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Research Article

Spectroscopic Studies on the Mechanism of Interaction between Vitamin B₁₂ and Vitamin C with Bovine   Serum Albumin

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Abstract: The interaction between vitamin C and vitamin B₁₂ with bovine serum albumin has been investigated. The binding mechanism was studied by UV-absorption and fluorescence spectroscopy. From spectral analysis both vitamins showed strong ability to quench the intrinsic fluorescence of BSA through a static quenching procedure. The binding constants are estimated to be 1.39×10^4 M^{-1} for vitamin C and 1.61×10^4 M^{-1} for vitamin B₁₂. FT-IR spectroscopy was used to determine the protein secondary structure. The observed spectral changes indicates an increase of intensity for HSA-vitamin C interaction and a reduction of intensity for HSA-vitamin B₁₂ interaction. While in the difference spectra of vitamin B₁₂-BSA complexes, intensity decreases as the concentration of vitamin B₁₂ increases for amide I. This variation of intensity is related indirectly to the formation of H-bonding in the complex molecules.

Keywords: BSA, Vitamin C, Vitamin B₁₂, UV-spectroscopy, Fluorescence spectroscopy, FT-IR

1. INTRODUCTION

Vitamins are vital as they play integral roles in hundreds of life-sustaining biochemical reactions and are also catalysts for all reactions using proteins, fats and carbohydrates for energy, growth and cell maintenance[1]. Vitamin B₁₂ (**Fig.1**) is needed in many body processes; in the manufacture and the maintenance of red blood cells, the synthesis of DNA, stimulate of nerve cells, the growth promotion and energy releases and the proper

functioning of folic acid. Characteristic signs of vitamin B_{12} deficiency include fatigue, weakness, nausea, constipation, flatulence (gas), loss of appetite and weight loss. It is the only vitamin to contain metal ion (cobalt (III))[2]. Vitamin C **(Fig.2)** known as ascorbic acid, represents the major water-soluble antioxidant in plasma and acts as a primary defense in the blood against free radical attack. It has a strong quenching ability for reactive oxygen species such as singlet oxygen and the superoxide anion radical by converting their hydro peroxides into stable products [3-7]].
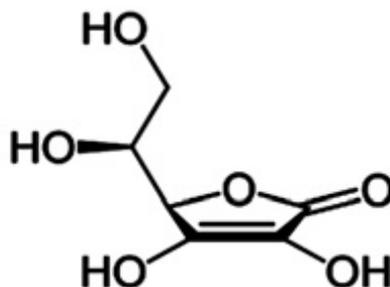

Figure 1. Chemical structure of Vitamin C

Serum albumin is the most abundant protein in blood plasma, it serve as depot proteins and transport proteins for a variety of compounds, like fatty acids, amino acids, bile salts, metals, hormones, drugs and pharmaceuticals, which has many physiological functions, such as maintaining the osmotic pressure and pH of blood and scavenging free radicals as an antioxidant[5]. It is an attractive macromolecular carrier, the lack of toxicity and immunogenicity make it an ideal candidate for drug delivery[6-9]. In addition, serum albumin is the most multifunctional transport protein and plays an important role in the transport and deposition of a variety of endogenous and exogenous substances in blood [10].
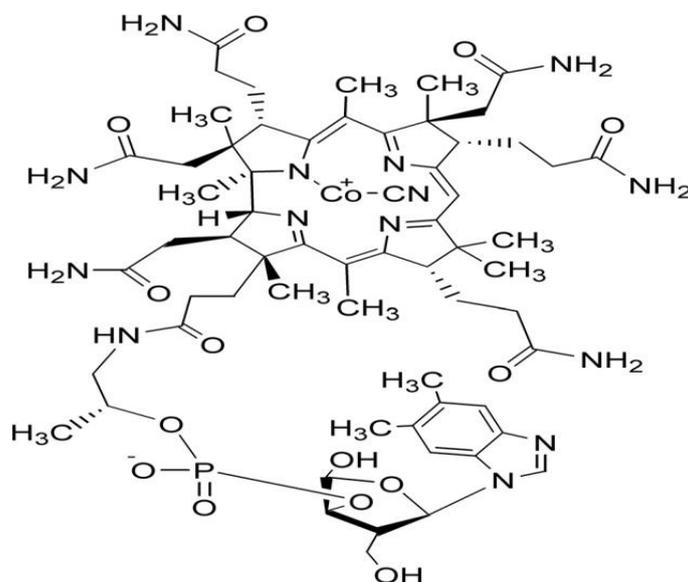

Figure 2. Chemical structure of vitamin B_{12}

Bovine serum albumin (BSA) has been one of the most extensively studied of this group of proteins, not only because of its medical importance, abundance, low cost, ease of purification, ready availability, unusual ligand-binding properties and it is widely accepted in the pharmaceutical industry, but also because of its structural

homology with human serum albumin[11] .

The binding properties of albumin depend on the three-dimensional structure of the binding sites, which are distributed over the molecule. Strong binding can decrease the concentrations of free drug/vitamin in plasma, whereas weak binding can lead to a short lifetime or poor distribution. Its remarkable capacity to bind a variety of drug/vitamin results in its prevailing role in pharmacokinetics and pharmacodynamics processes[12].

In recent years, studies on the mechanisms of interactions between drugs/vitamins and proteins have been performed with techniques such as spectroscopy[13-19]. Infrared spectroscopy provides measurements of molecular vibrations due to the specific absorption of infrared radiation by chemical bonds. Fluorescence and UV spectroscopy are commonly used because of their high sensitivity, rapidity and ease of implementation[18].The objective of this work was to investigate the interaction of BSA complexes with vitamin C and vitamin B_{12} by using Fourier transform infrared spectroscopy (FTIR), fluorescence and UV spectroscopy. Thus, the binding mechanism of two different vitamins interacting with BSA may be different and have to be studied.

2. MATERIALS AND METHODS

BSA was purchased from Sigma chemical company. Vitamin C and vitamin B_{12} in powder form were also purchased from Sigma chemical company and used without further purification.

