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Spectroscopic Studies of Carcinoembryonic Antigen in Malignant Mammary Tissues

دراسة طيفية للمستضد الجنيني السرطاني في الانسجة الثدية المصابة بالاورام الخبيثة

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Abstract

Characterization of CEA was carried out through spectroscopic studies using ultraviolet absorption molecules. Factor affecting the absorption properties of purified CEA such as, pH, polarity, effect of solvent perturbation and pH titration have been studied, the results indicates that there are different effects of these factors on the CEA spectrum. The pH titration of purified CEA shows that the amino acid tyrosine is located on the surface of the CEA. This study was aimed to elucidate the spectroscopic properties of purified CEA using spectroscopic techniques, Uvana FTIR.

الخلاصة

درست أطياف الأشعة فوق البنفسجية لتشخيص بروتينيات CEA المنقى من خلال تأثير الأس الهيدروجيني وقطبية المذيب والاضطراب المذيبي على البنية الثنائية والثلاثية لتركيب البروتين ومنه تمت معرفة الأحماض الأمينية الموجودة على السطح و استخرجت قيمة معامل الامتصاصية وكانت 4.16 مل/ ملغم.سم. استخدمت مطيافية الأشعة تحت الحمراء (FTIR) في تشخيص بروتينيات CEAالمعزولة من مجانس الثدى الخبيثة وتم مقارنتها مع بروتينيات CEA القياسية وأظهرت نتائج القياسات التماثل الواضح بين طيف الأشعة تحت الحمراء وكل من المستضد السرطان الجنيني المنقى و القياس .

Introduction

Carcinoembryonic antigen (CEA) is the name given by Gold and Freedman to a tumor – specific antigen found in adenocarcinoma of the digestive system [(1)].

That the CEA is a glycoprotein was evident from its solubility and apparent stability in perchloric acid [(2)]. In view of the heterogeneous nature of the glycoprotein it is impossible to define CEA precisely in terms of physical and chemical properties. A definition of CEA at the present time has to be based on its specific immunological reaction with a monospecific anti – CEA antiserum, which has been shown to be identical in specificity to the original antiserum prepared by Gold and Freedman [(3)]. This is important since it has been clearly established that CEA possesses at least two different immunogenic groups; a unique group, which defines CEA – likes molecules and second is common to CEA and the normal glycoprotein (NGP) that is also found in normal and tumor tissues [(4,5,6,7,8)].

There are several procedures available for the isolation and purification of CEA from different sources [(9,10,11)]. Most of these depends on routine methods by perchloric acid extraction and then using gel–filtration however, other methods such as affinity chromatography was used for purification of CEA from liver affected by metastasis of colon carcinoma[(12)].

The best spectroscopic methods for characterization of CEA are the solvent perturbation technique [(13)]. In the solvent perturbation method of probing the surface of protein molecules, advantage is taken from the fact that the spectra of chromophoric residue coming freely in contact with the solvent is sensitive to change in the physical properties of the solvent, such as refractive index, dielectric constant, and solvent-solute interaction, in the immediate vicinity of chromophores[(14)]. There are several studies on the application of solvent perturbation technique of different spectroscopy such as those related to studying the location of chromophoric side chains in globular proteins (milk proteins, α- lacta albumin, … etc) dissolved in aqueous media [(15,16,17,18)].

Materials and Methods

A - Ultraviolet Absorption: (UV)

The ultraviolet absorption of the purified human CEA and CEA provided (CEA IRMA Kit / Byksangetes Diagostic GmbH&Co. KG / France) were continuously scanned from 200 – 350 nm by a Shimadzu UV.

A volume of 50 µl of CEA (1.3 mg/ml) was completed to 1 ml with tris buffer pH 7.2, then the absorption spectrum was measured against the same buffer in reference beam.

B- Factors Affecting the Absorption Properties of Purified CEA

1- pH Effect on the spectrum of purified CEA:

Solutions:

1. 0.01 M tris hydroxyl methyl amino ethan buffer of pH 7.2 was prepared by dissolving 0.302 gm of the tris in 250 ml distilled water and required pH was adjusted by addition of 0.1 N HCl.
2. Citrate buffer (pH 3) was prepared as follows:

Solution **A**: Citric acid (0.1 M); 10.50 gm citric acid was dissolved in 500 ml distilled water.

Solution **B**: Sodium citrate (0.1 M); 14.7050 gm sodium citrate was dissolved in 500 ml distill water.

Citrate buffer pH 3 were prepared by mixing 46.5 ml of solution A with an appropriate amount (3.5 ml) of solution B to obtain the required pH.

3. Phosphate buffer (pH 11) was prepared as follows:

Solution **A**: Dibasic sodium phosphate (0.1 M); 4.4497 gm Na_2HpO_4. 2 H_2O was dissolved in 250 ml distilled water.

Solution **B**: Sodium hydroxide (0.1 M); 0.4 gm NaOH was dissolved in 100 ml distilled water.

Phosphate buffer (pH 11) was prepared by mixing 60 ml of solution A with an appropriate amounts of solution B to obtain the required pH.

Procedure:

Fifty microliter of human purified CEA preparation (1.3 mg/ml) was completed to 1 ml with different buffer at different pH values (3 to 11). The samples were then transferred to 0.5 cm cuvette in the sample beam and the buffer at the adjusted pH in reference beam, the absorption spectrum was scanned.

2- Polarity effect:

The CEA was studied spectrophotometerically by the addition of different solvents:

Ethanol, ethylene glycol, glycerol and poly ethylene glycol in a percent of 20% prepared in tris buffer pH 7.2 for example, CEA in 20% ethanol:

A volume of 50µl of purified CEA solution (1.3 mg/ml) was completed to 1 ml with tris buffer pH 7.2 in the presence of 20% ethanol. Each of these mixtures was placed in test cell against 20% ethanol prepared in the same buffer in the reference beam. The absorption spectrum was measured in the area of (200 – 350 nm).

The experiment was repeated by using other solvents individually.

