CORRELATION OF FTIR SPECTRA OF PROTEIN GELS TO RHEOLOGICAL MEASUREMENTS OF GEL STRENGTH

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STUDY OF PROTEIN GELS BY FTIR SPECTROSCOPY

ABSTRACT

Globular proteins are important ingredients in many food formulations because of their nutritional value and their various functional properties such as gel formation. Proteins form gels by polymerization into a three-dimensional matrix. The rheological properties of the resulting viscoelastic solids can be obtained by Instron measurements. In the present work, gels were obtained by heating solutions of bovine serum albumin (BSA) in D_2O and egg albumin in H_2O under different conditions (i.e., pH, salt concentration, protein concentration, time of heating and temperature), and their gel strengths were measured by a compression test (Universal Testing Machine LRX). The Fourier transform infrared (FTIR) spectra of the same gel samples were recorded in order to investigate the changes in protein structure at the molecular level accompanying gel formation. Intermolecular β-sheet formation was found to increase as gel formation progresses at the expense of intramolecular β-sheet and α-helix structures. For BSA, maximum gel strength was obtained around pH 7. The addition of salt had little effect on the gel strength while increase in protein concentration, time of heating and temperature increased the gel strength. The rate of denaturation of BSA and ovalbumin and of mixtures of these proteins in the ratios 9:1, 1:1, and 1:9 was also investigated by measuring the peak height of an aggregation band at 1618 cm^{-1}; some inhibitory effects of BSA on ovalbumin aggregation were observed. Correlations between the measured gel strengths and the amide I band profile in the FTIR spectra were examined using partial-least-squares (PLS) regression. These studies reveals that gel strengths of a particular protein gel could be adequately predicted from their infrared spectra without the need to carry out the rheological compression measurements.

RÉSUMÉ

Les protéines globulaires entrent dans la formulation de beaucoup d'aliments de part leur valeur nutritionnelle et leurs différentes propriétés fonctionnelles comme celle de la gélification. Ces protéines forment des gels en se polymérisant en une matrice tridimensionnelle. Les propriétés rhéologiques des solides visco-élastiques ainsi obtenus peuvent être mesurées rhéologiquement. Les gels utilisés dans cette étude ont été obtenus en chauffant une solution d'Albumine de Sérum Bovin (BSA) dans du deutérium (D_2O) sous diverses conditions de pH, concentration en sel, concentration protéique, temps de chauffage et température. La fermeté de ces gels a été mesurée par un test de compression (Machine Expérimentale Universelle LRX). Les spectres Infra-rouge à Transformée de Fourier (FTIR) des mêmes échantillons de gel ont été enregistrés pour déterminer quels sont les changements de structure protéique qui - au niveau moléculaire - sont à l'origine de la formation du gel. Les corrélations entre les mesures de la fermeté des gels et les bande d'absorption Amide I des spectres Infra-rouge ont été étudiées à l'aide de la Méthode des Moindres Carrés partiels (PLS). Il a été démontré que la formation de feuillets β-inter-moleculaires augmente au fur et à mesure de la formation du gel, aux dépens des structures irrégulières et en α-hélice. Concernant la BSA, la fermeté de gel maximale a été obtenue autour du pH 7 et elle augmente avec la concentration protéique, le temps de chauffage et la température. Le taux de dénaturation de solutions pures de BSA, d'Ovalbumine et de mélanges de ces deux protéines dans les proportions 9:1, 1:1 et 1:9 a également été étudié en mesurant la hauteur du pic à 1618 cm-1, correspondant à une agrégation protéique . L'expérience a montré que la BSA a un effet d'inhibition sur l'agrégation de l'Ovalbumine au cours du processus de gélification.

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LIST OF ABBREVIATIONS

ATR = Attenuated total reflectance

B1O1 = (1/1) BSA/ovalbumin mixture 50/50

B1O9 = (1/9) BSA/ovalbumin mixture 10/90

B9O1 = (9/1) BSA/ovalbumin mixture 90/10

BKG = Background

BSA = Bovine Serum Albumin

CaF_2 = Calcium fluoride

CLS = Classical least squares

DTGS = Deuterium triglycine sulfate

FTIR = Fourier transform infrared

ILS = Inverse least squares

IR = Infrared

M = Mean

PCR = Principal component analysis

PLS = Partial-least-squares

PRESS = Predicted Residual Error Sum of Squares

SD = Standard deviation

SE = Standard error

SUM = Summation of error

T^o = Temperature

ZnSe = Zinc Selenide

Chapter 1 Introduction

Globular proteins exhibit characteristics critical to the preparation of many food products such as water holding capacity, excellent foaming and emulsification properties, high nutritional value and ability to coagulate and form gels upon heating. Heat coagulation is one of the most important functional properties of proteins in terms of their application in the food industries as food ingredients.

The initial step in heat-induced gelation of globular proteins is heating of a protein solution above the denaturation temperature of the protein. The primary importance of the denaturation process is to expose the functional groups (such as CO and NH of peptide bonds, polar and hydrophobic groups of the side chain amino acids), in the interior of the protein to the surrounding solvent molecules. Under appropriate conditions, these functional groups also interact with each other through intermolecular interaction to form a three-dimensional gel network that entraps and restricts the motion of the solvents (Ziegler and Foegeding 1990). The aggregate of heat-denatured protein molecules can be formed through intermolecular hydrophobic interaction, disulfide bonds, hydrogen bonds or electrostatic interaction (Kinsella 1989). Hydrophobic interaction (Kohnhorst and Mangino 1985) and disulfide bonds (Matsudomi et al 1991) are considered the strongest contributors to developing stable aggregate and gel networks in whey proteins. Since proteins undergo structural changes during thermal gelation, the extent of denaturation and exposure of functional groups may affect the extent of network formation. Conversely, the state of protein conformation in gels may have a bearing on the ultimate physical characteristics of protein gels. Gel formation

depends on extrinsic factors such as protein concentration, pH, ionic strength and temperature at which the protein solution is heated. As a consequence the combination of these factors will give rise to different rheological properties for a given protein.

Rheological properties of gels, in general, are associated to texture. Texture of foods is regarded by the processing industry as a key quality parameter in the development and acceptance of new food products and formulations, as well as in the grading and quality control of traditional food products. The characteristics of perceived "texture" are determined by different physico-chemical properties of the food and by the unique and complex features of the human sensory systems. It can be argued, however, that the stimulus in texture perception is predominantly mechanical in nature (Moskowitz, 1987). Consequently, most, if not all, of the instrumental methods of texture evaluation can also be classified as mechanical tests. In order to be able to establish the relationship between rheological properties of the gel and their functionality, it is essential to utilize other instrumental techniques that can provide additional information on gel formation at the molecular level.

Spectroscopic methods are routinely used to study protein conformation. Fourier transform infrared (FTIR) spectroscopy, in particular, has been shown to be very useful for investigating secondary structural changes of proteins (Kato, 1987, Fen-nifu, 1994, Kirsch, 1989, Clark, 1981, Frushour, 1975, Bandekar, 1980). The amide group of polypeptides and proteins possess nine characteristic vibrational modes or group frequencies (Susi and Byler, 1986). Differences in geometric orientation of the amide group in α-helix, β-sheet, turns and random coil structures give rise to differences in vibrational frequencies and distinct IR

absorptions. With the use of resolution enhancement techniques, such as Fourier self-deconvolution, FTIR spectroscopy provides high sensitivity for protein secondary structure analysis.

In the present work, the changes in secondary structure accompanying the thermal denaturation of two important food proteins, bovine serum albumin (BSA) and ovalbumin, were investigated by variable-temperature FTIR spectroscopy. Gels were then obtained by heating solutions of BSA and egg albumin under different conditions, and their gel strengths were measured by a compression test performed using a Universal Testing Machine (Instron). The FTIR spectra of the same gel samples were recorded employing an attenuated total reflectance (ATR) accessory in order to investigate correlations between measured gel strength and the IR absorption bands of the proteins. Partial-least-squares (PLS) regression was employed to develop calibration models for the prediction of gel strength directly from the infrared spectra of gel samples. In order to understand the effect of various physicochemical parameters (i.e., pH, salt concentration, protein concentration, time of heating and temperature) on gel strength, each parameter was independently investigated and rheological properties of the protein gels were studied in detail. Gel strengths obtained by Instron for each gel prepared under varying physicochemical conditions were then compared to gel strengths predicted from infrared spectra through the application of PLS.

This research focuses on the factors affecting rheological properties of protein gelation, employing infrared spectroscopy to elucidate the changes in protein structure during the gelation process. This work also focuses on correlating the changes in infrared spectra of protein gels to their gel strength employing multivariate analysis techniques.

In the next chapter a short review on the nature of gels will be presented. Although no exact definition of a gel will emerge, it is hoped that what is said will make the essential nature of gels and gel character much clearer. Attention will then turn to processes available for generating gels from aqueous solutions of polymers, and to the underlying network-building mechanisms involved. Here, emphasis will be placed on the critical nature of the gelation event (at the gel point), and various theories of gelation (classical and modern) will be reviewed. At this point physical methods available for monitoring the gelation process will also be considered. Among these, rheological measurements will be singled out as particularly important means of identifying the gel properties and characterizing gel systems. This emphasis on mechanical behavior of gels will then be extended to include description of experiments designed to monitor gel behavior under different conditions.

In Chapter 4, the use of infrared spectroscopy to monitor the thermal denaturation of BSA and ovalbumin as well as of mixtures of these proteins will be described. The effect of physico-chemical conditions on gelation of BSA will be discussed in Chapter 5 while egg albumin gelation under different conditions will be examined in Chapter 6. These chapters will focus on the quantitative analysis of heat-induced coagulation or gelation of the proteins. Factors affecting gelation and the rheological properties of the gels are discussed, as well as attempts to correlate the infrared spectra of protein gels to their gel strengths measured by rheological compression tests. In the conclusion, some attempt will be made to assess what has been achieved so far by physical gel studies followed by what still remains to be done.

Chapter 2 Literature Review on Protein Gelation

2.1 Introduction

The term "*functional property*" has been defined as any physico-chemical property which affects the processing and behavior of proteins in food systems, as judged by the quality attributes of the final product (Kinsella 1976). Elsewhere, and less specifically, any property of a food or food ingredient except its nutritional ones that affects its utilization has been suggested (Pour-El 1981). Neither definition fully sums up the food manufacturer's view that functional proteins are used to produce or stabilize certain structures in foods which are attractive to customers. In particular, functional proteins are used to emulsify, foam, to heat-set or gel, bind fat and hold water or modify its flow properties. Similarly, proteins included in food to induce or promote color and flavor are seldom regarded in practical terms as being 'functional', although their use depends on attributes other than nutritional value. This section concentrates on the key functional properties of proteins from a food manufacturer's point of view, but also devotes some attention to structure. This is not itself a functional property, but is usually a prerequisite of functionality and, therefore, of importance in functional testing.

2.2 Protein Structure-Function Relationships

The determination and understanding of the relationship between structure and function of proteins plays an important role in the field of biochemistry. Substantial progress in identifying the functional properties of proteins has been made, while progress in obtaining structural information has been very limited

(Singh et al, 1994). Many workers have attempted to relate the molecular structure or physico-chemical properties of proteins to their functional properties (Kato *et al.* 1983; West *et al.* 1985; McWatters and Holmes 1979; Mangino 1984). The results are not always clear-cut. The presence of hydrophobic bonds and hydrogen bonds, while important in some gels, is detrimental to the strength of others (Howell and Lawrie 1985). Although surface hydrophobicity has correlated well with foam stability, the composite content of hydrophobic amino acids shows no such relationship (Kinsella 1981; Horiuchi 1978). Many workers have shown positive correlations between emulsifying activity and surface hydrophobicity (Kato *et al.* 1983; Shimizu *et al.* 1985; Kato and Nakai 1980; Nakai *et al.* 1981; Kato *et al.* 1981; Matsudomi *et al.* 1982). This is not always so, however; β-lactoglobulin displays relatively low emulsifying activity at acid pH values where its surface hydrophobicity is greatest (Shimizu *et al.* 1985).

Prediction of protein functionality from determination of molecular structural parameters may be possible for well-defined soluble, globular proteins. However, such predictions for commercial protein products are hampered in several ways. For example, commercial proteins are often heterogeneous. Whey protein concentrates contain β-lactoglobulin, α-lactalbumin, BSA, immunoglobulins and several minor components (Kinsella 1976; Kilara 1984; Morr 1985). Each of these may contribute to commercially useful functionality in a different way. Conditions which induce changes in protein structure affect functional properties too. Thus, the preparation and processing of proteins for ingredient use influences their functionality. Operations such as heating, cooling, ultra filtration and electrodialysis are known to have such effects (Kinsella 1976; Kinsella 1979; Aalbersberg and Moor 1985; Nash *et al.* 1971; De Wit 1983). These are in addition to the more obviously drastic changes which result from intentional chemical modification. The processing of commercial proteins may

result in their being mixtures of native, partially denatured and wholly denatured material. Whereas soluble single proteins such as BSA generally exhibit characteristic pH and salt-solubility profiles (Lehninger 1975; Mahler 1967), mixed protein preparations do not (Kinsella 1979; Kelly *et al.* 1983). In relation to partially denatured whey proteins, this difficulty has been put forward specifically as limiting the use of solubility data in the prediction of functional properties (De Wit 1983). Such prediction for many commercial proteins may thus be less than straightforward, even without considering the complexities of the food product itself.

It should be recognized that proteins often exhibit several functional properties and that many applications demand a combination of functional attributes. These properties may be required simultaneously or in a sequence dependent on processing. For example, in a cooked comminuted meat product, functional proteins may be called upon to emulsify the chopped mix, to gel on heating, and subsequently to hold water and fat in a matrix stable to refrigerated storage and, possibly, to subsequent re-heating before consumption. Such polyfunctionality is unlikely to be revealed by simple application of the more fundamental tests of physico-chemical behavior. Generally, however, since solubility and, often, hydrophobicity are essential for exhibiting functional properties, the determination of these parameters may give an indication of potential functionality (Patel and Fry 1987).

Structure formation can also be studied using a rheological scan. A nondestructive test producing continuous or intermittent data during the processing schedule is required. Results are in term of material rigidity (a modulus) and the measure of the proportion of input mechanical energy conserved (elasticity). Results depend on the functionality of the proteins and other ingredients,

processing parameters, including the time/temperature history, and test conditions (Hamann, 1987).

2.3 Gels and gelation

The importance of gels and gelling processes to food technologists is undeniable. In food materials, gelling components fulfill many functions, particularly in relation to texture and stability, and they of course significantly influence processing procedures. Their importance is especially great at the present time when the need to replace fat in foods has given rise to many new applications of water-based gelling systems.

An important function of proteins in food systems is gelation. This phenomenon involves the formation of a three-dimensional matrix mainly through inter-protein hydrogen bonding and allows the immobilization of water within the gel structure. The coagulation or gelation of proteins-in particular, the irreversible, heat-induced coagulation or gelation of egg proteins-often controls the success of certain cooked food products. It is of interest to the food scientist to be able to quantitatively monitor the gelation process to better predict end-product characteristics, as well as understand the mechanism of network formation. A better understanding of the gelation process will permit manipulation of variables to obtain a gel of desired textural characteristics and functional properties (Gossett et al, 1984).

Protein concentration is critical to network formation. Minimum protein concentrations required for gelation range from 4% for bovine serum albumin (BSA) (Paulsson *et al.* 1986) to 8-10% for whey protein (Mulvihill and Kinsella

1987) and 14% for casein based milk gels (Kalab and Harwalkar 1974). In addition, an increase in protein concentration above the critical protein concentration (CPC) results in increased values for either gel viscosity (Ehninger and Pratt 1974; Bau *et al.* 1985) or hardness (Beveridge *et al.* 1980; Mulvihill and Kinsella 1987; Ma *et al.* 1988) as well as the G' (storage modulus) obtained with more fundamental rheological studies (Beveridge *et al.* 1984; Bikbov *et al.* 1985; van Kleef 1986). Attempts have been made to account for both the CPC and variations in hardness or G' to get a master concentration dependence which is independent of the protein source. In this respect, a single curve has been obtained for BSA which fits the theoretical prediction based on the relationship:

$$p = (Kf/M)C$$

where p is the extent of conversion to a network, K is the affinity for cross-linking between functional groups, f is the number of functional groups, M is the molecular weight and C is the concentration (Clark and Lee-Tuffnell 1986). The suitability of this theoretical treatment to describe other protein gelling systems is unclear due to the limited amount of fundamental data available on food protein systems.

2.4 Terminology

A discussion of gelation necessitates defining some commonly used terms associated with the phenomenon. At first glance, some of these terms may appear to overlap in meaning. However, each has its particular role in this discussion.

- **Denaturation** is (1) the process in which a protein or polypeptide is transformed from an ordered to a disordered state without rupture of covalent bonds (Scheraga 1961), or (2) any process , except chemical modification, not involving rupture of peptide bonds which causes a change in the three-dimensional structure of a protein from its "native" in-vivo form (Haschemeyer and Haschemeyer 1973). Denaturation involves protein-solvent interactions and leads to changes in physical properties, such as loss of solubility of the protein. Sometimes unfolding of the protein structure is considered part of denaturation.

- **Aggregation** is a general term referring to protein-protein interactions, with formation of complexes of higher molecular weights (Hermansson 1979). Aggregation is usually governed by a balance between attractive and repulsive forces. Attractive forces can involve hydrogen bonds, covalent bonds such as disulfide linkages, and hydrophobic associations, whereas repulsive forces can involve coloumbic forces which are affected by the net charge of the protein molecule or the ionic strength of the solution (Egelandsdal 1980).

- **Coagulation** is the random aggregation of already denatured protein molecules (Hermansson 1979), in which polymer-polymer interactions are favored over polymer-solvent reactions (Schmidt 1981). The coagulum is often turbid and the formation of the coagulum is usually thermally irreversible (Shimada and Matsushita 1981). A coagulum may settle out of solution.

- **Gelation** is an orderly aggregation of proteins, which may or may not be denatured, forming a three-dimensional network (Hermansson 1979). Polymer-polymer and polymer-solvent interactions, as well as attractive and repulsive forces, are balanced such that a well-ordered matrix can be formed (Schmidt

1981). The gel may be turbid or translucent; in the latter case, the gel may be thermoreversible (Shimada and Matsushita 1981) The term gelation is also used in another context with respect to egg-yolk proteins. The phenomenon of egg-yolk gelation refers to the formation of an irreversibly gelled product upon freezing of the yolk (Cotterill 1977). However, in this thesis, the term gelation will refer to the former definition only.

2.5 Theory of gelation

The mechanisms underlying the formation of the three-dimensional networks characteristic of protein gels are not fully understood and have not been systematically studied. However, it seems that "some" protein denaturation is a prerequisite to the formation of an ordered protein-protein gel structure. (Ferry, 1948 ; Hermansson, 1979).

The classic explanation of heat-induced aggregation of protein molecules is the following two-step process (Ferry 1948):

Native protein	→	**denatured protein**	→	**aggregated protein**
		(long chains)		(associated network)

The first step is considered a denaturation process and the second step an aggregation process. Comparison of the rate of the denaturation step vs that of the aggregation step helps determine gel characteristics. For example, Ferry (1948) suggested that for a given rate of denaturation, the rate of aggregation will be slow if the attractive forces between the denatured protein chains are small. The resulting gel will be a finer network of protein chains, will be less opaque, and will

exhibit less syneresis than one with a faster rate of aggregation. A coarser network of protein chains yields an opaque gel with large interstices capable of holding solvent which is easily expressed from the matrix. Hermansson (1979) suggested that conditions favoring denaturation, such as high or low pH, have the opposite effect on aggregation of globular proteins, possibly due to the fact that at high net charge, protein-solvent interactions are favored, rather than protein-protein interactions leading to aggregation. A gel network with a certain degree of order can be attained if the aggregation step occurs more slowly than the denaturation step, thus giving the denatured protein molecules time to orient themselves before aggregation; such a gel is lower in opacity and higher in elasticity than one where aggregation is not suppressed. Schmidt (1981) suggested that if aggregation occurs simultaneously with denaturation, an opaque, less-elastic gel results.

Mulvihill (1987) proposed that the final gel state corresponds to aggregates of partly denatured protein and involves the following mechanism :

$$x\,PN \xrightarrow{\text{heat}} x\,PD \xrightarrow{\text{heat and/or cooling}} (PD)x$$

where x is the number of protein molecules, PN is the native protein, and PD is denatured protein. Thus, gelation is a two-stage process involving an initial denaturation or conformational alteration of the native protein induced by heat. This results in the presence in solution of several species of protein molecules in different states of denaturation and having different properties. These may gradually associate to form the gel matrix if attractive forces and thermodynamic conditions are suitable. The overall process requires that the proteins unfold or undergo some conformational change initially and that the second aggregation step

proceeds relatively more slowly than the first to allow the denatured protein molecules to orient themselves and interact at specific points, thus forming a three-dimensional network (Ferry, 1948 : Hermansson, 1979). When aggregation occurs very rapidly, a coagulum, characterized by high opacity, low elasticity, and considerable syneresis, is obtained. Tombs (1974) and Hermansson (1979) noted that coagulation occurs essentially when random protein-protein interactions predominate to form large molecular weight aggregates. Thus, when the heating conditions are extreme, protein molecules may not have time to align themselves in an ordered fashion ; under these circumstances, poorly hydrated aggregates or precipitates, which lack the continuous matrix of gels, are formed. The ability of denatured proteins to associate and coagulate, precipitate, or gel depends upon the protein ; its amino acid composition ; molecular weight ; net hydrophobicity ; and concentration (Shimada and Matsushita, 1980a) ; heating and cooling rates (Foegeding et al., 1986) ; and the critical balance between attractive and repulsive forces (Schmidt, 1981 ; Ferry, 1948; Kalab et al., 1971; Eagland, 1975; Egelandsdal, 1980; Babajimopoulos et al., 1983; Kinsella, 1984).

Strong attractive forces generally cause coagulation, while strong repulsive forces inhibit coagulation. Hydrophobic interactions between apolar segments of adjacent polypeptides are enhanced by heating and may be involved in the initial restructuring of the polypeptides. Hydrogen bonding (enhanced by cooling), particularly in hydrophobic regions, has been reported to be involved in soya and gelatin gels (Eagland, 1975; Babajimopoulos et al., 1983). Electrostatic interactions (bridges with Ca^{2+} and other divalent ions) between specific residues in adjacent polypeptides are known to be important (Kalab et al., 1971; Schmidt, 1981). Disulfide cross-links or thiol-disulfide interchange are involved in stabilizing several protein gels (Egelandsdal, 1980; Schmidt, 1981). Electrostatic

repulsion (especially at high or low pH) tends to keep polypeptide chains apart. Some water in gels may be hydrogen bonded to hydrophilic groups, involved in dipolar interactions with ionic groups or structured around hydrophilic groups (Labuza and Busk, 1979; Busk, 1984). This protein water interaction also tends to reduce protein-protein complex formation.

The pH range over which gelation occurs generally increases with increasing protein concentration. Thus, the numerous hydrophobic and disulfide bonds formed at high-protein concentrations can compensate for the repulsive electrostatic forces associated with pH values well removed from the isoelectric point (Circle et al., 1964; Shimada and Matsushita, 1980b, 1981). Large proteins (>60 kDa) which have high molar percentages of hydrophobic amino acids (>31.5%), such as egg albumin, exhibit gelation/pH ranges dependent on concentration (Shimada and Matsushita, 1980a). On the other hand, large proteins with low molar percentages of hydrophobic amino acids (22-31.5%), such as conalbumin, and small proteins such as β-lactoglobulin with a high molar percentage of hydrophobic amino acids (34.6%) are concentration-independent (Shimada and Matsushita, 1980a). The hydrophobic amino acid content also influences the changes which occur on heating. Gels may be classified as thermoset (thermo-reversible) and thermoplastic (thermo-irreversible) (Shimada and Matsushita 1980, Schmidt 1981). Thermoset gels are transformed to a progel condition on heating; this solution or progel 'sets' to form a gel on cooling, but may revert to the progel state on subsequent reheating, suggesting that the aggregation step is reversible (Hermansson, 1979). Thermoplastic gels such as whey protein gels do not revert to the progel state on reheating; they may, however, soften or shrink.

Based on the results of a study of heat denaturation of BSA, Lin and Koenig (1976) suggested a mechanism of denaturation not previously proposed. This mechanism, which indicates four stages each starting at a critical temperature, is shown in Diagram 2.1. The BSA solution remains in the native state up to 42 ^{o}C. Between 42 ^{o}C and 50 ^{o}C the conformational changes of BSA are reversible; i.e., the protein will return to the native state upon cooling. Between 50 ^{o}C and 60 ^{o}C the α-helical unfolding is only partially reversible. Gel formation takes place at 70 ^{o}C and above, and evidence for the presence of β-conformation is also found around this temperature. Therefore, it has been proposed that the β-conformation is formed by intermolecular hydrogen bonding subsequent to intermolecular disulfide exchange, which is probably responsible for the initial stage of aggregation. The unfolding of α-helices proceeds during the aggregation and gel formation processes.

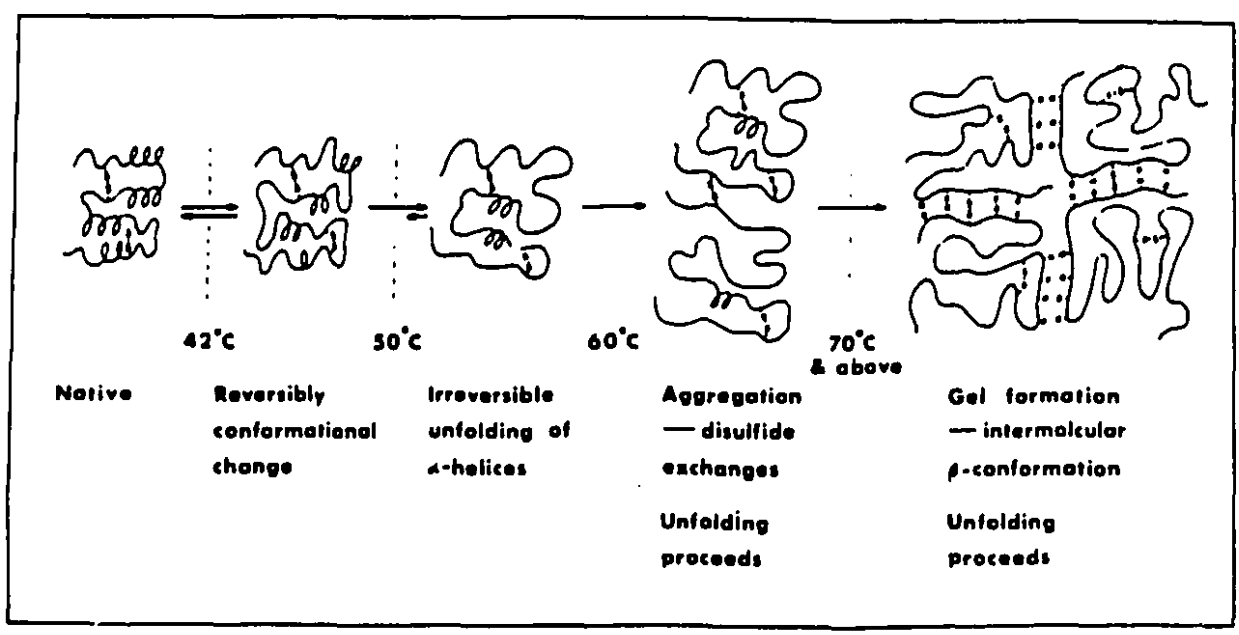

Diagram 2.1 : Mechanism of heat denaturation of bovine serum albumin

2.6 Some basic concepts of rheology

Mechanical properties may be defined as those having to do with the behavior of the material under applied forces. Following this broad definition, such properties as stress-strain behavior of a material under static and dynamic loading as well as flow characteristics of the material in air or in water can be classified as mechanical properties.

Rheology has been defined as "*a science devoted to the study of deformation and flow.*" Therefore, when the action of forces results in deformation and flow in the material, the mechanical properties will be referred to as rheological properties. Moreover, rheology considers the time effect during the loading of the body. Rheologically then, mechanical behavior of a material is expressed in terms of the three parameters of force, deformation and time. Examples of rheological properties are time-dependent stress and strain behavior, creep, stress relaxation, and viscosity. Mechanical properties other than rheological properties usually deal with the motion of the material under applied forces. Examples are drag coefficient and terminal velocity, rebound and coefficient of restitution in impact, and flow of the material in bulk

The objective of the rheological analysis of protein gels is usually texture characterization. Therefore, one is generally interested in material performances under different physico-chemical conditions. In order to meet this requirement, rheological evaluations of foods are usually carried out using test procedures that involve quite large deformations. However, it is well known that most foods exhibit nonlinear rheological behavior, even at small strains (Moskowitz 1987). Engineering properties derived from linear constitutive equations cannot be used to describe and predict material performance under those conditions to which foods

are submitted during texture evaluation, and adequate nonlinear rheological models must be adopted.

Sensory notes significantly correlate (correlation coefficient > 0.80) with rheological properties such as springness, firmness, cohesiveness, denseness, coarseness, and graininess (Foegeding 1992). In general, stress reflects the firmness (hardness) of the gel whereas strain is an indication of the cohesiveness properties. The combination of stress and strain can be used to determine whether formulation or processing variables alter textural attributes such as bitter, tough, mushy or rubbery aspects of product texture (Lanier 1986).

2.6.1 Rheological properties of gels

A definition that has been used to describe protein gels states that *"the one feature identified almost universally as an essential characteristic of a gel is its solid like behavior. When deformed its response is that of an elastic body"* .(Kinsella 1989). Most food gels are viscoelastic; a property which can be subjected to rigorous investigation by the techniques of rheology. Rheological properties determined at failure have been shown to correlate with sensory perception of texture (Mohsenin 1986). Shear stress at failure (shear force/area of shear surface) and shear strain at failure (shear deformation/thickness of the layer sheared) are physical properties of gels which can be determined by experimental analysis of samples of various simple shapes in compression, torsion or tension (Montejano et al, 1984). Conversely, Instron texture profile analysis parameters (force to fracture, strain, stress, elasticity) and other properties such as gel strength are empirical and dependent on sample size and shape. Furthermore, determination of shear stress and strain gives a more complete description of the texture than single property evaluations. For example, a soft deformable gel may have the same

failure stress (hardness) as a rigid, brittle gel; however, the failure strain (deformation) could vary greatly between samples. Methods which determine the force required for rupture of gels (hardness or gel strength) tend to be correlated with failure stress but give no information on failure strain. Rheological analysis of failure stress and strain can be used to distinguish between filled gel and mixed/complex gels. Fillers tend to increase the failure stress but not the strain; whereas mixed/complex gels would be indicated by changes in the stress and strain (Montejano et al, 1984).

The lowest concentration of protein to form a gel, sometimes designated critical concentration (Co) (Kinsella et al, 1989), can be used as a point of comparison among gelling proteins. This value can be determined by direct measurement, or by extrapolating to the concentration where a rheological parameter goes to zero.