2.1. Preparation of Stock Solutions: BSA was dissolved in phosphate buffer Saline and at physiological pH of 7.4), to a concentration of (80mg/ml), and used at final concentration of (40 mg/ml) in the final vitamin-BSA solution. Vitamin C (molecular weight of 176.13 g.mol^{-1}) and vitamin B_{12} (molecular weight of 1355.4 g.mol^{-1}), were dissolved in 25% ethanol in phosphate buffer Saline and, then the solution was placed in ultrasonic water path (SIBATA AU-3T) for one hour to ensure that all the amount of vitamins were completely dissolved. The final concentrations of BSA-Vitamins complexes were prepared by mixing equal volume of BSA to equal volume from different concentration of vitamins C and B_{12}. BSA concentration in all samples kept at 40 mg.ml^{-1}. However, the final concentrations of the vitamins in solutions are (40 mg.ml^{-1}, 20 mg.ml^{-1}, 10 mg.ml^{-1}, 5 mg.ml^{-1}, 2 mg.ml^{-1}and 1 mg.ml^{-1}).The solution of vitamins and BSA were incubated for 1 h (at 25^{0}C) before spectroscopic measurements were taken.

2.2. UV-VIS Spectrophotometer: The absorption spectra were obtained by the use of a Nano Drop ND-1000 spectrophotometer. It is used to measure the absorption spectrum of the samples in the range between 220-750 nm, with high accuracy and reproducibility. The absorption spectra were recorded for free BSA 40 mg.ml^{-1} and for its complexes with vitamin C and B_{12}solutions with the concentrations of (40 mg.ml^{-1}, 20 mg.ml^{-1}, 10 mg.ml^{-1}, 5 mg.ml^{-1}, 2 mg.ml^{-1}and 1 mg.ml^{-1}). Repeated measurements were done for all samples.

2.3. Fluorescence Spectrometer: The fluorescence measurements were performed by a Nano Drop ND-3300 Fluoro-spectrophotometer at 25°C. The excitation source comes from one of three solid-state light emitting diodes (LEDs). The excitation source options include: UV LED with maximum excitation 365 nm, Blue LED with excitation 470 nm, and white LED from 500 to 650nm excitation. A 2048-element CCD array detector covering 400-750 nm, is connected by an optical fiber to the optical measurement surface. The excitation is done at the wavelength of 360 nm and the maximum[20] emission wavelength is at 439 nm. The emission spectra were recorded for free BSA 40 mg.ml^{-1} and its complexes with vitamins C and B_{12} solutions with the concentrations of (40 mg.ml^{-1}, 20 mg.ml^{-1}, 10 mg.ml^{-1}, 5 mg.ml^{-1}, 2 mg.ml^{-1}and 1 mg.ml^{-1}).

2.4. FT-IR Spectroscopy: The FT-IR measurements were obtained on a Bruker IFS 66/Spectrophotometer

equipped with a liquid nitrogen-cooled MCT detector and a KBr beam splitter. The spectrometer was continuously purged with dry air during the measurements. The absorption spectra were obtained in the wave number range of 400- 4000 cm^{-1}. A spectrum was taken as an average of 60 scans to increase the signal to noise ratio, and the spectral resolution was at 4 cm^{-1}. The aperture used in this study was 8 mm, since we found that this aperture gives best signal to noise ratio. Baseline correction, normalization and peak areas calculations were performed for all the spectra by OPUS software. The peak positions were determined using the second derivative of the spectra[21]. The infrared spectra of BSA, and vitamins-BSA complexes were obtained in the region of 1000-1800 cm^{-1}. The FT-IR spectrum of free BSA was acquired by subtracting the absorption spectrum of the buffer solution from the spectrum of the protein solution. For the net interaction effect, the difference spectra [(protein and vitamins solutions) - (protein solution)] were generated using the featureless region of the protein solution 1800-2200 cm^{-1}as an internal standard[22-24]. The accuracy of this subtraction method is tested using several control samples with the same protein or vitamins concentrations, which resulted into a flat base line formation. The obtained spectral differences were used here, to investigate the nature of the vitamins-BSA interaction.

3. RESULTS AND DISCUSSION

3.1. UV-absorption Spectroscopy: The evolution of UV-absorption spectroscopy was registered and used to determine the binding constants between BSA and a drug (Vitamin C and Vitamin B$_{12}$). The strength of interaction between BSA and drugs is dependent on the binding constant which can be calculated using graphical analysis of the absorbance spectrum**.** The excitation has been done on 210 nm and the absorption is recorded at 268 nm for vitamin C and at 278 nm for vitamin B$_{12}$.
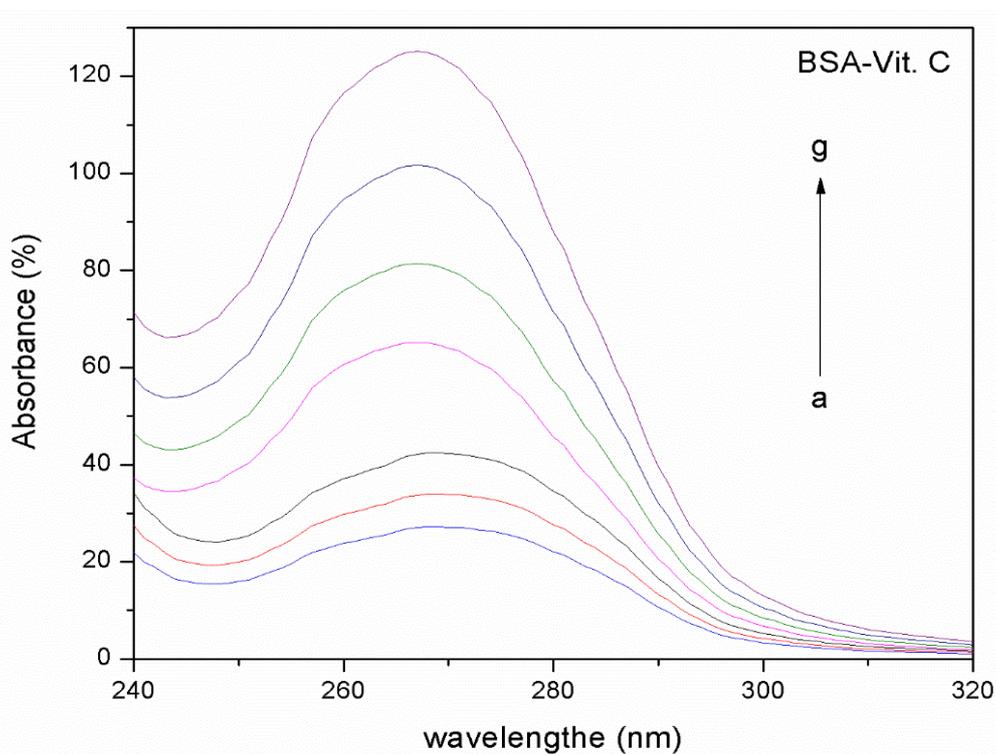