3- Effect of urea, KCl. and urea, KCl mixture on the spectrum of CEA:

Solutions used:

1- Eight molar of urea was prepared by dissolving 24.02 gm of urea in 50 ml of tris buffer at pH 7.2

2- 0.03 M KCl was prepared by dissolving 0.2737 gm of the salt in 50 ml of corresponding buffer.

3- Tris buffer solution was prepared as described in section (B-1).

Procedure:

Fifty µl of CEA was pipetted in a set of three tubes. The volume was completed to 1 ml with tris buffer pH 7.2 contains 0.03 M KCl, 8M urea and mixture 1:1 of both 8 urea and 0.03 M KCl respectively then each of which was placed in 1.0 cm cuvette in the sample beam and the buffer at the same pH in the presence of the same salt in the reference beam.
The absorption spectrum was scanned.

C - Structural Studies of CEA

Solution used:

Preparation of buffers used in these experiments was described in section (B):

Procedure

A series of the samples were prepared at pH ranged from 6 to 11 using different buffers.
The maximum absorbance of each sample was measured at wavelength of 295 nm, the absorbance of λ_{max} at each pH value was plotted versus the corresponding pH.
Another series of samples were prepared at pH ranging from 3 to 8 using different buffer.
The maximum absorbance of each sample was measured at wavelength of 211.0 nm. The absorbance of λ_{max} at each pH value was plotted versus in the corresponding pH.

D - Estimation of Absorption Coefficient (a_s) of CEA

The standard CEA solution with different concentrations (0.15, 0.3,0.5, 0.6, 0.8, 0.9, 1) mg/ml were prepared by serial dilution from a stock solution 2 (mg/ml) with tris buffer pH 7.2 . The maximum absorbance of each sample was measured at wavelength of 25 nm, the absorbance value for each sample was plotted versus the corresponding concentration.

Calculation:

Specific absorption coefficient (a_s) of human CEA at λ_{max} = 25 was calculated using Lambert Beer's law [19] :

$$\mathbf{A = a_s c \ell}$$

Where
A: Absorbance
C: Human CEA concentration (mg/ml)
ℓ: Length of light path in (cm)
$\mathbf{a_s}$:Specific absorption coefficient in ($ml.mg^{-1}.cm^{-1}$) at 25 nm

$$(a_s)_{25} = \frac{A}{c\ell} = \frac{\text{slope of stander curve}}{\text{length of light path in cm}} = (ml).(mg^{-1}).(cm^{-1})$$

E- Fouler Transform Infrared Spectroscopy (FTIR)

Bach of purified CEA sample with concentration (1mg/ml) for Human Mammary carcinoma and CEA standard were lyophoilized. FTIR absorption spectra of CEA were obtained by KBr films with approximately a 1:100 weight ratio of CEA sample to KBr. The water content was rigorously controlled, as this parameter is essntial to stabilize the structure.
All FTIR spectra were measured from 4000 to 250 cm^{-1} by spectrophotometer (FTIR – 8300) SHIMADZU.

Result and Discussion

The Ultraviolet Spectral Results

(Fig 1) shows UV spectra (200–350 nm) for two types of CEA molecules. Standard and purified CEA from human mammary carcinoma at pH 7.2.

The parameters, which are usually measured, at absorbance and wavelength corresponding to a peak of maximum absorption (λ_{max}) consist of multiple peaks, at 25 nm, 235 nm for purified CEA and 275 nm, 230 nm for standard CEA. As a result, purified CEA has characteristic spectra and can be identified by its peaks, the first peak (at 235 nm) is due to amide group in the polypeptide bond of purified CEA molecule with contribution of the histidyl residues, [20]. While the second peak (at 25) is assigned to the side chain chromophor of tyrosin and phenylalanine [14].

The spectrum curve had a shape like a shoulder at near by 290 nm from that tryptophan that gives an ultraviolet absorption spectrum with a maximum at 290 nm. It was suggested that CEA may contain tryptophan as amino acid component[21].

Factors Effecting the Absorption Properties of Purified CEA

The absorption spectrum of purified CEA is primarily determined by the chemical structure of the molecule. However, a large number environmental factors produce defectable change in λ_{max}.

Environmental factor consists of pH, the polarity of solvent or neighboring molecule[20]. These environmental factors effect provide the basis for the use of absorption spectroscopy in characterizing CEA. The general features of these environmental effects on the spectrum of molecule are the following.

1. pH Effect

The pH of the solvent determines the Ionization State of ionizable chromophore in the protein molecule [20].

The UV spectrum of purified CEA was determined at four pH's (1.6, 3, 9, 11) (Fig 2) shows these spectra.

It seems that in acid region (1.6, 3) there were a blue shift in $\lambda_{max,}$ for the peptide bond, as shown in Fig (2) in pH 1.6 shifted to 215 nm while in pH3 shifted to 225.

The blue shift is due to the increasing of hydrogen bond formed in the presence of highly positively charged state [22]. The result is in agreement with Al-kazzaz observation [23].

Also the decrease associated with dispersed λ_{max} 25 nm of tyrosine and phenylalanine due to the conformational changes and the chromophore in native molecule were buried in the interior of their molecules [24,25].

When the pH was increased from neutral to basic region (pH 9,11) There were a significant shift to a longer wavelength (red shift) in λ_{max2} from 25 to near 300 nm, while no significant changes in λ_{max1} value was observed as shown in Fig (1).

The red shift is due to the slightly increase in the energies of electronic transition of the aromatic rings from the formation of the electron - with drowning ammonium groups [26].

It is known that in the acidic region the phenolic OH groups are not ionized, so there is no $\mathbf{n} \rightarrow \pi^*$ or $\pi \rightarrow \pi^*$ transition.

In the neutral pH region, the native organization of CEA is fairly compacting, rigid, and has a remarkably hydrophilic exposed molecular surface. Conformational changes occur in CEA in acid and alkaline pH regions; extensive hydrophobic areas in CEA are exposed by both acid and alkaline transition [25,26].

2. Effect of Solvent Polarity on Purified CEA UV. Spectrum (Solvent Perturbation Studies)

The determination of whether an amino acid is internal or external is carried out by measuring the spectra of a protein in a polar and non-polar solvent. In fact, protein is rarely studied in completely non-polar solvents because most proteins are either insoluble or denatured in these solvents.