2.6.2 Elastic properties of the protein gel

The ideal rubber equation, Eq. (1), obtained from classic statistical thermodynamics (Flory and Rehner 1943a, Flory 1944. 1953a; Flory *et al.* 1949) has been applied by several investigators to the study of protein gels (Szczesniak and MacAllister 1964; Preston and Meyer 1971; Mitchell and Blanshard 1976; Mitchell 1976, 1980; Van Kleef *et al.* 1978; Laurent *et al.* 1980; Johnston 1984; and Oakenfull 1984, 1987).

$$\tau = RTv_e/V\,(\alpha - 1/\alpha^2) \qquad (1)$$

where τ is the stress (N/m^2); V is the deformed volume (m^3); R is the gas constant (8.314 Nm/g-mole °K); T is the absolute temperature (°K); α is the ratio of L/L_0

(length after compression/length before compression); and v_e/V is the moles of effective polymer chains per volume V (moles/m^3) in a real network structure or the concentration of effective cross-linked units.

A minimum of N-1 cross-links are required to connect N protein molecules into a single giant molecule if there are no closed loops. However, a gel structure with N-1 cross-links is not a gel network and could conceivably dissipate the energy of all orientation temporarily imposed on the system by a deformation. Additional interchain cross-links beyond the N-1 will result in the formation of interchain closed loops. Thus, in order to denote the effective cross-links in a given volume, $v_e/2$, one can calculate the actual number of cross-links, v/2, minus the N-1 cross-links ($\approx$ N primary molecules) that were required to setup the single giant molecules without creating the ability to absorb any elastic force. Therefore, $v_e/2 = v/2 - N \rightarrow v_e = v - 2N = v(1 - 2N/v)$, where $N = V/(s*Mn)$, $v = V/(s*Mc)$, s is the specific volume of the polymerized network structure as given by Flory (1943a,b,1944, 1949, 1953a); Mc is the average molecular weight (MW) of the internal chains; v is the moles of actual (total) polymer chains or the moles of polymer chains in a perfect network structure; Mn is the number-average MW of molecules before polymerization.

2.6.3 Rheological measurements

Parallel plate uniaxial compression using an Instron Universal Testing Machine to obtain force-deformation curves is commonly used for food texture investigations. A particular test may involve one compression or several consecutive compression cycles. Special attention has been attached to one-cycle compression, a method used in Texture Profile Analysis (TPA) according to the pioneering work of the General Foods research group led by Szczesniak (Brand et al. 1963; Szczesniak

1963;Szczesniak et al. 1963a, b). Universal Testing Machines have been used increasingly for TPA measurements since Bourne's (1968) studies.

In general, uniaxial parallel plate compression is a method in which a cylindrical or cubical sample with flat surfaces is placed between two flat plates and one of the plates is then made to move at a preselected constant speed. The force developed is recorded as a function of distance (deformation) or time. The resulting force-distance curve is then interpreted by assigning textural properties to one or more parameters derived from the curves. In TPA analysis the compressions are repeated using preselected fixed deformations.

The conditions under which the measurements of uniaxial compression have been made vary considerably (Breene 1975). It is evident that compression testing data for food materials which do not specify sample dimensions and test conditions, such as deformation speed and the degree of deformation in cyclic testing, are in most cases inadequate. The specification of instrumental conditions, especially the deformation speed, is important since most food materials, particularly protein gels, seem to be viscoelastic.

Dimensional effects of the sample size in uniaxial compression testing have been studied by Brinton and Bourne (1972), Peleg *et al.* (1976), and Culioli and Sherman (1976) and theoretically by Peleg (1977). The effect of deformation rate has been studied by, for example, Shama and Sherman (1973), Boyd and Sherman (1975) Olkku and Rha (1975), and Culioli and Sherman (1976). The failure patterns of materials also seem to be of importance in the interpretation of force-deformation curves (Voisey, 1976; Peleg, 1976b; Peleg et al., 1976; Culioli and Sherman, 1976;Peleg and Calzada, 1976). Shimada et al. (1980) and Woodward et al. (1986) reported that significant interaction of pH and protein concentration occurred while measuring the gel strength of egg albumin by a compression test,

and (Hsieh et al, 1989) looked at the concentration dependence of the hardness of egg albumin gels. The relationship between gel strength, as measured by Instron, and salt concentration of globular protein gels has been investigated by Kitabatake et al. (1988), Hatte (1986), and Kato et al. (1990). Studies of the relationship between molecular weight and hardness measurements by Instron for heat-induced albumin gels have been reported by Kato et al. (1990) and Nandi et al. (1972). Different rheological properties of egg albumin and whey protein concentrate gels have been studied by Kitabatake et al. (1988) and Hatta et al. (1986). The main factors contributing to different rheological properties of heat-induced protein aggregates have also been discussed by Gosset (1984), including the role of attractive and repulsive forces between denatured protein molecules. Van Klef (1986) investigated the effect of pH on gel strength and reported that rheological and other physical properties of heat-set protein gels were strongly dependent on the pH of the solvent. Effect of time and temperature on gel strength, as measured by Instron, has been studied by Woodward et al. (1984), who that at elevated temperatures and prolonged heating time, the gel strength of globular proteins could be enhanced.

2.7 Selected Studies on the Secondary Structure of Protein

In the native state globular proteins have a characteristic secondary structure, i.e., a specific content of α-helix, β-sheet and disordered peptide chain conformations. When a protein in solution is heated, some change in secondary structure is likely to occur. Cross-linking in protein gels is thought to arise through alignment of unfolded peptide chains and formation of extensive regions of β-sheet. By measuring the secondary structure profiles of both the native protein and protein gels, it should be possible to test such a postulate by determination of (a)

how much of the original native secondary structure was altered during the transformation, (b) whether this change involves an increase in an ordered conformation such as intermolecular β-sheet, and (c) whether factors influencing gelation such as pH and ionic strength also result in changes in the secondary structure.

Experimentally, the study of secondary structure in protein systems is relatively easy. Three spectroscopic techniques are available; of these, circular dichroism (CD), a chiroptical method related to optical rotatory dispersion (ORD), is potentially the most powerful as it lends itself readily to quantitative analysis and can operate at low protein concentrations. The other two methods are infrared and laser Raman spectroscopy; these are also useful in quantitative terms and have the advantage of being relatively unaffected by sample turbidity. This is a common property of protein gels and can seriously limit the applicability of the CD approach.

Because of the turbidity problem, infrared and Raman spectroscopy have been applied to the study of heat-set protein gels more often than circular dichroism. Infrared spectroscopy involves the measurement of absorption of infrared radiation, and Raman spectroscopy is based on the inelastic scattering of visible light. Both methods provide information about the vibrational frequencies of protein molecules, the infrared method being at its most effective when used to detect β-sheet, and the Raman technique being particularly suited to monitoring α-helix conformation. At present, infrared spectroscopy provides both qualitative and quantitative information; however, D_2O must be used as a solvent because of extensive H_2O absorption in the vicinity of important protein vibrational bands. In this, and in other respects, laser Raman spectroscopy is the more versatile technique as it can be applied to proteins in ordinary water, has at times been used

quantitatively, and can provide information about the vibrations of amino acid side chains and disulfide cross-links as well as about the fundamental vibrations of the peptide backbone.

2.8 Application of infrared spectroscopy to the study of protein secondary structure

The sensitivity of the infrared spectra of proteins to the secondary structure of the polypeptide backbone has been recognized for a long time (Surewicz and Mantsch 1993). Analysis of the normal modes of the peptide group vibrations in model components and in polypeptide systems lead to the characterization of a number of specific amide vibrational modes (Miyazawa et al. 1956, Susi 1967, Krimm and Bandekar 1986). Ideally, the deduction of protein structure from IR spectra should be based on the systematic comparison of the observed vibrational frequencies and intensities with those obtained from normal mode calculations for different hypothetical conformations. Such a rigorous approach has proven remarkably successful in predicting the spectra of simple peptides of known three-dimensional structure, as reviewed recently by Krimm and Bandekar (1986).

In view of the difficulty of normal mode calculations, the analysis of infrared spectra of proteins has been based on empirical correlation between the frequencies of certain vibrational modes and specific secondary structures. The modes most sensitive and best characterized in this respect are the so called amide I, amide II and amide III modes or bands. The amide I mode is primarily a stretching vibration of the C=O bonds, combined with the out-of-phase C-N stretching component and a small contribution from the C-C-N bending vibration. The amide II mode is primarily due to coupling of the in-plane N-H bending and

C-N stretching vibrations. The amide III mode, which consists of in-plane N-H bending and C-N stretching, with contribution from C-C stretching and C=O bending, is very weak in the infrared spectrum and appears of greater diagnostic value when studied by Raman spectroscopy. The most often used of the group vibrational modes is the amide I mode. The relationship between the position of the amide I band and the type of secondary structure may be best observed from the infrared spectra of homopolypeptides that adopt-well defined and often highly homogeneous secondary structures (Susi et al, 1967, Timasheff 1967, Itoh et al, 1976, Dwivedi et al 1984, Sengupta and Krimm 1985, Krimm and Bandekar 1986). In certain cases, these structures can even be interconverted from one virtually pure form to another pure form by changing the environmental conditions such as pH or temperature; the changes in the infrared spectrum that accompany these transitions are very helpful in establishing the spectrum-structure relationship for a given polypeptide system. The transfer of these spectrum-structure correlations to more complex systems such as heteropolypeptides or proteins is not straightforward, however. One problem is presented by the finite length of α-helices or β-sheet segments in proteins. Thus, although the secondary structure of homopolypeptides is usually highly regular and consists of very long arrays of identical subunits, the α-helical segments or β-strands in protein are usually short-sometimes as short as only a few amino acid residues. This irregular nature of secondary structure in real world proteins may result in significant deviations of the amide I frequencies from those observed experimentally for homopolypeptides, or calculated for infinite α-helices or β-sheets (Chirgadze and Nevskaya 1976, Krimm and Bandekar 1986, Byler and Susi 1986).

The assignment of the amide I component bands identified in infrared spectra of proteins to different type of secondary structure has been based on

experimental studies with proteins and peptides of known three dimensional structure (Byler and Susi 1986), as well as on normal mode calculations (Krimm and Bandekar 1986). Thus, bands in the wavenumber range between approximately 1620 and 1640 cm^{-1} are believed to represent extended β sheet structure. In certain cases, and particularly upon deuteration, β strands may also give rise to bands at wavenumbers even below 1620 cm^{-1} (Susi et al, 1967, Surewicz et al, 1987). Another infrared-active mode characteristic of antiparallel β structures occurs in the 1670-1695 cm^{-1} range. However, an unambiguous identification of this relatively weak, high frequency "β component" is often complicated by the presence of more than one band in the amide I region above 1670 cm^{-1} . The other bands in this region represent mostly turns. Turns are believed to be associated also with a characteristic band near 1665 cm^{-1} (Byler and Susi 1986). Polypeptide fragments in the α helical conformation give rise to an infrared band between approximately 1650 and 1660 cm^{-1} . Unfortunately, the "α-helix bands" often overlap with the bands originating from non-ordered structures. These two types of bands usually become better separated if the protein sample used for spectroscopic measurements is prepared in heavy water; an almost instantaneous hydrogen-deuterium exchange of the amide N-H groups in the non-ordered structures results in a shift of the respective infrared bands to a considerably lower frequency. For example, the amide I band in the spectra measured in D_2O of apparently orderless proteins such as α-casein or myelin basic protein are located near 1644 cm^{-1} (Byler and Susi 1986, Surewicz et al. 1987). Although the relationship between the major elements of protein secondary structure and their respective amide I frequencies is well established, certain less common or irregular structures, as well as those originating from amino-acid side-chains (Singh et al. 1991), may interfere with the assignments discussed above.

2.9 Spectroscopic studies of protein gels

In view of their complementary attributes, infrared and Raman spectroscopy are particularly effective when used together in studies of protein gelation. For example, Clark et al. (1981) in an extensive, if qualitative, study applied infrared and laser Raman spectroscopy to a range of gelling protein systems including insulin and BSA. Evidence was found for β-sheet formation in aggregates and gels formed from almost all of the proteins including BSA, and it was found that the proportion of β-sheet present varied considerably from system to system, being greatest for insulin, when heat-set into rod-like fibres at acid pH, and least for BSA gelled at pH's in the range 5.4 to 8.0. Heat-setting of BSA led to a decrease in intensity of the band at 1650 cm^{-1} and increase in intensity of the band at 1620 cm^{-1}, characteristic of intermolecular β-sheet. Since native BSA molecules have a high α-helix content and little β-sheet (Schechter and Blout 1964), the IR evidence points to a significant conformational change during heat-induced association, i.e. one which could play a part in cross-linking. Raman spectra obtained for gelling BSA solutions by Clark et al. (1981) and independently by Lin and Koenig (1976) confirmed this development of β-sheet structure, and changes in the low-intensity band at ~940 cm^{-1} suggested an accompanying drop in α-helix content. Indeed, on the basis of Raman evidence, Lin and Koenig (1976) proposed intermolecular β-sheet formation as a cross-linking mechanism in BSA gels.

In the paper by Clark et al. (1981), studies of the BSA system by infrared spectroscopy were taken further in an effort to better characterize the role of development of β-sheet structure in network formation. Measurement of the carbonyl band for BSA gels prepared under widely different conditions of pH and ionic strength gave virtually the same result, and concentration studies down to a limiting concentration of 1.0% w/w showed little variation in the contribution of

the ~1620 cm^{-1} shoulder to the amide I band. Since the average number of cross-links formed by a BSA molecule would be expected to vary considerably if pH, ionic strength, and concentration conditions were varied, the inference must be that β-sheet content in BSA systems does not vary in proportion to the level of aggregation. This in turn suggests that not all cross-links form via the chain alignment process implied by β-sheet formation, though a limited number could be of this type. From the data available, it seems that the formation of β-sheet is likely to be an intramolecular process, or one involved in dimer formation at most. Once the sheet has formed, however, there is always the possibility that other bonding mechanisms come into play if, for example, hydrophobic surfaces are generated at the same time and are available for less specific protein-protein associations.

The work published so far on BSA secondary structure in gels has been limited to IR and Raman investigations only. In a more recent re-examination of BSA aggregation, Clark and Lee-Tuffnell (1981) have attempted to apply the more quantitatively viable CD approach. Only solutions of aggregates made at sub-gelling concentrations and very clear gels could be studied, but for these systems the results of analyzing CD spectra using the method of Greenfield and Fasman (1969) are worth reporting. In this experiment aggregates were made by heating BSA solutions (pH 8.0) at 75°C for 2h and then cooling to 25°C. Structural characterization was carried out using electron microscopy and a metal shadowing approach as well as by CD studies The results show linear aggregates, with only small polymers being present at 2-3% w/w. The qualitative conclusions of the IR work were clearly substantiated, for the secondary structure content is roughly constant down to 2% concentration. Below this level, substantial changes in the proportions of α-helix, β-sheet and disordered structure occur, and when the concentration reaches the lowest level studied (0.01% w/w) the β-sheet content

falls rapidly. At this low concentration aggregation of heat-treated BSA molecules was inhibited, and some measure of helix content was restored to the unfolded molecules during cooling. Although β-sheet in BSA gels may be *inter*molecular, it seems from the CD data that even if it is, it is still a feature of the molecule in the aggregated condition; i.e. bonding to other molecules is necessary for it to remain stable in cooled solutions of gels.

At the present time, it is difficult to provide a more detailed description of the structural significance of β-sheet in BSA networks than that just given. As Clark and Lee-Tuffnell (1981) have found in their attempts to probe structure in protein gels, it is extremely difficult to obtain accurate data for fully-hydrated unaligned gels in X-ray diffraction experiments, because of the weakness of the protein scattering contribution. When sols and gels are dried, however, measurements of intensity I(2θ)-versus-2θ relationships using wide-angle powder diffractometry can easily be carried out. For BSA sols and gels, dried and powdered, it is obvious that the gel pattern is one of amorphous halos with no sign of sharp diffraction peaks. It may be dangerous to draw conclusions about the gel state from powder patterns but the data seems to provide yet more evidence that the β-sheet in BSA gels is neither very extensive nor substantially repeating. Also the similarity between the sol and gel data implies only limited unfolding prior to aggregation. Clearly, the X-ray data point to the same conclusions as were reached from spectroscopic experiments, but the overall picture of β-sheet in BSA gels is not made significantly more detailed.

Heat-set gels based on BSA have clearly received considerable attention both in terms of their rheological behavior, as described earlier, and in terms of their structural properties at the peptide chain level. Unfortunately, few other protein systems have undergone anything like the same examination. However, Clark et al. (1981) showed that development of β-sheet structure also occurs in

other proteins and varies greatly in amount from one system of gelling conditions to another, and Clark and Lee-Tuffnell (1981) likewise found evidence for β-sheet development in heat-set gels formed from the egg-white protein ovalbumin at pH's in the range 6.0 to 9.0. This last observation, which was based on IR data, confirmed conclusions from earlier Raman studies in which Painter and Koeing (1976) had demonstrated the presence of β-sheet in heat-treated ovalbumin systems. In the more recent work, however, IR spectra of BSA and ovalbumin were compared and revealed that the amount of β-sheet in the egg protein gels was greater than in comparable BSA systems (Clark et al. 1981). This observation was confirmed by X-ray powder measurements. For ovalbumin gels, there is a slight indication of a diffraction peak with a spacing characteristic of β-sheet. In ovalbumin gels at pH 6.3, the β-sheet present is evidently more extensive and repeating than in similar BSA gels, and protein unfolding, as judged by the relationship between sol and gel scattering data, is somewhat greater. Despite its more extensive character, however, the β-sheet in ovalbumin gels appears to have the same limited role in protein-protein bonding as it does in BSA networks for again IR spectra show little sensitivity to changes in gelling conditions.

Finally, in this discussion of the role of secondary structure in protein cross-linking it is interesting to examine spectroscopic data for networks made from mixtures of ovalbumin and BSA. At pH's greater than 6.0, and at low ionic strength, these two proteins form clear homogeneous gels over a complete range of mixture compositions, and IR spectra for a series of such materials, the total protein concentration being 10% w/w, show clearly that the β-sheet content of these systems varies in an approximately linear way with composition. This could be interpreted in terms of separate domains of BSA and ovalbumin in the networks formed, but could also be more evidence for the restricted cross-linking role assigned to β-sheet in the previous discussions, or yet another demonstration that

the model of Astbury et al. (1935) is too extreme to apply closely to the majority of heat-set protein gels.

This concludes discussion of cross-linking mechanisms and formation of intermolecular β structure in heat-set protein gel networks and of the methods available for their study, but before ending this review, it may be worth adding a few general remarks about the relevance of cross-linking studies to the investigation of protein gelation as a whole. At the simplest level, it might be hoped that an understanding of cross-linking behavior would lead to control of the gelation process, i.e., would enable the experimentalist to inhibit or accelerate cross-linking as well. Though there is some truth in this, influence of the unfolding process may also occur accidentally, and control may be much less predictable or effective than intended. In fact, the importance of studying cross-linking at a molecular level seems to have more to do with the way this activity can be combined with linear viscoelastic characterization.

Chapter 3 Instrumentation and Methodologies

3.1 Preparation of protein solutions

Solutions of egg albumin powder (Sigma Chemical Co., crude II, lot. 9006-59-1) in distilled water were prepared and stirred overnight at room temperature using a magnetic stirrer. NaCl was added to each solution of egg white to 0.1M final concentration to avoid stickiness of the gels formed. The solutions were incubated for 20 minutes at 0 ^{o}C, then stirred again to solubilize the insoluble materials for about 30 minutes at room temperature.

Bovine serum albumin, BSA (lot. A-6793), and ovalbumin (lot. A-5503) powder were purchased from Sigma and used without further purification. Solutions of the proteins in D_2O were prepared as described above.

3.2 Monitoring of thermal denaturation of BSA and ovalbumin by FTIR spectroscopy

For monitoring the effect of both time and temperature on protein secondary structure, 10% BSA and ovalbumin solutions were prepared in D_2O. Solutions were loaded in a transmission IR cell with a pair of CaF_2 windows separated by a 50μ Teflon spacer. The temperature of the IR cell was controlled by an Omega temperature controller. FTIR spectra were recorded at 4 cm^{-1} resolution every 30 minutes at a specified temperature.

For time experiments, solutions of BSA and ovalbumin were prepared as described above. FTIR spectra were first recorded at room temperature. Then the

temperature was increased (using a temperature controller) and samples were held at that particular temperature overnight. Additional spectra were recorded every 20 minutes thereafter to see the effect of time on the secondary structure of the protein.

For variable temperature experiments, solutions of BSA and ovalbumin were prepared as described above and the FTIR spectrum was recorded at room temperature. The temperature was then gradually increased from 30 to 95 °C at 5 °C intervals allowing a 20-minute delay for temperature equilibration. The temperature was then decreased to 30 °C at 10 °C intervals since major structural changes were expected to occur during the heating cycle rather than the cooling cycle (Kato et al. 1990). FTIR spectra were collected at each temperature in order to examine the effect of temperature on protein secondary structure and to investigate the effect of cooling.

3.3 Preparation of gels

Preparation of egg albumin gels was as described by Montejano et al. (1984) with minor changes. Six milliliters of centrifuged egg white solution were poured into a pre-prepared natural sausage casein (which was purchased from local stores). Both ends of the casein tube were closed tightly after the removal of remaining air from the casein. A metal clamp was attached to one end of casein while the other end was attached by a string, above the water bath in order to keep the casein tube in a vertical position in water, since the casein containing the protein solution tends to float after the gel is formed. Samples were removed from the water bath every 15 minutes and cooled down with running cold water, then the casein film was removed from the gel. Gel samples were sliced into 3 replicates using a modified egg cutter, and at least 6 gels were obtained at different

time intervals. A total of 18 gels were used for subsequent compression tests performed on a Universal Testing Machine .

BSA and ovalbumin solutions were also prepared in D_2O to avoid the interferences caused by water in the IR spectrum in the amide I region of the spectrum. Protein solutions were then centrifuged to remove air bubbles and then 3 ml of the solution was transferred to a standard polypropylene test tube (i.d. 10 mm) which was then tightly closed. The tubes were then transferred to a water bath at the desired temperature for subsequent gel formation. At 15-minute time intervals the tubes were removed from the bath and cooled with cold running water. Tubes were then cut from the bottom and opened from the top and gels were taken out carefully by inserting a rod (with a 10 mm diameter) into the tube from one side. Gels were sliced with a modified egg cutter into 3 identical pieces for rheological measurement.

Gel strengths of the samples prepared above were measured by a compression test with force being constant, employing a 50 N load cell mounted on the instrument (LLOYD instrument, model LRX). The FTIR spectra were then recorded on a Nicolet model 8210 Fourier transform infrared spectrometer, equipped with a 45° horizontal zinc selenide attenuated total reflectance (ATR) accessory and deuterated triglycine sulfate (DTGS) detector. The DX advanced operations system from Nicolet was employed for data processing. A total of 512 scans were collected for the clean ATR crystal (background) and 256 scans were collected for protein gel samples. The spectrometer was purged with CO_2-free dry air and was operated at 4 cm^{-1} resolution.

The composition of the protein solutions used in the preparation of gels was varied in order to make protein gels with different physical properties. The nature of the variability included changes in protein concentration, ionic strength and pH of the protein solution. Table 1. illustrates how these parameters were varied for egg albumin gels made in H_2O, and pure BSA gels in D_2O. Variations in pH of the protein solution were achieved by using NaOH or 0.1 N HCl.

Table 3.1. Variable parameters of protein solutions used for gel formation

Protein concentration [a]	10	15	20	30
Ionic strength [b]	0.1	0.5	1	2
Temperature [c]	70	80	90	95
pH [d]	5	7	9	

a- Percentage w/v

b- M NaCL

c- °C

d- 0.1 N NaOH/HCL

Gel samples were taken out of the water bath at different time intervals, i. e. 15, 30, 45, 60, 75, 90, 105, and 120 minutes, cooled down with cold running water and stored at 0 °C overnight. The gel strength of the samples were then measured by the compression test, and the FTIR spectra were recorded as described above. The correlation between the changes in gel strength and the changes in the FTIR spectra was carried out employing partial least squares (PLS) regression analysis.

3.4 Instrumentation : Principles and Applications

3.4.1 Application of Universal Testing Machine in the study of gelation

Heat-induced protein gels are generally the result of transition from a viscous solution to a rigid and elastic solid like structure. The mechanism involved in this transformation, however; has not been fully elucidated. Continuous measurement of an appropriate rheological property during heating presumably would yield valuable insight into the structure-development event in gel-forming proteins. This information would contribute to the understanding of the mechanism of protein gelation and should provide the basis for selecting appropriate conditions for the use of proteins as texture-binding components in heat-processed foods. Since texture is one of the important quality attributes of a food product, it might be expected that fundamental rheological characteristics of protein gel products would have been adequately studied. An examination of the literature, however, reveals that basic rheological knowledge of protein gel systems is limited. To truly take advantage of the potential structure and texture binding characteristics of gel-forming proteins one should be able, in addition to monitoring the changes during cooking, to express the deformation, fracture and flow characteristics of final products in term of fundamental rheological parameters. Thus, compression tests were performed on protein gels in the work described here to investigate their rheological properties.

3.4.2 Compression-decompression tests

If an ideal elastic body (e.g., metal spring) is subjected to a compression-decompression cycle (Diagram 3.1-a), it will always return to its original shape, and all the energy invested in the deformation process will be recovered. In most

non-ideal elastic materials (e.g., rubber), some energy is always lost mainly due to internal friction. The body, nevertheless, will return to its original shape and will exhibit the same or almost the same properties on being subjected to a subsequent deformation cycle. The energy that is dissipated in the process, usually in the form of heat, is represented by the area of the hysteresis loop of the force-deformation curve (Diagram 3.1-b).

Most solid food materials are neither ideally elastic nor rubbery. Part of the deformation will, therefore, remain permanent after decompression (plastic deformation). In such materials, a considerable portion of the energy invested in the specimen deformation is irrecoverable due to both internal friction and irreversible structural modifications (Diagram 3.1-c). The ratio between the recoverable and the total deformation was suggested as a "degree of elasticity" and, similarly, the ratio between recoverable and irrecoverable work can also be a characteristic of the material. It ought to be remembered that the magnitude of these parameters may strongly depend on the final strain level as well as on other factors (notably the specimen dimensions and the deformation rate). In such cases, the dependency of these parameters on the test conditions (e.g., strain, specimen diameter) can be considered as a rheological fingerprint of the material and be interpreted in terms such as continuous structural disintegration, or build-up of hydrostatic pressure.

In this study a computer was interfaced with a Lloyd Instrument (LLOYD, LRX) *material testing machine*. The computer interface controlled both the direction and speed of crosshead movement of the instrument. Signal conditioning hardware was installed to process the load cell signal and sense the crosshead position simultaneously. The system was used to perform small-deformation tests on gel samples.

For the compression test, the center probe of the universal testing machine was fitted to a 50 N compression load cell, mounted in the crosshead of the machine. A small cyclic motion force (from an upward-downward cyclic motion of the crosshead at 10.00 mm/min) was applied to the samples for one cycle to produce a constant peak-to-peak deformation in the cylindrical samples (10.00 mm in height, 15 mm in diameter for egg albumin and 10 mm in height, 10 mm in diameter for BSA gel samples). Gel strength was calculated as force (N) divided by deformation (mm). However, frictional forces between samples and the removable plate and the presence of a compressive force on the center probe caused by protein material at the base of the plate produced some deviation in this device (Montejano et al., 1984).

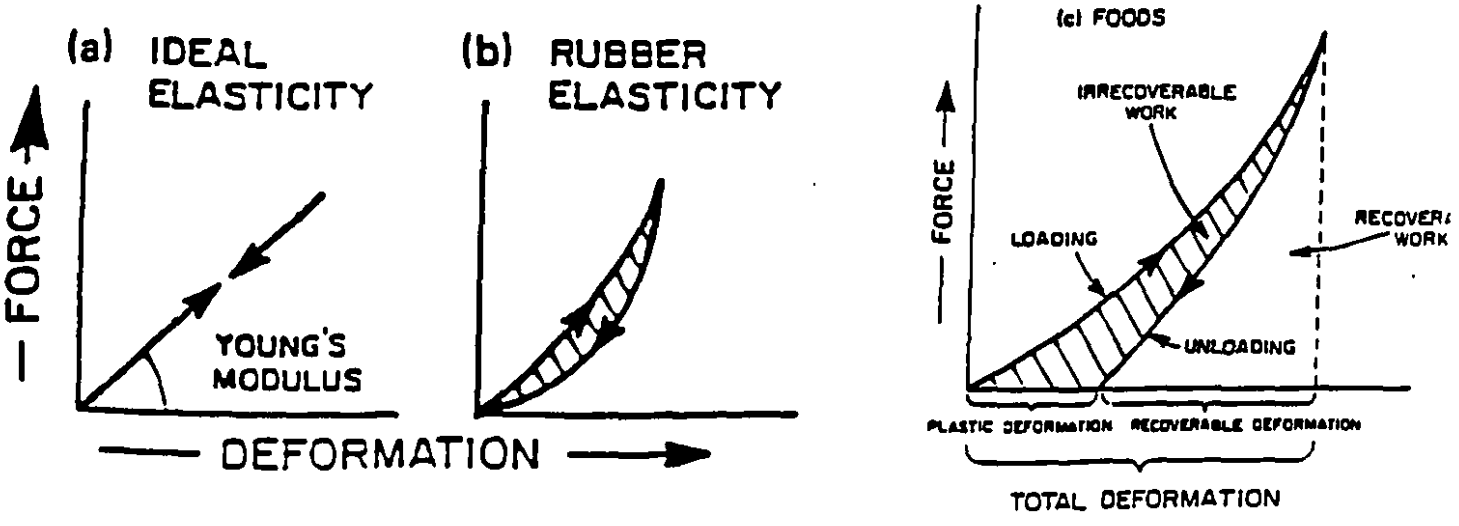

Diagram 3.1 : Different types of deformation curves caused by compression force

Most if not all solid foods, particularly protein gels, are known to be viscoelastic materials. *"Viscoelastic"* in this context means that their mechanical behavior is neither purely elastic nor purely viscous, but something in between that

shares the properties of both types of materials. One of the prominent characteristics of viscoelastic materials is that the stress they develop is not only a function of the strain, but also of the rate at which it is applied. Generally, the faster the rate, the higher the stress. There is, however, a theoretical limit to the rate effect. Beyond a certain rate, primarily determined by the material's relaxation time, the response of a viscoelastic body will converge to what is equivalent to the behavior of an elastic body. The exact nature of the rate dependency is a characteristic of the material. The more solid or elastic, the less is the rate effect. Thus, rigid gel samples are less rate-sensitive than are softer gels.

3.4.3 Theory of Infrared Spectroscopy

Infrared radiation is usually defined as that electromagnetic radiation whose frequency range falls between ~ 14,300 and 20 cm^{-1}. Within this region of the electromagnetic spectrum, chemical compounds absorb IR radiation providing there is a dipole moment change during a normal molecular vibration, molecular rotation, molecular rotation-vibration or a lattice mode or from combination, difference and overtones of the normal molecular vibrations. The absorption frequencies and their intensities for a chemical compound uniquely characterize the material, and thus the IR spectrum of a particular substance can be used to identify and quantify it in an unknown sample. Different classes of chemical compounds contain chemical groups which absorb IR radiation at essentially identical frequency(ies) and have essentially the same band intensity(ies), and these bands are termed *"group frequencies"* . Group frequencies are predictable and allow the IR spectroscopist to elucidate and identify molecular structures. In addition, IR spectra can be recorded rapidly from materials in the solids, liquid,

solution, and vapor phases over a wide range of temperature. Such studies aid in elucidating the molecular structure of materials in different physical phases.

