI treatment, there is an obvious correlation found between plasma Folate concentrations and the incidence of dichorionic twin pregnancies. In the end, corroborated that can be compensated these negative effects for in full by increasing the Folic Acid daily dose to at least 0.8 mg [62].

2.2 Principles of Spectroscopy

2.2.1 Electromagnetic Radiation

Electromagnetic radiation (EM) is defined as the flow of energy accompanied by electric and magnetic fields, which are the components of Electromagnetic waves. These components are in the form of oscillating fields, which are at right angles to each other and to the direction of propagation of the wave **(Figure 2.7)**. The physical characteristics of the time variation electromagnetic wave are the intensity and frequency, of the electric and magnetic fields [63].

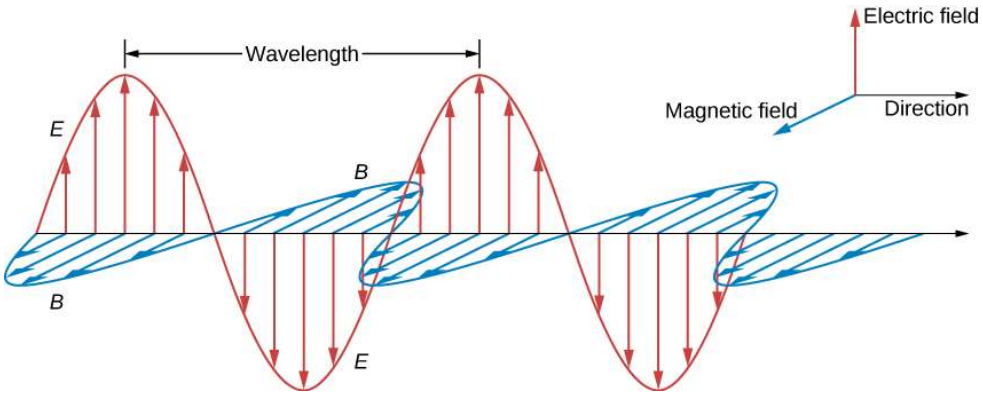


Figure 2.7: Scheme of an electromagnetic wave propagates along the x-axis, with electric (red) and magnetic fields (blue) perpendicular to each other [64].

The EM waves travel through space at the speed of light (2.997 925 × 10^8 m/s). Depending on their wavelength, electromagnetic waves can be classified as visible light, microwave, infrared, gamma-rays, etc. Wavelength λ, and frequency ν (the number of cycles per second) are quantities that are inversely proportional. The propagation speed serves as the proportionality constant. In free space, propagation speed equals light speed (c):

$$\lambda = \frac{c}{\nu} \tag{2.1}$$

Max Planck developed the hypothesis that EM radiation can act both like a massless particle and like a classical wave (1900). Albert Einstein supported this hypothesis with the discovery of the photo effect (1904). The fundamental basic units of electromagnetic radiation are photons. They are indivisible, stable, and lack electric charge. When a charged particle transitions from a higher energy state to a lower one, a photon is released. The following Planck-Einstein equation provides the photon's energy E in Joules (J) [65, 66].

$$E = h\nu = h\left(\frac{c}{\lambda}\right) \tag{2.2}$$

Where h is the Planck constant; $h = 6.62607 \times 10^{-34} J.s$.

Electromagnetic radiation can be arranged according to their wavelength and energy **(Figure 2.8)**. Due to the differences in energy, various parts of the spectrum may interact with matter and trigger various processes in atoms or molecules, such as Ionization, electronic transitions, vibrational transitions, and rotational [63, 67].

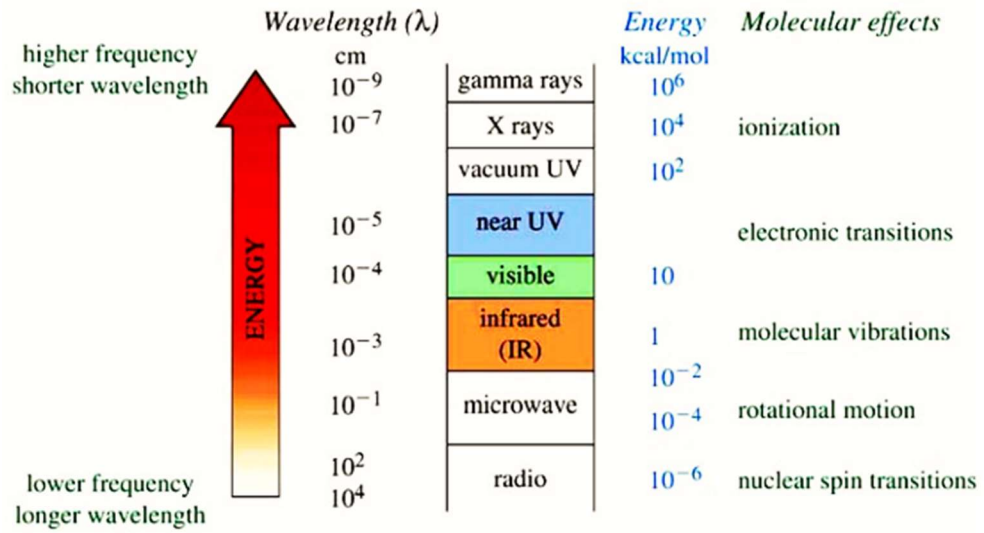


Figure 2.8: Electromagnetic spectrum based on wavelengths, energies, and their corresponding molecular effects [68].

When a molecule absorbs electromagnetic radiation, the molecule may undergo rotational, vibrational, electronic, or ionization reactions. Ionization or electronic transition processes may occur in the absence of rotational or vibrational degrees of freedom in an atom. In this experiment, visible or near-ultraviolet light is frequently used as the source of illumination. Electronic transition processes take place when an atom absorbs an ultraviolet photon or a visible light photon [69].

The spectrum provides a molecule's molecular structure by giving information about the spacing between its energy levels. Energy is absorbed during the process of excitation from a lower to a higher energy level. A simple spectrum exists when the wavelengths are few and separated, resulting in a single line (absorptive line). Each energy state typically has a number of energy sublevels; which causes these lines to be indistinguishable and the absorption to appear as a broad peak or band [70].

2.2.2 Molecular Vibrations

Different types of motion can be experienced by the atoms in a molecule, including vibrational and rotational motion. A molecule can only absorb electromagnetic radiation if the incident Infrared (IR) light matching the energy difference between two vibrational energy levels of the molecule.

Classically, a molecule's vibrational modes can be thought of as a series of balls representing different atoms' nuclei connected by massless springs, which represent the intramolecular interactions between the atoms [71]. The classical model of H_2O is depicted in **Figure 2.9**. Due to the stronger bonding forces between O and H, their springs are less extended than those between non-bonded hydrogens. The stronger spring resists stretching of O-H bonds, while the weaker spring resists changing the angle of HOH [69].

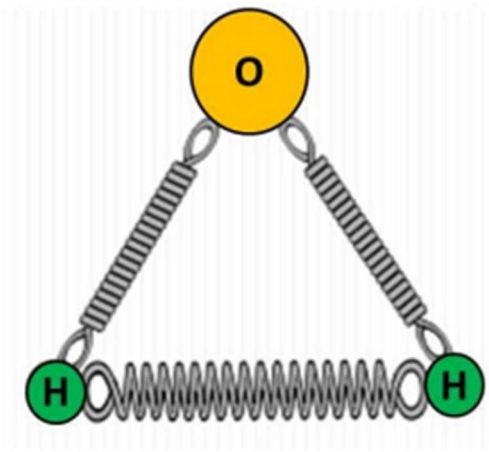


Figure 2.9: **Water's (H_2O) Classical Ball-Spring Model.** Classical Model of the water molecule (H_2O). Compressed springs are used to symbolize the powerful forces that hold the hydrogen and oxygen atoms together. While weaker springs that resemble a weaker bonding, force are used to bind hydrogen atoms.

Molecule atom connections can be linear or non-linear. A linear molecule is formed when atoms are connected in a straight line, their bond angles are 180 such as carbon dioxide and nitric oxide. Molecules with non-linear connections typically have V-shaped or bent connections [72]. Three of the degrees of freedom in a molecule with N atoms are for translational motion in perpendicular directions, and the other three are for rotating motion along the x, y, and z-axes. The remaining degrees of freedom, 3N-6, are the so-called normal modes of vibration, which are the number of vibrational modes present in a nonlinear molecule. These modes result in a net change in the dipole moment, which could lead to an IR activity [73]. According to **Table 2.2**, the total 3N degrees of freedom are decomposed.

Table 2.2: Degrees of freedom for linear and non-linear molecule.

Types of degree of freedom	Linear	Non-Linear
Translational	3	3
Rotational	2	3
Vibrational	3N-5	3N-6
Total	**3N**	**3N**

Diatomic molecules are always linear, while some triatomic molecules, such as carbon dioxide CO_2 and hydrogen cyanide HCN, also exhibit linearity. Molecules containing more than three atoms exhibit an increasing number of vibrational degrees of freedom, including torsions and fragment movements with respect to one another [74].

2.2.3 Normal Modes of Vibration

Normal modes are used to characterize the vibrational movements of molecules. All atoms move harmonically and oscillate at the same frequency but with different amplitudes in a normal mode of vibration. Changes in bond lengths and angles generate vibrations or normal modes [74].

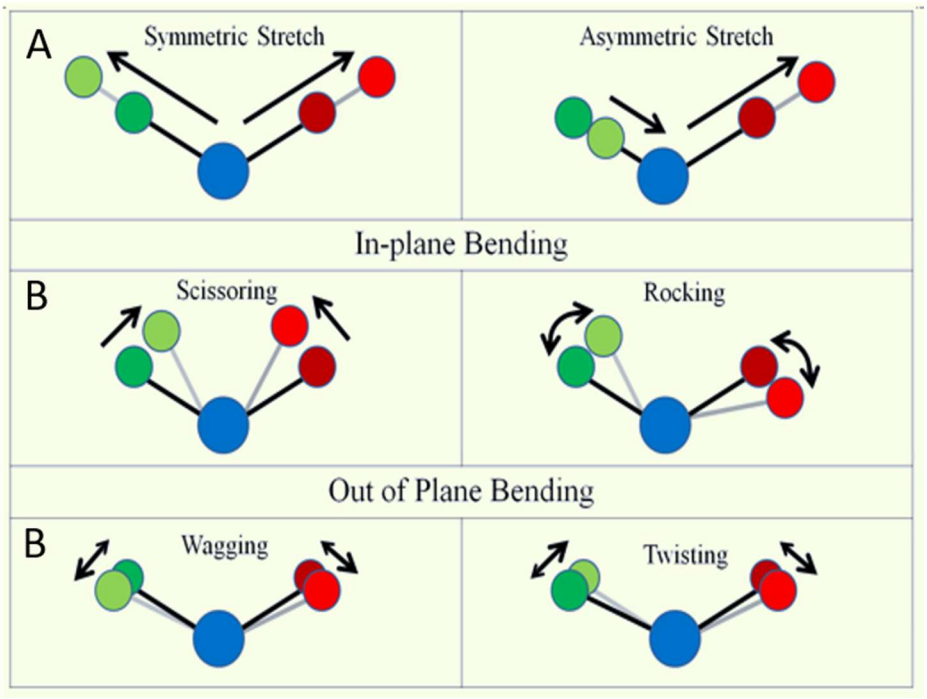


Figure 2.10: Molecular Vibration Types, stretching vibrations **(A)** and bending vibrations **(B)** [63].