The table (1) and (Fig 3) shows, the effect of different solvents on purified CEA at neutral pH 7.2 .

When comparing values of $\lambda_{max,}$ of purified CEA in presence of 20% ethanol, or other solvents with that obtained in the absence of these solvents, *(λ_{max1} = 235.0 nm obtained in pervious experiment)*, (Fig 3) shows that there is a shift towards longer wavelength at 240, 23.6 nm in the presence ethylene glycol and polyethylene glycol respectively. These alterations in the position at λ_{max1} are all due to the inter molecular hydrogen bonding between amide bond in CEA molecule with EG and PEG. The intermolecular hydrogen bonding increase as the concentration of solution increase and additional band started to appear at longer or shorter wavelength [(26)].

There was a significant blue shift (~ 12, 10 nm) in the λ_{max1} for CEA in the presence of 20% of ethanol and glycerol respectively. This blue shift indicated that the protein was defolded (rigid) and the exposed histidyl residues were buried interior the molecule in presence of ethanol and glycerol.

Unfortunately, it will be difficult to detect histidyl difference spectra in protein because of it's high absorbency occurred in the low wavelength region which contributed with peptide bond absorbance, there for high difference of spectrum is caused by tyrosine, tyrptophan and phenylalanin [(13)].

When comparing the effect of the different solvents that were used in this study, on the shift of λ_{max2} which is due to tyrosyl residues in the purified CEA spectrum. A red shift was noticed from 25 nm to 300 nm, which were assigned to new chromophore (tryptophyl residues) appeared on the surface of CEA molecule. These were embedded in an interior region of the protein in the absence of the polyhydroxyl solvent [(27)].

The effect of 20% chloroform, ether, and DMSO at pH 7.2 are shown in table (1). The absence of any λ_{max} was observed, this indicates the proteins were denaturated due to change in the secondary and tertiary structures of the protein[(14)].

3. The Effect of Urea, KCl and Urea - KCl Mixture on the CEA UV. Spectrum

Table (2) and Fig (4) show the effect of 8M urea, 0.03 KCl and a mixture of (1:1) of 8M Urea, 0.03 KCl on λ_{max} of the CEA UV. spectrum at pH 7.2. Comparing the values of λ_{max} of this molecule obtained in absence of urea or KCl with those obtained in presence of 8M urea in table (2), it seems that there was a significant red shift in λ_{max1} of the poly peptide bond, for purified CEA spectrum while λ_{max2} of aromatic amino acid i.e tyrosyl residues in CEA was disappeared.

These results indicate that the molecules solvated with urea (dipole-dipole interaction) produce a red–shift and new chromophore come to the surface. The red shift is due to the intermolecular hydrogen bonding between the oxygen of the amide group and the solvent [(13)].

When 0.03 MKCl was used, there was a slight blue shift (4 nm) in the λ_{max1} of the polypeptide bond as shown in Fig (4), and a new absorption peak appeared in the spectrum near 300 nm which could be assigned to the $\mathbf{n \blacktriangleright \pi^*}$ transition in the aromatic ring of the tryptophyl residues.

Such a blue or a red shift can arise by introducing positive (K^+) or negative (Cl^-) charge near the chromophore (the amide group) which might interact directly with the π electron system of the amide group [(28)].

When 8M urea was mixed with 0.03M KCl, the same shift in λ_{max1} was observed when 8M urea was used alone with CEA. This means that the shift caused by mixture may be due to the effect of urea, but not to 0.03 M KCl.

As was seen, the changes occurred in absorption were near 230 nm and near 25 nm. This was also observed by Glazer who noted that solvent perturbation or denaturation of protein produces many changes in absorption near 230 nm and 25 nm. Some of this change in absorption may be produced by change in the $\mathbf{n \rightarrow \pi^*}$ absorption of polypeptide bonds in the protein either because of a change in their geometrical arrangement, or because of an environmental changes[(29)].

Spectrophotometric pH Titration of Purified CEA (Structural Studies)

Spectrophotometric pH titration is followed the changes in absorbance of the chromophore with increasing pH. Many studies of protein structure require the determination of pka values for proton dissociation from ionizable amino acid side chains, because these values give an indication of the location of the amino acid in the protein. This can often be done spectrophotometrically because dissociation often changes the spectrum of one of the chromophores, the observation of tyrosine dissociation was performed by measuring the absorption at 295 nm (λ_{max} for the ionized form of tyrosine), and the observation of histidine dissociation was carried out by measuring the absorption at 211 nm [30].

(Fig 5) shows the pH titration curves of CEA for tyrosine and histidine respectively. (A) Curve show that the pka values for tyrosine is 9.02, while the pka values for histidine in (B) Curve was equal to 5.5

In (Fig 5), the spectral changes as a function of pH for ionizable groups (i.e., the OH of tyrosin, imidazol of histidine) have the same pka as it would be if they were free in solution then the amino acid tyrosine and histidine in CEA are located on the surface of the protein [14].

From the same fig it was found:

(1)About 53% of tyrosine are located on the surface of purified human CEA molecule.
(2)About 47% of tyrosine residues are buried interior the folded structure of purified CEA.
(3)About 67% of histidine residues are located on the surface of purified human CEA molecule.
(4)About 33% of histidine residues are embedded in the interior region of purified CEA molecule.

The internal tyrosine residues are in strongly non-polar environment. On the other hand, the histidine residues present on the molecule surface of purified CEA and internal histidine residues are likely to be in strongly polar environment.

Estimation of Absorption Coefficient (a_s)

Fig (6) show standard curve of purified CEA at (λ_{max} 25 nm. The specific absorption coefficient $(a_s)_{25}$ was found to be 4.16 $ml.mg^{-1}.cm^{-1}$ according to Lamber Beer's Law as described in section (D).

This is not very high absorption coefficient; its sensitivity would have been detected as more than one- percent.

FTIR Spectroscopic Results [26,31,32]

FTIR spectroscopy serves as another qualitative tool to characterize nondestructively the principal classes of chemical groups that compose the CEAs under study and complements the information obtained from UV spectroscopy.