Quantitative measurements are based upon the fundamental relationship in absorption spectroscopy between absorbance, path length and concentration. This relationship is commonly called Beer's law and expressed by the following equation.

$$A = a * b * c$$

Where

A = ***Absorbance***

a = ***absorptivity***

b = ***path length of sample***

c = ***concentration***

Absorptivity (a) is the property of a chemical that determines the amount of incident light that is absorbed at a given frequency. The path length (b) is the distance the light must travel through the sample, that is, the path length of the cell being used to enclosed the sample. Concentration (c) is the amount of the component in the sample. To obtain the concentration of the component, the Beer's law equation is rearranged as follows:

$$c = A / (a * b)$$

The absorptivity term in the equation is a constant for a given compound at a selected frequency. Additionally, for analysis run on the same instrument, using the same cell, the path length factor is also constant. Given this situation, a calibration curve can be easily generated which relates the infrared absorbance to the concentration of the component.

3.4.4 Infrared spectrometer

Infrared spectrometers function by passing a beam of infrared light through a sample and measuring the decrease in signal intensity at each frequency. However, the output of an infrared source is a continuous emission of all possible frequencies, and infrared detectors cannot discriminate individual frequencies of light. In a traditional infrared spectrometer, a monochromator separates the light from the source into its component wavelengths so that detector response can be directly related to specific light frequency.

An FTIR spectrometer has three basic components:

* a source
* a Michelson interferometer
* a detector

The Michelson interferometer, which consists of a beam splitter, a fixed mirror and a moving mirror, preserves both frequency and intensity information and replaces the conventional monochromator. A simplified schematic representation of an FTIR spectrometer is shown in Diagram 3.2.

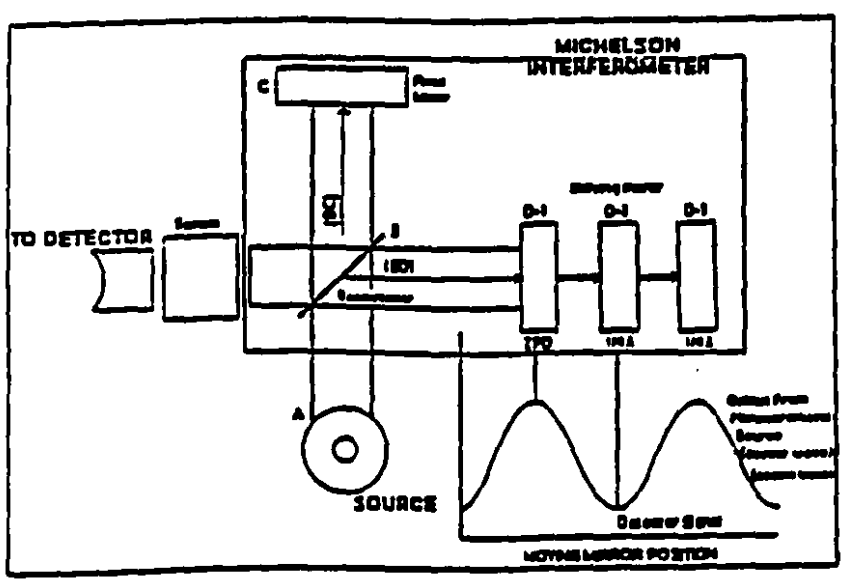

Diagram 3.2 : Schematic representation of an FTIR spectrometer.

Referring to Diagram 3.2,

1- Collimated radiation from the broad-band infrared source, A, is directed into the interferometer and impinges on the beam splitter, B .(basically a very thin film of germanium on a KBr substrate) As its name implies, the beam splitter splits the incoming beam into two "arms" of about equal energy.

2- Approximately 50% of the light is transmitted through the film and is directed onto the fixed mirror, C. The remainder of the light reflects off the beam splitter and is directed onto the moving mirror, D.

3- The beam reflects off the surfaces of the two mirrors and is recombined at the beam splitter. Here constructive and destructive interference occurs, depending on the position of the moving mirror relative to the fixed mirror.

4- The resulting beam passes through the sample, where selective absorption takes place, and then continues on to the detector, where it is digitized by an analog-to-digital converter. The digitized interference pattern or "interferogram" is transformed into a spectrum by a computer through the use of a fast Fourier transform algorithm.

For the work described in this thesis, a Nicolet model 8210 FTIR spectrometer, equipped with a 45° horizontal zinc selenide attenuated total reflectance (ATR) sampling accessory and a deuterated triglycine sulfate (DTGS) detector, operating under DX advanced operations system software from Nicolet, was employed.

3.5 Development of PLS calibration models

In the past few years it has become common to use infrared spectra for quantitative analysis of complex mixtures. Prior to this, infrared spectroscopy was used primarily as a qualititative tool and for relatively simple quantitative measurements. The complex spectral features of many multicomponent data sets often made it difficult or impossible to develop reliable infrared quantitative methods. The integration of high speed data processing into infrared spectrometers allowed data intensive applications, allowing the development of multivariate analytical solutions (Fuller et al., 1988). The simplest attempts at multiple wavelength methods involve the application of Beer's law, which states that absorbance is proportional to concentration and pathlength. By automatically drawing a calibration curve of absorbance at specific frequency vs. concentration, one can assign a concentration value to an unknown sample. This approach was, and still remains, a valuable way to solve many quantitative problems. This technique can be used if the components are to be quantified over a limited concentration range and if specific, nonoverlaping spectral features can be assigned to the components of interest. However, for more complicated systems, more sophisticated approaches are required. One such approach is the partial-least-squares (PLS) regression method.

PLS is a powerful multivariate statistical tool that is gaining importance in many fields of chemistry; including analytical, physical, and clinical chemistry. Industrial process control can also benefit from the use of the method (Geladi et al., 1986). The pioneering work in PLS was done in the late sixties by H. Wold in the field of econometrics. The use of PLS for chemical applications was pioneered by the group of S. Wold and H. Martens in the late seventies after the initial

application of the method by Kowalski et al. (1982). The PLS regression method for spectral analysis is related to other multivariate calibration methods such as classical least squares (CLS), inverse least squares (ILS) and principal component regression (PCR) methods which have been used often in quantitative spectral analyses (Haaland et. al., 1988). The quantitative information directly available from PLS analysis is superior to that obtained from PCR while PLS calibration can be broken down into steps that separately involve CLS calibration and prediction followed by ILS calibration. Thus, PLS has properties which combine some of the separate advantages of CLS and ILS methods while making some potential improvements over PCR. In addition, a detailed understanding of the PLS algorithm allows chemically interpretable information to be obtained from the intermediate steps of the PLS algorithm.

In this thesis, PLS was employed to investigate correlations between gel strength of protein gels measured by Instron and changes in FTIR spectra of the gel samples and to develop calibration models for the prediction of gel strength of protein gels from their FTIR spectra. A separate calibration model was developed for each protein concentration examined using a set of gel samples of different gel strengths, measured by Instron, as calibration standards. The spectral region included in the calibration model were selected by examining the variance spectra for the calibration standards, and the optimal number of factors for the model was selected as that giving the minimum PRESS (Predicted Residual Error Sum of Squares). The predictive accuracy of the calibration models was assessed by leave-one-out cross-validation.

Chapter 4 Comparison of the rates of denaturation of BSA, ovalbumin and their mixtures

4.1 Introduction

Variable temperature analysis of protein solution reveals information about secondary structure changes that occur during heating. Intermolecular interaction of denatured molecules is the most common event involved in heat-induced gelation of egg albumin proteins. Protein gels may be defined as three dimensional matrices or networks in which polymer-polymer and polymer-solvent interactions occur in a ordered manner resulting in immobilization of large amounts of water by a small proportion of protein. The mechanism underlying the formation of the three-dimensional networks of protein gels is not fully understood and has not been systematically studied. However, the conformational changes accompanying thermal treatment given to a protein solution can be investigated using infrared spectroscopy by looking at the amide bands in the infrared spectra of proteins.

In this chapter, studies of the thermal denaturation of bovine serum albumin, ovalbumin, and egg albumin by FTIR spectroscopy are described. The objective of these studies was to examine the changes in secondary structure occurring upon thermal denaturation of these proteins and to investigate their relative rates of denaturation. These studies were then extended to mixtures of BSA and ovalbumin in 1/1, 1/9 and 9/1 ratios in order to examine the cooperative or inhibitory effect of one protein on the other.

4.2 Thermal denaturation of bovine serum albumin

Bovine serum albumin (BSA) is composed of a single polypeptide chain with 582 amino acids and has a molecular weight of 66,000, crosslinked with 18 cystine residues (Lin et al., 1976). BSA has four thiol groups and 17 disulfide bonds and a pI of 5.1. The secondary structure of BSA is composed of 57% α-helix, 24% β-sheet and 19% unordered structures (Fen-nifu et al., 1994)

The effect of heating on the secondary structure of BSA was monitored by recording the spectra of BSA solutions in D_2O in a temperature-controlled transmission IR cell. D_2O solutions were employed in this work in order to examine changes in the amide I region of the IR spectrum, which would be obscured by solvent absorptions in the case of H_2O solutions. Fourier self-deconvolution (band width 13, K=2) was performed on all spectra in order to enhance the resolution of the amide I band.

The spectra recorded as the temperature of a 10% (w/w) solution of BSA in D_2O was increased from 35 °C to 95 °C are shown in Figure 4.1. At 35 °C, a broad, featureless amide I band centered at ~1650 cm^{-1}, assigned to α-helix structure (Anderle et al., 1978, Painter and Koeing, 1976, Kirsch et al., 1986), is observed. No changes in this band are observed until approximately 65 °C; at this temperature, the band at 1650 cm^{-1} changes shape and two well-defined peaks at 1618 and 1683 cm^{-1} appear. The 1683 and 1618 cm^{-1} bands continue to grow at the expense of the 1650 cm^{-1} band as the temperature is increased from 65 to 95 °C. The 1683 and 1618 cm^{-1} are referred to as aggregation bands and are associated with antiparallel intermolecular β-sheet (Yu et al., 1973, Anderle et al., 1978, Painter and Koeing, 1976, Kirsch et al., 1986, Ismail et al. 1992). The

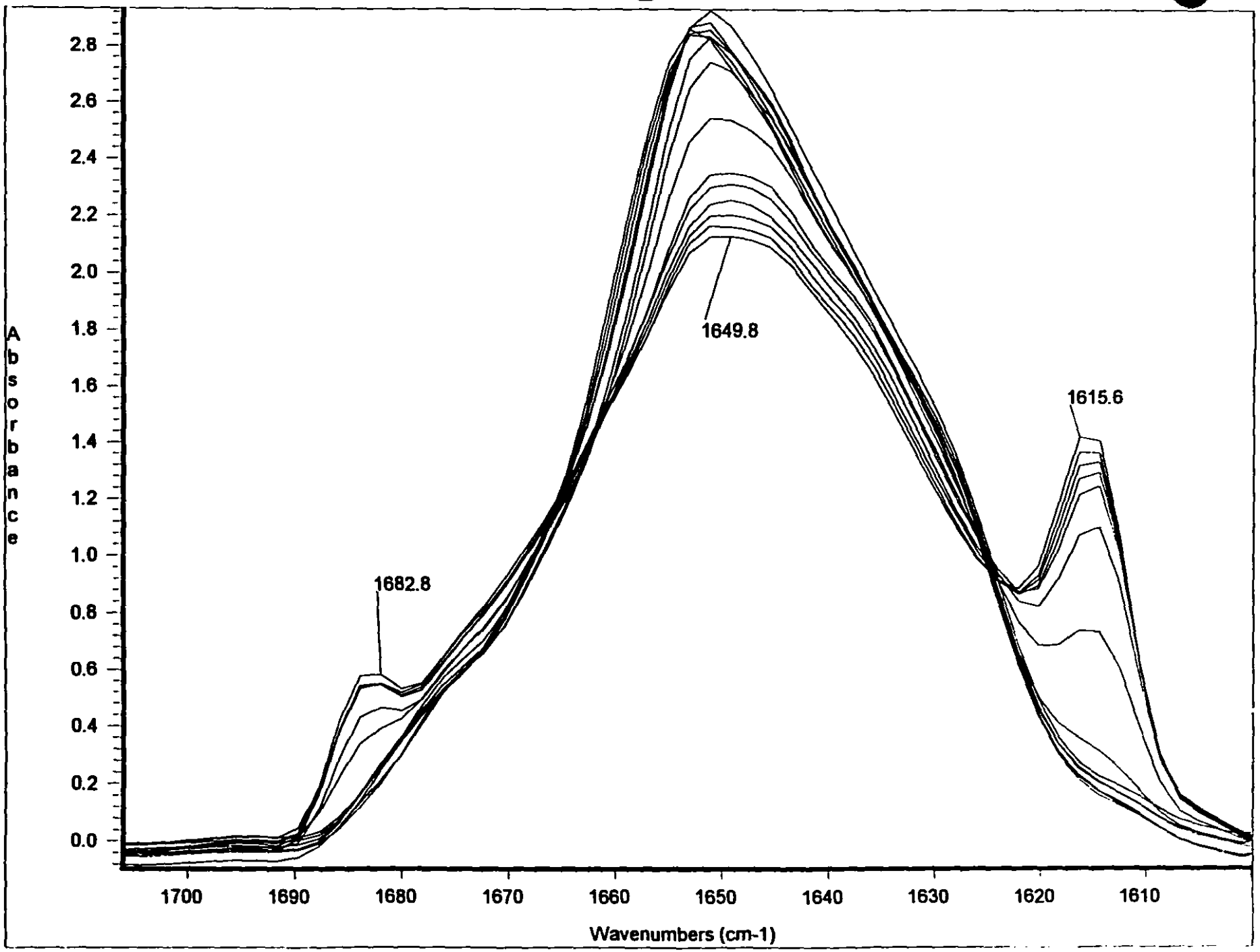

Figure 4.1 : The effect of heating on amide I band of BSA transmission spectra

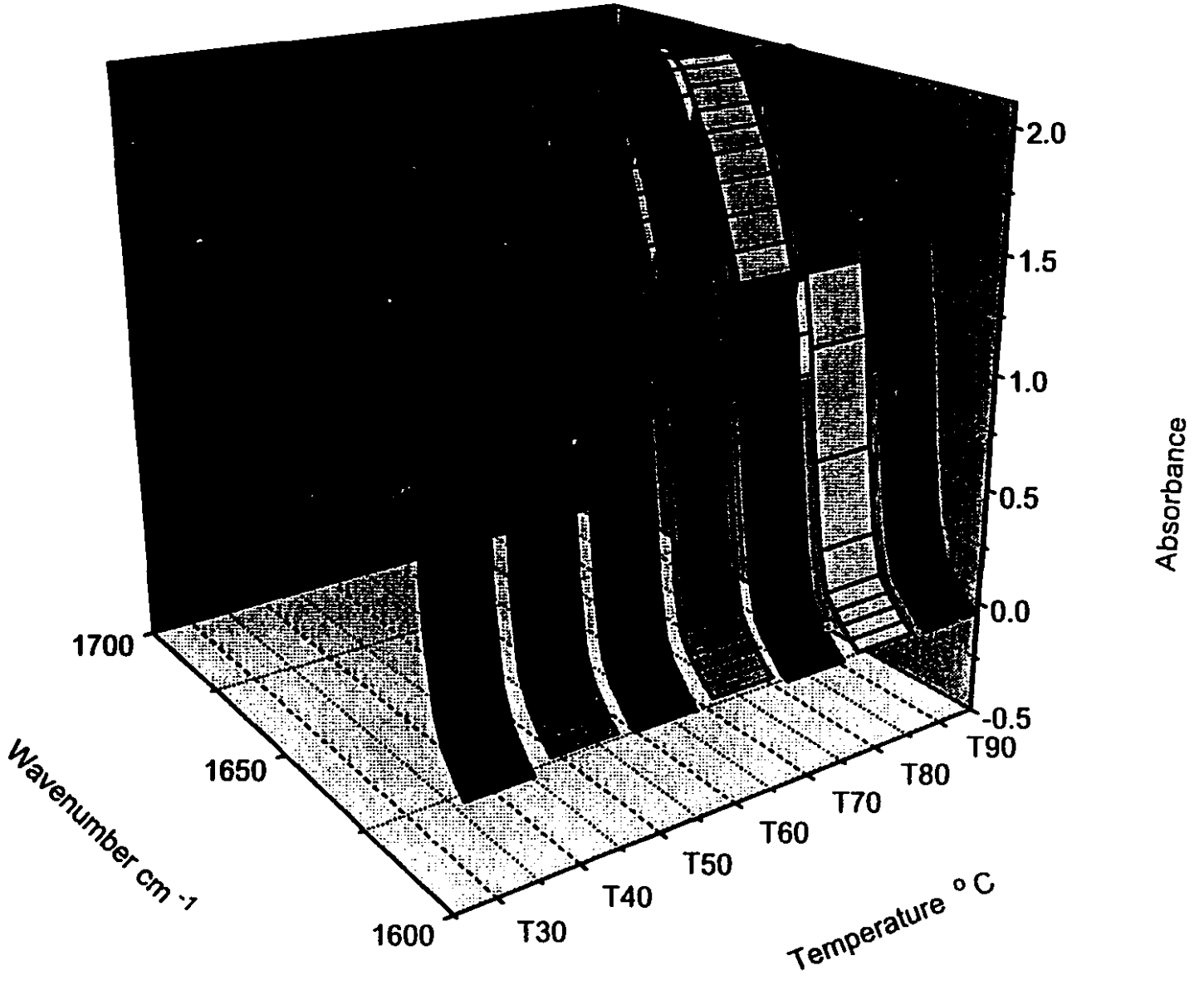

Figure 4.2 : The effect of cooling on amide I band of BSA transmission spectra

unfolding of BSA upon thermal treatment has been reported (Lin et al., 1976, Fennifu et al., 1994) to occur around 62.5 °C, which corresponds to the first appearance of the 1683 and 1618 cm^{-1} bands. The decrease in the 1650 cm^{-1}, which is assigned to α-helix structure, with heating; is consistent with the suggestion by Krimm and Bandekar (1980) that the formation of antiparallel β-sheet occurs at the expense of helical structure as well as disordered conformation. It is also evident from Figure 4.1 and peak height measurements of the 1618 cm^{-1} band, that BSA has only one transition temperature at around 65 °C, which corresponds to the transition of the BSA solution to a gel structure. However, this transition temperature in turn is dependent on the pH of the solution, being dependent on the balance between attractive and repulsive forces during gelation.

Cooling of BSA solutions from 90 °C to 20 °C (Figure 4.2) had very little effect on the FTIR spectra, indicating the irreversibility of heat-induced BSA denaturation.

4.3 Thermal denaturation of ovalbumin

Ovalbumin is a glycoprotein, carbohydrate being present to the extent of 3.2%, has a molecular weight of 45,000 and a pI of 4.5-4.6, and is composed of compact spherical molecules (Painter et al., 1976). Each molecule of ovalbumin has four thiol groups and one disulfide bond (Shimada et al., 1980). The secondary structure is comprised of 25% α-helix, 25% β-sheet, and 50% unordered structures (Gossett et al., 1984).

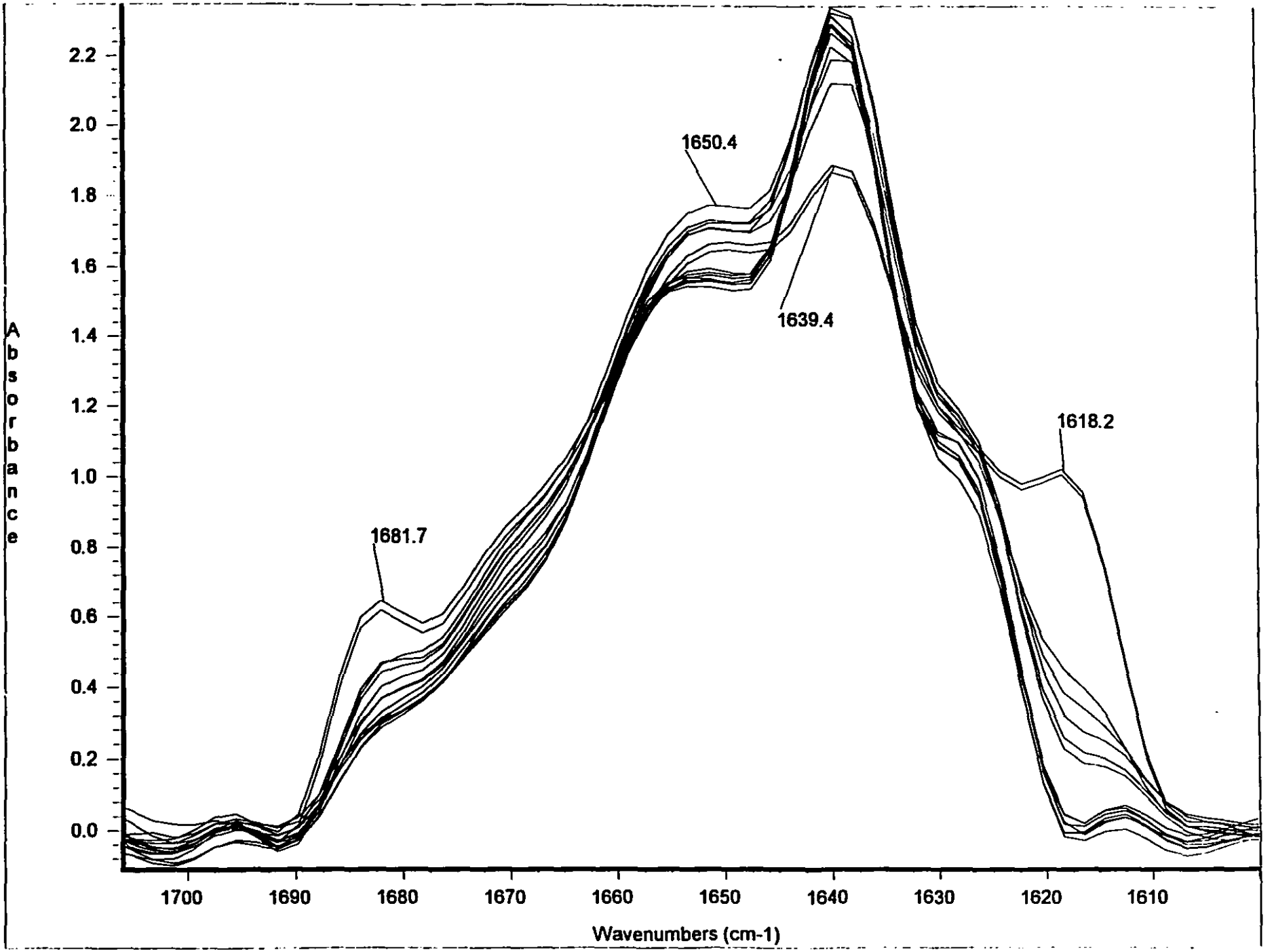

Figure 4.3 :The effect of heating on amide I band of Ovalbumin, transmission spectra

Figure 4.3 shows the FTIR spectra of a 10% (w/w) solution of ovalbumin in D_2O in the amide I region of the IR spectrum recorded as the temperature was increased from 35 °C to 95 °C . Ovalbumin unfolding is known to occur around 85 °C (Gossett et al., 1984, Painter et al., 1976). As can be seen in Figure 4.3, the aggregation bands at 1682 and 1619 cm^{-1} first appear at approximately 90 °C, accompanied by a decrease in the intensity of the band at 1639 cm^{-1}, which is associated with random structure. It seems that the helical content of ovalbumin (1649 cm^{-1}) is not affected to the same extent as other structures. However, it has been shown that, depending on the ionic strength of the ovalbumin solution, the α-helix content can vary in the gelation process (Egalandsdal et al., 1986). The reason that ovalbumin unfolds at such high temperature is thought to be due to its compact structure, which consists of spherical molecules comprised of 25% α-helix, 25% β-sheet and 50% unordered or random conformation (Gossett et al., 1984, Painter et al., 1976).

A closer look at Figure 4.3 shows that there are two transition temperatures associated with ovalbumin, the first one occurring at 65 °C. This may be suggested to be due to another residual protein mixed with ovalbumin in this case. Shimada et al. (1980) reported that at a heating rate of 10 °C/min, egg white at pH 7 showed two major endotherms at 65 and 84 °C, which were produced by the denaturation of conalbumin and ovalbumin, respectively, therefore; the first transition temperature may be due to the presence of a small amount of conalbumin.

As in the case of BSA, the spectra remain unchanged on cooling from 95 to 20 °C, which indicates the irreversibility of protein denaturation.

4.4 Thermal denaturation of mixtures of BSA and ovalbumin

Three different mixtures of BSA (B) and ovalbumin (O) in D_2O in 1/1 (B1O1), 9/1 (B9O1) and 1/9 (B1O9) ratios were heated. The degree of structural transformation was monitored by FTIR spectroscopy. For the 1/1 mixture, two distinctive peaks at 1640 and 1651 cm^{-1} are observed in the 30 °C spectrum, due to intramolecular β-sheet and α-helix structures. These bands are decreased in intensity in the 95 °C spectrum and new bands at 1617 cm^{-1} and 1682 cm^{-1} are observed due to the formation of antiparallel intermolecular β-sheet structure. This is the only mixture for which bands due to both α-helix and intramolecular β-sheet structures appeared in the spectrum with equal intensity at room temperature and decreased as the temperature of the solution was increased to 95 °C (Figure 4.4). The other two protein mixtures showed different intensities of the 1651 and 1640 cm^{-1} bands due to the different amounts of BSA and ovalbumin present (Figures 4.5 and 4.6). Denaturation of B9O1 was observed at approximately the same temperature as seen for BSA alone (65 °C) with the appearance of the aggregation bands at 1618 and 1682 cm^{-1}, which increased with further heating. In the case of B1O9, the same behavior was observed as for ovalbumin, whereby the intensity of the 1638 cm^{-1} band decreased appreciably with the growth of the aggregation bands at 1617 and 1681 cm^{-1}. However, the decrease in intensity of the 1638 cm^{-1} band in the spectrum of B1O9 occurred much more gradually than in the spectrum of ovalbumin, in which this band shows a sudden decrease in intensity. At the two highest temperatures, the spectra of B1O9 ratio are identical in their intensities and are superimposable. It has been suggested by Beveridge et al. (1980) that the aggregation behavior of heat-denatured ovalbumin is heavily dependent on the state of charge groups in the protein. When the amount of negative charge is decreased, the aggregate formation by denatured ovalbumin is