Figure 3.a: UV-absorbance spectra of BSA with different concentrations of Vitamin C (a=free BSA,

b=1mg.ml^{-1},c=2mg.ml^{-1}, d=5mg.ml^{-1}, e=10mg.ml^{-1}, f=20mg.ml^{-1}, g=40 mg.ml^{-1})

The absorption spectra of the different concentrations of vitamin C (**Fig.3.a**) and vitamin B$_{12}$ (**Fig.3.b)** with BSA showed an increase of the intensity as the vitamin C concentration increases; this is due to major ligand protein interaction at protein surface which does not limit the mobility of ligand around BSA molecule while the intensity deceases with increasing vitamin B$_{12}$ concentration which originates from the existence of aromatic amino acids being the components of a protein molecule as well as its complicated molecular structure.
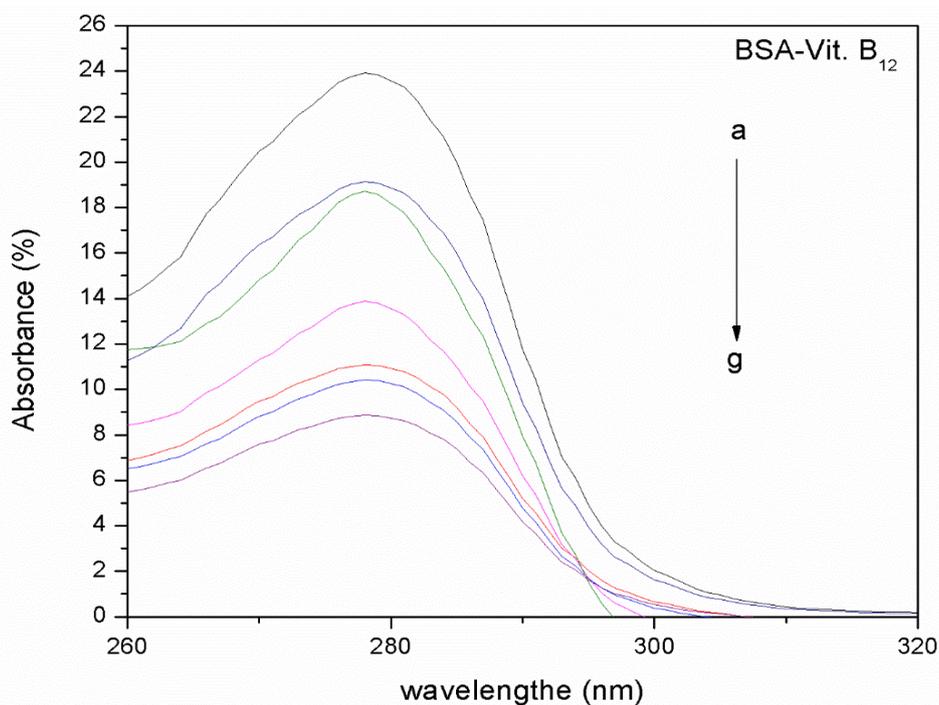

Figure 3.b. UV-absorbance spectra of BSA with different concentrations of Vitamin B$_{12}$ (a=free BSA, b=1mg.ml^{-1},c=2mg.ml^{-1}, d=5mg.ml^{-1}, e=10mg.ml^{-1}, f=20mg.ml^{-1}, g=40 mg.ml^{-1})

 The absorption data were treated using linear reciprocal plots based on the following equation [26].

$$\frac{1}{A-A_0} = \frac{1}{A_\infty - A_0} + \frac{1}{K[A_\infty - A_0]} \cdot \frac{1}{L} \tag{1}$$

Where A$_0$ corresponds to the initial absorption of protein at 280 nm in the absence of ligand, A$_\infty$ is the final absorption of the ligated protein, and A is the recorded absorption at different vitamin C concentrations (L). **Fig. 4.a and Fig. 4.b** represents the double reciprocal plots of 1/(A- A$_0$) vs. 1/L for HSA-vitamin C and HSA-vitamin B$_{12}$ complexes respectively. The binding constant (K) can be estimated from the ratio of the intercept to the slope. The obtained values of the binding constants indicates a relatively weak interaction of vitamin C (K= 1.39x10^4 M^{-1}) and vitamin B$_{12}$ (K= 1.61x10^4 M^{-1}) when compared to other drug-HSA complexes with binding constants in the range[27-29] of 10^5 and 10^6 M^{-1} . The reason for the low stability can be attributed to the presence of mainly hydrogen-bonding interaction or an indirect vitamin-HSA interaction through water molecules [30].
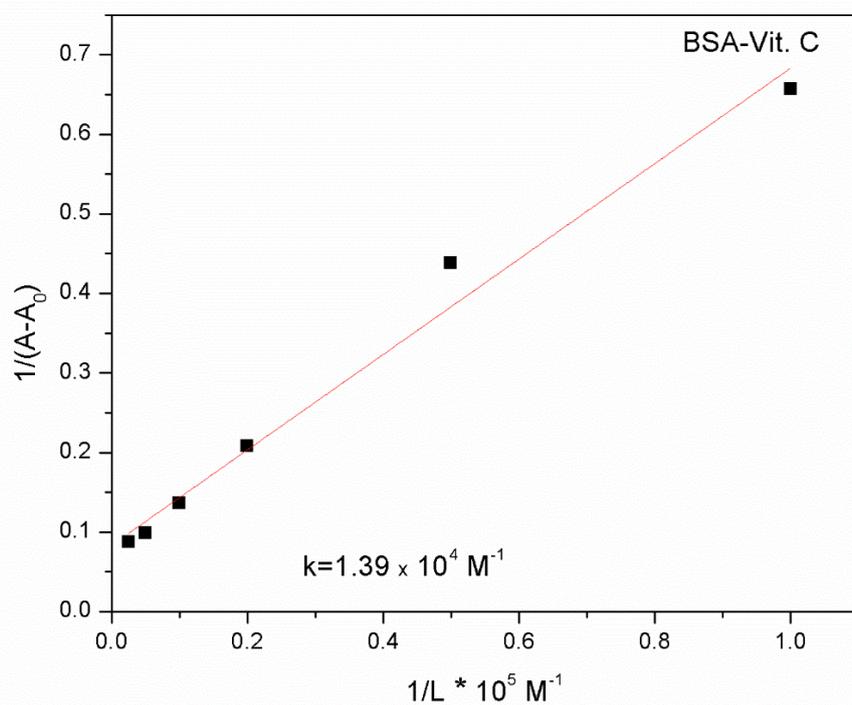
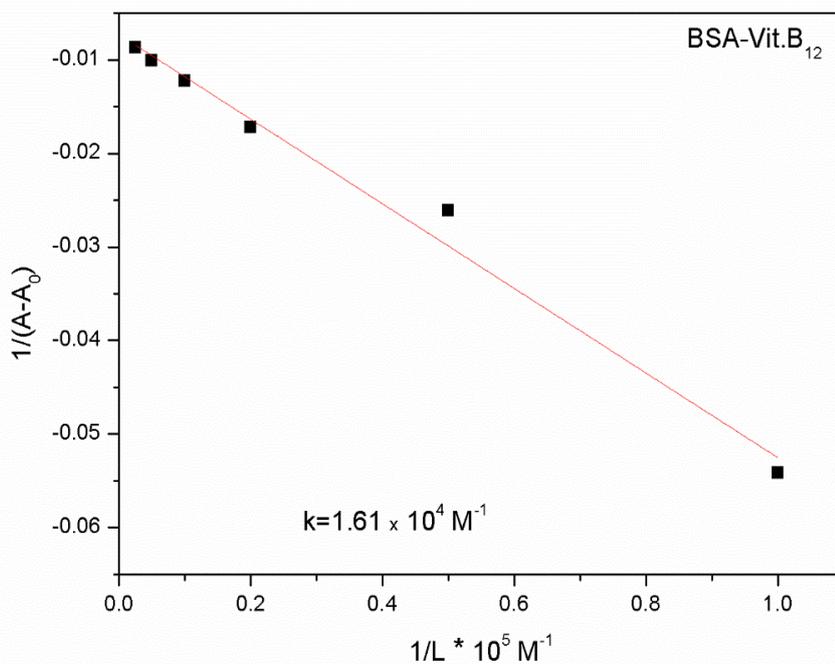**Figure 4.a:**   The plot of $1/(A-A_o)$ vs. $1/L$ for BSA with different concentrations of vitamin C.