Comparison of the FTIR spectra of standard CEA and purified CEA in (Fig 7,8), show that both of them exhibit a similar series of bands indicating that the major types of chemical groups present in each of these samples are similar. The frequency positions of the bands remain remarkably constant for all two types of CEA (Table 3).

In this section highlight the most obvious distinctions in the two spectra and related them to the information obtained by UV spectroscopy.

Few groups constitute the major portion of these proteins and thus produce relatively strong absorption in the IR spectra. These groups include alkyl moieties, both methyl ($-CH_3$) and methylene ($-CH_2-$); amides (-NH-(-C=O)-; aromatic ring; carbonyls (-C=O); alkoxy (-C-O-); and hydroxyl (-OH) (Table 3 and Fig 7,8). The spectra indicate that one other important constituent group, sulfhydral (-SH-), are also present, but, based on their relatively lower intensities, they probably occur in somewhat smaller quantities. For they're no marked distinction among the two type of CEA studied with respect to the identity of the major categories of functional groups present in the samples.

FTIR spectroscopy offers several advantages over older dispersive techniques. Among these are enhanced signal – to – noise ratios and higher frequency accuracy and reproducibility. These advantages are particularly important when one wishes to search for relatively small changes in a complex system [31,32].

Table (1): The effect of solvent polarity on λ_{max} value of purified CEA UV spectrum at pH 7
(All details are explained in the text)

solvent	λ_{max1} (nm)	λ_{max2} (nm)
Tris buffer (H_2O)	235	25
20% ethanol	222.4	300
20% EG	240	300.8
20% glycerol	225	300
20% PEG	23.6	300.6
20% chloroform	-	-
20% DMSo	-	-
20% Ether	-	-

Table (2): The effect of 8M urea, 0.03 M KCl and mixture of urea+KCl on the λ_{max} of CEA Spectra at neutral pH (7.2)
(All details are explained in the text)

solvent	λ_{max1} (nm)	λ_{max2} (nm)
Tris buffer (pH 7.2)	235	25
8 urea	249.6	-
0.03 KCl	230.4	297
Urea+KCl mix(1:1)	23	-

Table (3): infrared band of CEA (All details are explained in the text)

Band cm^{-1}	Proposed assignment [122]	Relative intensity *
3300 br	-OH stretch (hydrogen-bonded hydroxyl)	Vs
3068	-CH- stretch (aromatic; olefinic)	W
2930	$-CH_2$-asym stretch	W
235	$-CH_3$-asym stretch	Vw
2340	$-CH_2$-asym stretch	Vw
1658	Amide I (hydrogen-bonded-C=0 stretch	Vs
1539	Amide II (hydrogen-bonded-NH bend)	S
1450	$-CH_3$ asy bend, $-CH_3$ sym bend (methoxy); aromatic ring stretch (ortho-di substituted)	Ms
1394	CH_3 sym stretch at 2 or 3° carbon	S
1247	-C-O-stretch (aromatic ether, AOR; acid dimers with electron with drawing group); amide III (-C-N-Stretch)	Ms
1168	Aromatic ring bending (para-and ortho-disubstituted); C-O-C Stretch (alky / ether)	S
1118	-C-O-Stretch (hydrated polyols and carbohydrates)	Ms
933	-OH- bend carboxylic acid dimer	Ms
700	?	
621	?	
528	Ring bend caromatic, para disubstituted	Mw

* Relative peak intensity: vs, very strong; s, strong; ms, medium strong; m, medium; mw, medium weak; w, weak; vw, very weak.

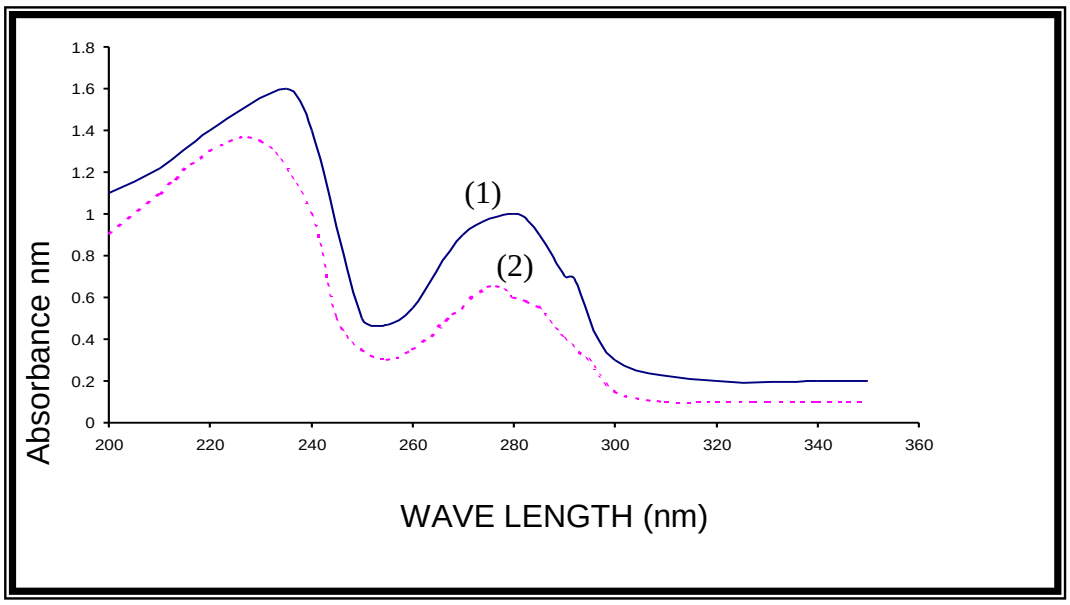

Fig (1) : The UV. spectrum of human CEA in Tris buffer at netural pH (7.2) ,(1)highly purified CEA from mammary carcinoma (2) standard CEA
(All details are explained in text)