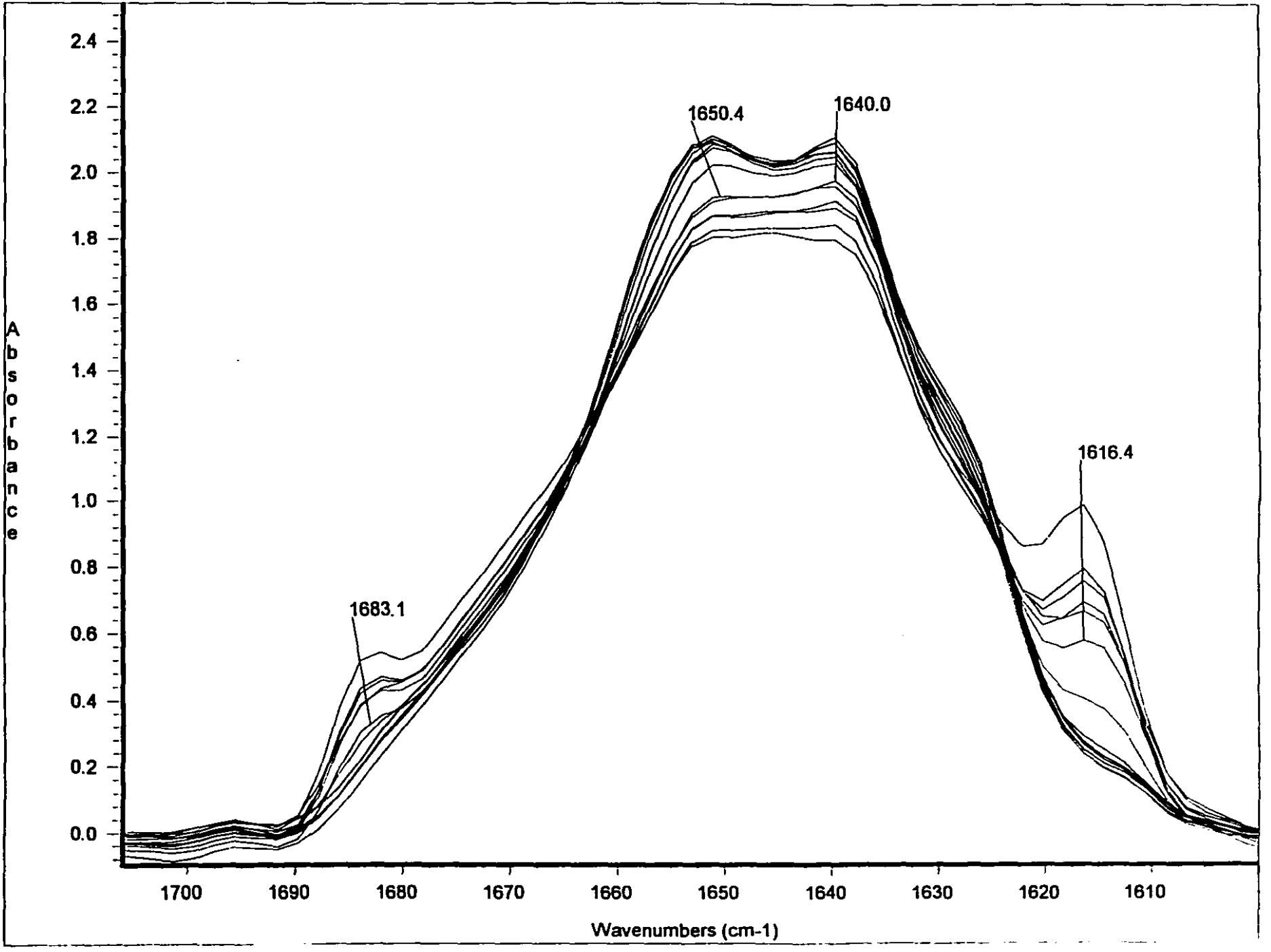

Figure 4.4 : The effect of heating on amide I band of B1O1, transmission spectra

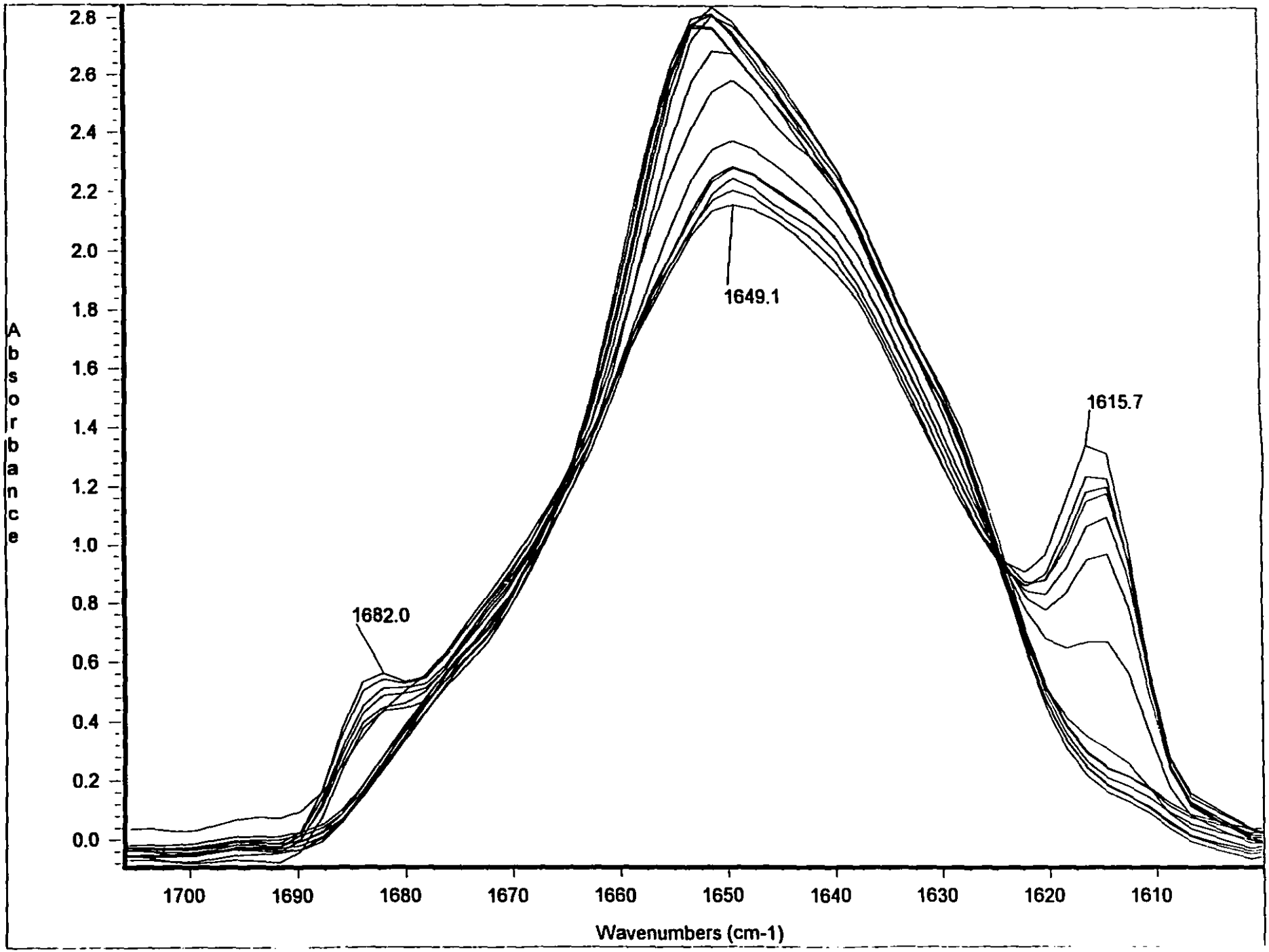

Figure 4.5 : The effect of heating on amide I band of B9O1, transmission spectra

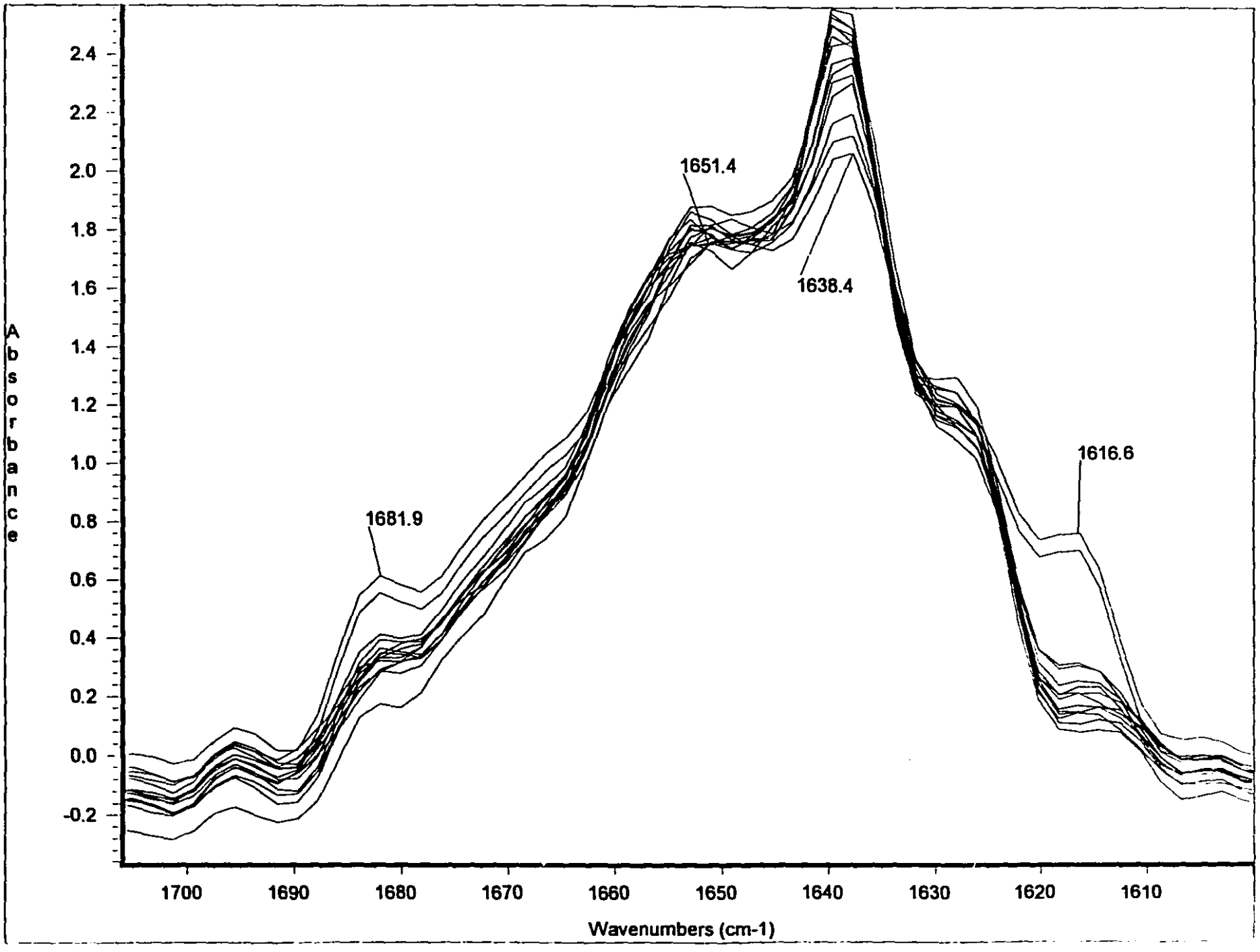

Figure 4.6 : The effect of heating on amide I band of B1O9, transmission spectra

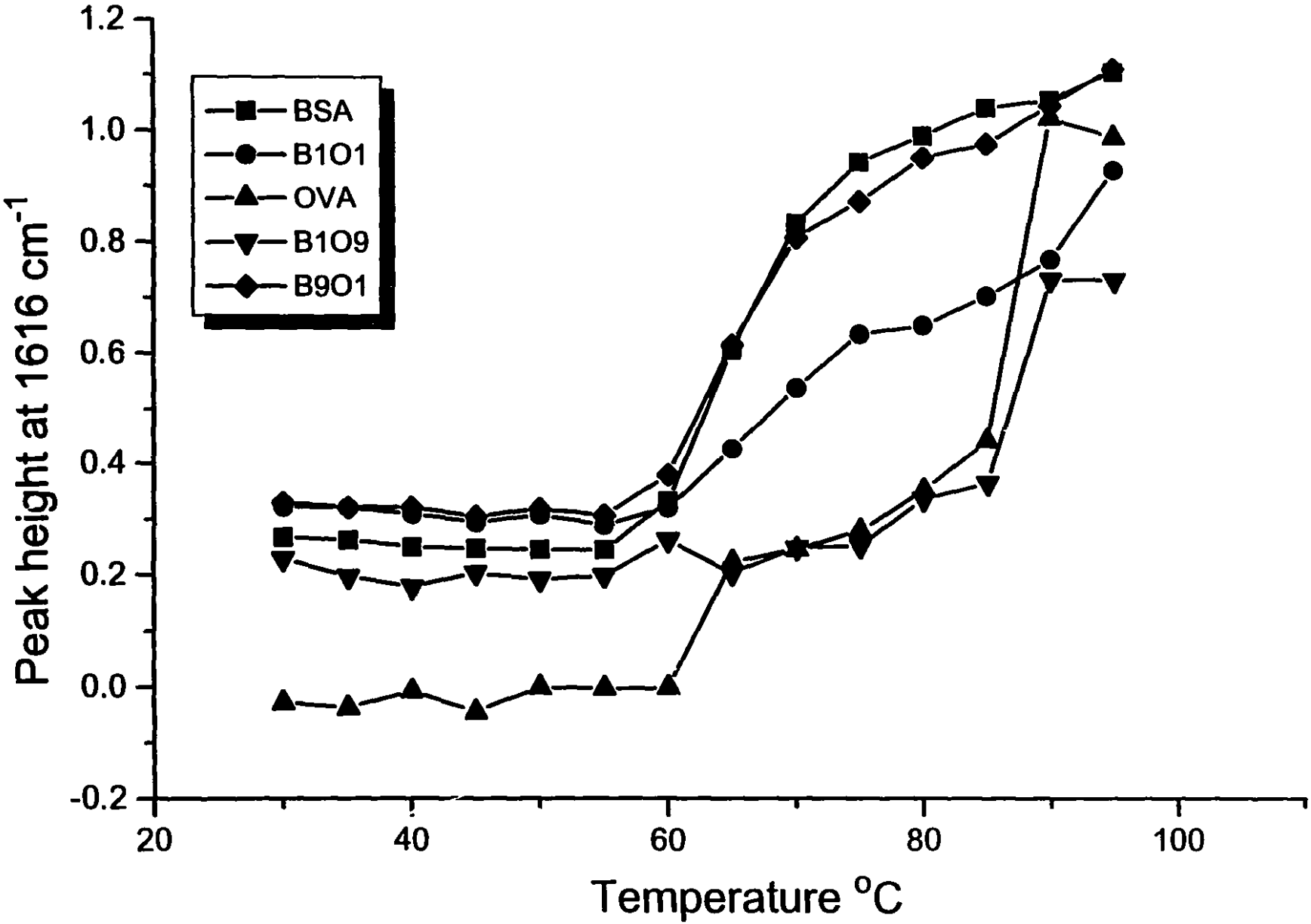

Figure 4.7 : Plot of peak height at 1618 cm^{-1} vs. temperature for BSA, Ovalbumin and their mixtures

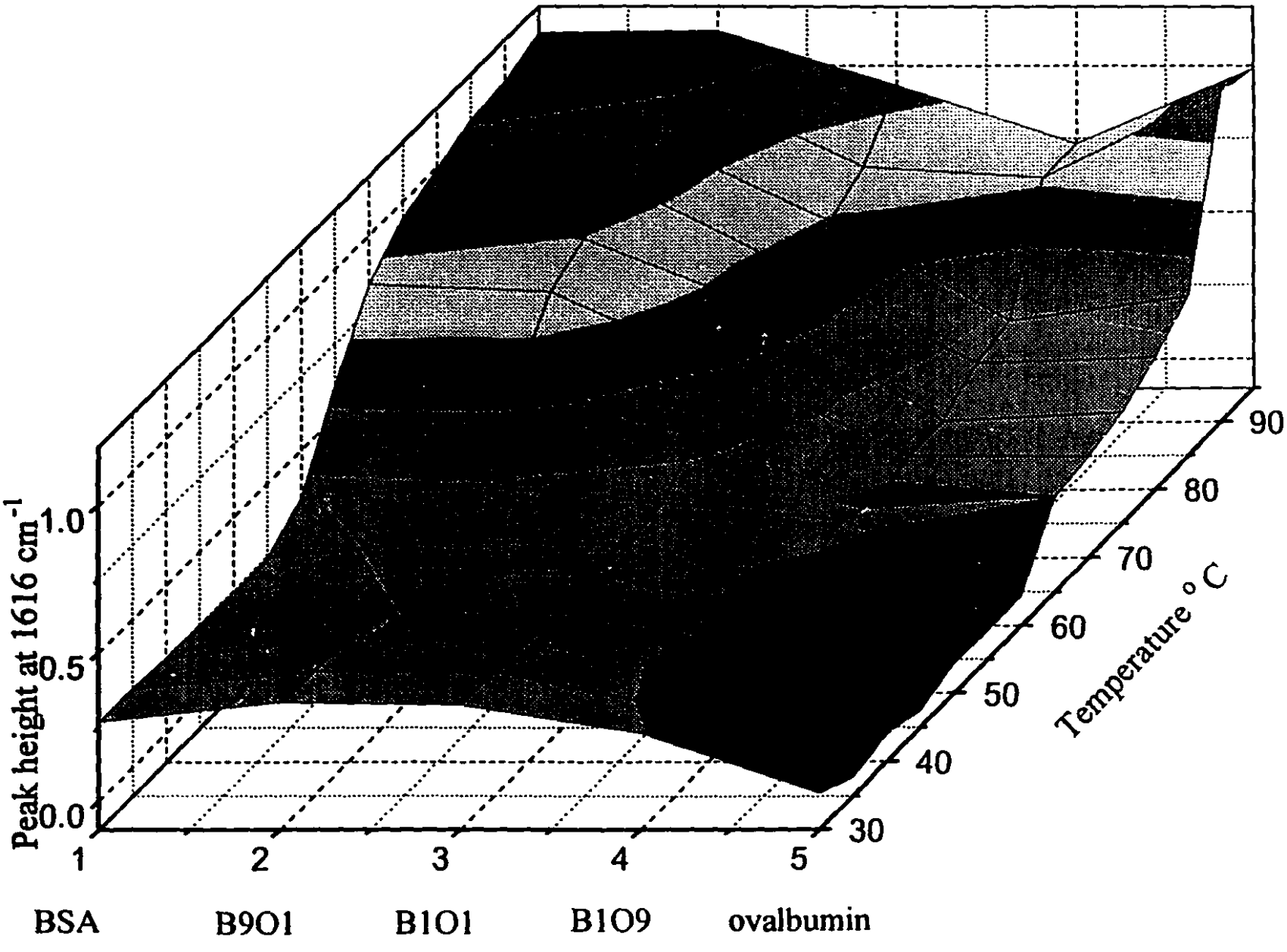

Figure 4.8 : 3D plot of peak height at 1618 cm^{-1} vs. temperature vs. protein compositions

accelerated. On the other hands, when the amount of negative charge is increased, the aggregate formation is decelerated. A similar tendency of aggregate formation was also observed in this study.

The extent of aggregate formation is reflected in the intensity increase of the 1618 and 1683 cm^{-1} bands. A plot of the peak heights of the 1618 cm^{-1} band as a function of temperature for BSA and ovalbumin and their mixtures is shown in Figure 4.7. BSA is known to denature at 65 °C, which is shown by the sharp increase in the intensity of the 1618 cm^{-1} band at 65 °C while there are two transition temperatures associated with ovalbumin aggregation, one at 65 °C and the other at 90 °C. As mentioned above, the lower transition may be due to denaturation of a small amount of conalbumin mixed with the ovalbumin. The B901 mixture exhibits similar behavior to BSA alone as a function of increasing temperature. However, the behavior pattern of the B109 mixture is somewhat different from that of ovalbumin alone. Ovalbumin has two transition temperatures at 65 °C (possibly due to conalbumin impurity) and 90 °C whereas the first transition is not observed in the plot for B109, the slight rise in the plot at 60 °C being due to denaturation of BSA. The second transition temperature at 90 °C is identical to that of ovalbumin, but the intensity of the 1618 cm^{-1} band at this temperature is almost half of that in the case of ovalbumin. This is indicative of a decrease in the formation of antiparallel β-sheet structure. Thus, it appears that the small fraction of BSA in the B109 mixture interacts with ovalbumin (and possibly conalbumin) and has an inhibitory effect on aggregation of the protein(s). A similar effect is observed in the plot for the 1/1 mixture (B101). At temperatures below 90 °C, this plot lies midway between the plots for BSA alone and ovalbumin alone, as would be expected for a 1/1 mixture of these proteins in the absence of any cooperative or inhibitory effects. However, the expected sharp transition at 90

°C, corresponding to the transition temperature of ovalbumin, is not observed, and the peak height for this mixture at this temperature is much lower than the half the sum of the peak heights for BSA alone and ovalbumin alone. At 95 °C, the peak height is closer to, but still less than, expected on the basis of additivity of the contributions of the individual proteins to the observed peak height. These results again suggest some inhibitory effect of BSA on ovalbumin aggregation.

Figure 4.8 shows a 3D plot of the peak height of the 1618 cm^{-1} band vs. temperature vs. protein composition, using different colors (which can be seen as different shades of black and white). In this representation, a valley is clearly observed along the protein mixtures axis at the upper end of the temperature axis, again showing an apparent inhibitory effect of BSA on ovalbumin aggregation in the 1/1 and 1/9 BSA/ovalbumin mixtures.

4.5 Thermal denaturation of egg albumin

4.5.1 Amide I spectral region

Figure 4.9 shows the deconvoluted amide I band of egg albumin in D_2O (band width = 13, k = 2). The spectrum of egg albumin is almost identical to that of ovalbumin (Painter et al., 1976) which is not surprising in that this protein comprises 54% by weight of the system (Parkinson et al., 1966).When the temperature is raised from 30 to 95 °C, four distinctive peaks at 1618, 1639, 1651 and 1683 cm^{-1} are seen to be affected by the changes in temperature. The bands at 1618 cm^{-1} and 1683 cm^{-1} are increased in intensity with increase in temperature while bands at 1639 cm^{-1} and 1651cm^{-1} decreased in intensity. The band at 1639 cm^{-1} has been assigned here to intramolecular β-sheet (Kaiden et al., 1987, Fen-

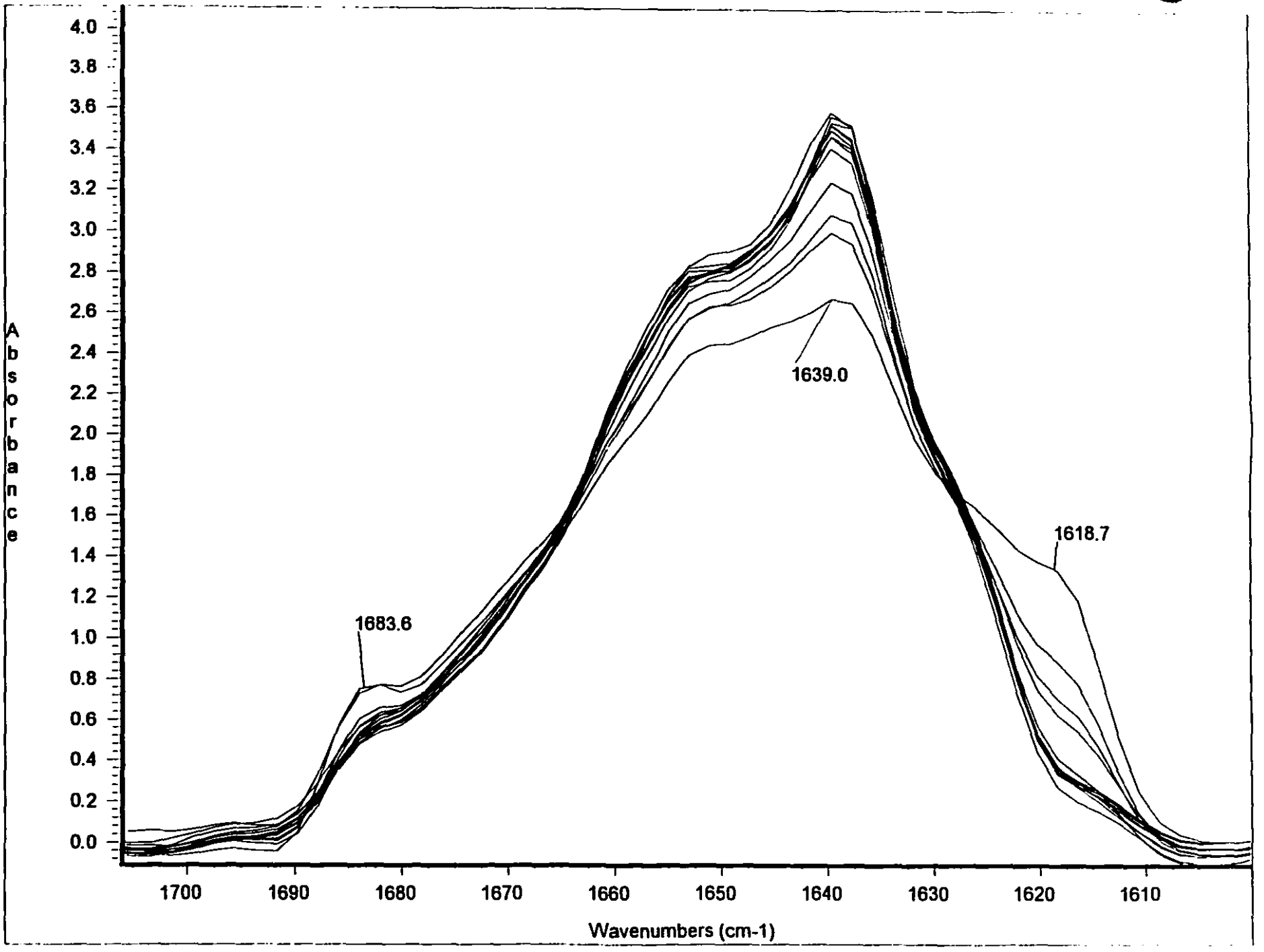

Figure 4.9 : Deconvoluted amide I band of egg albumin in D_2O, Temperature-run

nifu et al., 1994, Frushour and Koeing, 1974) Upon thermal denaturation, this structure unfolds and intermolecular β-sheet formation advances which can be seen by increase in intensity at 1618 cm^{-1} and 1683 cm^{-1}, followed by gel formation. The band at 1651 cm^{-1} has been assigned to α-helix (Kato et al., 1987, Kaiden et al., 1987, Anderle and Mendelson 1987, Jakobson et al., 1990 and Fennifu et al., 1994) which also decreased in intensity upon heating the protein. These data illustrate that intermolecular β-sheet structure increases and plays an important role in the formation of gel at the expense of α-helix and intramolecular β-sheet structure. Changes in the secondary structure of egg albumin can also be seen in Figure 4.10 when the temperature of a 10% solution of egg albumin in D_2O was raised up to 90 °C and spectra were then recorded as a function of time for ~12 h.. Bands at 1619 cm^{-1} and 1683 cm^{-1} due to intermolecular β-sheet increased while bands at 1638 cm^{-1} and 1650 cm^{-1} due to intramolecular β-sheet and α-helix, respectively, decreased. Thus, the mechanism of gel formation can be partly elucidated from examination of amide I vibration of proteins. It has been reported (Kato et al., 1987) that the force involved in the formation of the gel network from egg albumin are proven to be mainly the enhancement of intermolecular hydrogen bonding and hydrophobic interaction including the additive sulfydryl-disulfide interchange interaction .

The rapid increase in β-sheet structure was quantitatively observed during heat denaturation of ovalbumin, the main protein fraction of egg albumin (Kato et al., 1983, and 1988). The formation of β-sheet structure of heat denatured ovalbumin increased in higher protein concentration which the intermolecular interaction was enhanced. It seems likely that protein interaction would be primarily of a hydrophobic nature. β-sheet is generally though to be stabilized by free energy gained from increased hydrogen bond formation. It seems possible that

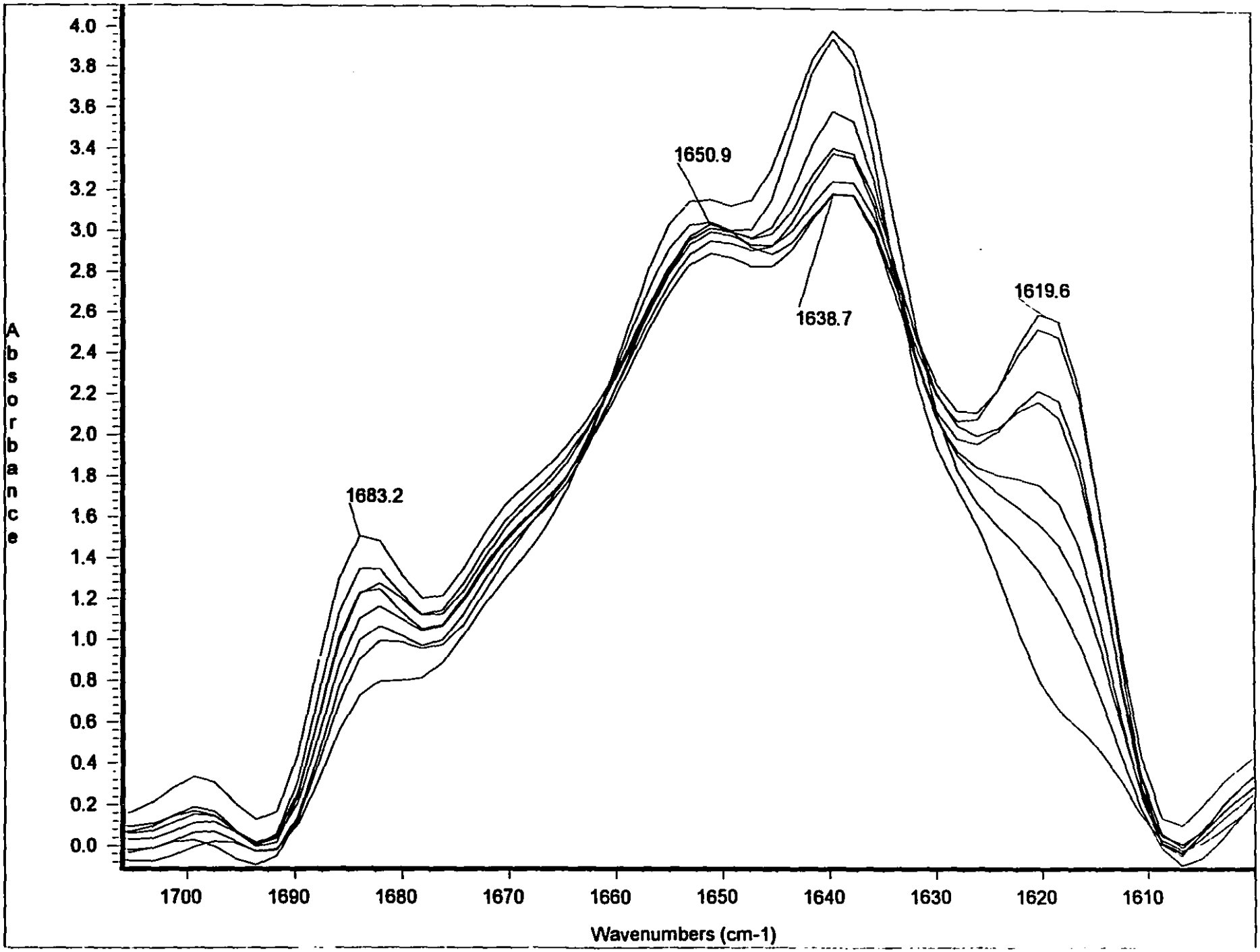

Figure 4.10 : Deconvoluted amide I band of egg albumin in D_2O, Time-run

increased β-sheet structure was stabilized by hydrophobic interaction. In addition, the content of β-sheet structure was increased in proportion to the molecular weight of the heat-denatured ovalbumin aggregates (Jakobson et al., 1990). Therefore; it is apparent that the β-sheet is intermolecularly formed between unfolded molecules. It is probable that partially unfolded form with significant amount of secondary structure is stabilized by intermolecular interaction. Thus, the irreversibility of the heat denaturation of ovalbumin is derived from the intermolecular interaction formed by β-sheet structure. Taking into account the result that the exposed hydrophobic residues on the molecular surface remarkably increased with the heat denaturation of ovalbumin (Kato et al., 1987), the β-sheet structure may be liable to form in the hydrophobic environment. That is, the intermolecular β-structure may be strengthened by the exposed hydrophobic residues that exclude the water in the spaced between unfolded molecules.

4.6 Conclusion

Infrared spectroscopy is an extremly useful method for studying gelation of food proteins. Development of ATR calibration in PLS allows the prediction of gel strength to be carried out in reasonable fashion. Infrared looks at molecular level of protein gelation and can reveal valuable information about structural and functional properties of food proteins. The strength of a given gel sample can be derived from correlating the rheological properties of that gel to the infrared spectra of the same gel sample. The extend of these rheological prediction by PLS is still not fully explored, but the discovery of this technique allows the prediction of rheological properties of food proteins to be carried out on a routine basis for industrial purposes.

Chapter 5 Gel Strength Measurements of Bovine serum albumin in D_2O

5.1 Introduction

Globular proteins as ingredients posses useful functional properties for variety of application in the food industry. The capacity to form heat induced gels is one of the important properties. Proteins forms gel by polymerizing into a three dimensional matrix which converts a viscous liquid into viscoelastic solid. The polymerization process can be caused by heat denaturation or by any other process that converts proteins to a state that favors intermolecular protein interactions (Kinsella et al., 1989). Bovine serum albumin have been investigated here for its gel forming properties under different condition by compression test of universal testing machine. The gelation behavior of BSA is known to be affected by the heating temperature and time, the protein concentration, and the conditions of medium such as pH, salt concentration and type of salt. The gelling behavior is also sensitive to a critical balance between attractive and repulsive forces (Matsudomi et al., 1991). As the pH of protein medium varies, the attractive and repulsive forces governing the protein solution varies and gels with different physical properties are obtained. In order to understand the effect of these factors on gelling behavior of proteins, it is often desirable to study them independently, to minimize the overlap effect of other factors. Thus, the effect of protein concentration, pH and temperature as a function of time of heating have been investigated by FTIR spectroscopy of protein gels. Infrared analysis of BSA gelation process under varying conditions can elucidate the molecular structural changes that take place during thermal denaturation of proteins. In BSA, gelation involves transformation of α-helix and aperiodic structures into β-sheet

conformations (Jakobson et al., 1990). Conditions that decreased the formation of β-sheet structures decreased the gel strength (Paulsson et al., 1986). It is hypothesized that, in globular protein gels, intermolecular hydrogen bonding between segments of β-sheet oriented either in parallel or in antiparallel configurations may act as junction zones in the gel net work (Kato et al., 1987).

5.2 Gelation at different protein concentrations

There is a minimum concentration of a protein required for formation of heat induced gels. Normally high concentrations of protein are frequently used in studies reported on protein gelation (Clark et al., 1981, Matsudomi et al., 1991, Richardson et al., 1981 and Lee et al., 1993). Even if heat induced gelation of globular proteins is dependent on experimental conditions used, determination of the lowest protein concentration for gel formation might be employed as criterion for, or as a classification of the ability of specific protein to gel (Paulsson et al., 1986).

The hardness of heat induced gels of BSA is known to be affected by the protein concentration (Matsudomi et al., 1991, Yasuda et al., 1986, Hamman et al., 1987). The minimum concentration of protein required for gel formation is an important criterion of the gel forming ability of BSA. The hardness of the gel made with various concentration of BSA was measured (Matsudomi et al., 1991) using 20% and 70% compression, the BSA gels were reported to be transparent, had a smooth texture and exhibited good water holding capacity with little syneresis. The hardness of BSA gels increased exponentially to approximately the 2.2 to the power of protein concentration. This was similar to previous work of Ferry, (1948) who plotted gel hardness against the square of BSA concentration,

and found a linear relationship. A minimum concentration of 4.4% of BSA was required for gel formation. This is close to the BSA concentration of 5% (w/v) in this study and 4.5% concentration has been reported in another study (Matsudomi et al., 1991) for the formation of self supporting gel from BSA.