Figure 4.b: The plot of $1/(A-A_o)$ vs. $1/L$ for BSA with different concentrations of vitamin B_{12}.

3.**2. Fluorescence Spectroscopy:** It was found that the protein fluorescence quenching can be the result of both the interactions, collisional or binding, with other molecules, as well as the inner filter effect pertaining to the absorption of light at the excitation or emission wavelength by the compounds presented in the solution. Fluorescence measurements can give some information on the binding mechanism of small molecule substances to protein, including binding mode, binding constants, binding sites and intermolecular distances. Various molecular interactions can decrease the fluorescence intensity of a compound such as molecular rearrangements, exited state reactions, energy transfer, ground state complex formation, and collisional quenching[31, 32]

The intrinsic fluorescence of many proteins is mainly contributed by tryptophan alone, because phenylalanine has very low quantum yield and the fluorescence of tyrosine is almost totally quenched if it is ionized or near an amino group, a carboxyl group, or a tryptophan residue[17, 32, 34]. In this work for BSA-vitamins complexes excitation wavelength at 360nm was used. The fluorescence sensor is based on intramolecular charge transfer (ICT), which is highly sensitive to the polarity of microenvironment. Therefore, it is expected to act as fluorescent probe for some biochemical systems like proteins[35].

It was observed that the BSA fluorescence spectrum exhibiting the peak maximum at 440 nm (**Fig.5.a**) and the intensity increases as the vitamin C concentration increased while the peak maximum appears to be at 430 nm (**Fig.5.b**) and the fluorescence intensity decreased regularly with increasing of vitamin B12 concentration. The peak positions shows little or no change at all.
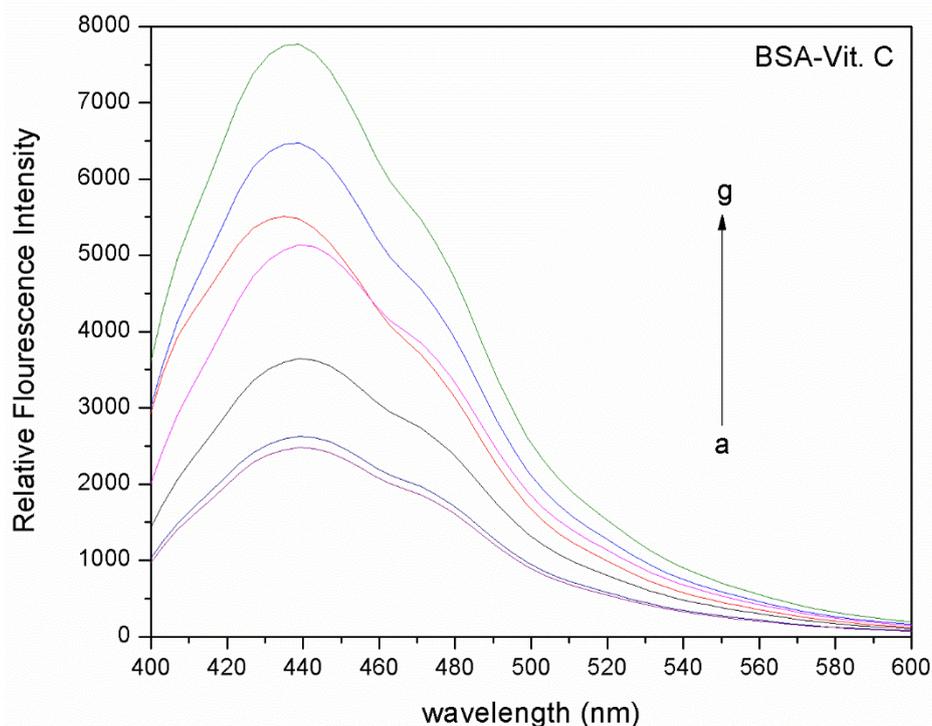