When the IR radiation is absorbed, the energy is converted into bond vibrations. These vibrations are classified as either stretching vibrations or bending vibrations. Stretching vibrations are classified as symmetrical (motion in the same direction) or asymmetrical (motion in opposite directions). Asymmetrical Stretching requires higher energy than Symmetrical Stretching to occur. Bending vibrations are categorized as scissoring,

rocking, twisting or wagging as shown **in Figure 2.10**. The number of valence bonds found in a molecule indicate the number of stretching vibrations, while the remaining vibrations are called deformation modes [75].

2.2.4 Harmonic Oscillator of Normal Modes

All the nuclei in a normal mode of vibration move harmonically, oscillate at the same frequency, and move in phase, though typically with different amplitudes. Since each mode of atomic vibration involves harmonic displacement of atoms from their equilibrium positions, the harmonic oscillator model can be utilized to treat atomic vibrations. The potential energy V(r) of a harmonic oscillator is a function of the interatomic distance r for every mode (i) the atom vibrates with frequency (ν_i) **(Dashed line in Figure 2.11)**. In accordance with Hook's law, atoms vibrating in a simple harmonic pattern will have the following vibrational energy states:

$$E_{vib} = h.\,\nu_i\,(n_i + ½) \qquad (2.3)$$

Where h is the Planck's constant, ν_i is the fundamental frequency for a specific mode and n_i is the vibrational quantum number for the i^{th} mode (n=0, 1, 2,…) [73].

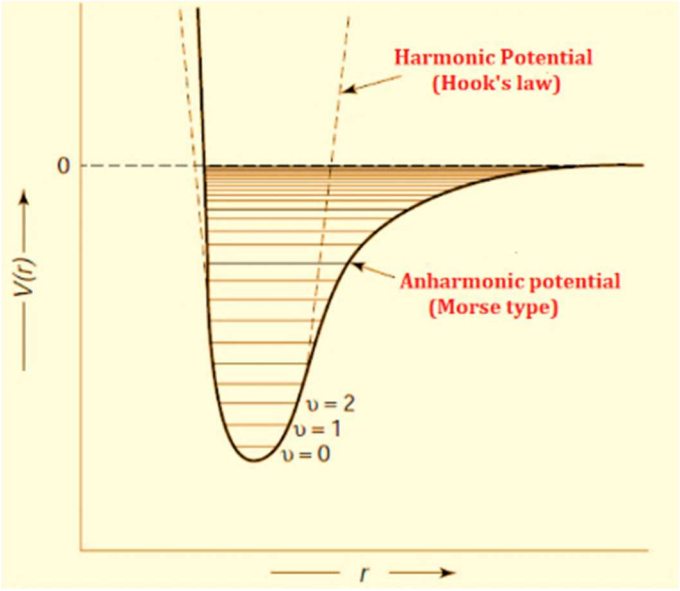


Figure 2.11: A diatomic molecule's Potential Energy. An-harmonic potential (solid line) and harmonic potential (dashed line) [76].

The energy of radiation in the mid-infrared spectrum (4000 to 400 cm^{-1}) corresponds to the energy difference between the ground state (n_i=0) and the first excited state (n_i=1) of the majority of vibrational modes. However, using the harmonic oscillator model to treat normal modes has several limitations. E_{vib} must be characterized using an an-harmonic (morse-type) potential function because it is only viable for low values of the vibrational quantum number **(Figure 2.11 solid line).** The potential energy is given by:

$$E_{vib} = h.\,\nu_i\,(n_i + ½) + h.\,\nu_i.\,x_i\,(n_i + ½)^2 \qquad (2.4)$$

Where x_i is anharmonicity constant.

In many normal modes, the frequency is determined by the specific functional groups. Thus, spectral features detected in a particular spectrum region are often indicative of specific chemical functional groups in molecules [73]. Listed below **Table 2.3** are the wavenumbers assigned to the most common functional groups.

Table 2.3: Vibration wavenumbers for bond stretching and angle bending group [69].

Bond-stretching		Bond-stretching		Angle-bending	
Group	ω/cm^{-1}	Group	ω/cm^{-1}	Group	ω/cm^{-1}
≡C–H	3300	–C≡N	2100	≡C–H	700
=C(H)–	3020	>C–F	1100	=C(H)–H	1100
except: O=C(H)–	2800	>C–Cl	650	–C(H)(H)–H	1000
>C–H	2960	>C–Br	560	>C(H)–H	1450
–C≡C–	2050	>Cl–I	500	C≡C–C	300
>C=C<	1650	–O–H	3600[a]		
>C–C<	900	>N–H	3350		
>Si–Si<	430	>P=O	1295		
>C=O	1700	>S=O	1310		

2.2.5 Vibrational Energy Levels and Transitions

A set of 3N-6 vibrational quantum numbers $n_1, n_2, n_3, \ldots n_{3N-6}$ serve as a representation of the vibrational energy for a N-atom molecule. All quantum numbers are equal to zero at the vibrational level of the ground state, and this energy is known as zero point energy E_ν (0, 0, 0, …), which is not equal to zero. The level is known as the fundamental or first harmonic level for a polyatomic molecule if only one vibrational quantum number $n_k = 1$ is non-zero. A normal mode of vibration can be produced by separately excitation of each of the 3N-6 vibrational quantum numbers to the fundamental level from the ground state.

The polyatomic molecule has a total vibrational energy (E_ν) for 3N-6 vibrations, which is equal to the sum of individual energies ($E_1 + E_2 \ldots + E_{3N-6}$):

$$E_\nu = \sum_{i=1}^{3N-6} \left(n_i + \frac{1}{2}\right) h\nu_i \qquad (2.5)$$

Where h is the Planck's constant, ν_i is the normal vibrational frequency, and n_i is the vibrational quantum number.

The zero point energy E_v (0, 0, 0, …) for N-atom is given by:

$$E_\nu^0 = \frac{1}{2}\sum_{i=1}^{3N-6} h\nu_i \qquad (2.6)$$

For Polyatomic molecule, the vibrational energy with the anharmonicity correction is:

$$E_{vib} = \sum_{i=1}^{3N-6} \left(n_i + \frac{1}{2}\right) h\nu_{e,i} + \sum_{i\geq k}\sum_{k}^{3N-6} hx_{ki} \left(n_k + \frac{1}{2}\right)\left(n_i + \frac{1}{2}\right) + hG_0 \qquad (2.7)$$

Where $\boldsymbol{\nu_e}$ is the harmonic frequency, and G_0 is a small constant that is that is often disregarded [77-80].

2.3 Spectroscopy Approach

2.3.1 Infrared Spectroscopy

Infrared radiation has wavelengths ranging from 0.7 μm to 1000 μm in the electromagnetic spectrum. Due to their location in relation to the visible spectrum, the infrared spectrum can be divided into three regions **(Table 2.4)**.

For chemical analysis, the Mid-infrared region (MIR) with wavelengths between 2.5 μm and 30 μm (4000 to 400 cm^{-1}) is the most attractive. MIR corresponds to the fundamental vibrations of virtually all organic functional groups [81].

Table 2.4: Infrared Radiation regions and their corresponding wavelength and wavenumber.

Region of IR	Wavenumber (cm^{-1})	Wavelength (μm)
Near-infrared	14000-4000	2.5-0.7
Mid-infrared	4000-400	30-2.5
Far-infrared	400-10	1000-30

2.3.2 Infrared Absorption Process

When IR radiation falls on a particular sample, absorption may take place causing vibrational transitions between the atoms of molecules forming the sample. Absorption of IR radiation occurs when the frequency of applied IR equals the natural frequency of atomic vibrations. The different functional groups within the sample absorb characteristic frequencies of IR radiation, hence obtaining an IR spectrum that is a fingerprint of a molecule for it's identification [63].

The absorption of infrared radiation induces changes in the energy on the order from 8 to 40 KJ/mole. This amount of energy corresponds to the frequencies of stretching and bending vibration of the bands in most covalent molecules. However, not all the bonds are capable to absorb infrared energy. Only those bonds which have changes of the dipole moment as a function of time [82].

The IR absorption spectrum is represented in a graph of wavelength or wavenumber versus transmittance or absorption intensity. The transmittance (T) is equals a ratio of power transmitted by the sample (I) to the power incident on the sample (I_o). The absorbance (A) is the logarithm to the base 10 of the reciprocal of the transmittance T as shown in the equation:

$$A = \log_{10}\left(\frac{1}{T}\right) = -\log_{10}(T) = -\log_{10}\left(\frac{I}{I_0}\right) \tag{2.8}$$

Molecules with two different atoms (heteronuclear diatomic molecules) can be thought of as vibrating masses connected by a spring. The potential energy of the system increases as the spring is extended or compressed beyond the bond equilibrium distance. As a result, when a link vibrates, its vibrational energy continuously transform from kinetic to potential energy.

As may be seen here, the total bond energy is directly proportional to the vibrational frequency:

$$E_{osc} \propto h \,.\, \nu_{osc} \tag{2.9}$$

The spring's force constant (K), along with the masses of the two bound atoms, m_1 and m_2, define this energy. The natural frequency of a bond's vibration can be determined by using Hooke's law, which is as follows:

$$\bar{\vartheta} = \frac{1}{2\pi c}\sqrt{\frac{K}{\mu}} \tag{2.10}$$

Where μ is the reduced mass of the system, is given by:

$$\mu = \frac{m_1 m_2}{m_1 + m_2} \tag{2.11}$$

The force constant K varies depending on the type of bond, being three times greater for triple bonds than single bonds and twice as great for double bonds as single bonds. Bonds that are stronger vibrate at higher frequencies than those that are weaker. While, bonds between larger masses vibrate with less frequencies that those with lighter masses. Since the bending force has a lower value of K than the stretching force, bending motions often occur at lower energies (or frequencies) than stretching motions. C-H stretching, for instance, happens at ~3000 cm^{-1}, while C-H bending happens at ~1340 cm^{-1} [83].

A pure liquid, a solid embedded in KBr a disk, or a solution utilizing a non-polar solvent can all produce infrared spectra that are used for IR analysis. Since polar solvents can influence band intensities and group vibrations, they may also form hydrogen bonds with the solute [84].

Infrared spectra provide information about the molecular structural Bond absorptions such as N-H, C-H, O-H, C=O, C-O, C-C, C=C, C=N, and others only take place in specific regions of the vibrational infrared spectrum. For instance, the absorption in the range of 1715 ± 100 cm^{-1} is caused by the carbonyl group (C=O), while a peak in the region of 3000 ± 150 cm^{-1} corresponds to C-H bonds [82].

2.3.3 Fourier Transform Infrared Spectroscopy (FTIR)

The interferometer is the most important part of the FTIR instrumentation, which makes FTIR different from traditional IR dispersive spectroscopy. The interaction of waves is the fundamental concept behind the Michelson interferometer. Two waves'

peaks and troughs will add up if they have the same frequency, creating constructive interference. On the other hand, interference caused by waves that are out of phase is known to be destructive [73].