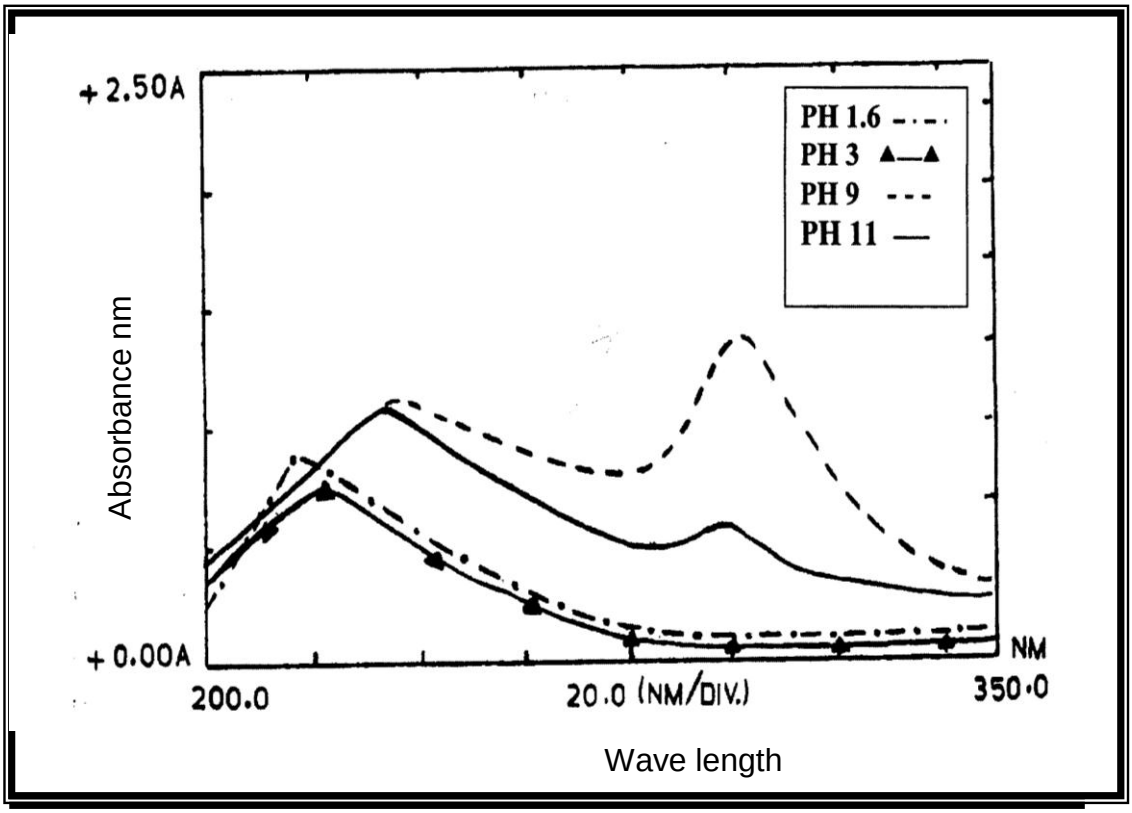

Fig. (2): The effect of pH on UV Sepectrum of human mammary Carcinoma
(All details are explained in the text)

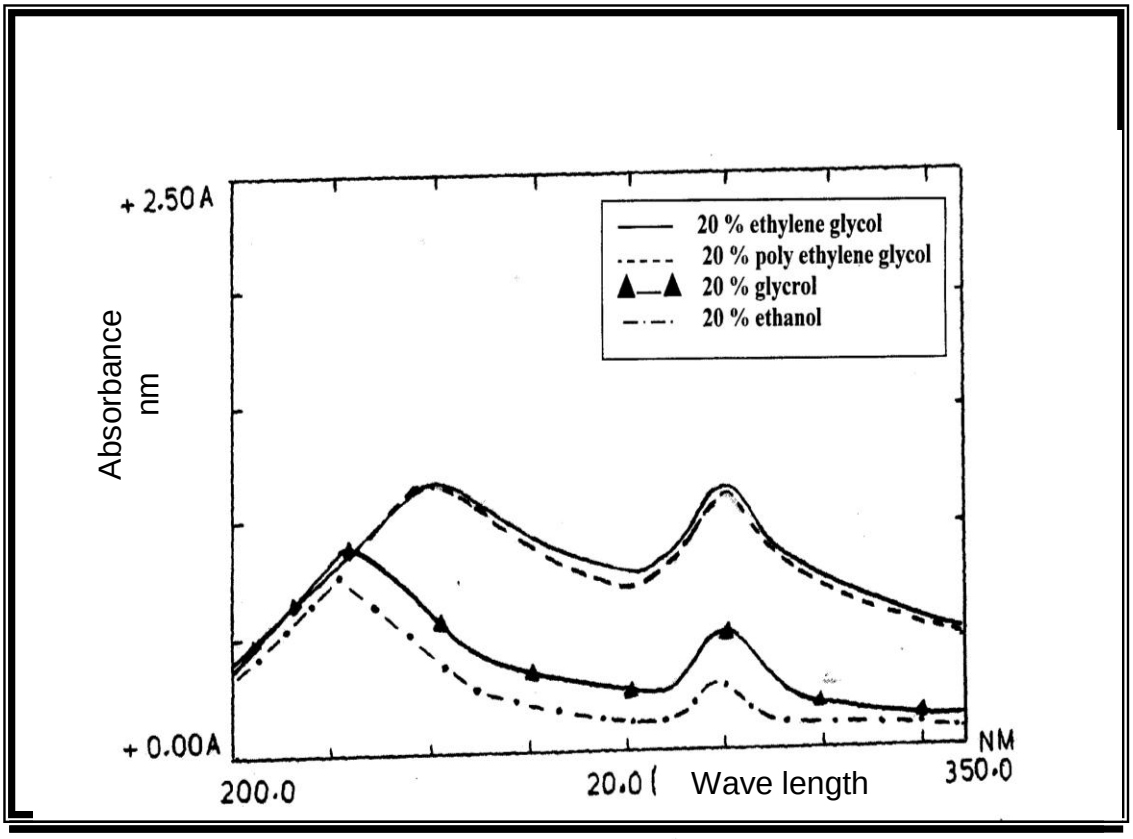

Fig. (3): The difference spectra obtained with human CEA from mammary carcinoma at neutral pH (7.2) in presence 20% ethylenglyycol, 20% polyethylenglyycol, 20% glycrol, 20% ethanol.
(all details are explained in the text)