Figure 5.a: Fluorescence emission spectra of BSA in the absence and presence of vitamin C in these concentrations (a=free BSA, b=1mg.ml^{-1},c=2mg.ml^{-1}, d=5mg.ml^{-1}, e=10mg.ml^{-1}, f=20mg.ml^{-1}, g=40 mg.ml^{-1}).
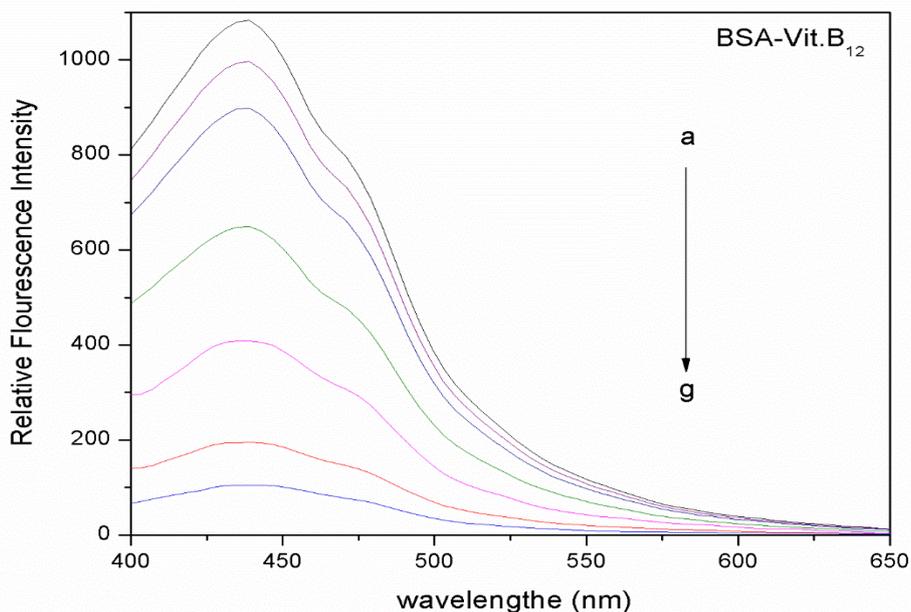
Figure 5.b: Fluorescence emission spectra of BSA in the absence and presence of vitamin B_{12} in these concentrations (a=free BSA, b=1mg.ml^{-1},c=2mg.ml^{-1}, d=5mg.ml^{-1}, e=10mg.ml^{-1}, f=20mg.ml^{-1}, g=40 mg.ml^{-1}).

To elucidate the mechanism of fluorescence quenching, the steady state fluorescence quenching data were examined based on the classic Stern-Volmer equation [36]:

$$\frac{F_0}{F} = 1 + K_{sv}[L] = 1 + k_q \tau_0 [L] \tag{2}$$

where F_0 and F are the BSA fluorescence intensities in the absence and presence of quencher (Vitamins C and B_{12}); k_q is the BSA bimolecular quenching rate constant; τ_0 is the average fluorescence lifetime of the BSA molecule without quencher (of the order of 10^{-8} [37]); [L] is the concentration of quencher, k_{sv} is Stern-Volmer fluorescence quenching constant.

The process of fluorescence quenching can proceed via two mechanisms: dynamic (collisional, diffusion-limited) and static (diffusion-independent). The Stern-Volmer plots, which are linear within certain concentration, may either expose the presence of a single type of quenching, or show the occurrence of just a single binding site for quencher in the fluorophore neighborhood[38-40].

The Stern-Volmer plots demonstrate sometimes an upward curvature, mainly at higher values of quencher concentration, what could be ascribed to the combined type of quenching (both static and dynamic)[41]. It appeared that in proteins case the positive deviations from the Stern-Volmer equation are also recorded when the extent of quenching is large or the fluorescence process is dominated by one single residue (distance-dependent quenching due to a single molecular interaction)[42]. **Fig. 6.a and Fig.6.b** represents the Stern-Volmer plot of BSA fluorescence intensities of Vitamin C and vitamin B_{12} respectively. The curves are linear, suggesting the existence of a single type of quenching (dynamic or static) and/or a single binding site for both vitamins in the BSA neighborhood., the values of the fluorescence quenching constant determined applying Eq. (2) are equal to (4.78×10^3, 2.42×10^4) L mol^{-1} for vitamins C and B_{12} respectively, both values are much lower than other k_{sv} values for the similar systems signalized earlier in literature[1,44]. The calculated k_{sv} values

are much too large to be caused by collisional quenching in water (two-three orders of magnitude larger), as well as the values of kq, which are equal to $(4.78 \times 10^{11}, 2.42 \times 10^{12})$ L mol^{-1} s^{-1} for vitamins C and B$_{12}$ respectively . These values confirm clearly the existence of static (diffusion- independent) mechanism of fluorescence quenching [14, 19, 43].
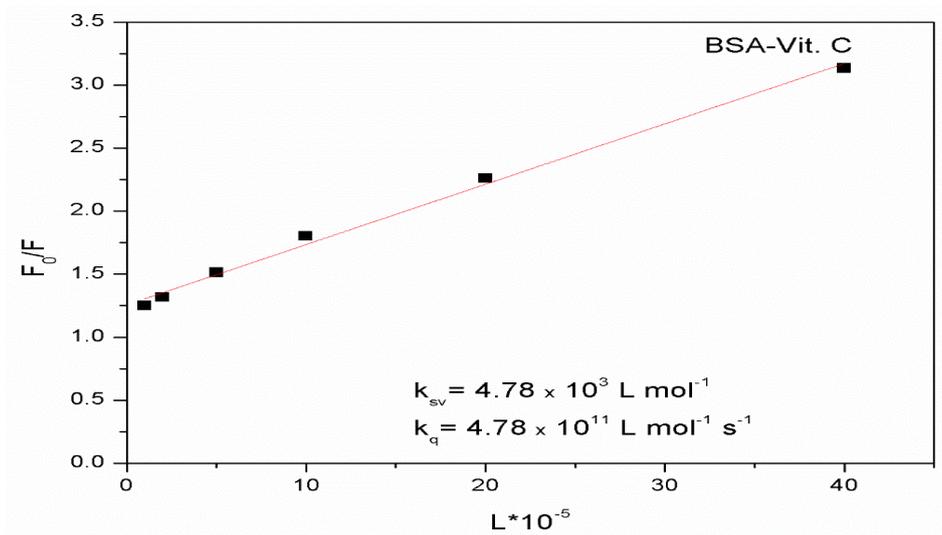

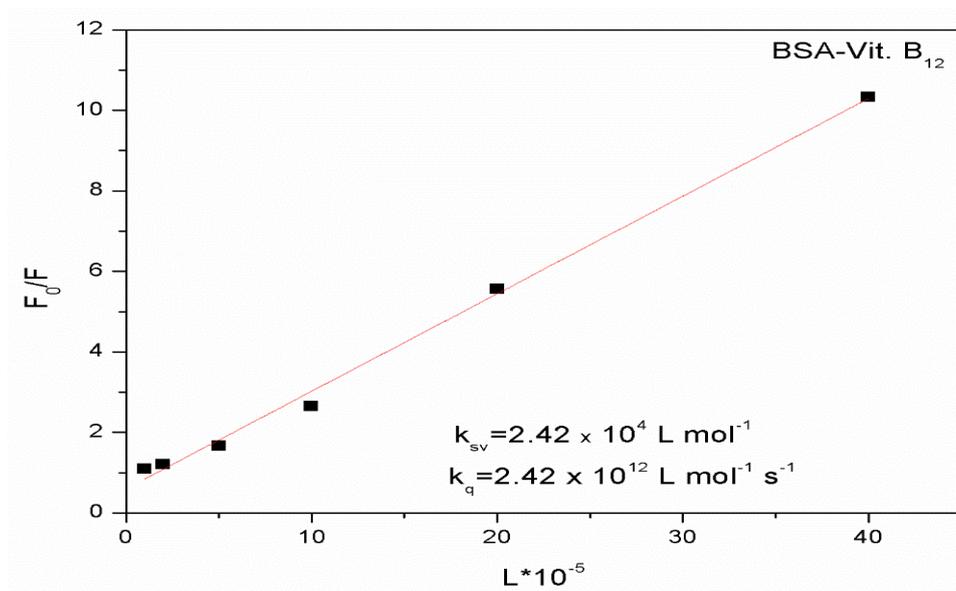**Figure 6.a:** The Stern-Volmer plot for vitamin C- BSA complex