The interferometer consist of fixed and movable mirrors as well as beam splitter. The interferometer split incoming light from a source into two portion as seen in **Figure 2.12**. The fixed mirror reflects half of the split light, which is then sent to the beam splitter and the detector. The moveable mirror returns the other light component to the beam splitter and detector [85].

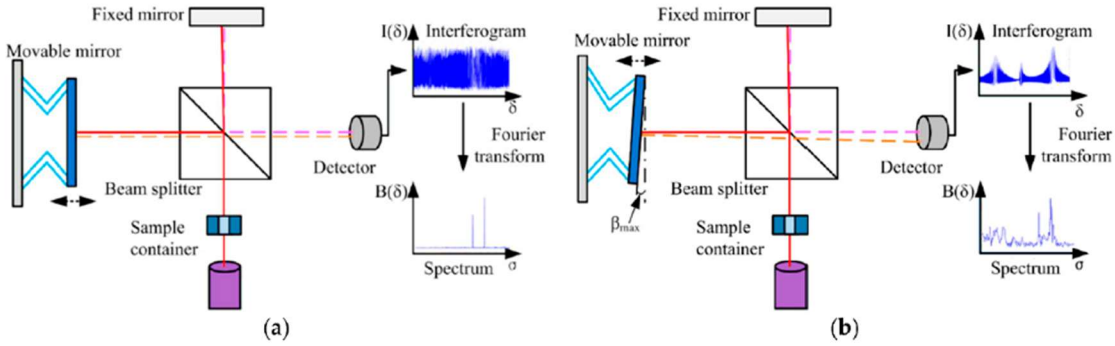


Figure 2.12: Schematic diagram of a Michelson interferometer configured for FTIR. **(a)** An ideal Michelson interferometer; **(b)** a Michelson interferometer with the movable mirror tilting. The continuous and dashed lines represent the different directions of light [86].

The sample is illuminated there by a light source. The light is refocused on the detector as it leave the sample compartment. Mirror movement causes path length differences between two beams, called retardation or optical path difference (OPD) [86]. Thus, interference can be either constructive or destructive, resulting in a pattern known as an interferogram. As clearly demonstrated in **Figure 2.13**, an interferogram is a plot of light intensity against time (where each sampled time interval relates to a specific optical path difference). The interferogram is then subjected to a Fourier-transform to provide a plot of intensity against wavenumber, or an infrared spectrum [73].

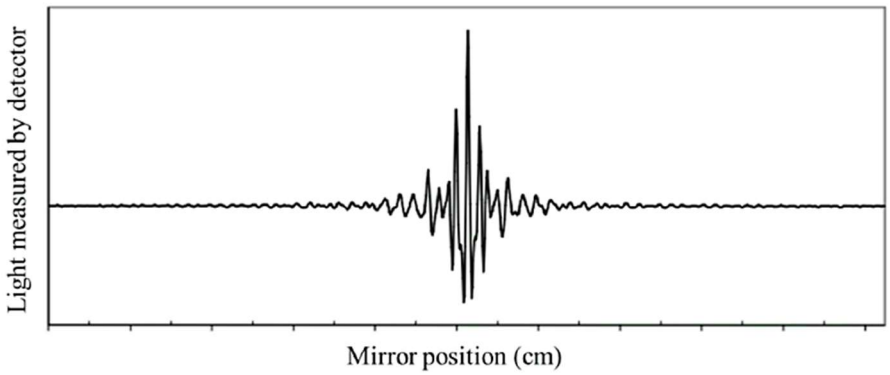


Figure 2.13: FTIR interferogram. The central peak is the location of maximum light transmission through the interferometer (zero path difference or zero retardation) [87].

The time domain plot of a Fourier transform spectrometer that records radiant power as a function of time $\boldsymbol{t}$ is transformed into a frequency domain spectrum. A Fourier Transform theorem relates the intensity of a falling light on a detector $\boldsymbol{I(x)}$, to its spectral power, expressed as $\boldsymbol{A(\bar{\nu})}$ at a specific wavenumber $\boldsymbol{\bar{\nu}}$.

$$F(x) = I(\infty) = \int_0^{\infty} A(\bar{\nu})\cos 2\pi\bar{\nu}x \, dx \tag{2.12}$$

$$A(\bar{\nu}) = 2\int_{-\infty}^{\infty} F(x)\cos 2\pi\bar{\nu}x \, dx \tag{2.13}$$

$$I(\bar{\nu}) = 2\int_{-x}^{x} f(x)\cos 2\pi\bar{\nu}x \, dx \tag{2.14}$$

Where $\boldsymbol{\bar{\nu}}$ is the radiation's wavenumber and $\boldsymbol{I(\bar{\nu})}$ is the radiation's intensity at wavenumber incident on the beam splitter [88].

The sample's spectrum can be either a transmission or an absorption spectrum, as seen below:

$$A(\nu) + T(\nu) = 1 \tag{2.15}$$

Each frequency's $\boldsymbol{\nu}$ transmission spectrum $T(\boldsymbol{\nu})$ and absorption spectrum $A(\boldsymbol{\nu})$ add up to one.

Chapter 3: Material and Data Acquisitions

In this chapter, there are two main sections, the first section relates to describing the materials used throughout this thesis and the preparation conditions needed for the measurement. The second section describes the implementation of biophysical approaches which are used in this study.

3.1 Materials and Sample Preparations

3.1.1 Samples of Serum Albumin From Pregnant Women

Serum albumin from healthy pregnant women of reproductive age in various gestational ages was used (25-35 years). These pregnant women were taking folic acid supplements during the First and Third Trimesters of pregnancy (5 mg per day). The samples were supplied by the Arab Health Center-Bethany branch- Palestine.

Blood samples from pregnant women were collected in the morning with fasting at least 2 hours. The blood was drawn from a peripheral vein and placed into a plain tube and was left to clot at 37^{o}C for 5 min. Blood serum was obtained by centrifugation (3500 rpm for 5 min). All serum samples were collected in the sample cups and frozen at -20^{o}C until analysis.

3.1.2 Determine The Albumin Concentration in Pregnant Women Samples

After completion of centrifugation, 0.5 ml of each sample was placed in Cobas c 111 analyzers to determine the albumin concentration of pregnant women. The Cobas c 111 analyzer automatically determines the concentration of albumin in each sample; during 5 minutes. Results are given in either g/L or μmol/L[6] units.

3.1.3 Determine The Folic Acid Concentration in The Serum Albumin of Pregnant Women

After completion of centrifugation, 25 μl of each sample was placed in Cobas e411 analyzers to determine the Folic Acid concentration in the serum albumin of pregnant women. The Cobas e411 analyzer automatically determines the concentration of Folic Acid in each sample; during 27 minutes. Results are given in either nmol/L or ng/mL units.

3.1.4 Thin Film Preparation

Optical grade silicon windows (NICODOM Ltd) are used as spectroscopic cell windows. The optical transmission is high with little or no distortion of the transmitted signal. The windows were purchased from Sigma Aldrich Company.

The measurements were performed using the samples in the form of thin films of serum albumin of pregnant women. The 25 µL of each sample is applied on a silicon window. Then the sample evaporates to complete dryness overnight before measurements.

3.2 Instruments

Here are the terminologies and the concepts associated with Labofuge 400 centrifuge, Cobas e411, Cobas c 111 and FTIR spectroscopy used in this work:

3.2.1 Labofuge 400 Centrifuge

The centrifugation technique is very commonly used to separate solid particles dispersed in a liquid medium, such as blood components [89]. The components of the blood are separated due to the centrifugal force, based on their size and density

differences (molecular weight) [90, 91]. In this thesis, the Labofuge 400 Centrifuge **(Figure 3.1)** was used to separate the components of clotted blood samples. After centrifugation, the clotted blood will be at the bottom of the tube and the serum albumin will be on top.

Figure 3.1: Labofuge 400 Centrifuge [92].

G-force or relative centrifugal force (RCF), is the acceleration generated by the spinning of the rotor and applied to the sample. When RCF will be greater than the buoyant and frictional forces in the sample, the particles moved away from the axis of rotation and was sediment at the bottom. The relative centrifugal force acting on particles is exponential to the speed of rotation (defined as revolutions per minute; rpm) [93].

RCF depends on the speed of rotation and the distance between the center of rotation and the particles, which can be calculated using the equation 3.1 [94, 95].

$$\mathrm{RCF} = 11.18 \times r \times \left(\frac{\mathrm{Q}}{1000}\right)^2 \qquad (3.1)$$

Where r is the distance (cm), and Q is the speed of rotation (rpm).

3.2.2 Cobas e411 Analyzer

The Cobas e411 analyzer **(Figure 3.2)** is an automated analyzer that depends on ElectroChemiLuminescence (ECL) technology for immunoassay analysis. It is designed for both qualitative and quantitative in vitro assay determinations for a wide range of applications (including infectious diseases, anemia; cardiac, bone, and tumor markers; hormones; maternal care; and critical care). The analyzer is available as a rack or disk sample handling system [96].

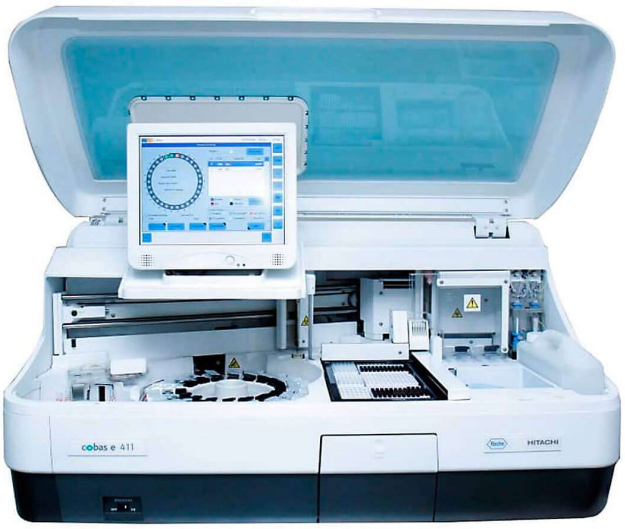


Figure 3.2: Immunology Analyzer Roche Cobas e411 Disk [97].

Cobas e411 was used as a quantitative determinate of Folic Acid in the serum albumin of pregnant women. Five Reagents were needed during the determination of the Folic Acid concentration; the pretreatment reagents (PT1, PT2) and the rackpack reagents (M, R1, R2) and:

1. **Pretreatment reagent 1 (PT1):** Sodium 2-mercaptoethanesulfonate (MESNA) 40 g/L, pH 5.5.
2. **Pretreatment reagent 2 (PT2):** Sodium hydroxide 25 g/L.
3. **Streptavidin-coated microparticles (M):** Streptavidin-coated microparticles 0.72 mg/mL; preservative.

4. **Folic Acid binding protein~Ru(bpy)$_3^{2+}$ (R1):** Ruthenium labeled Folic Acid binding protein 75 µg/L, pH 5.5; preservative.
5. **Folic Acid~biotin (R2):** Biotinylated Folic Acid 17 µg/L; biotin 120 µg/L, pH 9.0; preservative [98].