When protein concentration is increased from 5 to 20% the gel hardness of the BSA gels is shown to increase (Figure 5.1) from 0.25 to 4 N/mm as a function of time of heating. The effect of time on gel hardness is more pronounced at higher protein concentration (i.e.; 15-20%) since as time of heating increases, gel hardness increased at a much faster rate than that observed for gels formed from lower protein concentration. The BSA gel in absence of salt were transparent with a smooth texture and shown resistance to extrude water under compression test.

Figure 5.2 shows the stacked FTIR spectra in the amide I region of five gel samples obtained from a 20% BSA solution after different times of heating. As the gel strength increases with time of heating, several changes in the amide I band are observed. The aggregation bands at 1683 and 1614 cm^{-1} increase relative to the main component of the amide I band. In addition, there is a shift of the maximum of the band from 1647 to 1641 cm^{-1}, which may be attributed to a decrease in the helical component at 1650 cm^{-1}. These observations indicate that changes in gel strength give rise to discernible changes in the amide I band. Figure 5.3 shows a plot of actual gel strength of BSA gels obtained by compression test of Instron vs. values predicted from their FTIR spectra by a PLS calibration model for all four protein concentrations in D_2O where 48 samples are plotted and an excellent correlation is obtained, where R = 0.99 and SD = 0.13 are a good indication of the linearity of the PLS regression model. The calibration model was developed through the correlation of the spectral changes observed in the amide I region

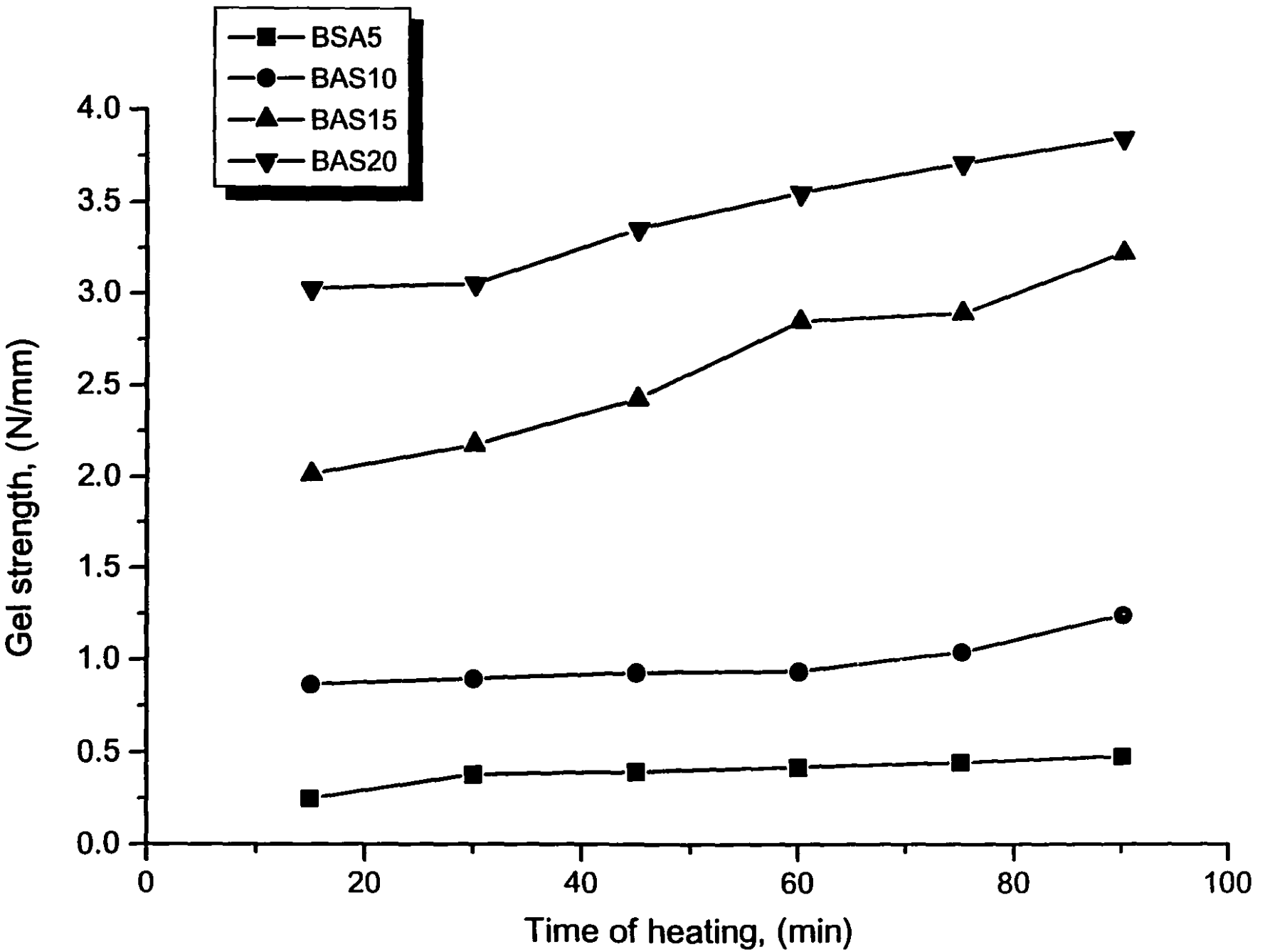

Figure 5.1 : Plot of gel strength vs. time of heating for protein concentrations

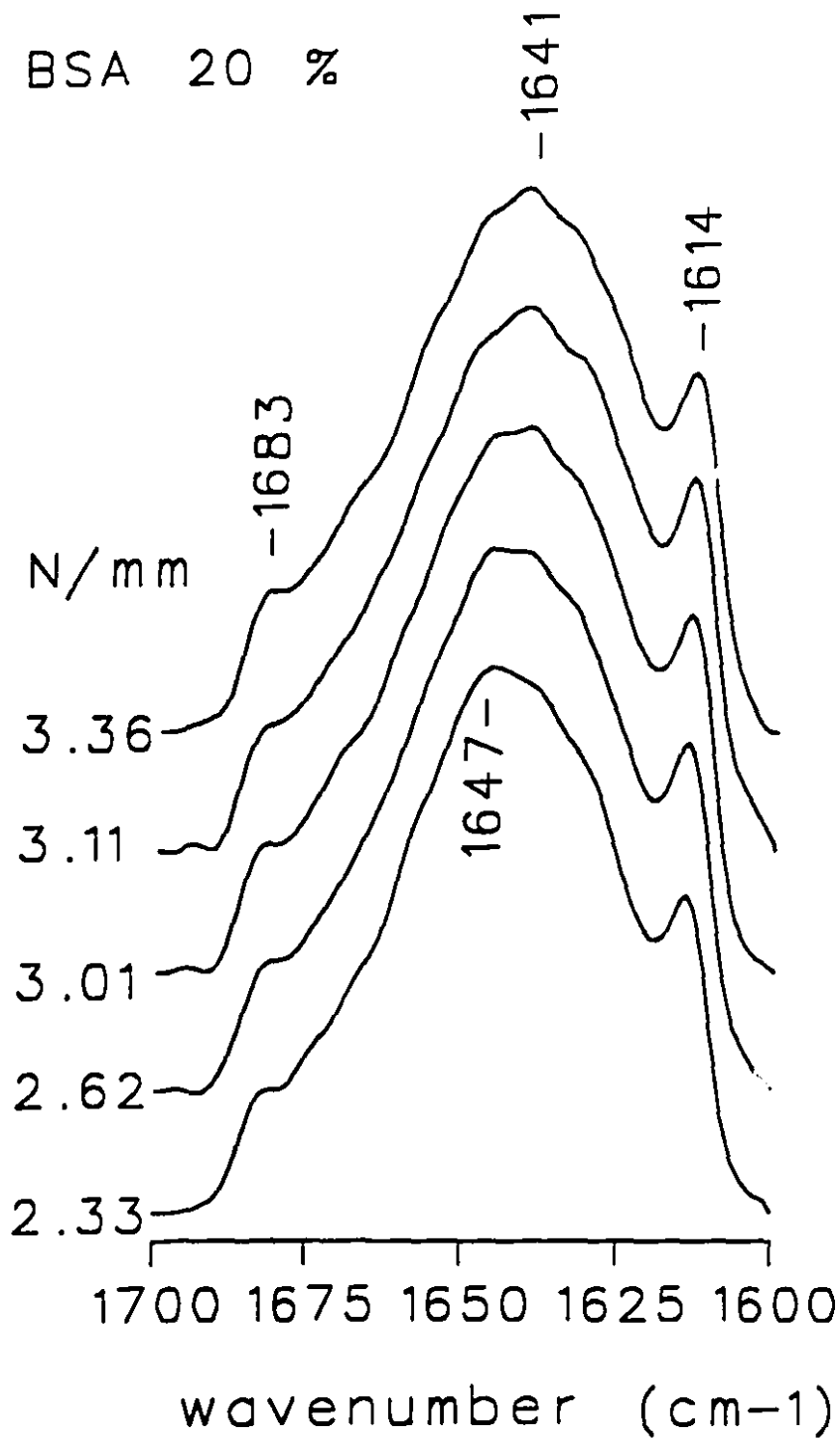

Figure 5.2 : spectra in the amide I region of 20% BSA gels

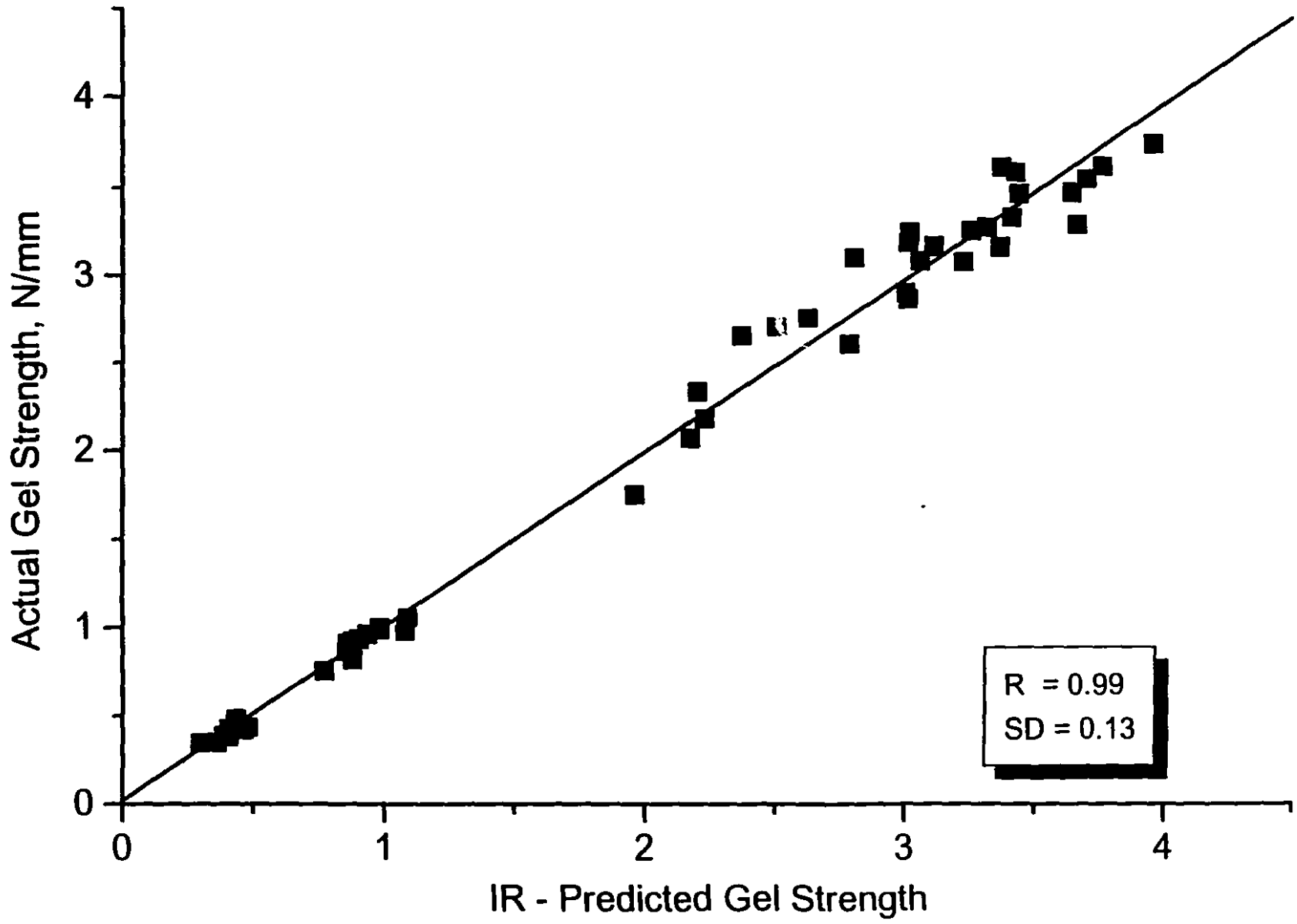

Figure 5.3 : Plot of actual gel strength vs. IR predicted for protein concentrations

(1700 - 1600 cm $^{-1}$) as it can be seen in Figure 5.3 and the changes in gel strength measurement as shown in Figure 5.2.

Table 5.1 Error between Actual and Predicted values of gel strength of BSA gels.

Protein Conc.	Mean	sd(yEr^{+})	se(yEr^{+})	Sum
5% Conc.	0.0005	0.0322	0.0102	0.0052
10% Conc.	0.0041	0.0470	0.0141	0.0455
15% Conc.	0.0005	0.1915	0.0512	0.0079
20% Conc.	0.0135	0.1720	0.0459	0.1892
Combined Conc.	0.0047	0.1365	0.0195	0.2320

Table 5.1 shows the statistical calculation of the error between actual gel strength obtained by compression test against predicted values by PLS, where mean, standard deviation (sd), standard error (se) and sum of the error for each experiment is tabulated. Smallest mean error and standard deviation are obtained for 5% protein concentration while largest mean error and standard deviation are obtained for 20 and 15% protein concentration respectively.

In comparison with different proteins, minimum BSA concentration were found to be 5% for gel formation (Matsudomi et al., 1994) the same results were found in this study, where below this level no gel hardness could be detected. However when BSA was mixed with β-Lactoglobulin hardness of the gels made with mixture increased. Indication of intense intermolecular interaction is accompanied the gel formation when a mixture of protein is used. The same type of interaction could be responsible for gel formation when a single protein i. e; BSA is used. Addition of α-Lactalbumin to BSA is reported (Hines et al., 1993,

Matsudomi et al., 1994) to cause significant increase in gel hardness and the gel obtained were transparent. BSA and α-Lactalbumin are thought to interact and form soluble aggregate through thiol-disulfide interchange reaction during gel formation.

5.3 Effect of pH on gel formation

The net charge on proteins markedly affects the gel formation and hardness of the gel. BSA gels possessed maximum hardness at pH 7 (Figure 5.4), followed by pH 9 and the weakest gel obtained at pH 5 close to the isoelectric point of BSA i.e; pI 5.1 (Matsudomi er al., 1991, Yasuda et al., 1986, Richardson et al., 1981, Hegg, 1982). Time of heating also has a pronounced effect on the gel hardness and the effect of time is more obvious for pH 7 since increase in heating time as was expected increased the gel hardness. This trend can be seen for all gels at different pH levels when compression test was applied to BSA gels. For pH value below 7, gel hardness decreased markedly and white, opaque sponge like aggregate was observed while at alkaline side of pI, gel hardness increased and above optimum pH (7) gel strength decreased back again, but still was higher than gel hardness close to pI of protein. These observations indicate that a certain number of electrostatic and repulsive forces between unfolded protein molecules plays an important role in gel network structure (Hermansson et al., 1992, Ferry, 1948). When the negative forces exceed a critical number, protein-protein interactions are reduced and weaker gel are obtained (Hillier 1980).

Figure 5.5 shows the stacked spectra in the amide I region of gel samples obtained after different times of heating from solutions at pH 7 and 9. In both cases, similar changes are seen, primarily an increase in the intensity of the 1683 and 1614 cm^{-1} bands with increase in gel strength together with a decrease in the

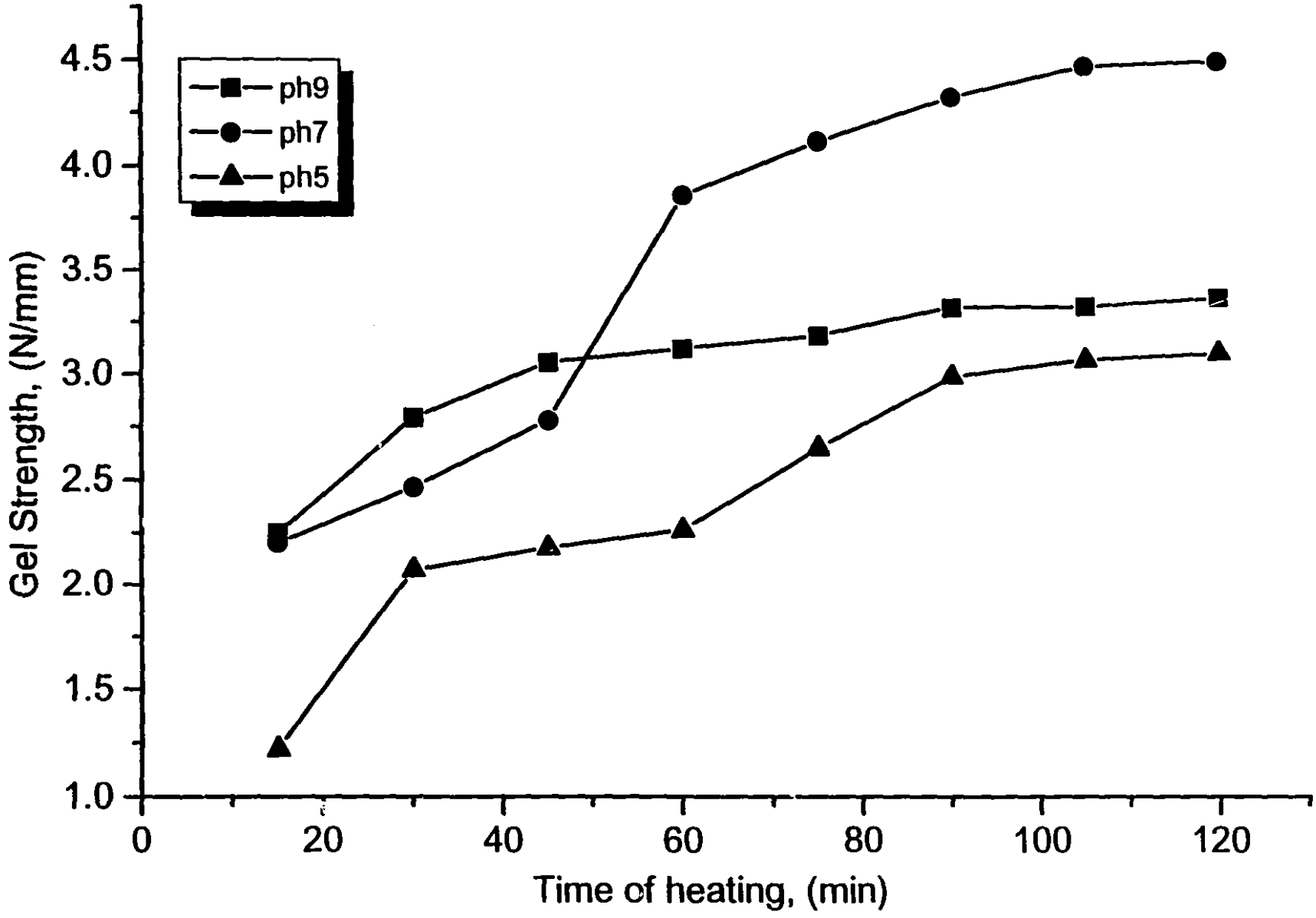

Figure 5.4 : Plot of gel strength vs. time of heating for pH

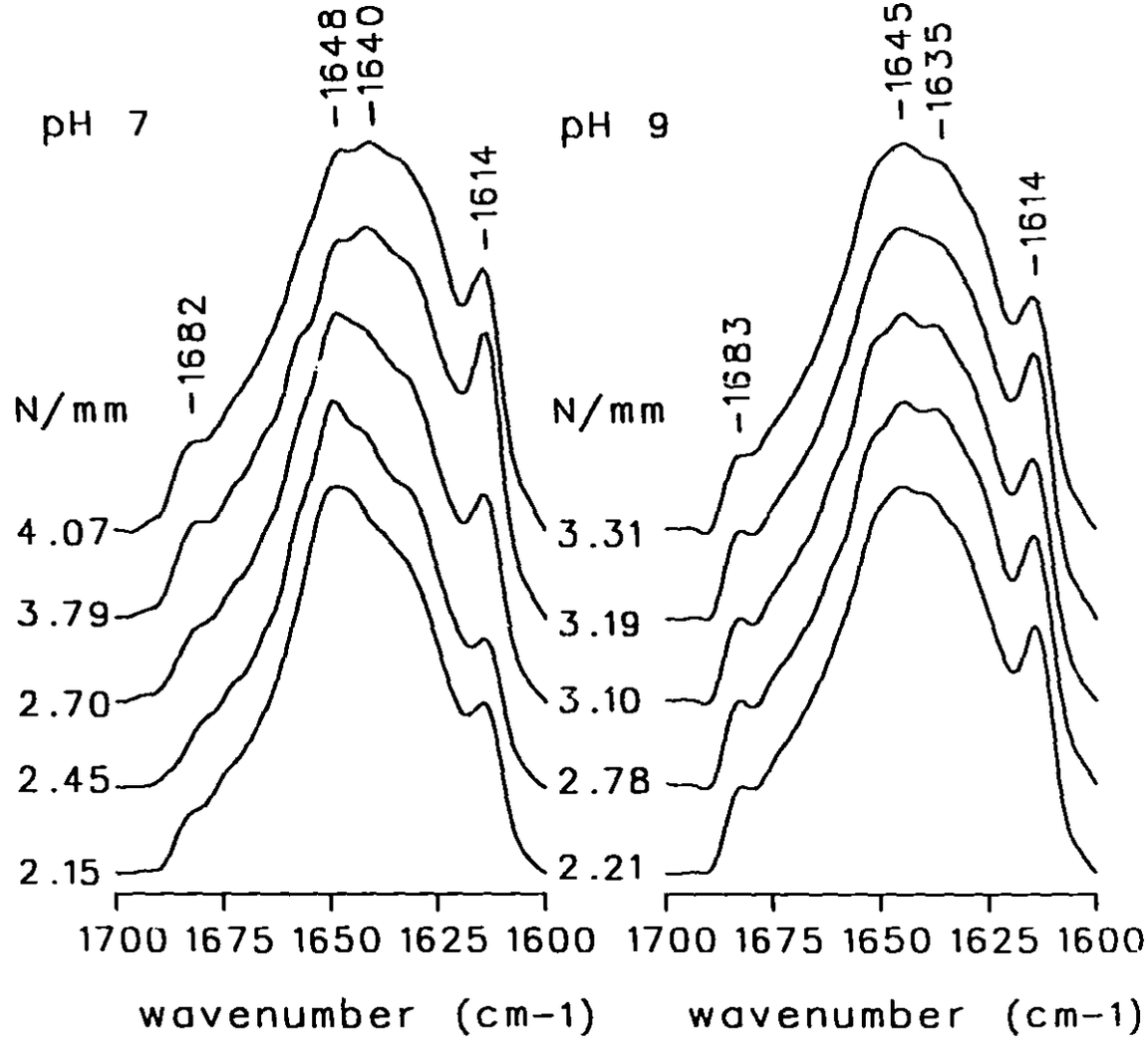

Figure 5.5 : Stacked spectra in the amide I region of BSA, pH 7 & 9

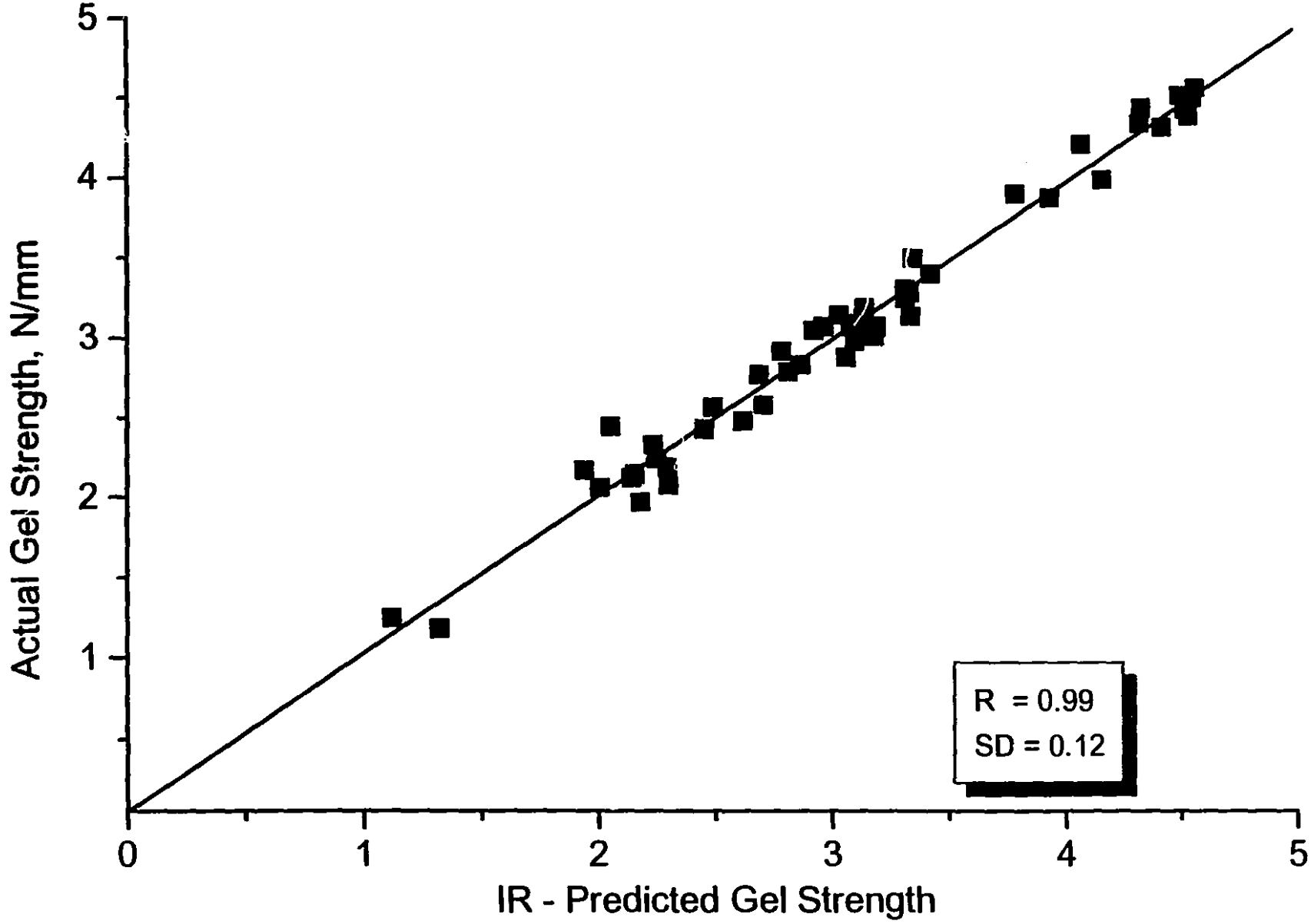

Figure 5.6 : Plot of actual gel strength vs. IR predicted for pH

intensity of the helical component of the amide I band at ~1650 cm $^{-1}$. These changes are similar to those seen in Figure 5.2. Other changes in the secondary structure include the interconversion of the helical structure to more unordered structure as observed from the increase in intensity of a band at 1640 and 1648 cm $^{-1}$ with increasing temperature. Table 5.2 shows the statistical information when actual values of gel strength are compared with predicted values obtained by PLS regression. The smallest mean error and standard deviation is associated with pH 7 (the optimum pH for gel strength of BSA gels) and the largest mean error and standard deviation is obtained when the three pH experiments were combined together in a single PLS calibration. Figure 5.6 is the plot of actual gel strength versus the predicted values for all pH levels where 50 samples are represented. An excellent correlation (R = 0.99 and SD = 0.11) was obtained.

Table 5.2: Error between Actual and Predicted values of gel strength of BSA gels

pH values	Mean	sd(yEr^{+})	se(yEr^{+})	Sum
pH 5	0.0058	0.1325	0.0331	0.0930
pH 7	0.0019	0.0896	0.0211	0.0350
pH 9	0.0063	0.1286	0.0321	0.1020
Combined p	0.0229	0.1967	0.0278	1.1472

The effect of pH on the attractive forces of BSA is found to be small in comparison with the effect on repulsive net charge (Hegg, 1982), consequently it would be possible to predict the boundaries between solubility and aggregation for any globular proteins by knowing the titration curve, the amount of salt present in the sample and simple physical data such as isoelectric point and pH induced

transition of the proteins. The forces which keep the framework of the gel together must instead be found in the hydrophobic and hydrogen bonds, which becomes available during thermal denaturation of the globular proteins (Hegg, 1982). These forces counteract the repulsive net charge of the proteins, and a delicate balance between attractive and repulsive forces seems to be a prerequisite for the formation of a gel net work (Frushour and Koeing, 1975). The difference in the ability to form gel might reflect different type of intermolecular interaction in the aggregation of the proteins. It has been proposed (Clark et al., 1981) that β-sheet hydrogen bonding might be important in aggregation formation. The β-sheet content in the native state of serum albumin, for instance, is reported as low (Yu et al., 1973) and that of denatured state as high. The α-helix content of native state is reported to be high (Wang et al., 1991) and upon heat induced gelation decreased along disordered conformation. There is therefore, some indication that high content of α-helix structure in the native state would facilitate the formation of intermolecular β-sheet formation in the denatured state.