Figure 6.b: The Stern-Volmer plot for vitamin B$_{12}$- BSA complex

The values of kq were greater than that of the maximum dynamic quenching constant. This suggested that the fluorescence quenching was not the result of dynamic quenching, but the consequence of static quenching[40,44]. When static quenching is dominant, the modified Stern- Volmer equation could be used[36].

$$\frac{1}{F_0 - F} = \frac{1}{F_0 K_{(L)}} + \frac{1}{F_0} \qquad\qquad (3)$$

where K is the binding constant of vitamin-BSA. To determine the binding constants of BSA- vitamins system, a plot of $\frac{1}{F0-F}$ vs $\frac{1}{L}$ for different vitamins ratios are made and shown in **Fig. 7.a and Fig. 7.b** for vitamins C and B$_{12}$ respectively. The plots are linear and have a slope of $\frac{1}{F0\,K}$ and intercept $\frac{1}{F0}$ according to eq. (3). The values of K were found to be (1.6×10^4 M^{-1}, 1.44×10^4 M^{-1}), which agrees well with the values obtained earlier by UV spectroscopy and supports the effective role of static quenching.
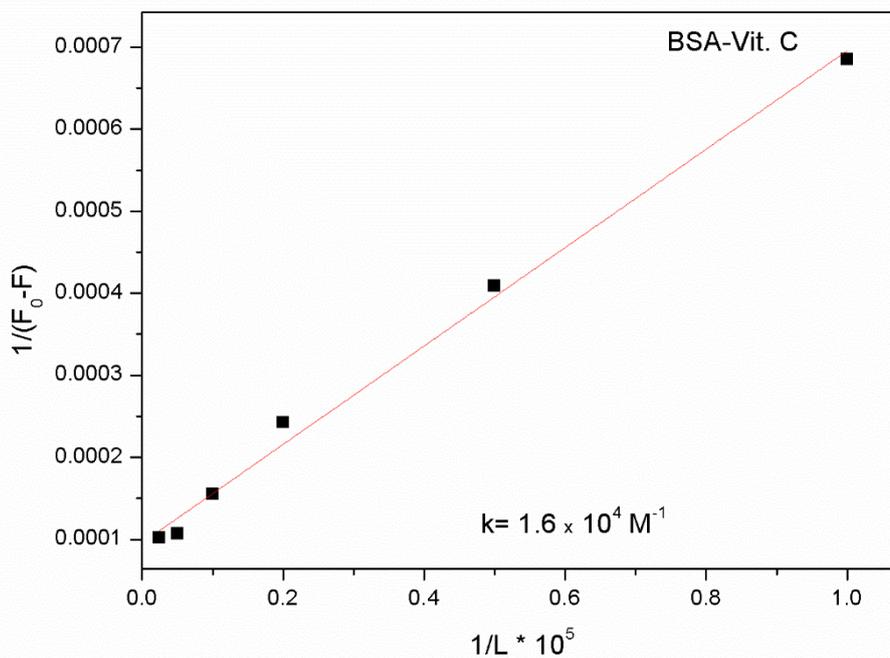

Figure 7.a: The plot of $1/(F_0$-F) vs. $1/L \times 10^5$ for BSA-Vitamin C
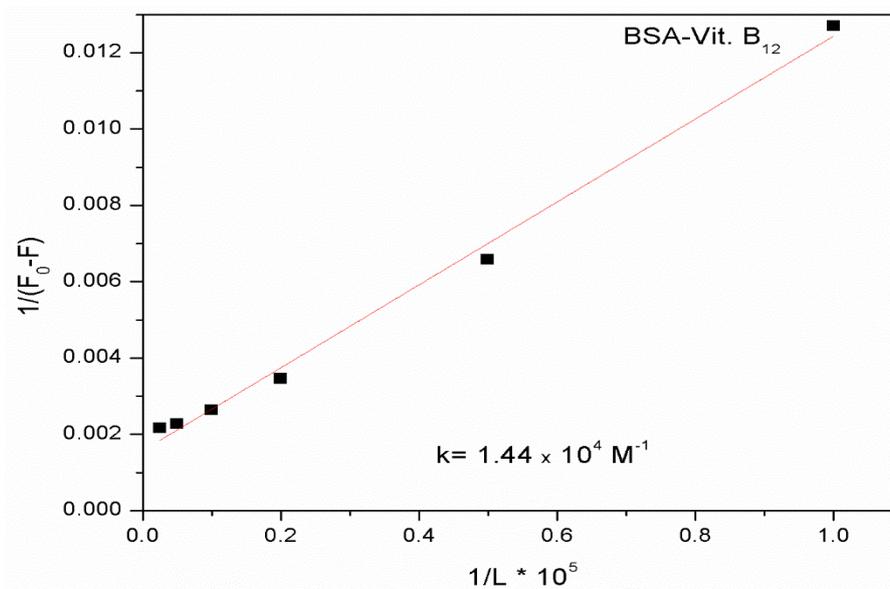

Figure 7.b: The plot of $1/(F_0$-F) vs. $1/[L \times 10^5]$ for BSA-Vitamin B$_{12}$

3.3. FT-IR Spectroscopy: FT-IR spectroscopy is a powerful technique for the study of hydrogen bonding [44], and has been identified as one of the few techniques that is established in the determination of protein secondary structure at different physiological systems[12, 33]. The information on the secondary structure of proteins could be deduced from the infrared spectra. Proteins exhibit a number of amide bands, which represent different vibrations of the peptide moiety. The amide group of proteins and polypeptides presents characteristic vibrational modes (amide modes) that are sensitive to the protein conformation and largely been constrained to group frequency interpretations[8, 42].