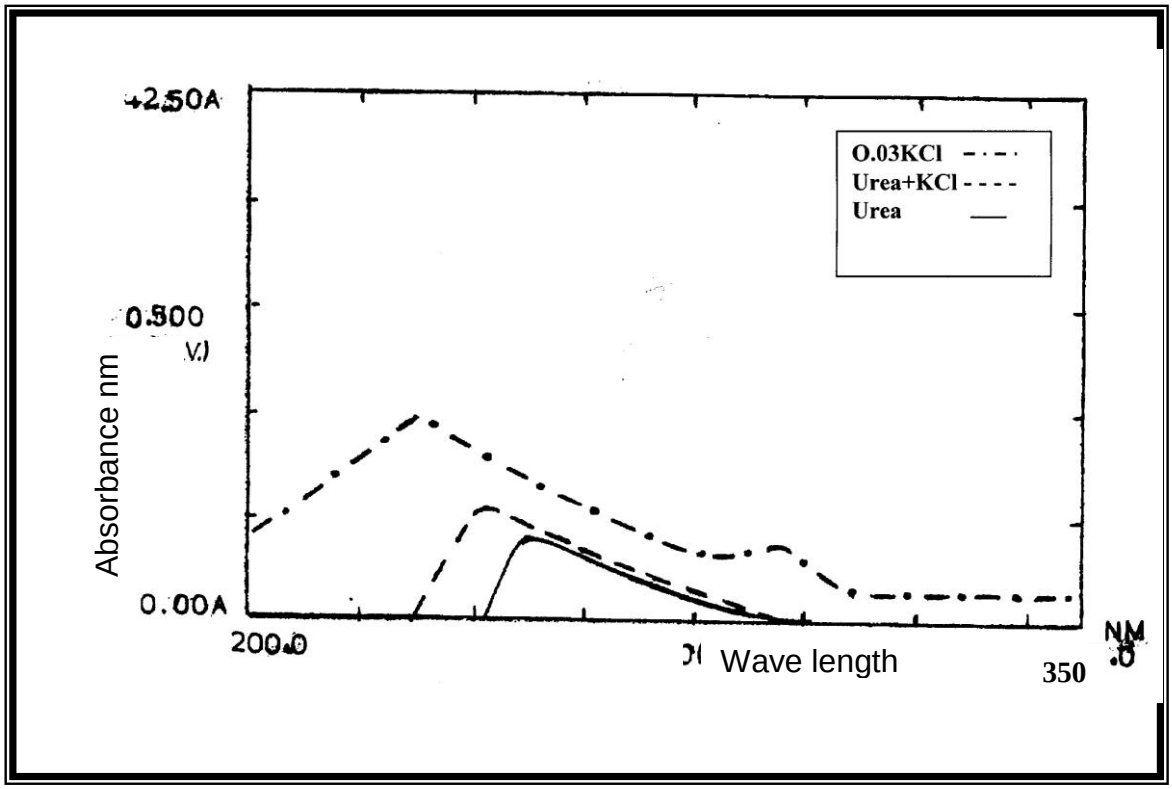

Fig. (4): The effect of 8M-Urea, 0.03 MKCl and mix (1:1) of 8M urea+0.03 MKCl on the human CEA UV spectrum at pH 7.2
(All details are explained in the text)

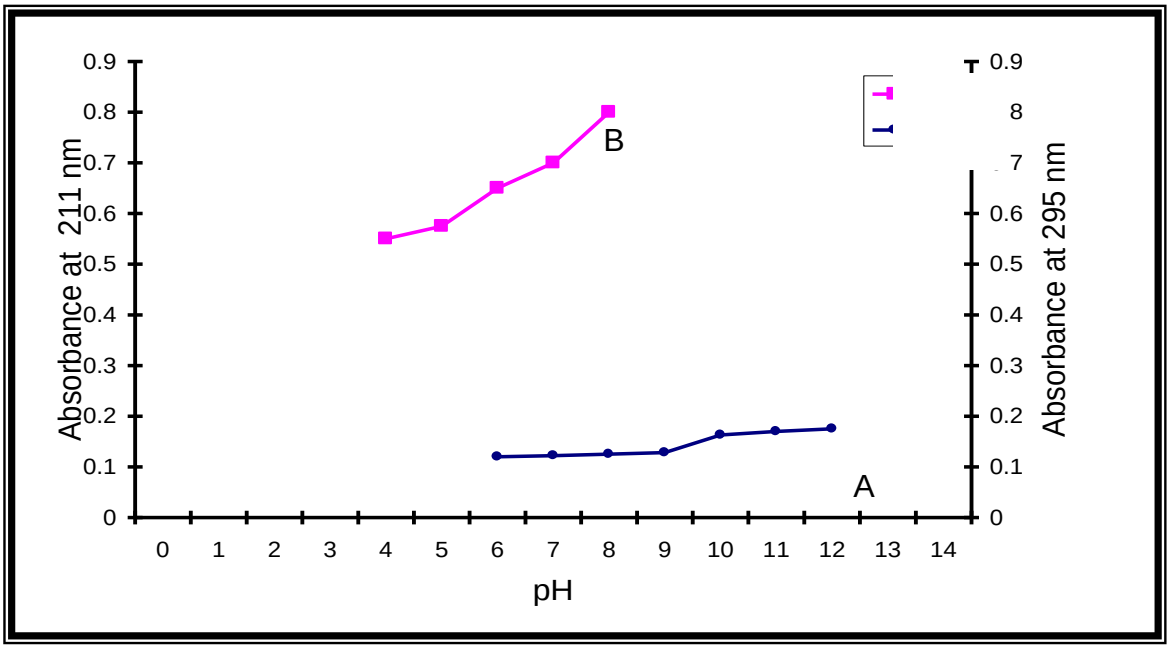

Fig. (5): Spectrophotometric titration of human CEA for:
(A) Tyrosine residues
(B) Histidien residues
(All details are explained in the text)