3.2.2.1 Cobas e411 Test Principle

The total duration of assay is about 27 minutes; it passes through three main steps of incubation:

1. **First incubation:** By incubating 25 µL of sample (serum albumin of pregnant women) with the Folic Acid pretreatment reagents 1 and 2, bound Folic Acid is released from endogenous Folic Acid binding proteins.
2. **Second incubation:** By incubating the pretreated sample with the ruthenium labeled Folic Acid binding protein and forming a Folic Acid complex. The amount of complex depends on the analyte concentration in the sample.
3. **Third incubation:** After the addition of streptavidin-coated microparticles and Folic Acid labeled with biotin, the unbound sites of the ruthenium labeled Folic Acid binding protein become occupied, with the formation of ruthenium labeled Folic Acid binding protein-Folic Acid biotin complex. The whole complex becomes bound to the solid phase via the interaction of biotin and streptavidin.

The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

Results are determined via a calibration curve which is instrument specifically generated by 2-point calibra. The Cobas e411 analyzer automatically calculates the analyte concentration of each sample in either ng/mL or nmol/L. The conversion factors is: ng/mL x 2.27 = nmol/L, nmol/L x 0.44 = ng/mL. The measuring range is 0.6-20.0

ng/mL or 1.36-45.4 nmol/L (defined by the Limit of Blank and the maximum of the master curve) [98].

3.2.3 Cobas c 111 Analyzer

Cobas c 111 **(Figure 3.3)** is a tabletop instrument designed for automated clinical chemistry tests. It is used for the in-vitro quantitative determination of clinical chemistry parameters in plasma, serum, urine, or whole blood. The main components of Cobas c 111 are a reagents, optics and calibrators. Two Reagents were needed during the determination of albumin concentration:

1. **R1** (Citrate buffer): 95 mmo|/L, human serum albumin stabilizers, pH 4.1, preservatives.
2. **SR** (Citrate buffer): 95 mmol/L, Bromcresol green: 0.66 mmol/L, pH 4.1.

Figure 3.3: Cobas c 111 analyzer, clinical chemistry analyzer [99].

3.2.3.1 Cobas c 111 Test Principle (Colorimetric Assay)

At a pH value of 4.1, the albumin becomes able to bind with an anionic dye called bromcresol green (BCG) to form a blue-green complex (see equation 3.2). The color intensity of the blue-green color is proportional to the albumin concentration in the sample and is measured photometrically.

$$\text{Albumin} + \text{BCG} \xrightarrow{\text{pH 4.1}} \text{Albumin-BCG complex} \qquad (3.2)$$

The Cobas c 111 analyzer automatically calculates the albumin concentration of each sample in either g/L or µmol/L[6]. The conversion factor is g/L × 15.2 = µmol/L[6]. The expected values range of the adults is 39.7-49.4 g/L or 603-751 µmol/L[6] [99-101].

3.2.4 Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy **(Figure 3.4)** is a fast and non-destructive tool. It provides information about the molecular composition of samples, it gives spectral fingerprint (in the mid-infrared (MIR) region), with the characteristic absorption peaks corresponding to the functional group of proteins [102, 103].

The FTIR measurements were obtained on a Bruker IFS 66/S spectrophotometer (Bruker GmbH, Germany) equipped with a liquid nitrogen-cooled MCT detector and a KBr beam splitter. 25 µl of each sample was applied on a silicon window and dried overnight. Scanning was performed at room temperature, in the range 4000-400 cm^{-1} with a resolution of 4 cm^{-1}, and is the average of 256 scans. For each sample spectra were recorded in duplicate or triplicate. Over 200 spectra were collected in this study. Results were analyzed using the OPUS software and the Origin Pro version 2019b program.

Figure 3.4: FTIR imaging system containing a FTIR microscope (Hyperion 3000, Bruker Optics) with FTIR spectrophotometer (Tensor 27, Bruker Optics) [104].

FTIR spectroscopy has three basic spectrometer components: Infrared radiation source, Michelson interferometer, and the detector. The Michelson Interferometer technique is adapted for FTIR, in which the light from the IR source is collimated and directed onto a beam splitter. 50% of the photons are directed to the fixed mirror, and the remaining 50% are transmitted by the movable mirror. In this configuration, light is reflected from the two mirrors back to the beam splitter. The interference signals are produced, which contain infrared spectral information generated after passes into the sample compartment (detector) [86].

Chapter 4: Results and discussion

In this study, the structural changes and the molecular mechanism of the interaction of Folic Acid with human serum albumin (HSA) taken from pregnant women during the First and the Third Trimester of pregnancy was investigated on a single molecular level (single functional group). In this study, the protein concentration was almost constant in all samples in both First and Third Trimester periods with various Folic Acid contents. FTIR total Absorption Spectroscopy, second derivative and Fourier self-deconvolution methods were used to analyze the Folic Acid binding sites and the effect on pregnant blood protein (HSA) stability and structural conformations.

4.1 Molecular Mechanism of Folic Acid (FA) and Pregnant's Human Albumin Interactions in The First and Third Trimester of Pregnancy

The function of the Folic Acid-binding blood proteins and the molecular mechanism of the interaction between Folic Acid and the carrier protein, i.e human serum albumin taken from Pregnant's women are not addressed yet. Our research study will result in a full understanding of the molecular mechanism of Folic Acid function as an inhibitor or reducer of many of the birth defects of the brain and the spinal cord using native blood protein taken from pregnant women during pregnancy.

In this part of study, the major aim is to reveal the mechanism of the biomolecular interaction between Folic Acid (FA) and Pregnant's human albumin at physiological conditions using the Total Infrared Absorption method in FTIR Spectroscopic technique. FTIR total absorption spectra were measured of serum albumins collected from pregnant women taken Folic Acid as a supplement in the First and the Third Trimester of pregnancy. Structural Comparison of the FTIR spectra of all samples in the two groups were examined in the wavenumber range between 3700-2600 cm^{-1} and 1800-800 cm^{-1}.

Figure 4.1 shows the total FTIR absorption spectra of serum albumin of the pregnant women in the First (A) and Third Trimester (B) at constant serum albumin

concentration (40 mg/ml) and with different contents of Folic Acid as the following: (5, 7, 9, 11 and 16 ng/ml) in the First trimester and (5, 7, 9, and 11 ng/ml) in the Third Trimester.

The IR Absorption spectra showed in **Figure 4.1 A and B** displays the major characteristic vibrational bands due to vibrations of protein, mainly the blood-plasma protein albumin and polypeptide chains. These bands are known in literature as Amide A, Amide B observed in the range (3000-3600 cm^{-1}), C-H Band (3000-2800 cm^{-1}), and Amide (I, II, III) that are observed in the range (1700-1200 cm^{-1}) [15, 16, 105].

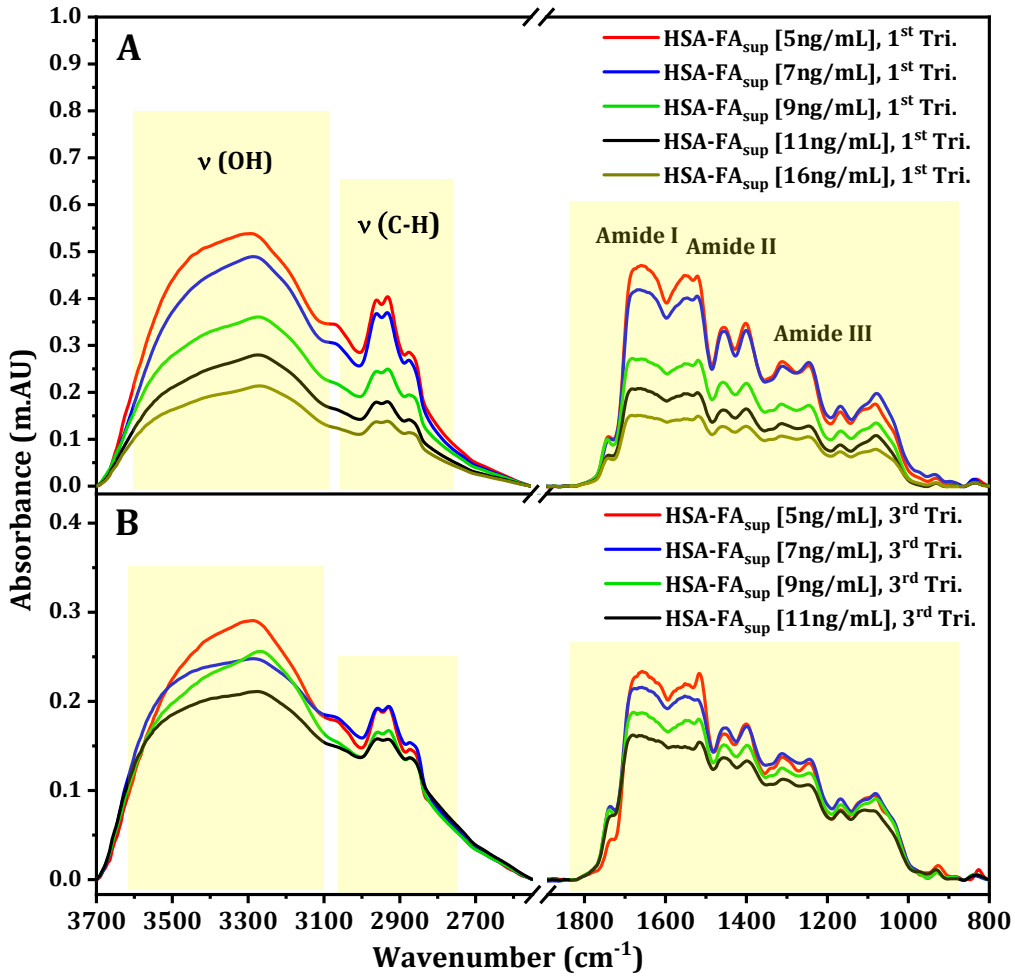


Figure 4.1: FTIR Total Absorption Spectra of FA-serum albumin complexes taken of pregnant women taken Folic Acid as supplement: **(A) in the First Trimester, (B) in the Third Trimester** of pregnancy. The in-vivo FA contents are: (5, 7, 9, 11 and 16 ng/ml) and the concentration of native serum albumin is about 40 mg/ml.

The results here showed that HSA act as carriers for Folic Acid by delivering it to the target molecules in the human body and the Folic Acid binds Pregnant's Serum Albumin in the First Trimester **(Fig.4.1 A)** more strongly than in the Third Trimester **(Fig.4.1 B)** in the major regions of protein structure (Amide A, Amide B) and (amide I, II and III). As can be seen from **Figure 4.1 A & B**, as Folic Acid concentration increases, the absorption intensity of native serum albumin decreases in both Trimesters. Hence, the observed decrease in absorption intensities in all major bands suggests that the interaction between Folic Acid and serum albumin get stronger with increasing Folic Acid contents in the Pregnant's blood.

Importantly, these major blood protein regions that are directly correlated with the biomolecular interactions of Folic Acid with serum albumin in both First and Third Trimester will be analyzed extensively in the next sections.