The modes most widely used in protein structural studies are amide I, amide II and amide III. Amide I band ranging from 1700 to 1600 cm^{-1} and arises principally from the C=O stretching[40] has been widely accepted to be used. The amide II band is primarily N-H bending with a contribution from C-N stretching vibrations; amide II ranging from 1600 to 1480 cm^{-1} while amide III band ranging from 1330 to 1220 cm^{-1} which is due to the C-N stretching mode coupled to the in-plane N - H bending mode[43].

The second derivative of free BSA is shown in **Fig. 8.a** , where the spectra is dominated by absorbance bands of amide I and amide II at peak positions 1653 and 1545cm^{-1}. **Figures (8.b&8.c)** respectively shows the spectrum of BSA-vitamin C and BSA-vitamin B$_{12}$ complexes with different concentrations.
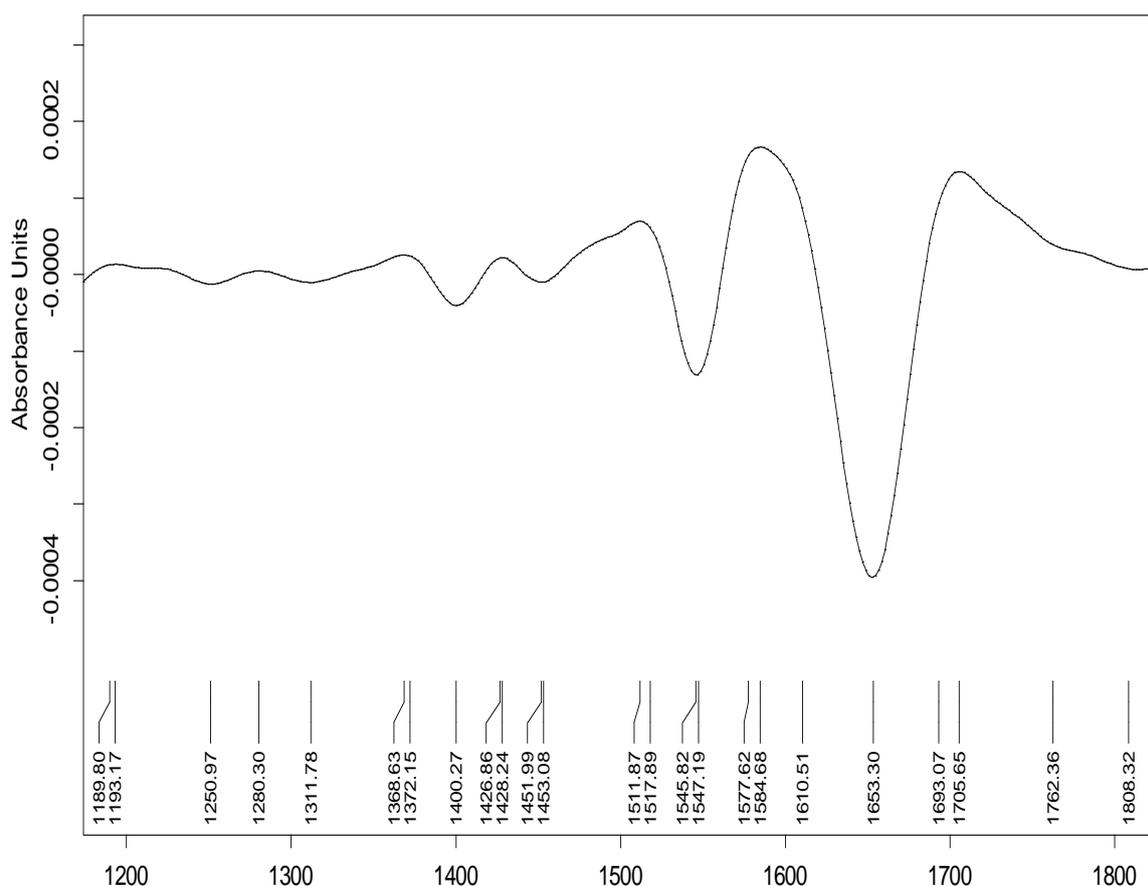

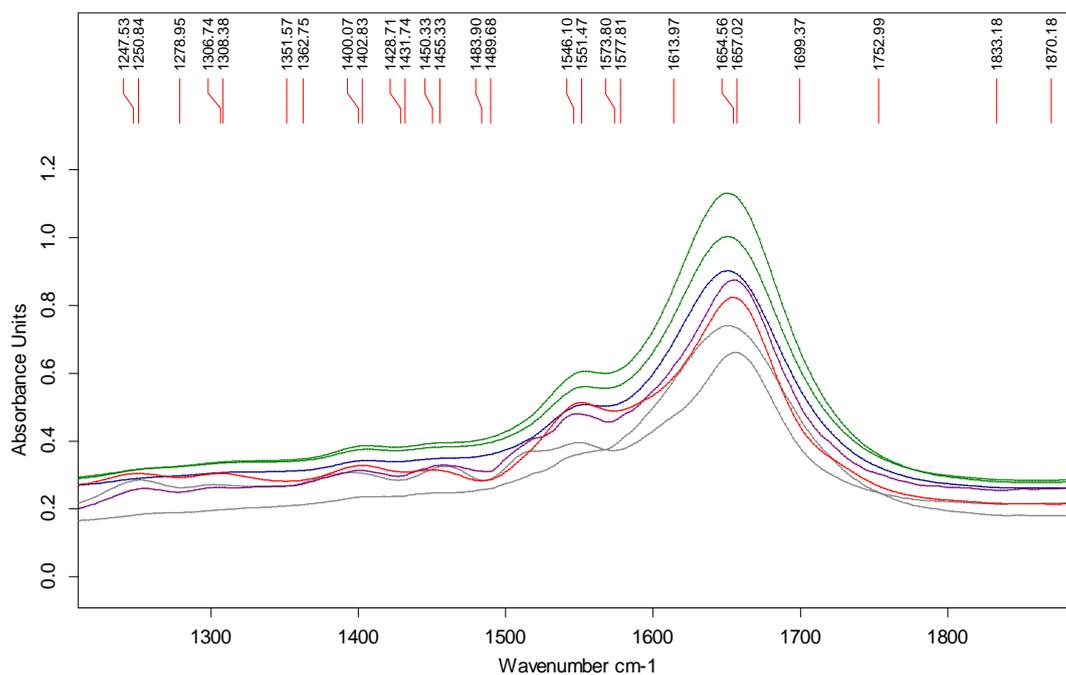**Figure 8.a:**   Second derivative of free BSA.