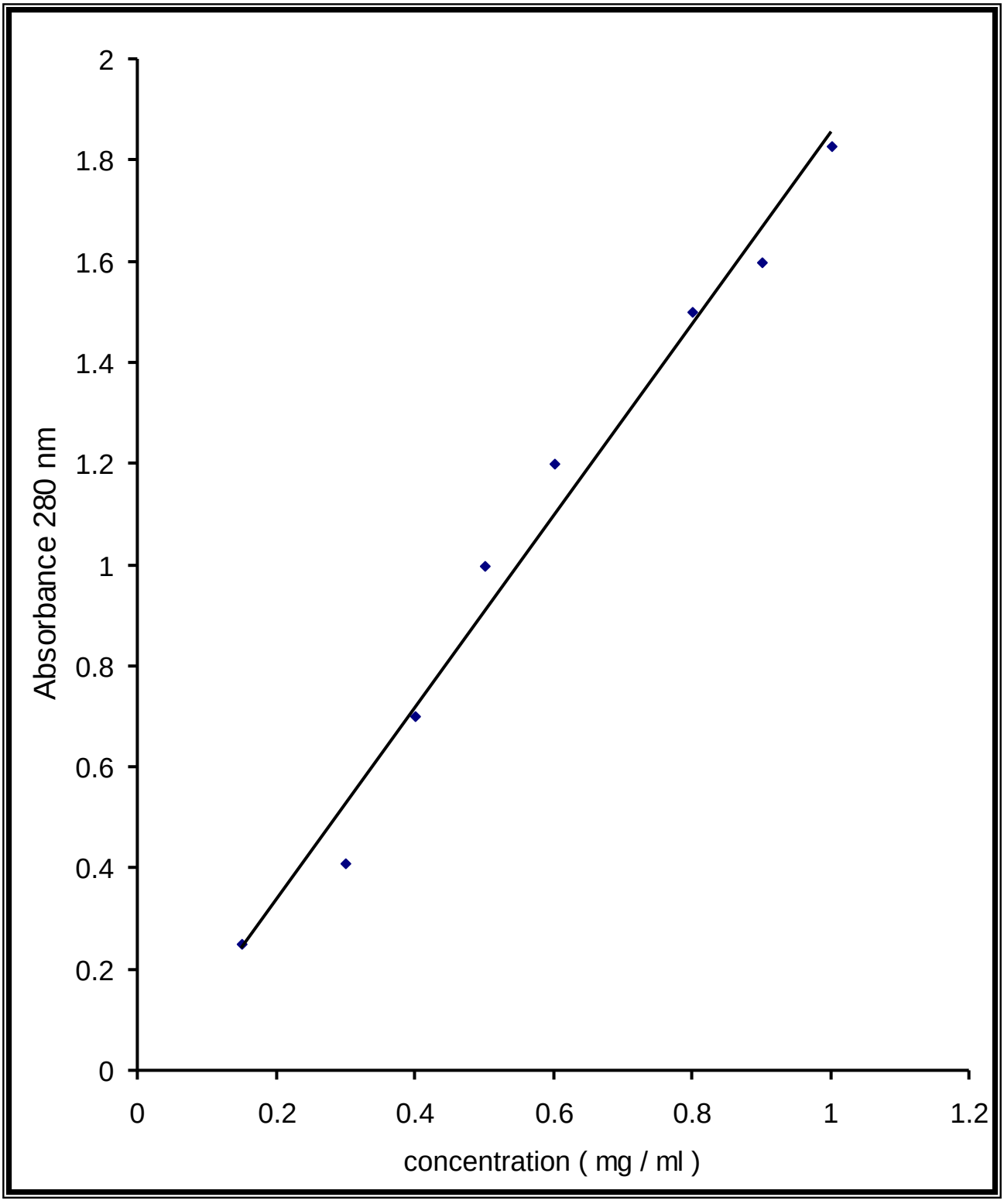

Fig. (6): Calibration curve for estimation of Absorption Coefficient (a_s)
(All details are explained in the text)