4.1.1 Structural Changes of Native Protein Amide A & Amide B Bands upon Folic Acid Complexation During First & Third Trimester

The IR vibrational regions (3600-3000 cm^{-1}) represents O-H stretching bands and N-H stretching bands (Amide A & Amide B) of proteins [106]. The N-H stretching region is an important band that indicates the intermolecular H-bond interactions between the drug and carrier proteins.

Figure 4.2 A and B show the effect of increasing the content of FA in the native HSA-FA complexes taken from Pregnants in the First and Third Trimester, respectively in the vibrational range 3000-3600 cm^{-1}. The FTIR Spectra of HSA-FA complexes in both stages show a strong band (Amide A) with a maximum at 3298 cm^{-1} and a relatively weak absorption band at 3061 cm^{-1} (Amide B) that overlapped with it. Both, Amide A and Amide B bands represented the N-H stretching vibrations in (N-H···O=C) H-bonds in the polypeptide chains of blood protein. As shown in **Figure 4.2**, the Amide A band

in the FTIR spectra of FA-HSA complexes showed on the high frequency range a broader shoulder with a maximum at 3507 cm^{-1} and 3508 cm^{-1} in the First and Third Trimester, respectively. This was due to the formation of H-bonds between the hydroxyls (OH groups) of Folic Acid and serum albumin in FA-HSA complexes.

Importantly, Amide A band is exclusively localized on the NH group of the protein backbone and is therefore insensitive to the structural conformations of the polypeptide backbone. Hence, its vibrational frequency depends on the strength of the hydrogen bond formed between protein NH groups and OH groups of the bonded Folic Acid.

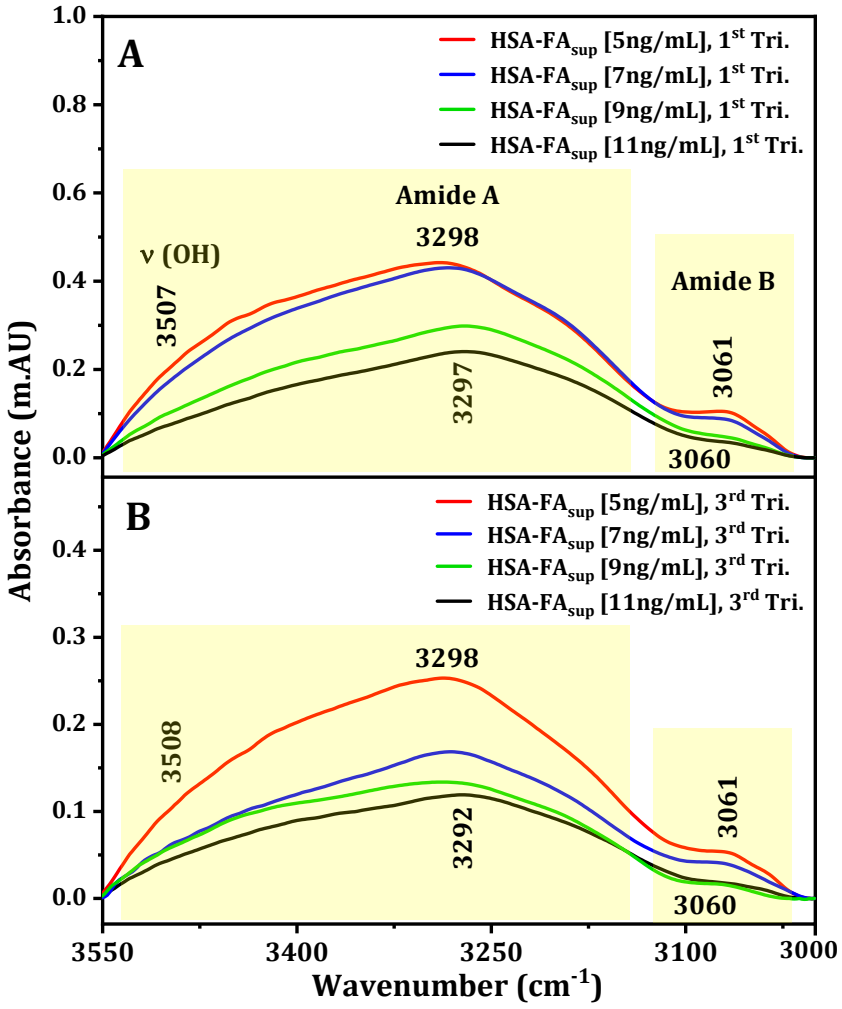


Figure 4.2: FTIR Absorption Spectra of **the Amide A & Amide B regions** of FA-Serum Albumin Complexes of pregnant women taken Folic Acid as supplement: **(A) in the First Trimester, (B) in the Third Trimester** of pregnancy. The in-vivo FA contents are: (5, 7, 9 and 11 ng/ml) and the concentration of native serum albumin is about 40 mg/ml.

When we compared the vibrational frequencies of Amide A of FA-HSA complexes in both Trimesters, shift in the peak positions was visible from 3298 to 3292 cm^{-1} in the Third Trimester **(Fig.4.2 B)** as the FA concentration increased in the Pregnant's blood. Furthermore, the intensity of Amide A and Amide B vibrational bands **(Table 4.1)** was much lower in FTIR spectra of FA-HSA complexes of Pregnant's women in the Third Trimester than of FA-HSA complexes of Pregnant's women in the First Trimester. These findings indicate less loading capacity of Folic Acid on Pregnant's blood protein as drug carrier in the late stages (Third Trimester) of gestation compared with early stages of gestation (First Trimester).

Table 4.1: IR Absorption Intensity of the **Amide A**, **CH_3** and **CH_2** & **Amide I** vibrational bands of FA-HSA complexes of pregnant women in the First and Third Trimester at different concentrations of Folic Acid.

	Abs. Intensity Amide A		**Abs. Intensity ν_{as} CH_3**		**Abs. Intensity ν_{as} CH_2**		**Abs. Intensity Amide I**	
Samples	**1st Tri.**	**3rd Tri.**	**1st Tri.**	**3rd Tri.**	**1st Tri.**	**3rd Tri.**	**1st Tri.**	**3rd Tri.**
HSA-FA (5ng/ml)	0.694	0.384	0.530	0.271	0.539	0.274	0.566	0.281
HSA-FA (7ng/ml)	0.628	0.329	0.489	0.270	0.493	0.273	0.504	0.263
HSA-FA (9ng/ml)	0.461	0.337	0.332	0.237	0.339	0.240	0.329	0.229
HSA-FA (11ng/ml)	0.367	0.294	0.252	0.242	0.253	0.243	0.267	0.198

A quantitative measurement of the strength of binding interactions between Folic Acid and serum albumin as a drug carrier of major vibrational bands of blood protein have been made and shown in **Table 4.2**. In **Amide A** band, the difference (delta) of the maximum Absorption intensity of the lowest and highest concentration of Folic Acid in FA-HSA complexes ($\Delta I = I_{5ng/ml} - I_{11ng/ml}$) were 0.327 in the First Trimester and 0.090 in the Third Trimester **(Table 4.2)**. The large decrease in the absorption intensity of FA-HSA complexes as the FA concentration increases is account for increases H-bonds between the Folic acid and the blood protein as drug carrier in the First Trimester.

Table 4.2: The Differences (delta) in the Absorption intensity of the lowest (5ng/ml) and the highest (11ng/ml) concentrations of Folic Acid contained in FA-HSA complexes of Pregnant women in the in the First and Third Trimester.

Protein Functional Groups	$\Delta I_{1st} = I_{5ng/ml} - I_{11ng/ml}$	$\Delta I_{3rd} = I_{5ng/ml} - I_{11ng/ml}$
Amide A	0.327	0.090
ν_{as} (CH_3)	0.278	0.029
ν_{as} (CH_2)	0.286	0.029
Amide I	0.299	0.083
Amide II	0.279	0.080
Amide III	0.120	0.026

These results allow to conclude the formation of strong binding between the blood protein side chains (Amide A & Amide B) and Folic Acid in-vivo through H-bond mechanism (hydrophilic interaction) in the early stage of pregnancy.

4.1.2 Structural Changes of Native Protein CH_3, CH_2 Bands Upon Folic Acid Complexation During First & Third Trimester

The structural changes of blood protein CH_3 and CH_2 stretching bands that induced by Folic Acid in the First and Third Trimesters was investigated in the range of 3000-2800 cm^{-1} using FTIR Spectroscopy. The position (wavenumber) of these bands were assigned using the method of second derivative. In **Figure 4.3**, four overlapping IR absorption bands with maxima at 2958, 2922, 2871, and 2852 cm^{-1} were observed in both FTIR spectra of FA-HSA complexes in the First **(Fig.4.3 A)** and Third Trimester **(Fig.4.3 B)**. The IR bands at 2958 and 2871 cm^{-1} belong to asymmetric and symmetric CH_3 stretching vibrations, while the bands at 2922 and 2852 cm^{-1} were due to CH_2 stretching vibrations [107-109].

The asymmetric and symmetric CH_3 and CH_2 bands of blood protein showed no significant wavenumber shift in the overlapped bands as the Folic Acid concentration

increases in both groups. But, there were a significant reduction of the absorption intensity of the four bands in FA-HSA complexes in the First Trimester relative to intensity reduction in FA-HSA complexes in the Third Trimester as clearly shown in **Figure 4.3 A & B**.

Additionally, quantitative FTIR analysis have been performed to determine the strength of binding interaction between Folic Acid and the hydrocarbon bands of blood protein in the range of 3000-2800 cm^{-1}. Hence, the difference (delta) of the maximum Absorption intensity of the lowest and highest concentration of Folic Acid in FA-HSA complexes ($\Delta I = I_{5ng/ml} - I_{11ng/ml}$) of asymmetric CH_3 and CH_2 bands have been calculated from **Table 4.1** and listed in **Table 4.2**. The results show that the difference (delta) of the maximum Absorption intensity of asymmetric CH_3 (ν_{as} CH_3) band in the First Trimester relative to the Third Trimester were 0.278 and 0.029, respectively. Also, this intensity difference of asymmetric CH_2 (ν_{as} CH_2) band were 0.286 in the First Trimester and 0.029 in the Third Trimester of pregnancy **(Table 4.2)**. This decrease in the absorption intensity upon increasing of Folic Acid contents in Pregnant's blood in the First Trimester was another evidence (section 4.3.1) of the strong binding and high loading of Folic Acid molecules on the blood protein due to the Folic Acid hydrophobic interaction with HSA.