Figure 8.b: Different spectra of BSA and its complexes with different vitamin C concentrations in the region 1800-1200 cm^{-1}
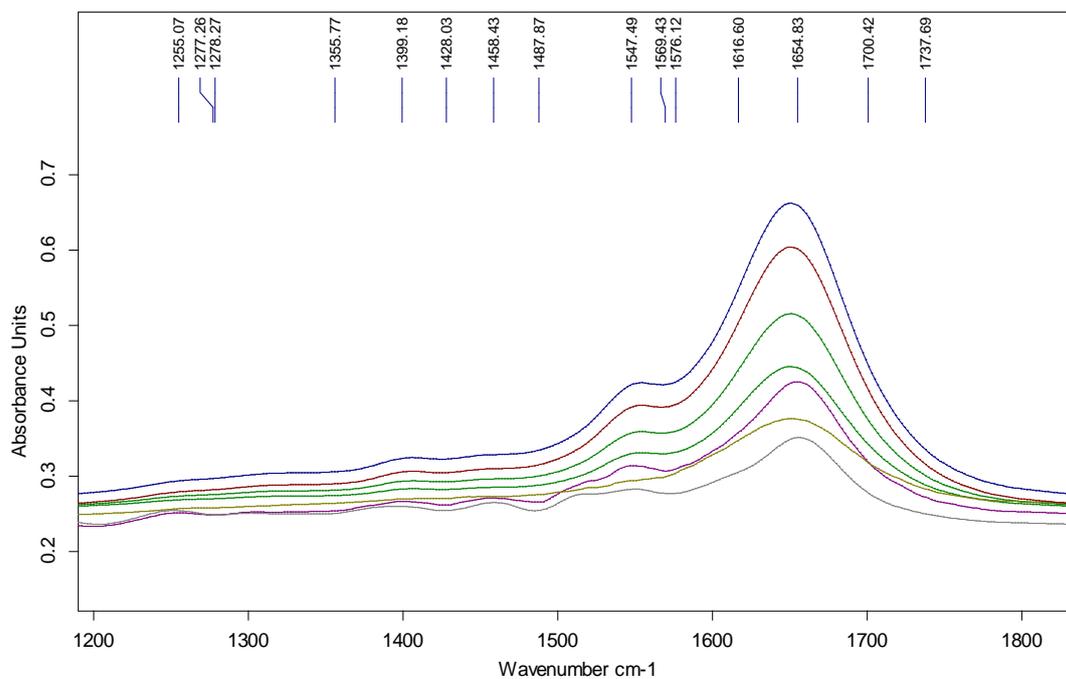

Figure 8.c: Different spectra of BSA and its complexes with different vitamin B$_{12}$ concentrations in the region 1800-1200 cm^{-1}.

The peak positions of BSA with different concentrations of vitamins C and B_{12} are listed in **tables 1 & 2** respectively. It is clearly that the amide bands of BSA infrared spectrum are shifted in two different manner.

The peak positions of amid I bands in BSA infrared spectrum as listed in **table (1):** 1609-1614 cm^{-1}, 1656-1661 cm^{-1}, 1698-1705 cm^{-1} after the interaction with vitamin C. The changes of these peak positions and peak shapes demonstrated the secondary structure of BSA had been changed by the interaction with vitamin C. By the same manner in amid II region some of the peak positions have shifted, while in the amid III region little change of the peak positions has been observed. The minor changes in peak positions can be attributed to the effect of the newly imposed H-bonding between vitamin C molecules and the protein[44].

In **table (2)**, the peak positions of amid I band infrared spectrum had been shifted as the following: 1603-1612 cm^{-1}, 1641-1652 cm^{-1}, 1684-1689 cm^{-1} after the interaction of vitamin B_{12}. This improves that the secondary structure of the protein had been changed. In amid II and III regions a minor or no change of the peak positions has been observed.

Table 1:   Band assignment in the absorbance spectra of BSA with different vitamin C concentrations for Amid I-III regions.

Band regions (cm^{-1})	BSA Free	BSA-Vitamin C 1mg.ml^{-1}	BSA-Vitamin C 2mg.ml^{-1}	BSA-Vitamin C 5mg.ml^{-1}	BSA-Vitamin C 10mg.ml^{-1}	BSA-Vitamin C 20mg.ml^{-1}	BSA-Vitamin C 40mg.ml^{-1}
Amid I (1600-1700)	1610	1609	1611	1610	1609	1611	1614
	1653	1657	1658	1656	1658	1659	1661
	1693	1699	1701	1698	1703	1704	1705
Amid II (1480-1600)	1589	1490	1491	1491	1490	1492	1493
	1545	1546	1545	1545	1546	1547	1547
	1573	1574	1573	1574	1576	1573	1578
Amid III (1220-1330)	1248	1248	1249	1248	1249	1249	1249
	1280	1279	1278	1280	1278	1277	1276
	1311	1309	1310	1311	1312	1312	1313

Table 2: Band assignment in the absorbance spectra of BSA with different vitamin B_{12} concentrations

Band regions (cm^{-1})	BSA Free	BSA-Vitamin B_{12} 1mg.ml^{-1}	BSA-Vitamin B_{12} 2mg.ml^{-1}	BSA-Vitamin B_{12} 5mg.ml^{-1}	BSA-Vitamin B_{12} 10mg.ml^{-1}	BSA-Vitamin B_{12} 20mg.ml^{-1}	BSA-Vitamin B_{12} 40mg.ml^{-1}
Amid I (1600-1700)	1610	1612	1609	1605	1604	1604	1603
	1653	1651	1650	1648	1646	1645	1642
	1693	1689	1689	1688	1686	1687	1684
Amid II (1480-1600)	1589	1587	1588	1586	1581	1584	1582
	1545	1542	1542	1539	1543	1538	1540
	1573	1597	1597	1596	1596	1597	1595
Amid III (1220-1330)	1250	1250	1249	1248	1248	1248	1246
	1309	1305	1305	1305	1302	1302	1303
	1311	1312	1311	1312	1312	1309	1309

4. SMMARY AND CONCLUSIONS

The interaction between vitamin C and vitamin B_{12} with bovine serum albumin has been investigated by UV-absorption spectroscopy, fluorescence spectroscopy and by FT-IR spectroscopy. The binding constant values indicate a relatively weak binding of both vitamins with BSA and the quenching constant indicate that the intrinsic fluorescence of BSA was quenched by both vitamins through static quenching mechanism. Analysis of FT-IR spectra reveals that BSA-vitamin C interaction induces intensity increment while BSA-vitamin B_{12} interaction induces intensity reduction.

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