5.4 Effect of time and temperature on gel formation

In order to determine the effect of temperature and time on gelation, solution of (20% w/v) BSA in D_2O (pH unadjusted) was heated at various temperature and time (Figure 5.7). BSA solutions did not form a self supporting gel by heat treatment below 70 °C although, the solutions becomes viscous, and some aggregation occurred. The hardness of BSA gels increased rapidly above 70 °C and 60 minutes of heating. BSA is known to denature by heat treatment above 65 °C (Harper et al., 1978, Matsudomi et al., 1991) and the first self-supporting gel can be seen at 70 °C in which the hardness can be measured. These results indicates that heating above the denaturation temperature of the protein is required

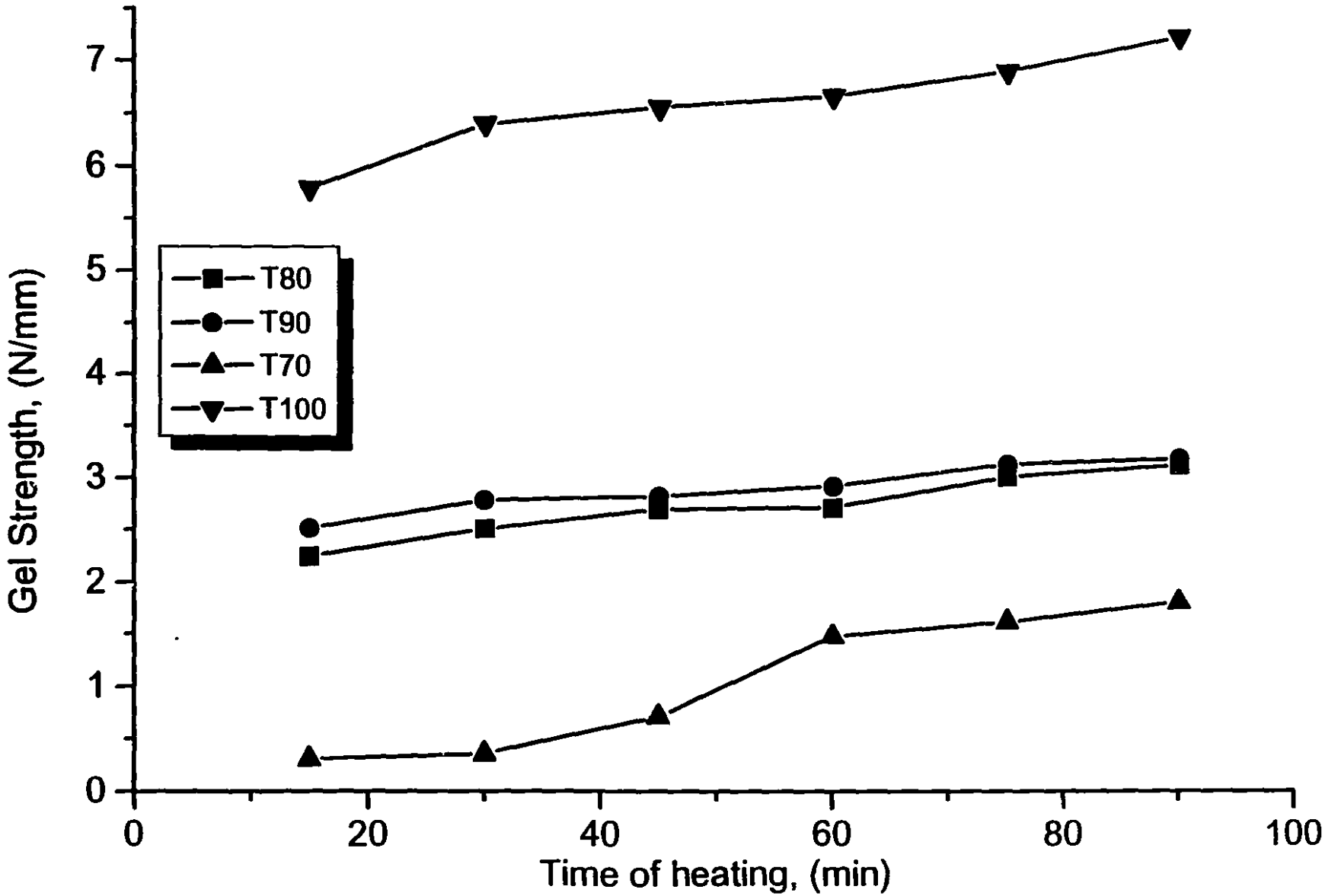

Figure 5.7 : Plot of gel strength vs. time of heating for temperatures

for gel formation. The effect of heating time at 70 °C on gel hardness is increased rapidly after 1 hour of heating and continues to grow, whereas at 80 and 90 °C this effect is less pronounced, although the same trend can be seen. Gels formed above 70 °C are initially much stronger even after only 15 minutes of heating than gels formed at 70 °C. The gel strength of BSA gels made at 80 and 90 °C reached almost the same values after 90 minutes of heating and the effect of temperature above 90 minutes of heating can not easily be distinguished. Increasing time or temperature above this point, would not increase the gel strength.

Figure 5.8 shows three sets of stacked spectra of gel samples obtained by heating at 70, 80, and 100 °C. In all three cases, the intensities of the aggregation bands at 1683 and 1614 cm^{-1} again increase with increase in gel strength. A shift of the α-helical band at 1647 cm^{-1} to 1642 cm^{-1} may be indicative of increase in unordered structure with increasing of time of heating. Figure 5.9 shows the PLS results obtained when the combined data of actual and predicted values from all temperature runs are plotted. An excellent correlation R = 0.99, SD = 0.17 is obtained. Table 5.3 shows the statistical data on the error, (error between actual and predicted) for different temperature experiments.

Table 5.3 Error between Actual and Predicted values of gel strength of BSA gels.

Temperatures	Mean	sd(yEr)	se(yEr)	Sum
70 °C	0.0083	0.1112	0.0262	0.1507
80 °C	0.0147	0.1052	0.0271	0.2205
90 °C	0.0025	0.1660	0.0415	0.0400
Combined temps.	0.0021	0.1242	0.0177	0.1034

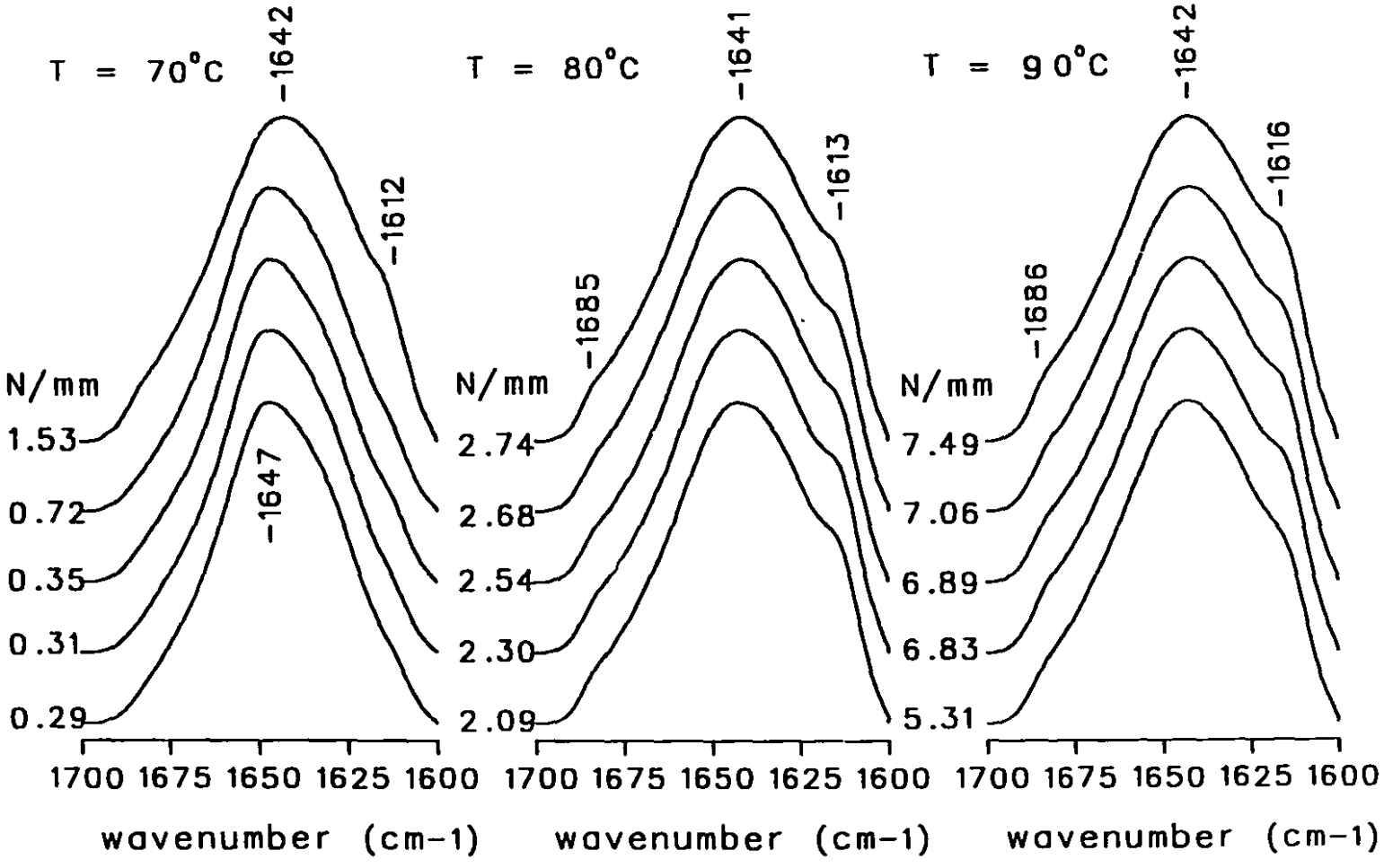

Figure 5.8 : Stacked spectra in the amide I region of BSA, T=70,80,100 °C

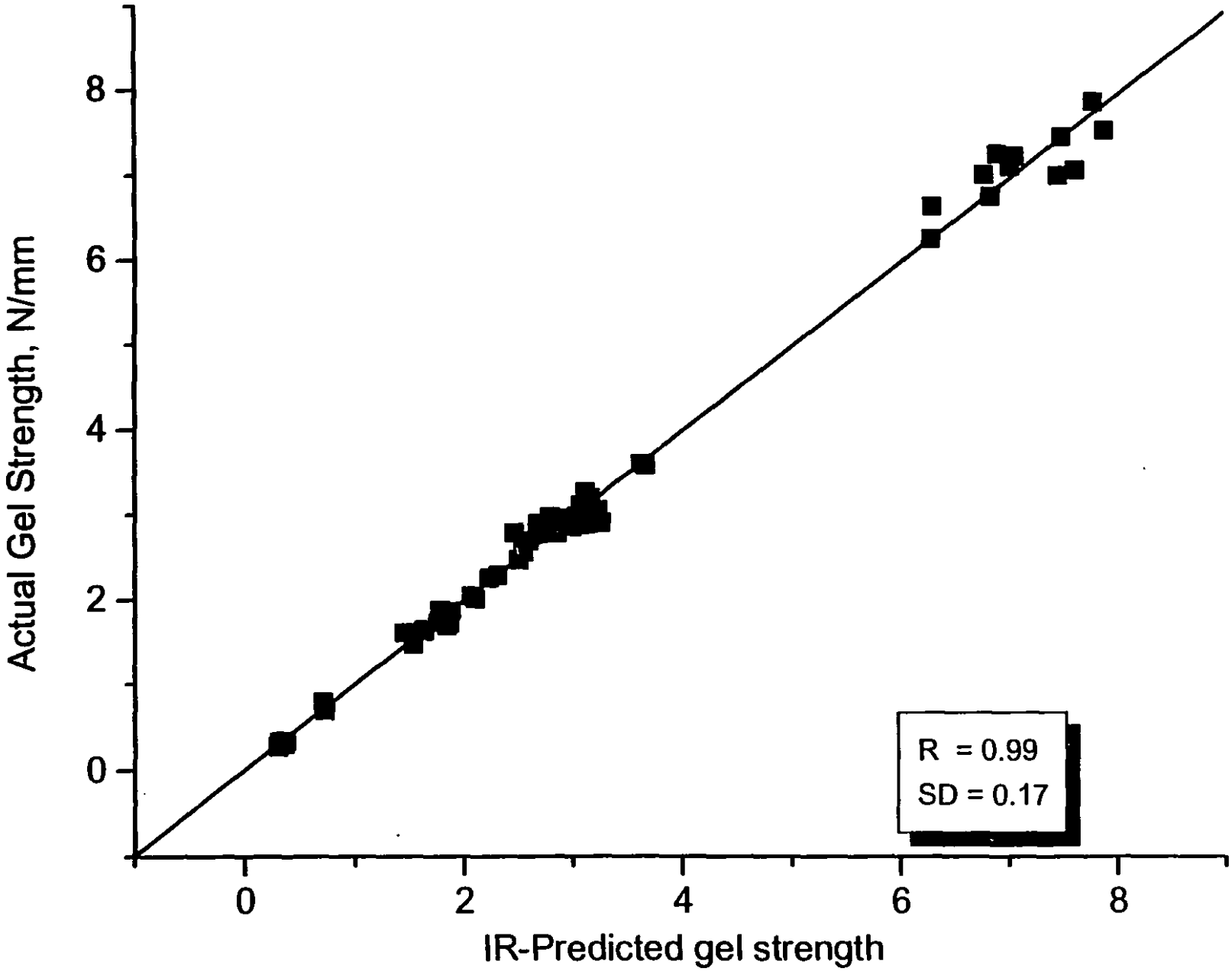

Figure 5.9 : Plot of actual gel strength vs. IR predicted for temperatures

A maximum mean error is associated with 80 °C temperature runs and maximum standard deviation is associated with 90 °C temperature runs. The overall prediction of gel strength values are very close to actual values. These results suggest that prediction of physical measurements of protein gels could be carried out practically and with good accuracy, by employing infrared spectroscopy to predict gel strength.

Exponential relationship was obtained (Harper et al., 1978) between time and temperature of heating and the extend of gelation of BSA. The extend of gelation increases with increase in time of heating and temperature at which solution of protein is heated. Water binding capacity which represents the gel strength (Hermansson et al., 1992, Yasuda et al., 1986) for instance, is reported to increase with increasing the temperature of heating of the protein solutions from 75 to 95 °C and no gel formation is obtained below 70 °C which again indicates a temperature above unfolding is required for initial stage of gel formation.

Lower temperature of heating was required for gel formation of BSA when higher protein concentration was used and when gel formation was carried out in optimum pH of protein, either a harder gel is obtained at the same temperature or lower temperature was required for the same gel strength under the same condition (Paulsoon et al., 1986). Gel properties such as gel time and rigidity modulus are reported (Kinsella et al., 1982) to be dependent on temperature and time of heating of protein solution when concentration of BSA is held constant, the gelation time (time required to form a gel) decreases with temperature increase. The effect of heating temperature on gelation of protein also is reported by (Matsudomi et al., 1992) to be significant and gel hardness increased gradually above denaturation

temperature and self supporting gel was formed above 70 °C. The hardness of the gel is reported to increases gradually with heating between 75 and 95 °C.

Major forces involved in the formation and stabilization of a gel matrix is suggested (Mulvihill et al., 1988) to be non covalent bonding such as hydrophobic interaction, hydrogen bonding and also disulfide bonding may have contributed (Richardson et al., 1981) considerably to the interaction involved during their heat induced gelation process.

5.5 Prediction of gel strength from transmission spectra of BSA by FTIR/PLS model

The feasibility of predicting the strength of BSA gels formed during the monitoring of thermal denaturation of BSA in a transmission cell (see Chapter 4) by using the PLS calibration models described in this chapter was investigated. As these calibration models were developed using spectra acquired by the ATR sampling technique, the spectra of the calibration standards were first mathematically converted to transmission spectra, and then a recalibration was performed. The transmission spectra were then predicted as unknowns. However; all the calibration standards were gels with different gel strength values, while the transmission spectra covered a temperature range from 30 to 95 °C while no gels are formed up to 65 °C. Thus, the problem of not having a representative sample in the calibration for samples prepared below 65 °C arises. Although PLS predicted gel strengths for the spectra below 65 °C, it is interesting to note that these predicted values of gel strength are negative because the transmission spectra prior to unfolding (below 65 °C) have no aggregation bands. After unfolding, the aggregation bands appear and continue to grow as the temperature increases (see Chapter 4). The PLS calibration seems to recognize and differentiate these

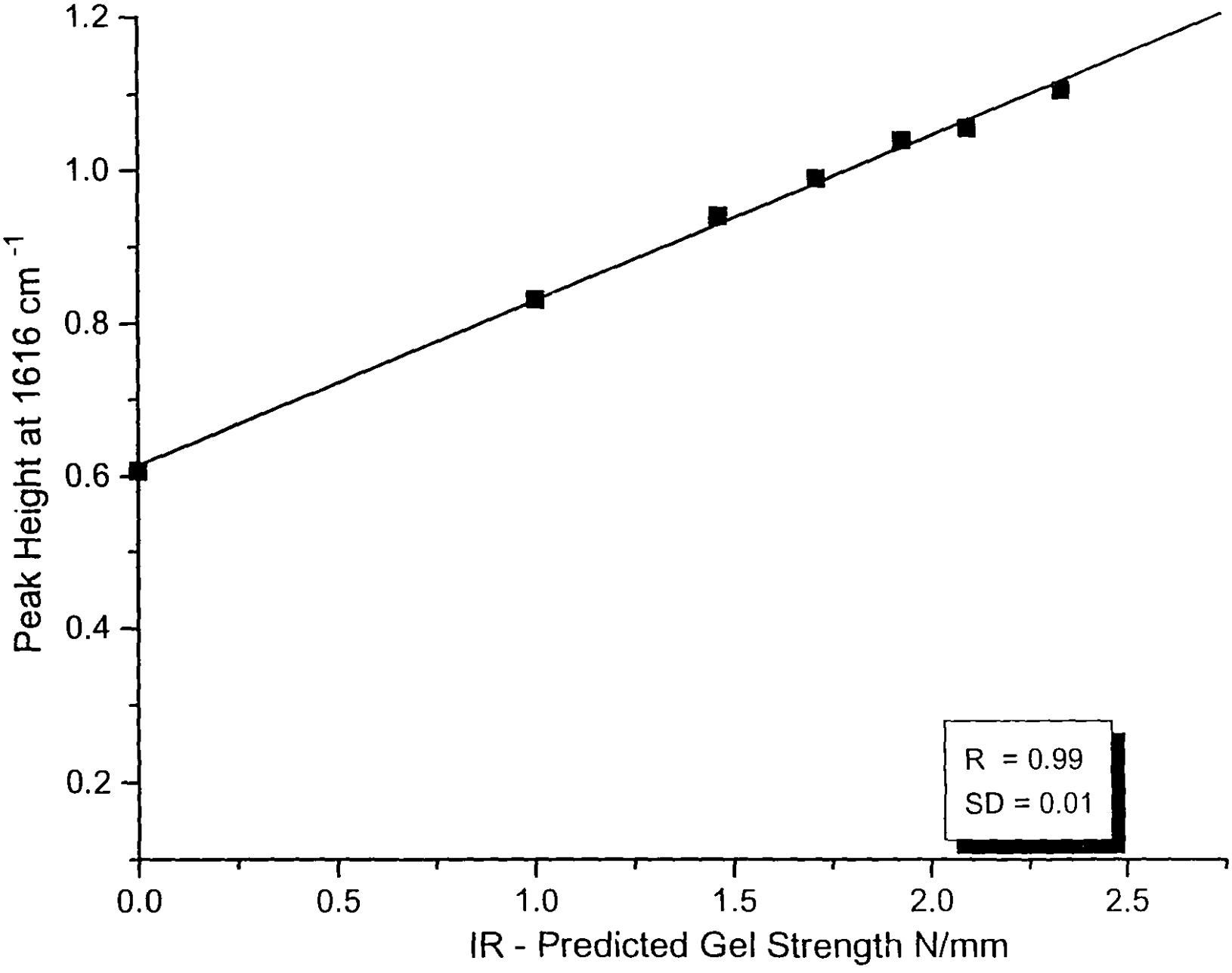

Figure 5.10 : Plot of peak height at 1618 cm^{-1} vs. IR predicted gel strength

transmission spectra, for those that have undergone denaturation and aggregation predict a positive number and for those spectra that resemble the native protein, predict a negative gel strength.

When IR-predicted gel strength for transmission spectra are plotted against the peak height of the aggregation band at 1618 cm^{-1}, a linear plot (R = 0.99) is obtained (Figure 5.10) when the negative IR-predicted gel strengths are treated as zero. Utilization of this ATR calibration for prediction of gel strength is performed on all BSA spectra and is reliable for any given conditions (i.e, pH, protein concentration, ionic strength, time and temperature) but this ATR calibration of BSA spectra is limited to BSA gelation. For other situation or proteins, a separate calibration should be developed.

5.6 Conclusion

Findings in this study tentatively suggests that the strength of BSA gel may be directly related to the extend of transformation of α-helical and aperiodic structures into intermolecular β-sheet during the heating step. The formation of intermolecular β-sheet structure is definitely a prerequisite for formation of aggregates and cross-linked network in a BSA gel network. It has also been found here that the formation of intermolecular β-sheet of heat denatured BSA is increased in higher protein concentration due to more availability of the protein molecules and higher intermolecular interactions such as hydrogen bonding. A PLS model was successfully developed to predict gel strength from the infrared spectra of gels prepared under a variety of physicochemical conditions.

Chapter 6 Denaturation and gelation of egg albumin

6.1 Introduction

Thermally induced gelation of egg albumin is dependent on extrinsic factors such as protein concentration, pH, ionic strength and the temperature at which the solution of egg albumin is heated. In the present work, the effect of each of these parameters has been investigated independently, and the rheological properties of the gels obtained have been studied by a compression test performed on an Instron. The FTIR spectra of the same gel samples have been recorded in order to investigate the relationship between changes in the secondary structure of the protein and its gel formation properties. These structural changes can also be monitored in amide III region of the spectrum and the usefulness of this region compared to the amide I region is described in the following section.

6.2 Amide III spectral region

The amide I, II, and III bands, which appear at 1600-1700 cm^{-1}, 1480-1574 cm^{-1} and 1200-1350 cm^{-1}, respectively, are among the most studied bands of proteins. The amide III spectral region has not been recognized as a potentially very useful region for secondary structure analysis, primarily because of the weak protein signal in this region (Fen-nifu et al., 1994). However, a great advantage of using the amide III region for structural analysis of proteins is the absence of interference from OH vibration of water, which masks the amide I band. Therefore, use of amide III protein bands eliminates the need for D_2O, which is commonly used to deal with H_2O interferences (Byler and Susi, 1986). The error introduced from subtraction process of water and water vapor present in the light

path is also eliminated (Anderle et al., 1987). Furthermore, ambiguity associated with the assignment of the bands to a specific protein secondary structures in the amide I region is absent in the amide III region (Singh et al., 1990, Surewicz et al., 1993). For instance, bands that are located between 1650 and 1655 cm^{-1} can be assigned to either α-helix or a random coil structure in the amide I region (Fin-nifu et al., 1994). In the amide III region, the frequency at which the different amide bond vibrations occur are more localized and provide a better resolved protein spectrum. This factor allows greater ease in the band assignment. In the amide III region, distinct peaks are identified without the need for resolution enhancement techniques. These characteristic bands afford a simple illustration of the secondary structure content of protein.

PLS variance spectra (within an experiment) reveals valuable information about the spectral features that changes the most within a set of spectra. Variance spectra have been calculated as $\Sigma(X_{1,2,3,16}\ldots - \bar{X})^2$, if 16 spectra were to be analyzed (Haaland et al., 1988, Li-Chan et al., 1987,.Lee et al., 1990). Variance spectra could be interpreted in the same manner as sample spectra since they contain peaks. However, these peaks are not due to compounds or bands within molecules as is the case for sample spectra. They are instead due to the changes that occur from one standard to another. In terms of proteins, the peaks are due to secondary structure changes, i.e. in α-helix or β-sheet, occurring the most within the region of the IR spectrum. Thus, if a secondary structure of a protein is not affected by the treatment given to the sample, there would be a band due to that structure in the sample spectra but not in the variance spectra. Then, by looking at different peaks in the variance spectra for a given parameter, i.e. one of the pH experiments, one could interpret the effect of pH at different conditions on protein gels at the molecular level. Figure 6.1 shows the mean, variance, and standard

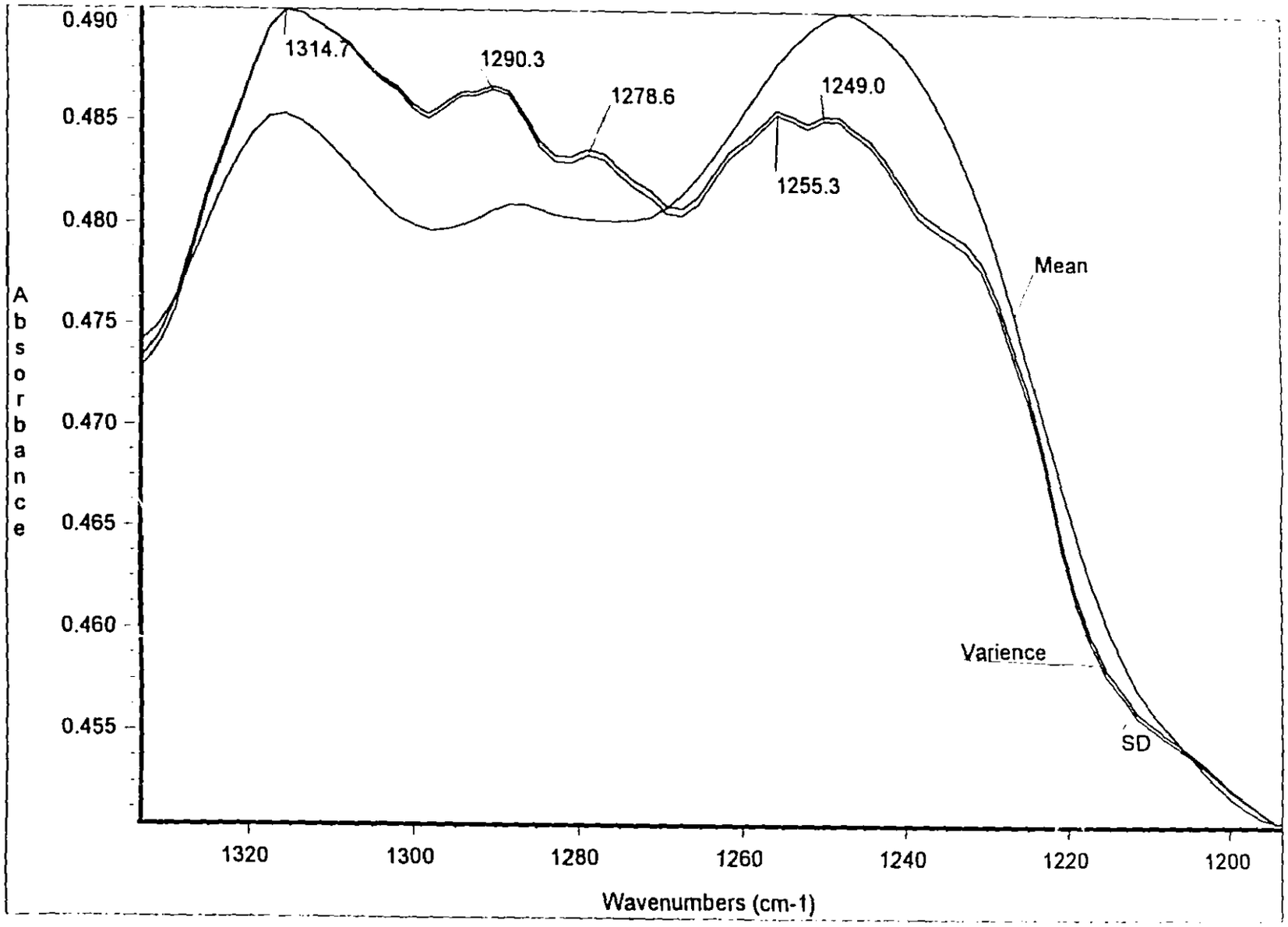

Figure 6.1 : Statistical spectra of amide III band of egg albumin

deviation spectra in the amide III region of egg albumin. The mean spectrum is simply the mean of all standards and it looks like a sample spectra while standard deviation and variance spectra are alike, pointing out the region of amide III band that changes the most among the spectra. In this case, these changes occur in the bands associated with α-helix, β-sheet and disordered structure of egg albumin: two bands associated with changes in β-sheet structure at 1249 cm^{-1} and 1255 cm^{-1}, another band associated with disordered structure at 1287 cm^{-1}, and two other bands associated with α-helix structure at 1290 cm^{-1} and 1314 cm^{-1} (Fen-nifu et al., 1994).

Three bands in the amide III region of the spectrum of egg albumin exhibit changes after 15 minutes of heating (Figure 6.2). The band at 1242 cm^{-1} increases in intensity as heating time increases while the 1317 cm^{-1} band decreases in intensity. It seems that one type of secondary structure has been converted to another one upon heat treatment. The 1242 cm^{-1} band has been assigned to β-sheet (Kato et al., 1987, Kaiden et al., 1987, Fen-nifu et al., 1994, Painter et al., 1976, Frushour et al., 1974, Yu et al., 1973 and Koeing et al., 1975) and to some extent a combination of β-sheet and disordered structures (Lenk et al., 1989 and Jakobson at el., 1990) but the extent of this combination is still unclear. The band around 1317 cm^{-1} has been assigned to α-helix structure in almost all globular proteins (Kato et al., 1987, Kaiden et al., 1987, Anderle and Mendelson 1987, Jakobson et al., 1990 and Fen-nifu et al., 1994). The band around 1286 cm^{-1} has been assigned to disordered structures (Jakobson et al., 1990 and Fen-nifu et al., 1994). The fact that a band near 1286 cm^{-1} only appears after extensive unfolding of the protein suggests that it is due to disordered structure. Thus, the band at 1286 cm^{-1} may be due to a type of disordered structure that forms after extensive unfolding. In general, the band at 1317 cm^{-1} due to α-helix decreases in intensity where increase in β structure at 1244 cm^{-1} is more indicative of intermolecular β-

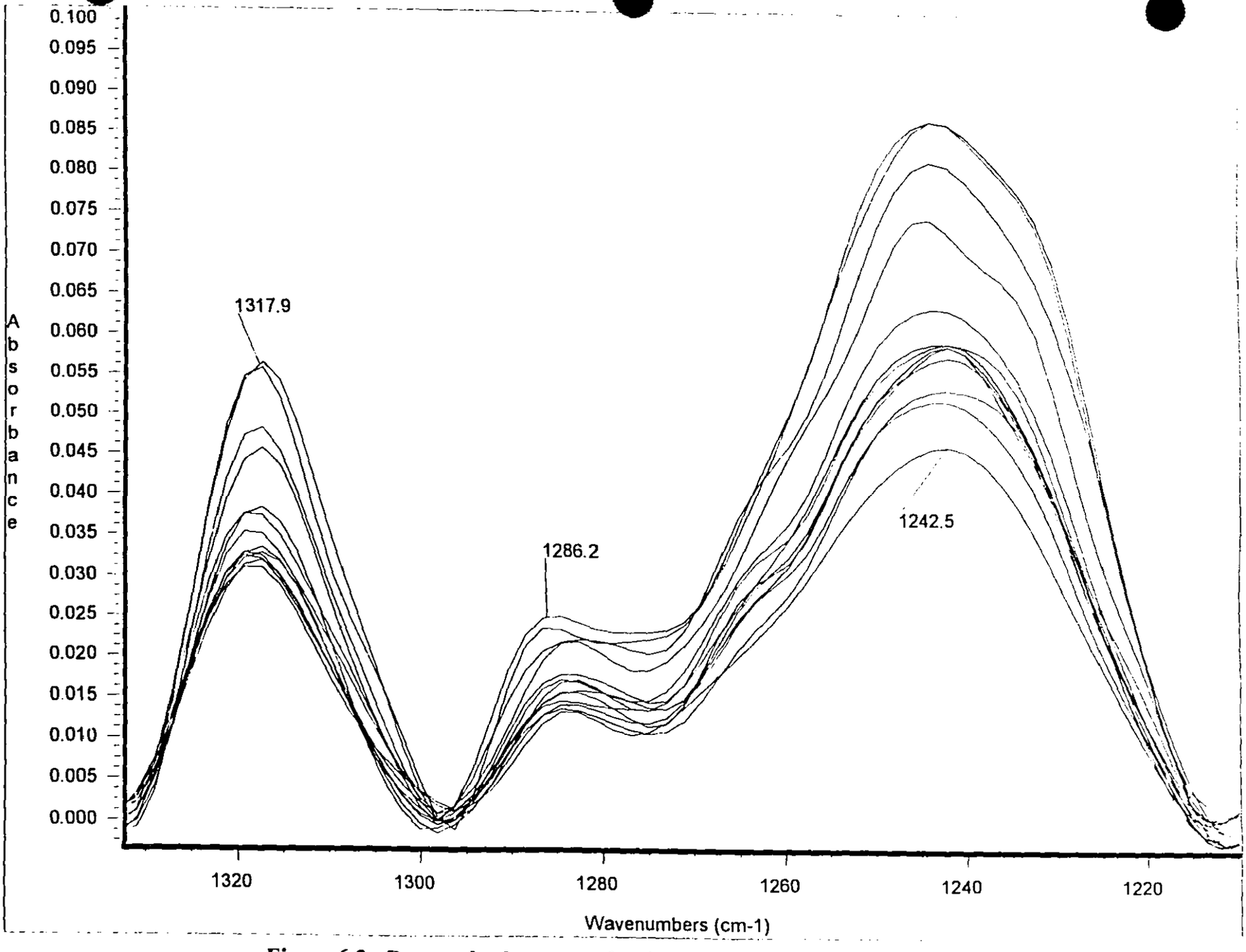

Figure 6.2 : Deconvolved spectra of amide III band of egg albumin

sheet formation, mainly responsible for gel network formation. It has been reported (Kato et al., 1988) that the β-sheet structure increases with heat denaturation in sacrifice of α-helix and these changes are irreversible.