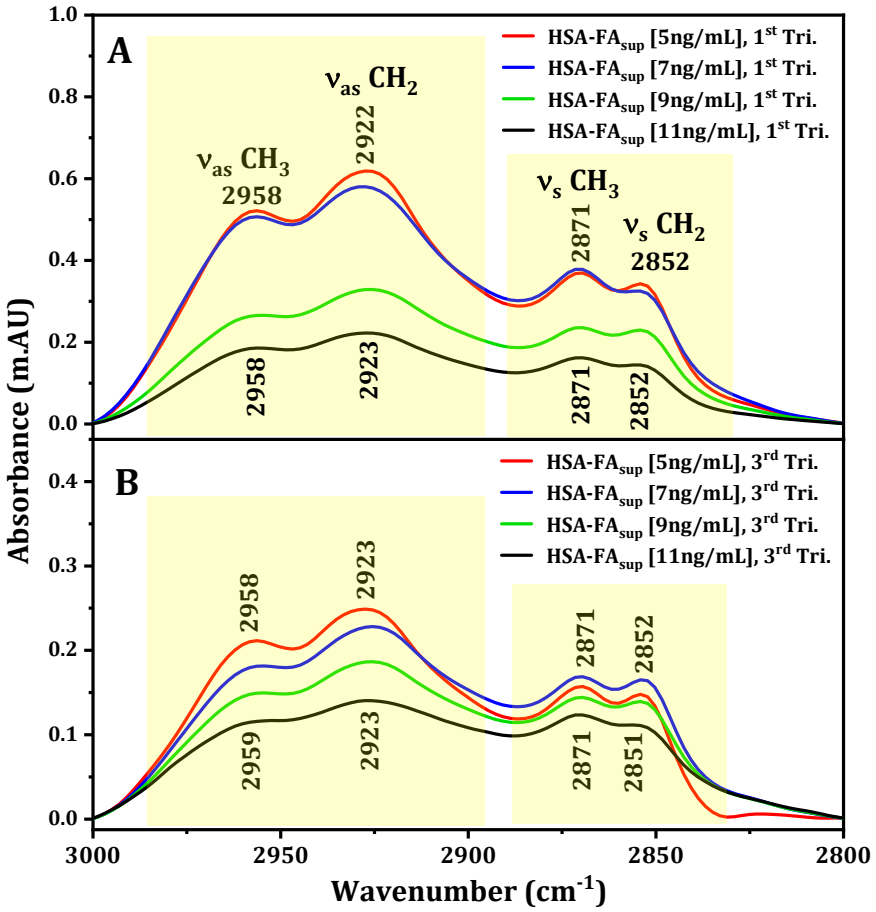


Figure 4.3: FTIR Absorption Spectra of **the region (3000-2800 cm^{-1})** of FA-Serum Albumin Complexes serum albumin of pregnant women taken Folic Acid as supplement: **(A) in the First Trimester, (B) in the Third Trimester** of pregnancy. The in-vivo FA contents are: (5, 7, 9 and 11 ng/ml) and the concentration of native serum albumin is about 40 mg/ml.

4.1.3 Structural Changes of Native Protein Amide (I, II, and I) Bands Upon Folic Acid Complexation During First & Third Trimester

Serum albumin is a the major soluble protein constituents of the circulatory system, made up of three structurally similar domains; called Amide (I, II and III) that are observed in the range of 1800-1200 cm-^{1} [110, 111]. Amide I band (1700-1600 cm^{-1}) is due to stretching vibrations of C=O groups in polypeptide chains. The Amide II band (1600-1500 cm^{-1}) is mainly caused by bending vibrations of peptide NH groups and side groups certain amino acids. While, Amide III (1350-1200 cm^{-1}) is determined by NH bending and CN stretching of the polypeptide backbone. The amide I, amide II, and amide III bands are sensitive to changes in the secondary structure of proteins.

However, the amide I band is more sensitive to the change of protein's secondary structure than amide II and III [112, 113].

Figure 4.4 shows the FTIR absorption spectra of Amide (I, II, and III) bands of FA-HSA complexes of Pregnant's women in the First **(Fig.4.4 A)** and Third **(Fig.4.4 B)** Trimester with various concentrations of Folic Acid. In both Trimesters, it has been observed a clear decrease in the absorption intensity of amide I band (1655 cm^{-1}), amide II band (1543 cm^{-1}), and amide III band (1317 cm^{-1},1242 cm^{-1}) once the Folic Acid concentration increased in the Pregnant's blood. The intensity reductions of the amide I and amide II bands were due to the binding of Folic Acid to the C=O and N-H groups of protein backbone through formation of H-bonds.

Based on these findings, a quantitative comparison has been employed to investigate the strength of H-bonding interactions between Folic Acid and blood protein in the range of 1800-1200 cm^{-1} in the First and Third Trimesters. The calculated intensity difference of the maximum Absorption of the lowest and highest concentration of Folic Acid in FA-HSA complexes ($\Delta I = I_{5ng/ml} - I_{11ng/ml}$) have been listed in **Table 4.2**. As Folic Acid concertation increased in Pregnant's blood, the reduced intensity of Amide (I, II, and III) were 0.299, 0.279 and 0.120 respectively. While, in the Third Trimester were 0.083, 0.080 and 0.026 respectively. The obtained values showed much higher reduction in the absorption intensity in the First Trimester with comparison with the reduction occurred in the Third Trimester due to the formation of strong binding via H-bonds between Folic Acid and C=O and N-H groups of protein backbone [114]. Hence, at the molecular level, this gives evidence regarding major alteration occurring in the native serum albumin of Pregnant's women as a result of Folic Acid interaction.

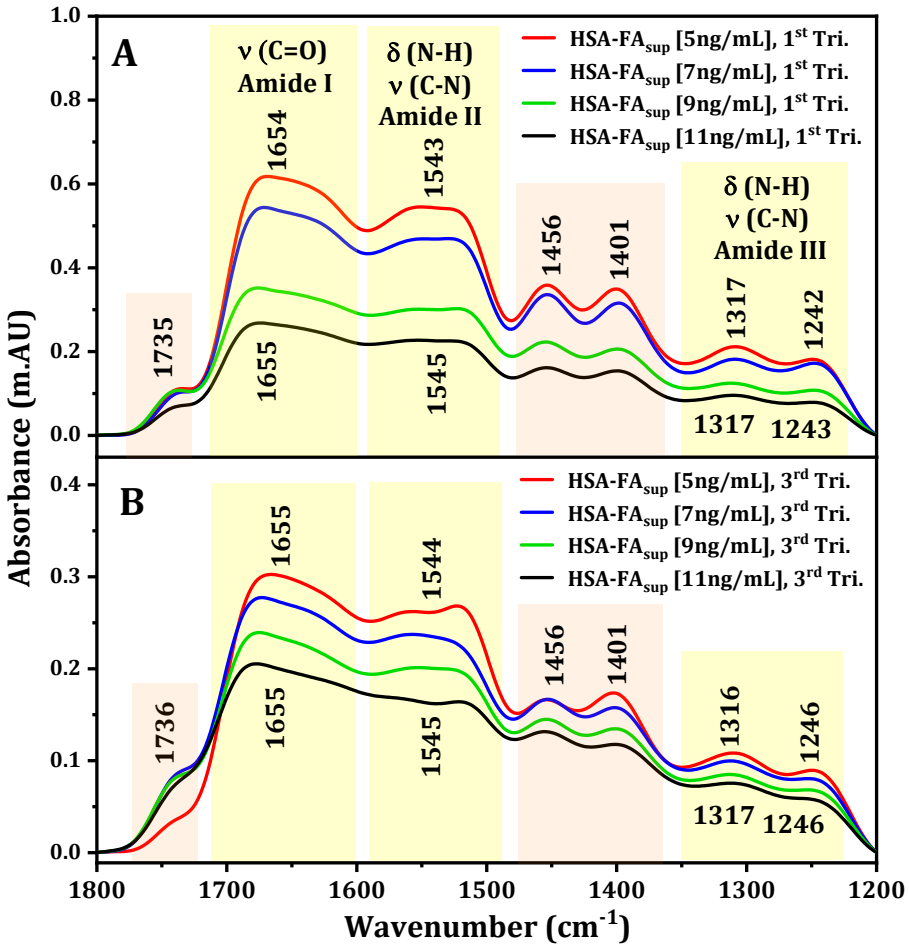


Figure 4.4: FTIR Absorption Spectra of **the region (1800-1200 cm^{-1})** of FA-Serum Albumin Complexes serum albumin of pregnant women taken Folic Acid as supplement: **(A) in the First Trimester, (B) in the Third Trimester** of pregnancy. The in-vivo FA contents are: (5, 7, 9 and 11 ng/ml) and the concentration of native serum albumin is about 40 mg/ml.

In Conclusion, based on all results obtained from the FTIR structural analysis in the previous three sections above, we could confirm that Folic Acid binds native serum albumin of Pregnant's via hydrophobic, hydrophilic and H-bonding interactions in both Trimesters. Also, our results confirmed that maximum loading capacity of FA on Pregnant's blood protein occurred during the First Trimester of pregnancy. These novel Spectroscopic findings in agreement with G. Miriam (2016) and M. Zannat (2016) that presented evidence for the protective function of Folic Acid in the prevention of neural tube defects, when Folic Acid is taken prior to conception and throughout the First Trimester of pregnancy. Therefore, taking Folic Acid as supplement by Pregnant's women can efficiently contribute to the public health action to decrease severe, disabling and potentially lethal Neural Tube Defects (birth defects) [57].

4.2 Determination of Secondary Structure Conformations of Native Blood Protein Induced by Folic Acid

FTIR spectroscopy is the preferred method for the investigation of the conformational changes of the secondary structure of proteins and their dynamics. The amide I band is uniquely useful for the analysis of protein secondary structure compositions and conformational changes due to its high sensitivity to small changes in molecular geometry and hydrogen bonding patterns [115-117]. It is well-known that the amide I band of serum albumin in the region 1700–1600 cm^{-1} are caused by C=O stretching vibration [116].

In this study, a quantitative analysis of the conformational changes of the native protein secondary structure induced by Folic Acid in the Amide I region (1700–1600 cm^{-1}) have been made using Fourier self-deconvolution methods on the basis of the procedure described by Byler and Susi [118]. Baseline corrections were carried out in this vibrational range of FA-HSA complexes of pregnant women in the First and the Third Trimester of pregnancy.

Both, Fourier self-deconvolution and second derivative methods were applied to this vibrational range to increase spectral resolutions and therefore to estimate the number, position (wavenumber in cm^{-1}) and the area of the individual component bands. Based on these parameters, a curve-fitting process was performed using OriginPro software (version 2019b) to obtain the best Gaussian-shaped curves that fit the original serum albumin spectra. The individual bands are specified with their representative secondary structure, and the content of the secondary structure of serum albumin was calculated by the area of its respective component bands. The procedures were carried out using the components that are observed in the second derivatives.

By means of the second derivative in the spectral region 1700-1600 cm^{-1}, six major peaks for FA-HSA complexes were resolved in the First Trimester **(Figure 4.5)** and Third Trimester **(Figure 4.6)**. These peaks correspond to α-helix (1645-1665 cm^{-1}), β-

sheet (1603-1630 cm^{-1}), turn (1664-1677 cm^{-1}), random coils (1630-1645 cm^{-1}) and anti-parallel β-structure (1675-1700 cm^{-1}) were adjusted and the area was measured with the Gaussian function. After that, the area of all the component bands assigned to a given conformation were then summed up and divided by the total area of the overlapped amid I band [60] of both FA-HSA complexes and listed in **Table 4.3** and **Table 4.4**.