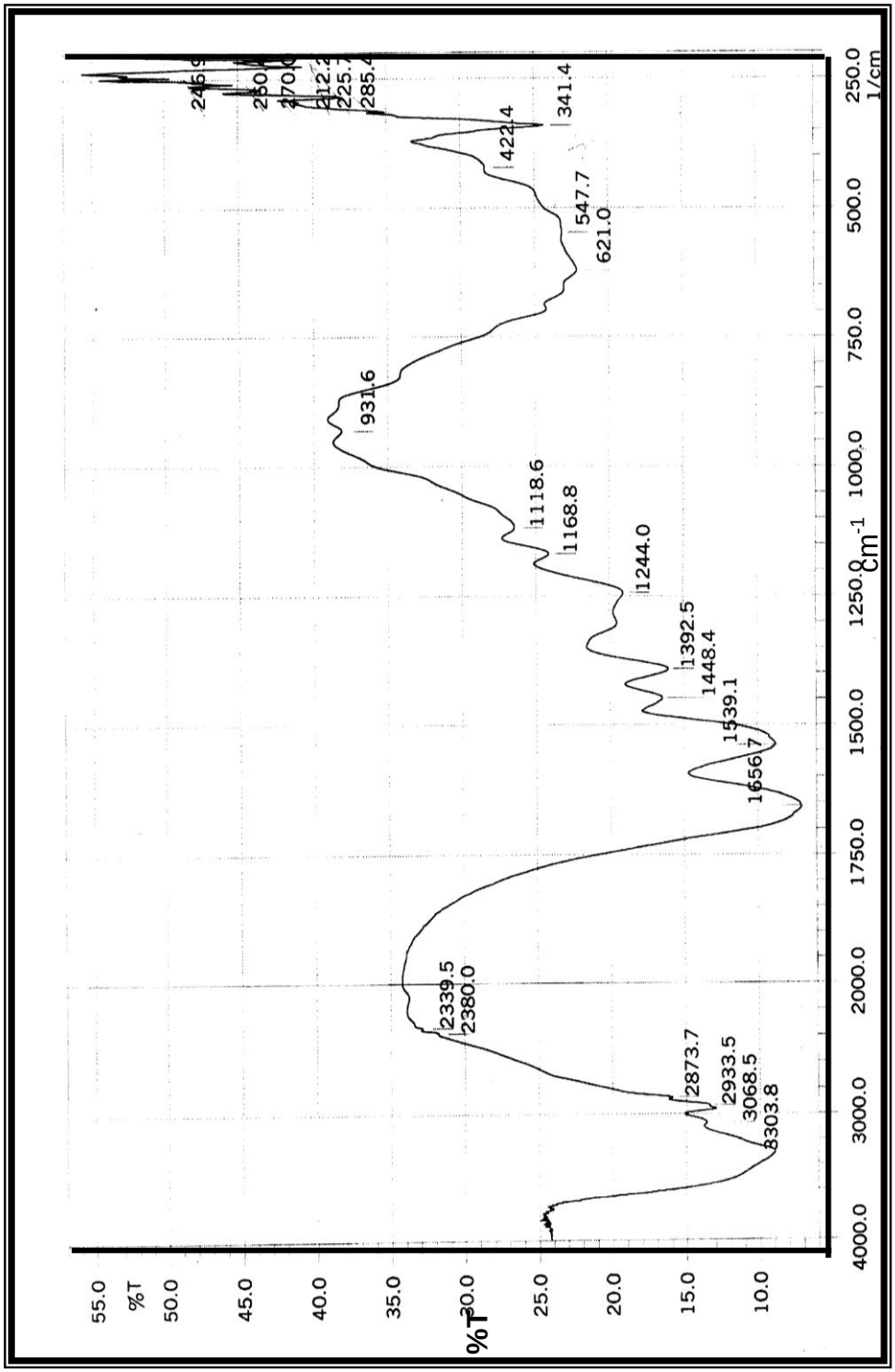

Fig. (7): FTIR Spectra of human CEA from mammary carcinoma
(All details are explained in the text)

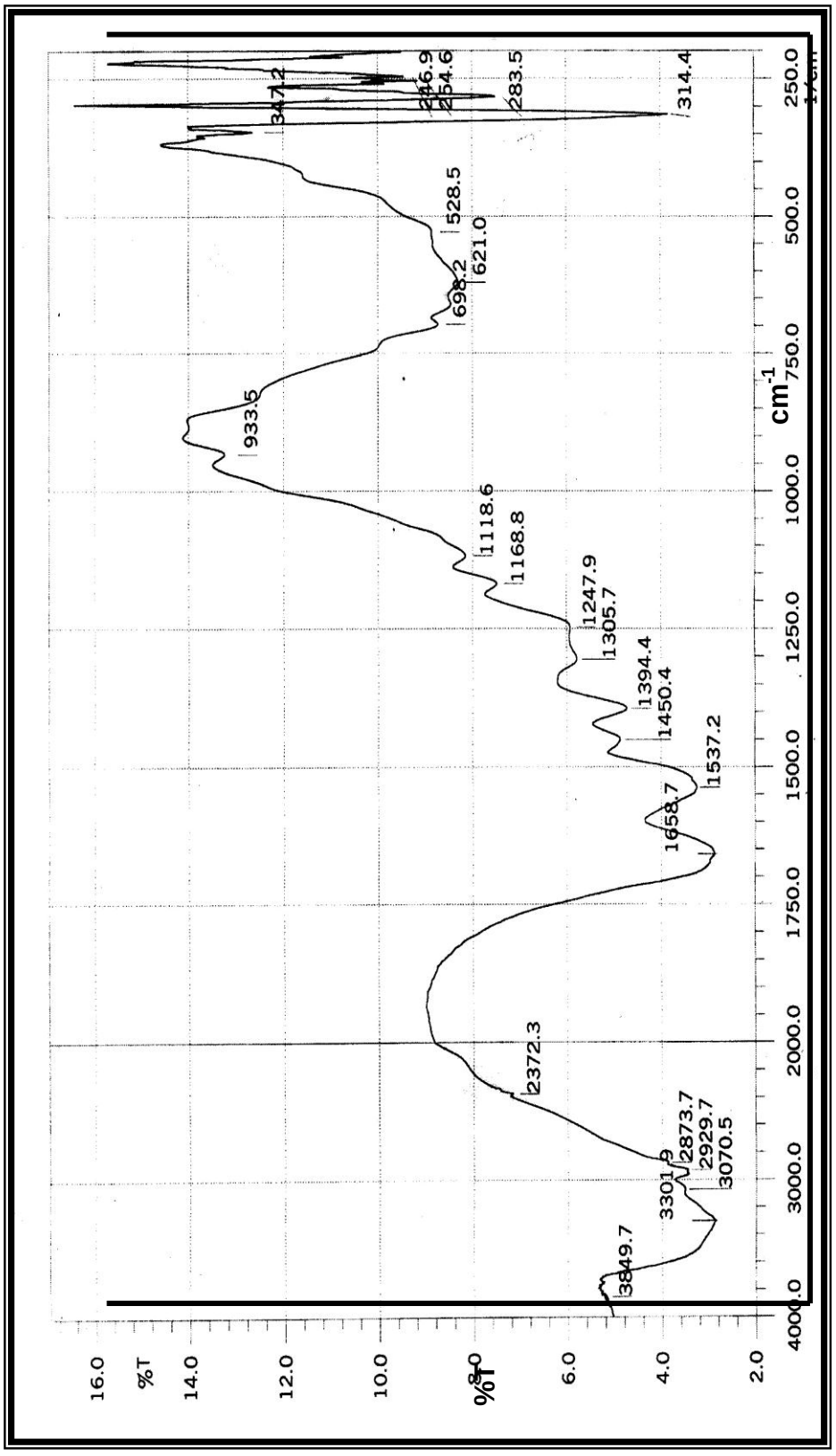

Fig. (8): FTIR Spectra of standard CEA
(All details are explained in the text)

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