Evidence for the role of H-bonding in gel has been given by Kato et al. (1988) who reported that intermolecular β-sheet structure increases in heat induced soluble aggregate of ovalbumin in proportion to increase in their molecular size. Accordingly, it is possible that exposure of large number of hydrophobic residue to the surface of ovalbumin (Kato et al., 1988 and 1990, Hisham et al., 1990) might enhances the formation of intermolecular hydrogen bonds through β-sheet conformation and thus, lead to the formation of a firm and stable gel network. They also reported that a good correlation was obtained between the molecular weight and β sheet content of ovalbumin and concluded that heat induced ovalbumin aggregate are formed by intermolecular β sheet structure.

6.3 Effect of protein concentration

When solutions of egg albumin in water of different concentrations were heated at 80 °C, gels were formed which possess different physical characteristics and gel strengths. Throughout this study, a linear relationship was observed between the gel strength and protein concentration. The gel strength values of protein gels heated for different amounts of time markedly increased with an increase in protein concentration (Figure 6.3). The effect of heating time on gel strength was more pronounced at 30% protein concentration than at lower concentrations. The gel strength of egg albumin greatly increased with an increase in time of heating. The dependency of gel strength upon protein concentration was

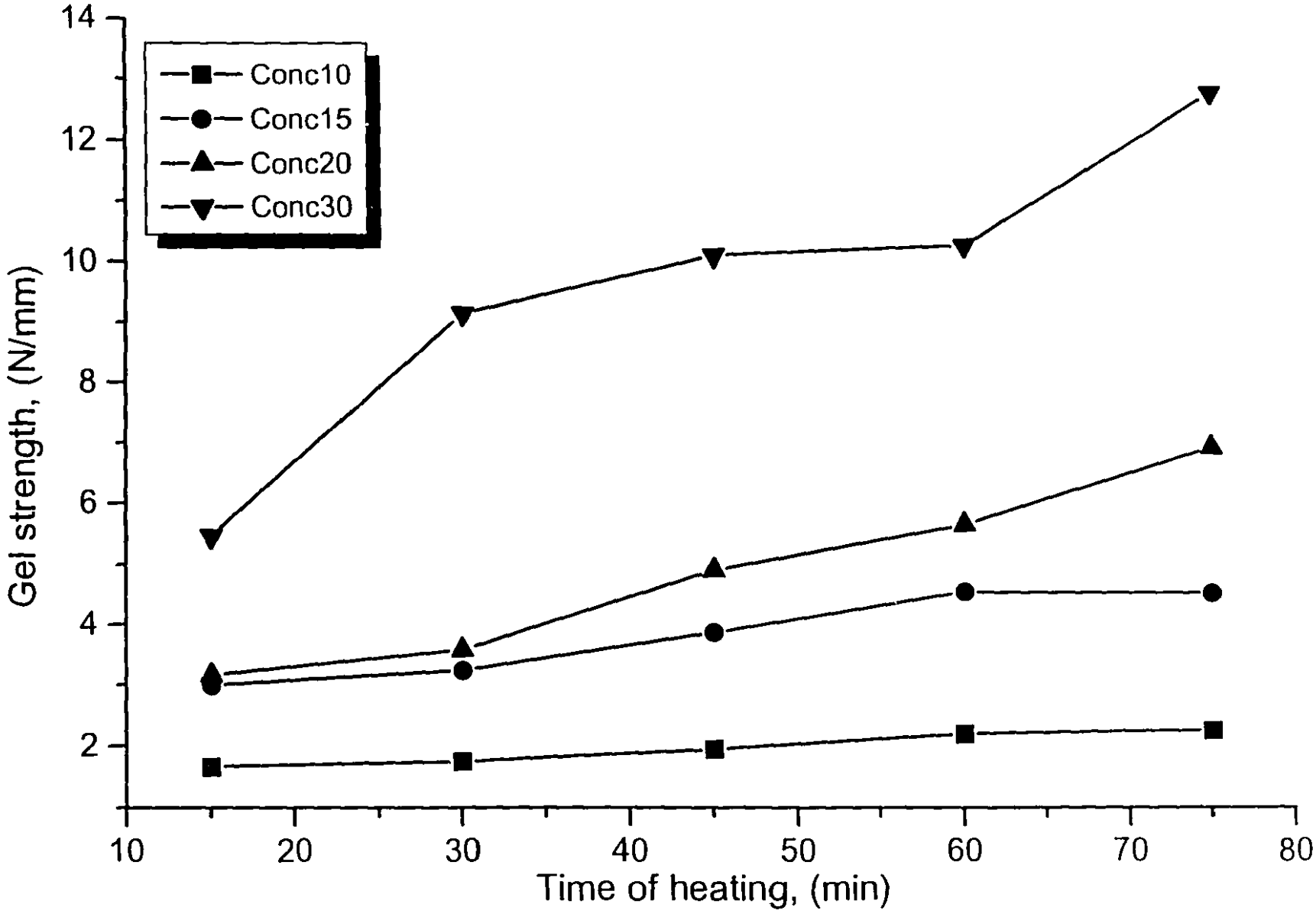

Figure 6.3 : Plot of gel strength vs. time of heating for protein concentrations

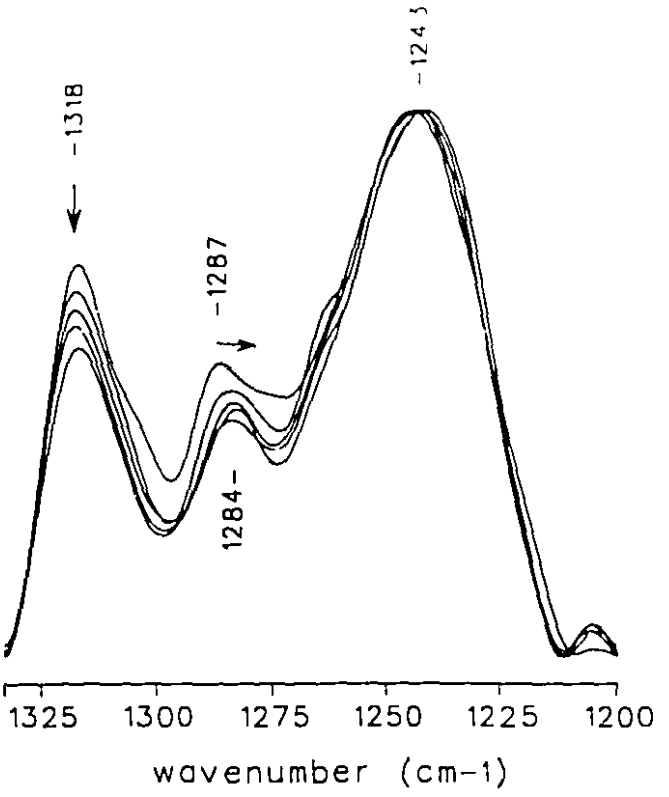

Figure 6.4b : Overlaid spectra of amide III region of egg albumin gels

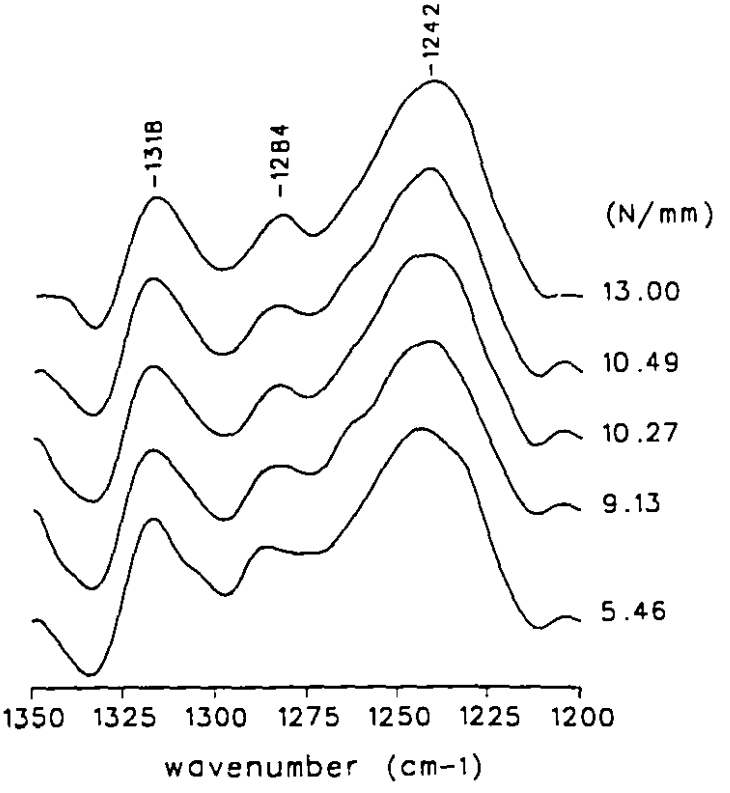

Figure 6.4a : Stacked spectra of amide III region of egg albumin gels

substantial for sample heated with 30% concentration in the early stages of heating (i.e.; 30 minutes). These results indicate that increase in heating time is an effective method to reduce the amount of protein required to yield a gel with particular gel strength.

The FTIR spectra of the gel samples obtained after heating a 30% solution of egg albumin for different times were recorded using an ATR sampling accessory. Because these gels were prepared from solutions in H_2O, the amide I region in the spectra is obscured by water absorption bands. However, information on changes in secondary structure can also be obtained from the amide III region (1350-1200 cm-1). Figure 6.4a shows a stacked plot of the amide III band in the spectra obtained from these gels, while these spectra are overlaid in Figure 6.4b to provide a clearer view of the relative intensity changes. The major change that is observed with increasing gel strength is a decrease in the band at 1318 cm^{-1}, assigned to helical structure (Section 4.5.1). Spectroscopic prediction of gel strength obtained through FTIR/PLS calibration, compared to actual values obtained by instron, indicates that there is a 3.81% error in the prediction of protein gels formed from a 10% solution (mean average error for all samples within an experiment), 5.05% error for 15% concentration, 5.29% error for 20% concentration and 5.53% error for 30% protein concentration which means on average, predicted gel strength obtained correlates very well to actual values of the gel strength of heat induced egg albumin gels. This high degree of accuracy may be increased to 98-99% if optimization of experimentation in all stages (sample preparation and handling, instron and FTIR alignment) is carried out. Figure 6.5 shows the plot of actual gel strength of protein concentration vs. predicted values obtained by PLS in which all the data for the four concentrations are combined together, and provide an excellent correlation (R = 0.99, SD = 0.46) between predicted and actual gel strength values. Table 6.1 shows a summary of the errors

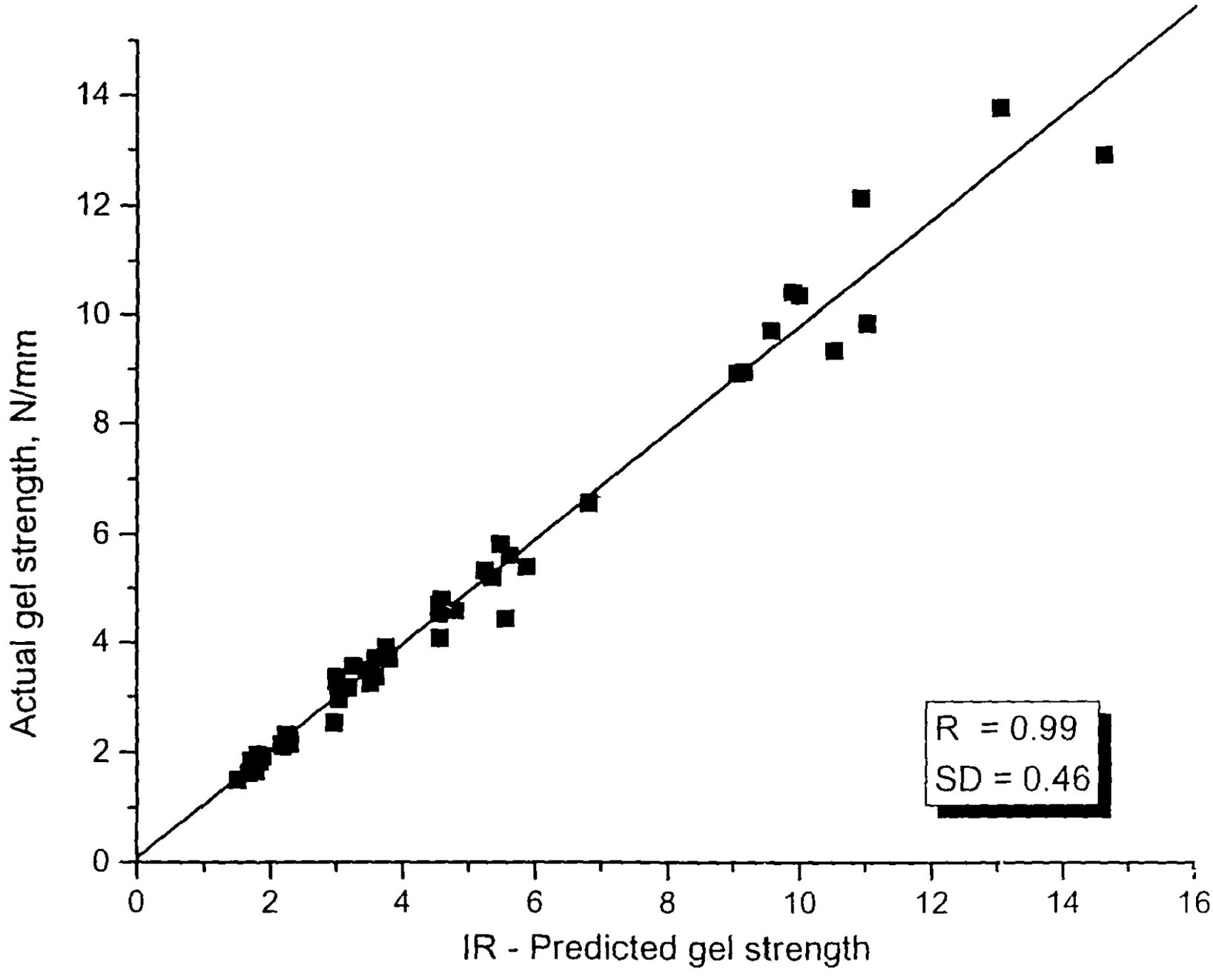

Figure 6.5 : Plot of actual gel strength vs. IR predicted for protein concentrations

(error between actual values obtained by compression test vs. predicted values by PLS) for all parameters studied. For protein concentration mean error obtained was 0.0168 for all samples predicted from the different concentrations.

Table 6.1: Statistical calculation of error (actual vs. predicted) values for gel strength

Parameters	Mean [a]	sd(yEr +) [b]	se(yEr +) [c]	Sum [d]
Protein conc.	0.0168	0.4641	0.0649	0.8579
pH	0.0048	0.2396	0.0357	0.2125
Salt conc.	0.0249	0.2023	0.0305	0.1095
Temperature	0.0439	0.2595	0.0426	1.6237

[a] Mean of error of actual Vs. predicted.

[b] Standard deviation of error

[c] Percentage error; A-B/A*100

[d] Summation of error

Woodward et al. (1986) reported that significant interaction of pH and protein concentration occurred while measuring the gel hardness of egg albumin and concluded that pH and protein concentration influences texture and water binding independently. Increasing protein content of egg albumin gels caused significant increase in hardness. Cohesiveness decreased slightly with increased fracturability at higher protein level. A slight but significant increase in springiness occurred as protein level was increase from 9-12 %. Good correlation was obtained (Hsieh et al. 1989) between hardness and egg albumin concentration with 30% and 65% Instron compression. A computer simulation of the hardness value for the 65% Instron compression from 0 to 99.9% egg albumin concentration

indicates a sharp increase between 15% and 30% egg albumin concentration. They concluded that the gel strength increased with increasing protein concentration. The egg albumin monomers complexes which were established by the coagulation of 8-10 protein monomers contributed predominantly to the hardness of the gels (Shimada et al., 1980). The combined effect of acidity or alkalinity with an increase in protein concentration resulted in turbid solutions. At low protein concentration turbidity was observed only within the narrow pH range around the pI, but at higher protein concentration, it was observed in a wider pH range. The pH when relative turbidity is 0.5, is expressed as critical pH for coagulation. Shimada et al. (1980) also established a linear relationship between protein concentration and the hardness of coagulum. When protein concentration increased hardness also increased under both alkaline and acidic conditions. At relatively low protein concentration, the thermally-induced protein gels formed at low pH are very soft, granular and prone to release water very easily, both granularity and water exudation decreases with increasing protein concentration (Van Kleef 1986).

6.4 Salt concentration effect

The transparency, opacity and rheological properties of heat induced egg albumin gel depends on NaCl concentration and pH of the protein solution. Most research in this area have been carried out, studying interrelationship of these two parameters for protein gels together, since both of these factors can alter electrostatic charges of protein. The relationship between gel strength and NaCl concentration was investigated by Kitabatake et al. (1988) and by Hatta et al. (1986), who showed that maximum gel strength was obtained at about 125 mM NaCl concentration along with a sharp increase in turbidity. Egg albumin first gave

transparent solution, next a translucent gel and then a turbid gel as NaCl concentration increased. These findings is in agreement with our results that maximum gel strength of egg albumin was obtained with 0.1M NaCl concentration, we also found that when NaCl concentration increased above 0.1M , the gel strength decreased.

When NaCl concentration was varied different rheological properties of heat induced egg albumin gel were obtained. At low concentration the heated samples were transparent, as concentration of NaCl increased the turbidity of sample increased and above 0.2M NaCl completely turbid gel were obtained. Kitabatake et al. (1988) and Hatta et al. (1986) reported that the interaction between denatured ovalbumin molecules is controlled by the balance of attractive and repulsive forces. When the repulsive forces between denatured protein molecules were stronger than the attractive forces i.e.; low ionic strength and/or far from pI of the molecules, the heat-denatured molecules could not form a large aggregate, therefore they exist as a solution. When attractive forces increase a little and repulsive forces decrease slightly i.e.; the binding sites of molecules appeared, each molecules bound together at a few points to give linear aggregate, forming the network structure of the gel. When the salt concentration is increased, the attractive forces further increased forming a random aggregate, a coagulum was produced which become fixed in the gel, in this case translucent gels or turbid gel were formed. At higher concentration of salt a turbid suspension is formed, indicating the salting out effect. Holt et al., 1984 reported that increased NaCl concentration produced only slight effect on gel strength and elasticity of heat induced egg albumin gel, resulting in minor increase in these parameters with increase in NaCl concentration. However viscosity was significantly affected by the linear component of NaCl concentration. The surface shows a decrease in

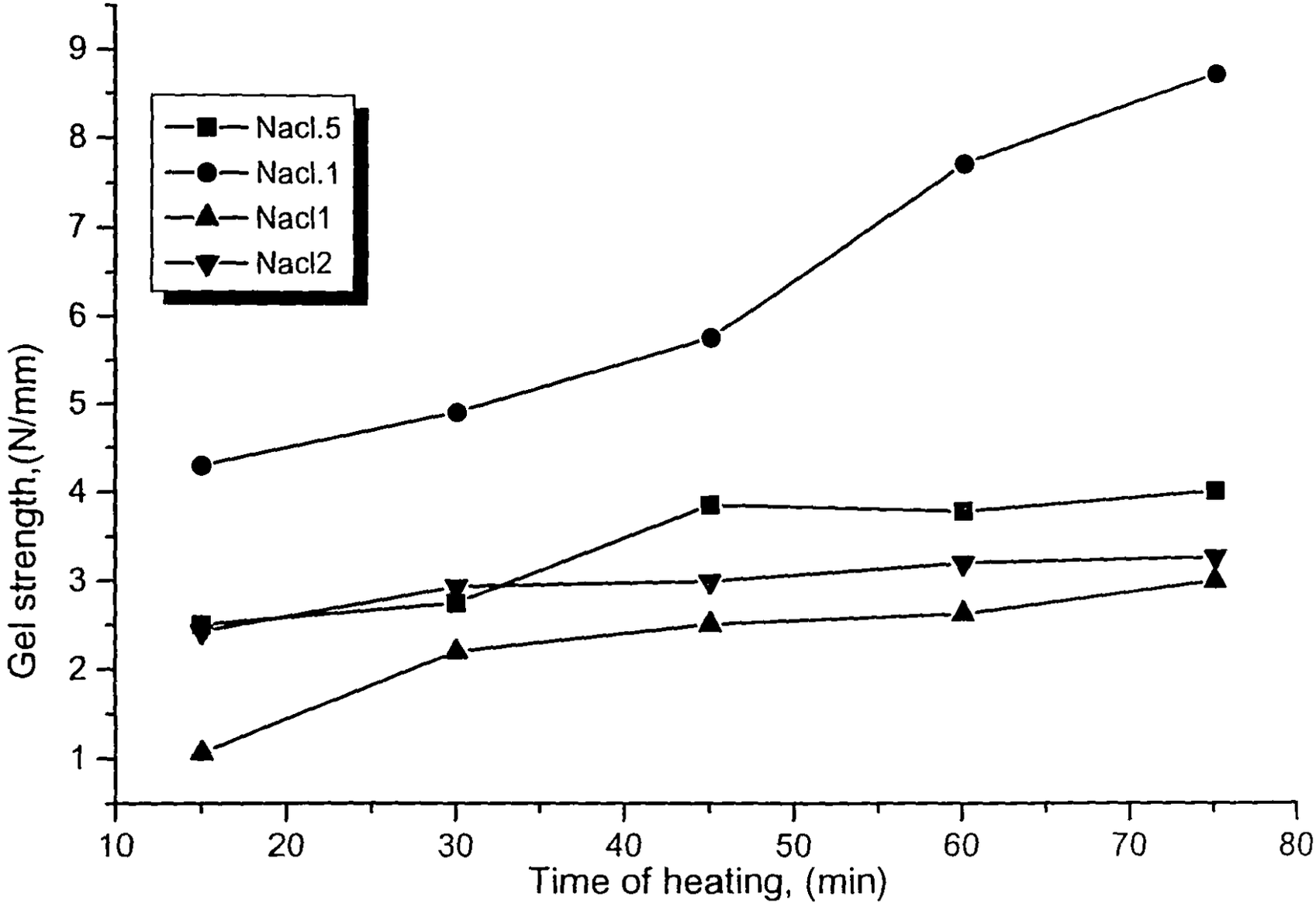

Figure 6.6 : Plot of gel strength vs. time of heating for ionic strengths

viscosity with increasing NaCl concentration from 0-0.02M and rapid increase in viscosity with NaCl concentration from 0.02-0.1M. This finding is in agreement with our results since as maximum gel strength of egg albumin was obtained with 0.1M NaCl concentration, we also found that when NaCl concentration increased above 0.1M, the gel strength decreased.

Figure 6.6 shows the plot of gel strength of egg albumin gel vs. time of heating for ionic strength. Maximum gel strength was obtained with the lowest salt concentration i.e.; 0.1 M NaCl, and as the salt concentration increased the hardness of the gel decreased. It is also evident that time of heating has a pronounced effect on the lowest salt concentration since, as the time of heating is prolonged, gel strength increases drastically, while this trend is absent in other salt concentration experiments.

FTIR spectra in the amide III region of protein gels prepared from solutions of varying NaCl concentrations are shown in Figure 6.7. Examination of the stacked spectra for gels obtained from the 0.5M and 1M NaCl solutions shows a decrease in the band at 1318 cm^{-1} and an increase in the band at 1247 cm^{-1} as the gel strength increases. The error involved in spectroscopic prediction of gel strength obtained by FTIR/PLS calibration model is 3.87% for 0.1M NaCl concentration, 2.03% for 0.5M, 3.54% for 1M and 3.06% error for 2M NaCl concentration. A mean error of 0.0048 was obtained between actual and predicted values from a combined calibration for the four sets of gels prepared from solutions of varying ionic strength, which indicates the workability of the PLS calibration. Figure 6.8 shows the plot of actual vs. predicted gel strength for all salt concentration experiments. Significant correlation (R=0.99, SD=0.19) is obtained from the regression curve.

Kato et al. (1990) and Hisham et al. (1990) reported the effect of salt concentration on molecular weight of heat induced egg albumin aggregate as a

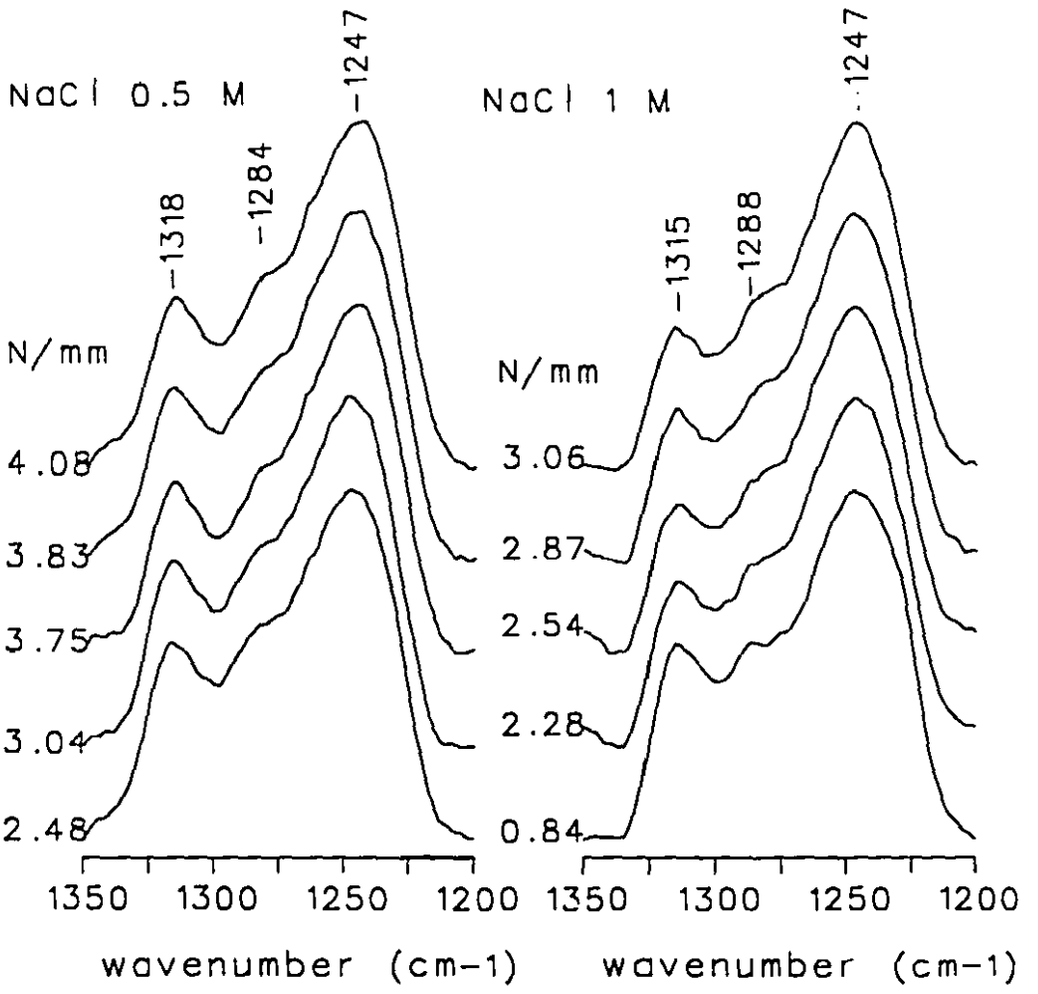

Figure 6.7 : Stacked spectra of amide III region of egg albumin gels, 0.5, 1 M NaCl

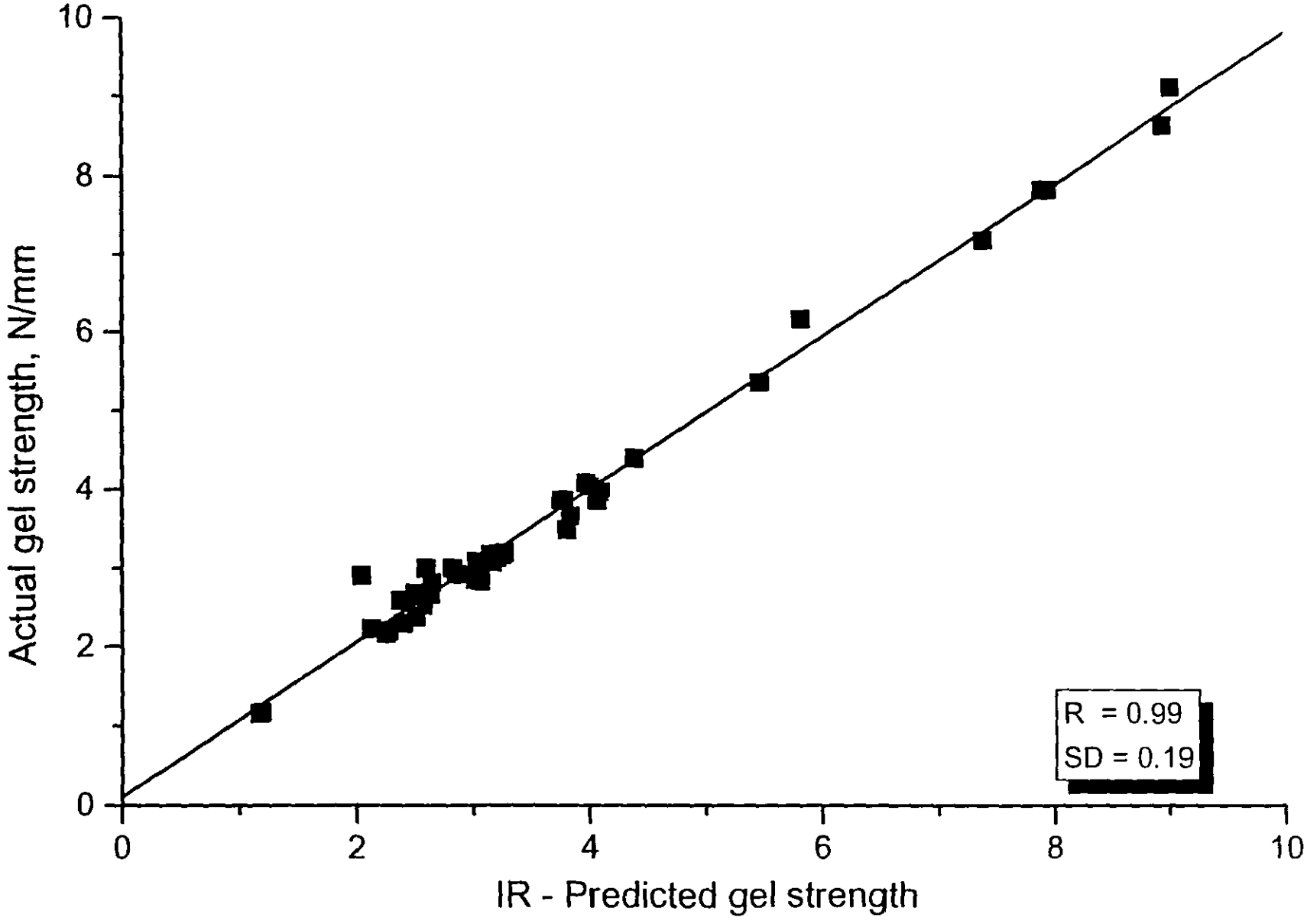

Figure 6.8 : Plot of actual gel strength vs. IR predicted for ionic strengths

function of preheating time in dry state. Adding salt to the protein medium from 2 to 10 mM NaCl caused a progressive and rapid increase in the molecular weight of heat induced egg albumin aggregate in the dry state. The molecular weight of heat induced egg albumin aggregate decreased rapidly with an increase in heating temperature up to 76°C, where it reached a constant value. They concluded that the apparent decrease in the degree polymerization of the preheated egg albumin in dry state upon gelation might be attributed to surface charge factors since salt is known to rupture ionic attraction between protein molecules and indirectly enhances hydrophobic interaction. It has also been reported that, as molecular weight of heat induced egg albumin gel increases, the gel strength decreases due to different degree of polymerization and subsequently a gel can be obtained which possesses a weaker gel strength (Nandi et al., 1972, Hisham, 1990).