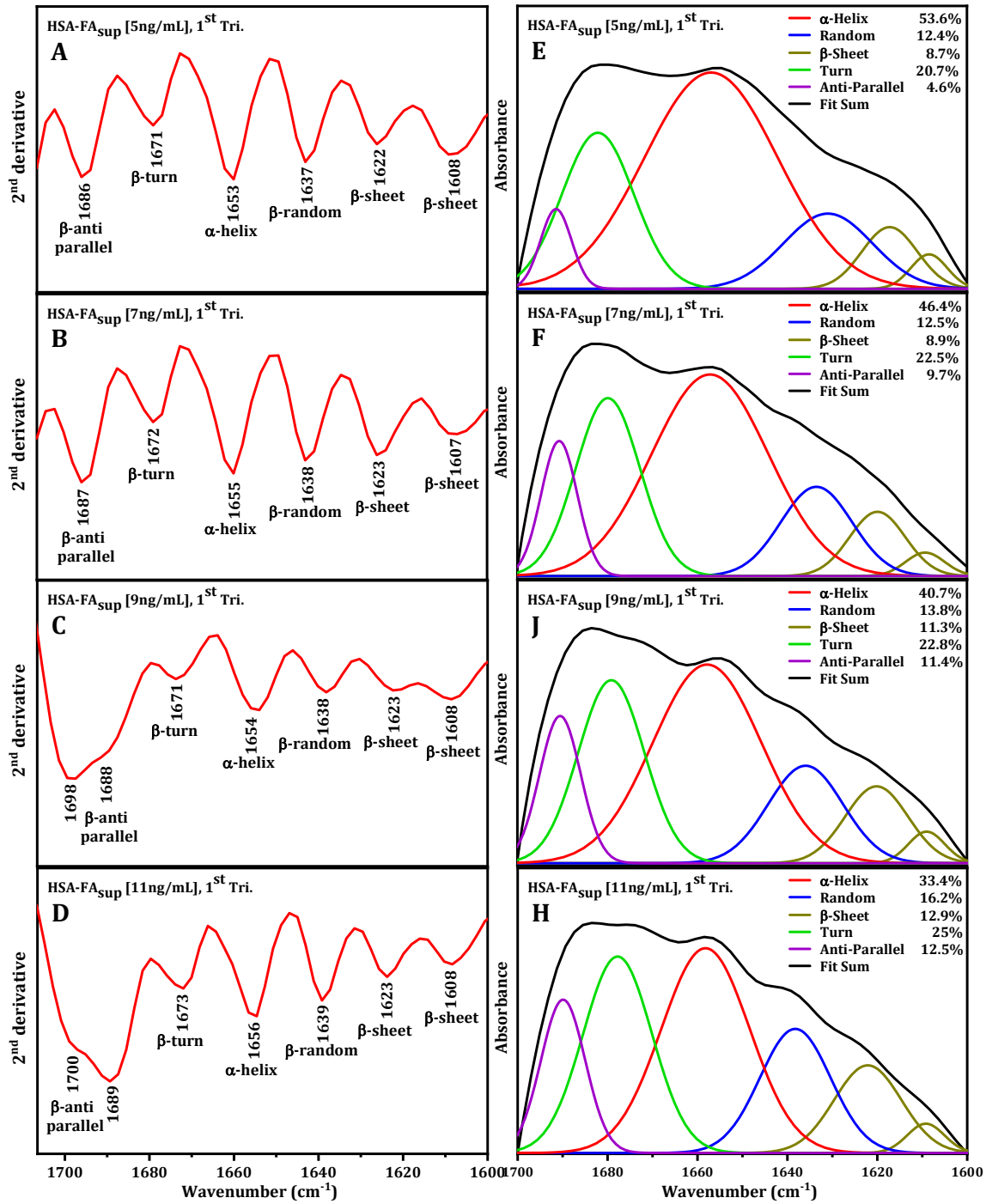


Figure 4.5: Secondary structure determination of the serum albumin of pregnant women in **First Trimester** of pregnancy at different concentrations of Folic Acid (5, 7, 9, 11ng/ml). **(A, B, C and D)** Second-derivative resolution enhancement and **(E, F, J and H)** curve-fitted amide I region (1700-1600 cm^{-1}).

Table 4.3: Secondary structure determination of amide I regions in serum albumin of pregnant women in **First Trimester** of pregnancy.

Folic Acid Concentration	α-helix 1635-1664	Random coil 1630-1645	β-Sheet 1603-1616 1619-1630	Turn 1664-1677	Anti-parallel 1673-1689
HSA-FA (5ng/mL), 1st Tri.	53.6%	12.4%	8.7%	20.7%	4.6%
HSA-FA (7ng/mL), 1st Tri.	46.4%	12.5%	8.9%	22.5%	9.7%
HSA-FA (9ng/mL), 1st Tri.	40.7%	13.8%	11.3%	22.8%	11.4%
HSA-FA (11ng/mL), 1st Tri.	33.4%	16.2%	12.9%	25%	12.5%

The second derivative resolution enhancement, curve–fitted of amide I region and secondary structure determinations of the conformational changes in serum albumin induced by different Folic Acid concentrations are shown in **Figure 4.5** and **Figure 4.6** respectively. The area percentages of these secondary structure conformations of FA-HSA complexes in the First and Third Trimesters are listed in **Table 4.3** and **Table 4.4**, respectively.

Figure 4.5 and **Table 4.3** show a major decrease of α-helix structure from 53.6% to 33.4% as the Folic acid contents increased in the Pregnant's women in the First Trimester with an increase in all other secondary structure components. There was an increase in random coil from 12.4% to 16.2%, β-sheet from 8.7% to 12.9%, turn from 20.7% to 25%, and β-anti parallel from 4.6% to 12.5%. On the other hand, the α-helix structure decreased from 50.6% to 30.6% when the Folic acid contents increased in the Pregnant's women in the Third Trimester (**Figure 4.6** and **Table 4.4)**. While there was an increase in random coil from 13.4%% to 16.4%, β-sheet from 9.8% to 12.9%, turn from 21.2% to 27.7%, and β-anti parallel from 5% to 12.4%. This major reduction of α-helix structures and the increasing of all types of β-sheet forms upon Folic Acid binding were in consistent with previous studies on model Human Serum Albumin (HSA) [15, 16, 60].

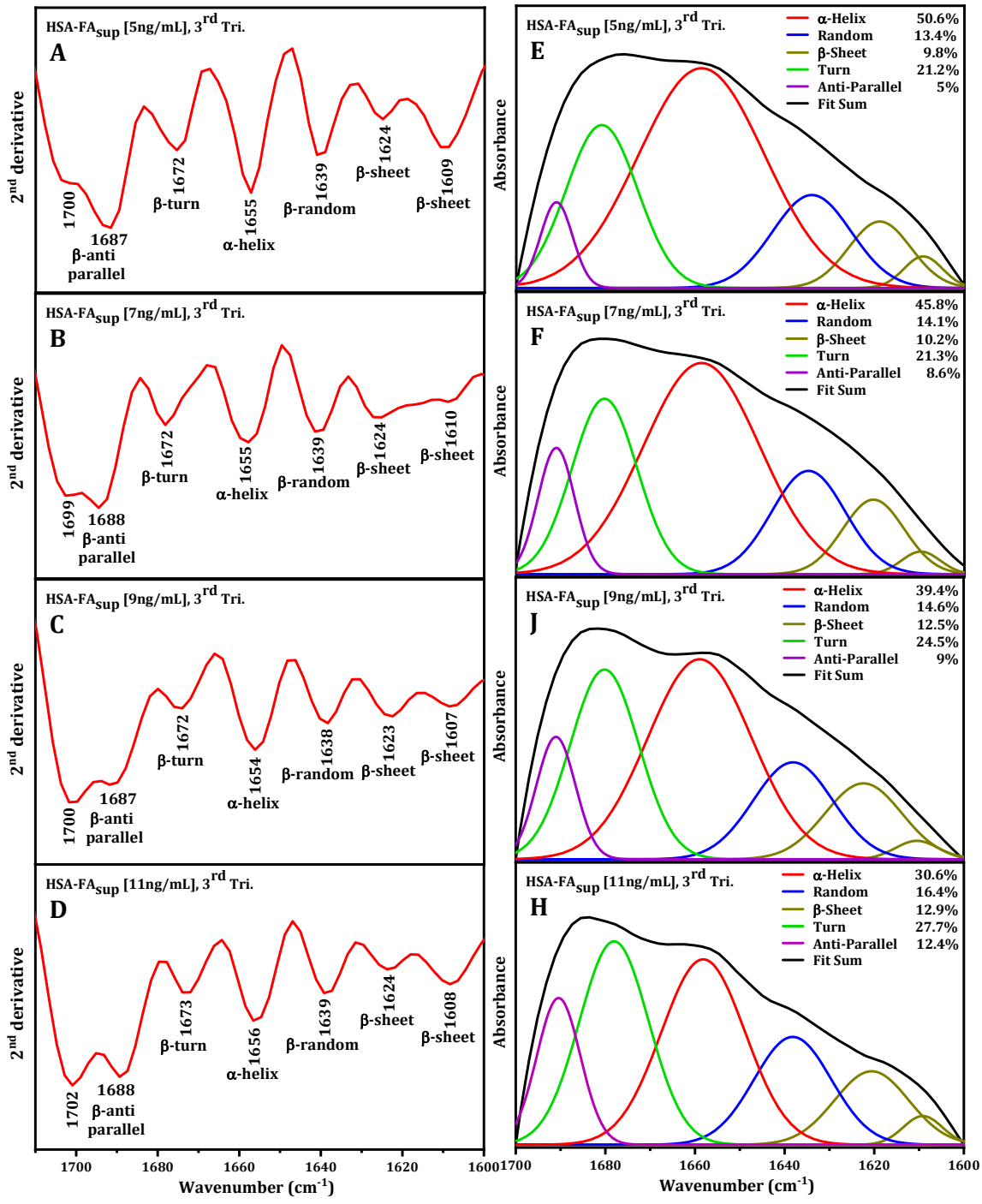


Figure 4.6: Secondary structure determination of the serum albumin of pregnant women in **Third Trimester** of pregnancy at different concentrations of Folic Acid (5, 7, 9, 11ng/ml). **(A, B, C and D)** Second-derivative resolution enhancement and **(E, F, J and H)** curve-fitted amide I region (1700-1600 cm^{-1}).

Table 4.4: Secondary structure determination of amide I regions in serum albumin of pregnant women in **Third Trimester** of pregnancy.

Folic Acid Concentration	α-helix 1645-1664	Random coil 1630-1645	β-Sheet 1603-1616 1619-1630	Turn 1664-1677	Anti-parallel 1673-1689
HSA-FA (5ng/mL), 3rd Tri.	50.6%	13.4%	9.8%	21.2%	5%
HSA-FA (7ng/mL), 3rd Tri.	45.8%	14.1%	10.2%	21.3%	8.6%
HSA-FA (9ng/mL), 3rd Tri.	39.4%	14.6%	12.5%	24.5%	9%
HSA-FA (11ng/mL), 3rd Tri.	30.6%	16.4%	12.9%	27.7%	12.4%

Evidently, these induced conformational changes of the secondary structure of native serum albumin taken from Pregnant's women in early and late stages of pregnancy by suggest a partial protein folding occurred at high Folic Acid concentrations as a result of formation of H-bonds between the carbonyl group (C=O) of serum albumin and the Folic Acid. In both Trimesters, the Folic Acid caused a rearrangement of the polypeptide carbonyl (C=O) hydrogen bonding network and hence, lead to the reduction of the protein α-helix structure in FA-HSA complexes.

Chapter 5: Conclusion

Folic Acid is recognized as an important component of the care of women of child-bearing age; it plays an important role in the prevention of birth defects. The strongest justification for Folic Acid supplementation during pregnancy comes from the association between adequate intake of Folic Acid and reduced risk of having a child with neural tube defects. Therefore, it is required for the normal development and closure of the neural tube in the fetus. So, the study of the interaction of Folic Acid with the native serum albumin of pregnant women is of prime significance.

The molecular mechanism of the interaction of Folic Acid with native serum albumin of pregnant women in the First and Third Trimesters was extensively investigated in all major structural regions of protein using FTIR spectroscopy. Three characteristic vibrational bands in the FTIR absorption spectra: Amide A and Amide B region (3600-3000 cm^{-1}), C-H stretching region (3000-2800 cm^{-1}) and C=O stretching and N-H bending region (1700-1500 cm^{-1}) have been investigated. Clearly, the absorption intensity of these major bands of serum albumin was reduced as Folic Acid concentration increased in the Pregnant's blood in both Trimesters.

A quantitative measurement of the strength of binding interactions between Folic Acid and serum albumin as a drug carrier of major vibrational bands of blood protein have been made**.** The intensity difference (delta) of the maximum Absorption of the lowest and highest concentration of Folic Acid in FA-HSA complexes ($\Delta I = I_{5ng/ml} - I_{11ng/ml}$) were calculated. For the Amide A and Amide B band (3500-3000 cm^{-1}) in the First and Third Trimesters were 0.327 and 0.090, respectively. In C-H Band (3000-2800 cm^{-1}), the intensity difference of ν_{as} CH_3 in the First and Third Trimester were 0.278 and 0.029, respectively, and of ν_{as} CH_2 in the First and Third Trimester were 0.286 and 0.029, respectively. Also, in Amide (I, II, III) bands, the intensity difference in the First Trimester were 0.299, 0.279 and 0.120, respectively. While in the Third Trimester were 0.083, 0.080 and 0.026, respectively. Interestingly, Amide I has the highest Intensity

reduction in the early and late stages of pregnancy; which is an indication that the main effect induced by Folic Acid was on amide I (C=O) region of Pregnant's protein.

These findings suggested that the large decrease in the absorption intensity of FA-HSA complexes as the Folic Acid concentration increases is account for increases H-bonds between the Folic Acid and the blood protein as drug carrier in the First Trimester. Moreover, these results indicate that the maximum loading capacity of FA on Pregnant's blood protein occurred during the First Trimester of pregnancy. Also, based on the overall all results obtained from the FTIR structural analysis in the first three sections above, we could confirm that Folic Acid binds native serum albumin of Pregnant's via hydrophobic, hydrophilic and H-bonding interactions in both Trimesters. Hence, at the molecular level, this gives evidence regarding major alteration occurring in the native serum albumin of Pregnant's women as a result of Folic Acid interaction in the early stages of pregnancy.

Finally, determination of the conformational changes of the native protein secondary structure induced by Folic Acid in the Amide I region (1700–1600 cm^{-1}) have been made using Fourier self-deconvolution methods. Folic Acid conjugation alters protein conformation by major alterations of α-helix and β-structures. The calculations of the protein secondary structure components showed alteration in the serum albumin conformations, characterized by a marked decrease in the α-helix from 53.6% to 33.4% in the First Trimester, and from 50.6% to 30.6% in the Third Trimester accompanied by increased β-structure and random coil when Folic Acid contents was increased Pregnant's blood.

Based on these detailed analyses, it has been found that Folic Acid binding to native serum albumin induce conformational and microenvironmental changes on protein secondary structure by major alternation in the α-helix (reduced) and β-sheet (incraesed) structures in First and Third Trimesters. While, the occurrence of major structural alterations of blood protein for the Pregnant women was in the early gestation ages (First Trimester) than later ages (Third Trimester). Therefore, taking Folic Acid as

supplement by Pregnant's women during the First Trimester of pregnancy can efficiently contribute to the public health action to decrease severe, disabling and potentially lethal Neural Tube Defects (birth defect).

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دراسة فيزيائية حيوية لتوصيل حمض الفوليك بواسطة بروتين الدم للنساء الحوامل في الثلث الأول والثالث من الحمل

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الملخص

بروتين الدم البشري (HSA) هو البروتين الأكثر وفرة في بلازما الدم. له أهمية بيولوجية كبيرة، حيث يرتبط بمجموعة متنوعة من الأدوية والجزيئات الصغيرة الأخرى مثل الفيتامينات، وله دور مهم في نقل وتوزيع الأدوية في جسم الإنسان، بما في ذلك حمض الفوليك.

حمض الفوليك هو أحد أنواع فيتامينات ب المركبة والذائبة في الماء، ويعرف كذلك باسم فيتامين ب 9. يُعد حمض الفوليك مهم بشكل خاص للنساء الحوامل لأنه يلعب دورًا مهماً في الوقاية من التشوهات الخلقية للجنين. إن السبب الأساسي لإدخال مكملات حمض الفوليك في النظام الغذائي للمرأة الحامل هو وجود ارتباط بين تناول كمية كافية من حمض الفوليك وتقليل نسبة إنجاب طفل مصاب بأحد عيوب الأنبوب العصبي. مما يعني أن حمض الفوليك ضروري للتطور الطبيعي والتكوّن السليم للأنبوب العصبي للجنين. لذلك، فإن دراسة التفاعل الجزيئي لحمض الفوليك مع بروتين الدم للمرأة الحامل له أهمية كبيرة لفهم وظيفة حمض الفوليك في تقليل نسبة إنجاب طفل مصاب بالتشوهات.

هناك تركيز هام على الحمل البشري في البحوث أكثر من الحمل عند باقي أنواع الثدييات. عيوبُ الأنبوب العصبي هي أحد العيوب الخلقية التي تظهر على الجنين عند الولادة والتي تؤثر على النخاع الشوكي والدماغ، وأكثر أنواعها شيوعاً هي: السنسنة المشقوقة، وعدم وجود الدماغ. وهناك الكثير من الأدلة الواضحة على أن حمض الفوليك قد يقلل من نسبة أنجاب أطفال مصابة بعيوب الأنبوب العصبي، وأن الأم التي تعاني من نقصه تكون أكثر عرضة للإجهاض وانقطاع المشيمة وتسمم الحمل والولادة المبكرة.

في هذه البحث، تم دراسة التغيرات الهيكلية والآلية الجزيئية لتفاعل حمض الفوليك مع بروتين الدم المأخوذ من النساء الحوامل في المرحلة المبكرة (الثلث الأول) والمرحلة المتأخرة (الثلث الثالث) من الحمل. كان تركيز بروتين الدم ثابت تقريبًا في جميع العينات في كل من الثلث الأول والثالث مع اختلاف تركيز حمض الفوليك في العينات.

تم استخدام جهاز مطياف تحويل فوريير للأشعة تحت الحمراء (FTIR) مع تطبيق عدد من التقنيات الموجود في هذا الجهاز مثل (Second derivative) و (Fourier-self deconvolution) بالإضافة إلى تقنية (curve-fitting) لتحليل مواقع وآليات الربط والتأثيرات الهيكلية لحمض الفوليك على بروتين الدم للمرأة الحامل.

تم استخدام التحليل الطيفي FTIR لدراسة الآلية الجزيئية لأنواع تفاعل حمض الفوليك مع بروتين الدم للنساء الحوامل في الثلث الأول والثالث من الحمل في جميع المناطق الهيكلية الرئيسية للبروتين. ومن نتائج الدراسة تبين أن هناك انخفاض واضح في شدة الامتصاص (Maximum Intensity) لكل من المناطق: Amide A, C-H amide I, II, and III في الثلث الأول والثالث. بعد ذلك تم حساب فرق شدة الامتصاص لبروتين الدم المرتبط بحمض الفوليك بين أقل وأعلى تركيز لحمض الفوليك ($\Delta I = I_{5ng/ml} - I_{11ng/ml}$) لكل من الثلث الأول والثالث. بالنسبة لمنطقة Amide A ($3500\text{-}3000\ cm^{-1}$)، كانت قيم فرق شدة الامتصاص 0.327 و0.090 في الثلث الأول والثالث على التوالي. أما منطقة C-H ($3000\text{-}2800\ cm^{-1}$)، كان القيم للقمة $\nu_{as}\ CH_3$ تساوي 0.278 و0.029 في الثلث الأول والثالث على التوالي. وللقمة $\nu_{as}\ CH_2$ تساوي 0.286 و0.029 في الفصل الأول والثالث على التوالي. بالإضافة إلى ذلك، في نطاقات Amide (I, II and III)، كانت قيم فرق شدة الامتصاص في الثلث الأول 0.299 و 0.279 و 0.120 على التوالي. بينما كانت في الثلث الثالث 0.083 و0.080 و0.026 على التوالي. تشير هذه النتائج إلى أن التفاعل بين حمض الفوليك وبروتين الدم للمرأة الحامل في المراحل المبكرة (الثلث الأول) أكبر من التفاعل في المراحل المتأخرة (الثلث الثالث) من الحمل.

استنادًا إلى النتائج التي تم التوصل إليها من التحليل الهيكلي للأطياف التي تم الحصول عليها من جهاز FTIR، يمكننا تأكيد أن حمض الفوليك يرتبط مع بروتين الدم للنساء الحوامل من خلال التفاعلات الكارهة للماء والمحبة للماء والروابط الهيدروجينية في كل من الثلث الأول والثالث. على المستوى الجزيئي، تقدم هذه النتيجة دليلًا على التغيرات الرئيسية التي تحدث في بروتين الدم للنساء الحوامل نتيجة تفاعله مع حمض الفوليك في المراحل المبكرة من الحمل.

بالإضافة إلى ذلك، تم تحديد التغييرات في البنية الثانوية لبروتين الدم نتيجة تفاعله مع حمض الفوليك في منطقة Amide I ($1700\text{–}1600\ cm^{-1}$) باستخدام تقنية (Fourier self-deconvolution)، وجد أن تفاعل حمض الفوليك مع بروتين الدم يؤدي إلى تغييرات في البنية الثانوية للبروتين عن طريق الانخفاض في نسبة حزم

الامتصاص التابعة ل (α-helix) مقابل زيادة نسبة حزم الامتصاص التابعة ل (β-sheet) في الثلث الأول والثالث. من ناحية أخرى، لوحظ أن التغيرات الهيكلية في بروتين الدم للنساء الحوامل قد حدثت بشكل أكبر وأوضح في المراحل المبكرة (الثلث الأول) من المراحل المتأخرة (الثلث الثالث) من الحمل. لذلك، فإن إدخال حمض الفوليك كمكمل في النظام الغذائي للنساء الحوامل في الأشهر الثلاثة الأولى من الحمل قد يساهم بشكل كبير في انخفاض نسبة إنجاب أطفال مصابة بعيوب الأنبوب العصبي التي قد تسبب تشوهات خلقية أو قد تكون مميتة للجنين.