6.5 pH effect

Electrostatic charges is one of the most commonly investigated factors in the study of heat-induced gelation of egg albumin. The pH as well as ionic strength of protein environment can alter the charge distribution among the amino acid side chains and can either increase or decrease the protein-protein interaction. The main factor contributing to the heat induced aggregation is the degree of electrostatic repulsion among the denatured protein molecules (Gosset et al., 1984). Different researchers reported different results for pH measurements in relation to gel hardness due to different, 1) conditions at which coagulum was formed and 2) the mechanism of gel measurements.

The rheological and other physical properties of heat set protein gels strongly depend on the pH of the solvent with which the protein is mixed and heated. The ionic strength of the solvent has less pronounced effect. When heat-

setting take place at high pH (pH 9), the protein unfolds (decreasing number of intramolecular protein-protein interaction) during heating and is cross linked at high temperature mainly by intermolecular non-covalent (and covalent) bonds. This leads to the formation of polymer network, in which flexible mobile unfolded chain is present. Since the protein molecules in the high pH gels are not so strongly interacting, there can be more protein-water interaction (water binding), and thus, the high pH gels show no synergesis. There are fewer cross links (intermolecular protein-protein interaction) at high pH than at low pH, due to ionic repulsion between negatively charged molecules. The storage modulus of high pH gels is therefore lower than that of the low pH gels. The presence of unfolded chains in the high pH gels result in larger value of deformation at break in a more homogeneous and perfect network and consequently, in a much stronger gel. The gel is almost transparent and smooth (Watanabe et al., 1985, Van Kleef, 1986).

When heat-setting takes place at low pH (pH 5), the protein molecules do not unfold to a large extent. This leads to the formation of an aggregated particle network, in which the compact rigid protein molecules strongly interact (high level of both intra and intermolecular protein-protein interaction). The storage modulus is therefore, higher for low pH gels. The level of protein water interaction is low resulting in considerable water exudation, depending on protein concentration and applied pressure. The presence of compact rigid protein aggregate results in low value of deformation at break, in a less homogenous network and therefore, in a much weaker gel. The gel is opaque and granular (Van Kleef, 1986, Egalandsdal, 1986).

A high net charge repulsion between proteins, limited intermolecular interaction and a low dry matter content have been suggested (Hegg Per-Olof, 1982) as condition required for formation of translucent gels. It appears that the

increase in the net negative charge of the protein delays and/or lowers the rate of protein aggregation allowing for more denaturation and unfolding. The particular conformation and spatial arrangement of the protein-water aggregates is responsible for translucent characteristics to the structure.

When the pH of the protein system was varied, the change in the turbidity and hardness of the coagulum was noticed, (Shimada et al., 1980), above pH 11, the turbidity of heated egg albumin in solution was not observed, but below pH 11, it increased and above pH 8, it reached a plateau. Coagulum began to form at about pH 9.5 and hardness reached its highest at pH 8.5 and decreased gradually below pH 8.5. In this work, protein solution at pH 9 gave the hardest gel and as the pH decreased, the gel strength decreased. These observations suggest that the coagulum exclude water and become fragile as the pH approaches the isoelectric point. The key to formation of a gel with fine structure and optimum water binding properties is balancing the attractive and repulsive forces. At low net charge, attractive forces predominate and protein aggregates. As the pH increases the net charge increases and repulsive forces balance attractive forces to an optimum in gel formation ability which in this study was found to be pH 9 for egg albumin portions. Beyond the optimum pH, the repulsive forces may be so strong that fewer protein interaction are possible, and a weak gel result (Woodware et al., 1986).

When different factors involved in gel formation of egg albumin is combined, maximum gel strength have been obtained at 85°C, pH 9 and 0.08M NaCl (Holt et al., 1984). Additionally, at higher pH levels, the ability of protein to cross link through sulfydryl disulfide exchanged is enhanced, possibly contributing to a structural integrity of the gels, which is in agreement with consistent results for all temperature, pH and salt concentration carried out in this study.

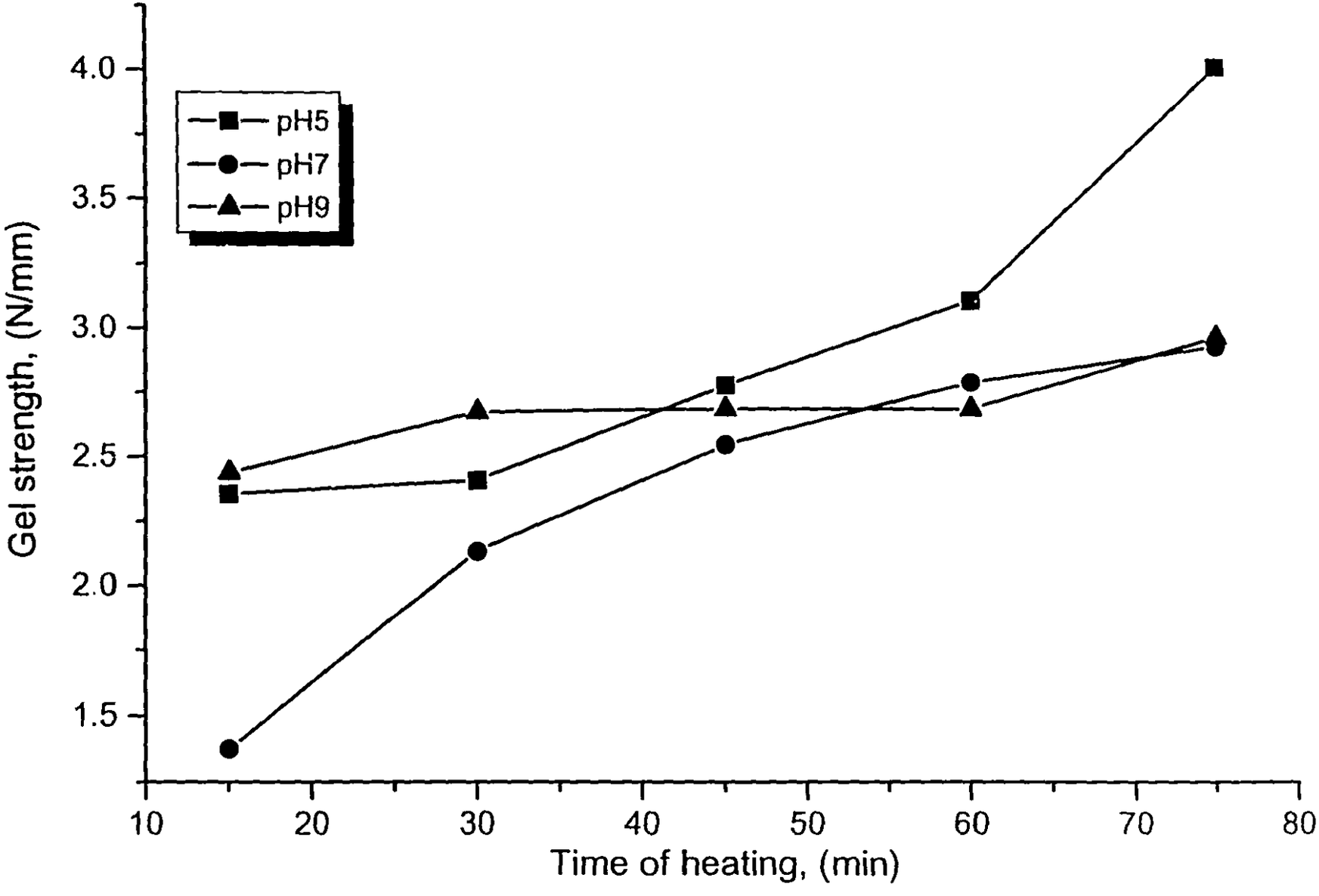

Figure 6.9 : Plot of gel strength vs. time of heating for pH

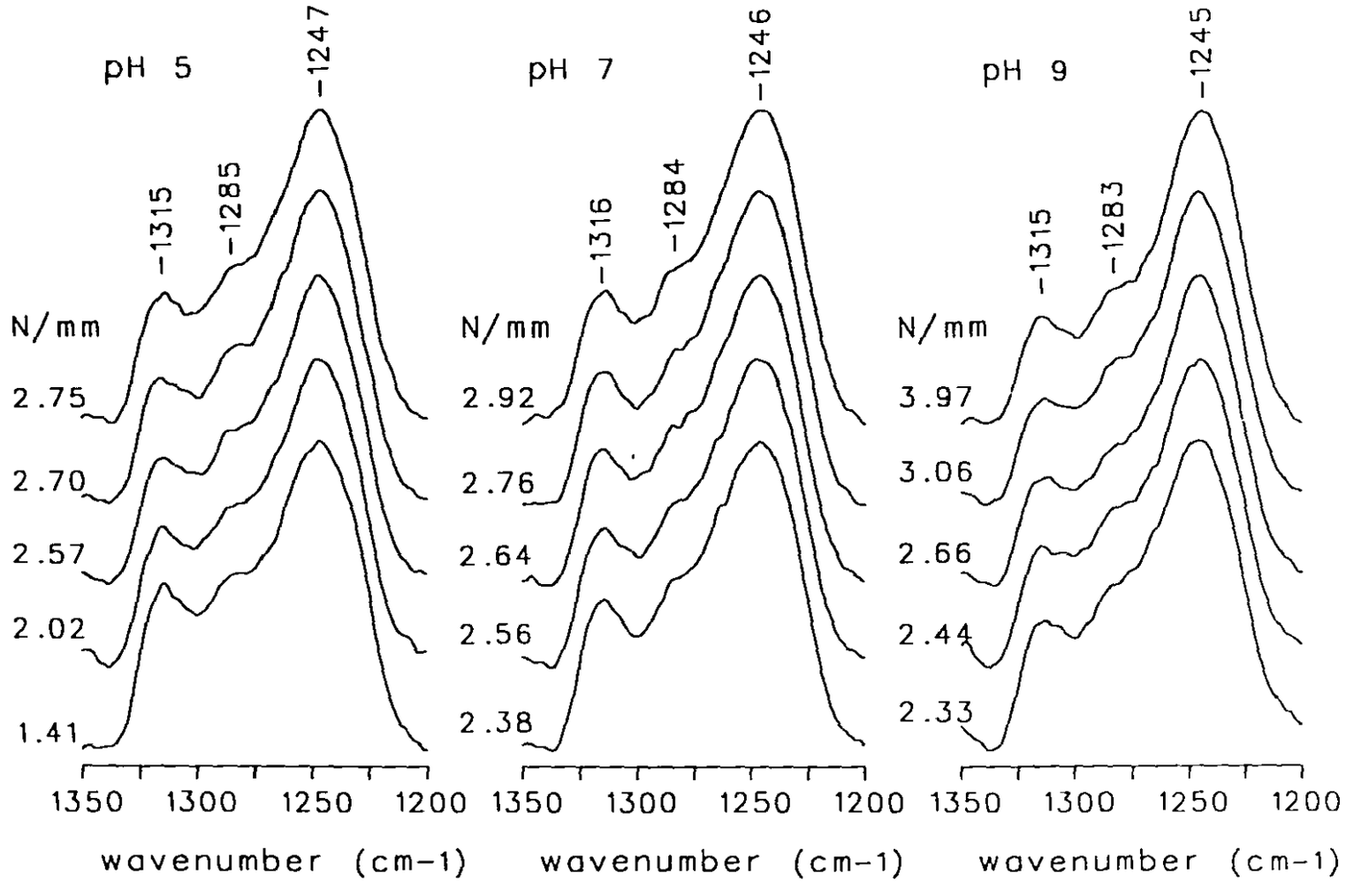

Figure 6.10 : Stacked spectra of amide III region of egg albumin gels, pH 5,7,9

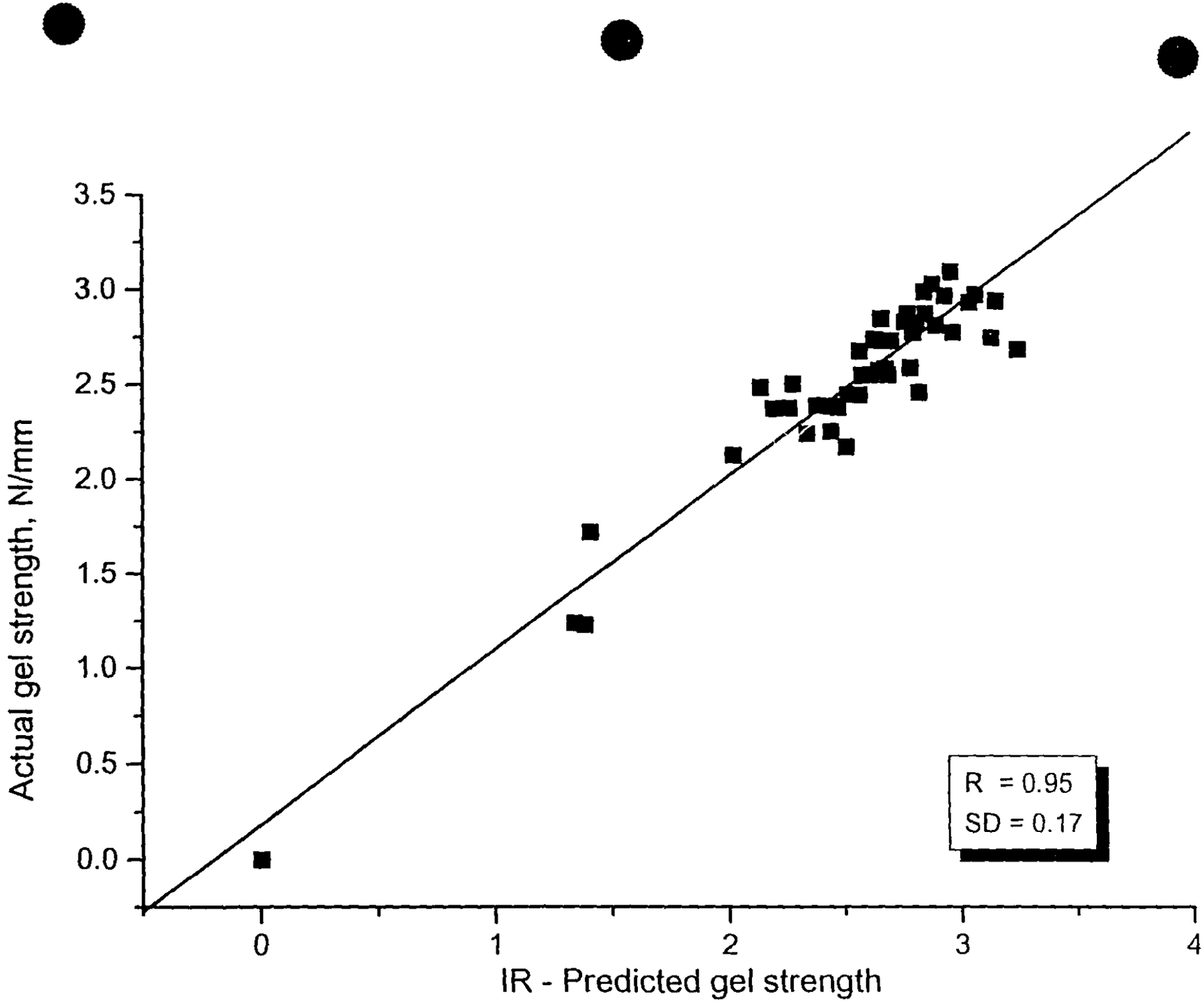

Figure 6.11 : Plot of actual gel strength vs. IR predicted for pH

Data obtained in this study indicates that protein gels at pH 9 has the maximum gel strength while pH 7 and 5 at the same conditions showed lower but similar strength with pH 5 specially at low temperature having the lowest gel strength as shown in Figure 6.9 . However the gel strength of all pH experiments increased as a function of time of heating from 15 to 120 minutes especially with pH 9 since early stage of gel formation is similar to other pH experiment but as time of heating progresses gel strength increases with time.

Figure 6.10 shows the stacked spectra in the amide III region obtained from gel samples prepared from solutions of pH 5, 7, and 9. Because of the very small variation in gel strength for these samples, no major differences between these spectra are observable; however, a slight decrease in the band at 1315 cm^{-1} is seen with increasing gel strength. Figure 6.11 shows a plot of actual vs. predicted gel strength for all pH experiments, from which R = 0.95 and SD =0 .17 have been obtained. Calculated error between predicted and actual shows in table 6.2 a mean of 0.0048 for all pH experiments. FTIR/PLS prediction of gel strength in this study gave 2.19% error for pH 9, an error of 1.79% was found for pH 7 and 4.77% error for pH 5, while cross validation of all pH spectra together indicates 5.43% error.

6.6 Temperature effect

Temperature is one of the most important factors influencing the rate at which a protein gel is formed as well as the firmness of the gel. When a 20% solution of egg albumin is heated at 70°C, the rate of gel formation increases sharply after 45 minutes of heating (Figure 6.12). When the temperature is increased to 80 and 90 °C, a more stable rate of gel formation is observed. After 90 minutes of heating, samples at the two different temperatures (i.e., 80 and 90 °C) gave almost the same gel strength. Gel hardness also increased as time of heating

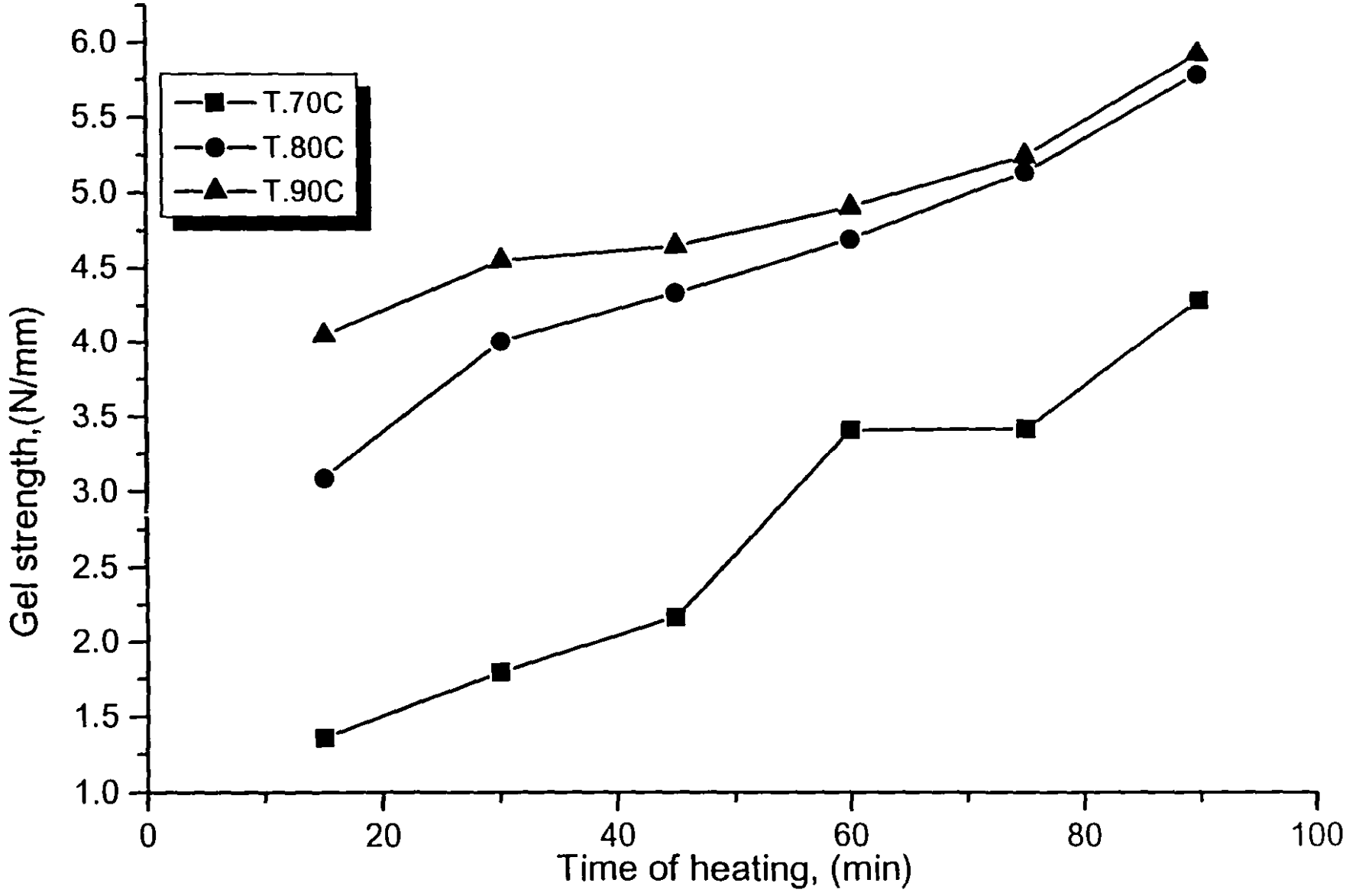

Figure 6.12 : Plot of gel strength vs. time of heating for temperatures

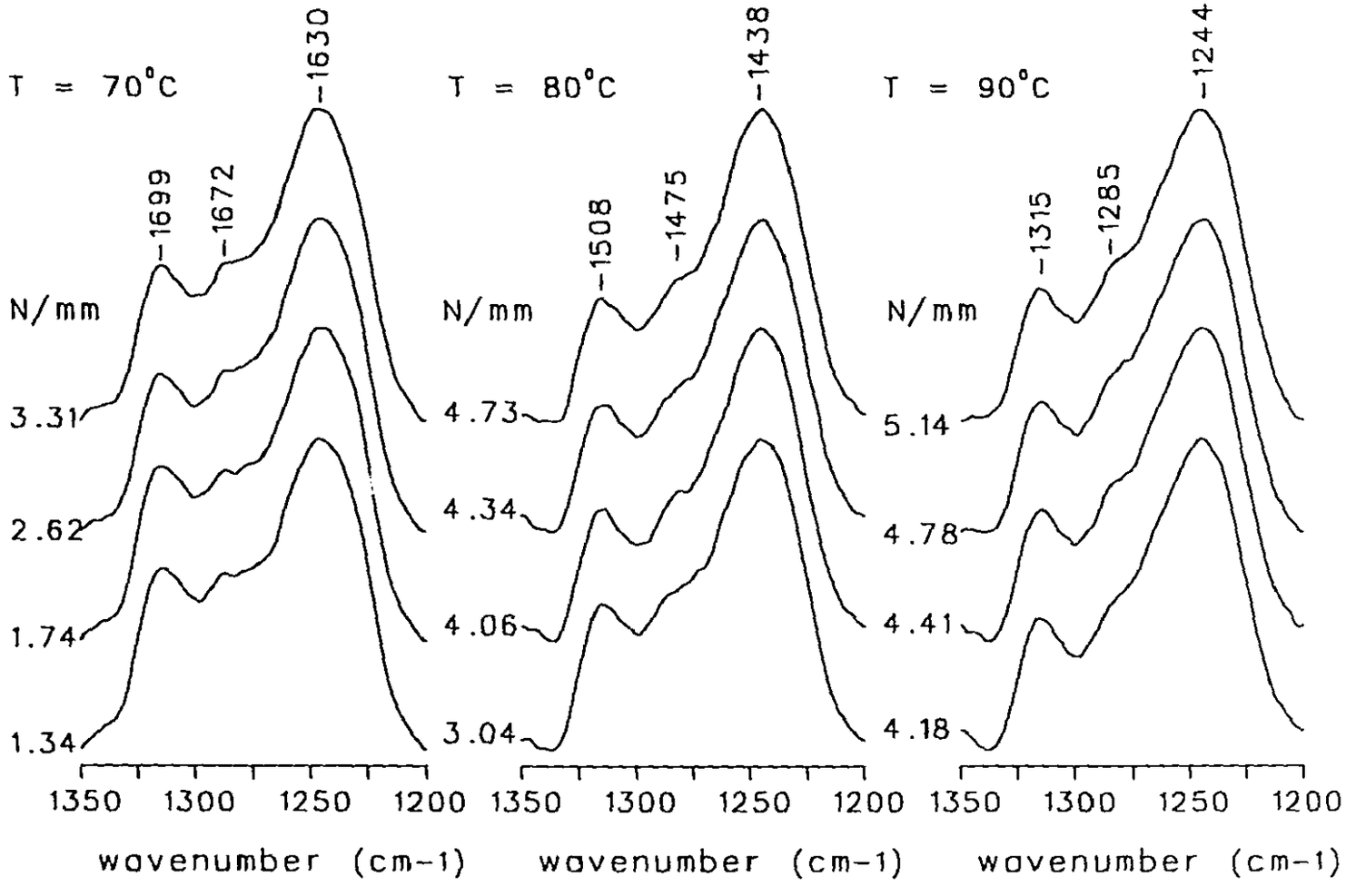

Figure 6.13 : Stacked spectra of amide III region of egg albumin, T=70,80,90 ^{0}C

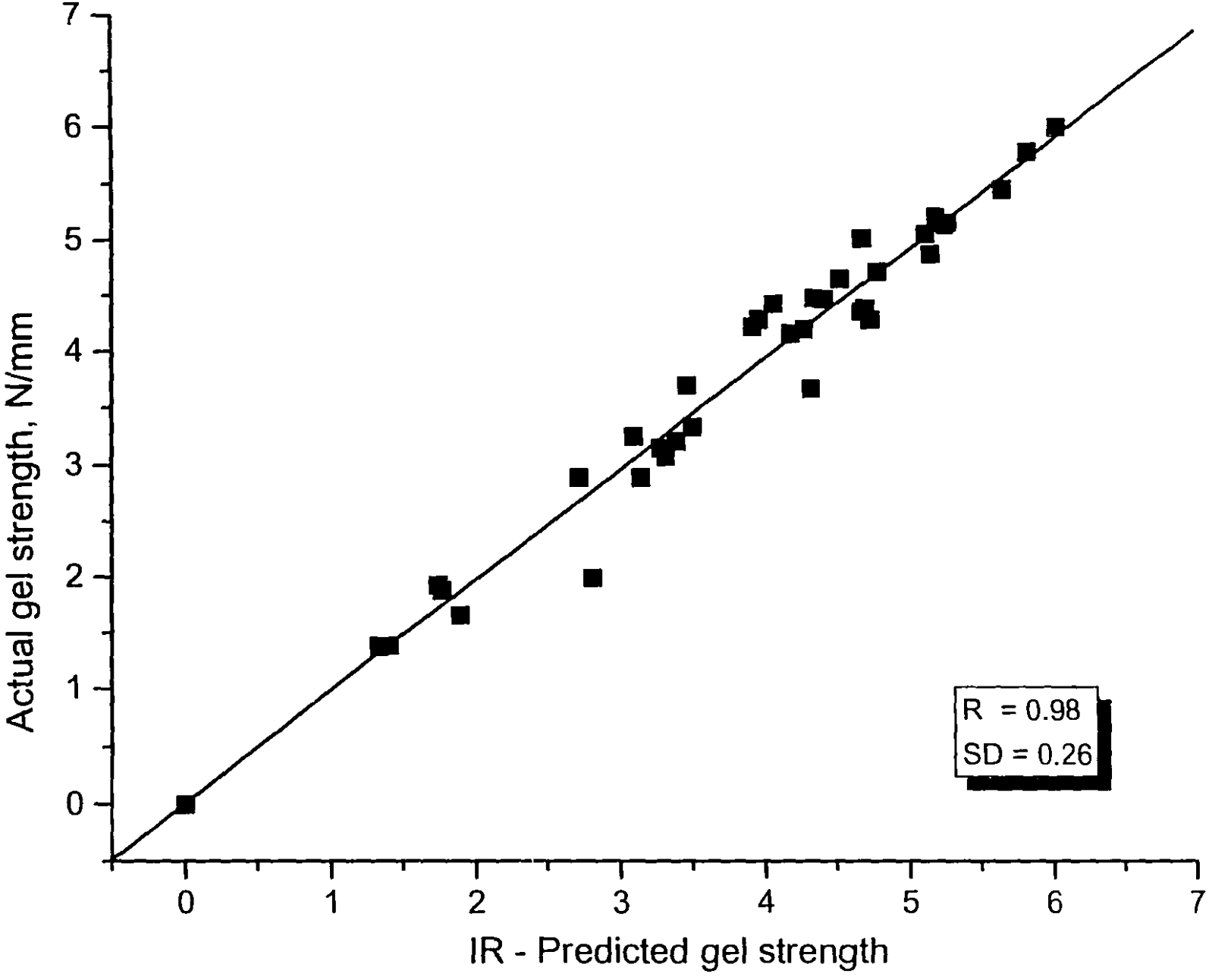

Figure 6.14 : Plot of actual gel strength vs. IR predicted for temperatures

increased from 15-120 minutes. Figure 6.13 shows the changes in the amide III region of the FIIR spectra of gels formed at 70, 80, and 90 °C as the gel strength increased with time of heating. In particular, the bands at 1244 cm^{-1} showed an increase with increasing gel strength, whereas the band at 1315 cm^{-1} showed a decrease. Figure 6.14 shows a plot of actual gel strength vs. predicted values for the temperature experiments. Regression curve shows an excellent correlation between two sets of values with R = 0.98, SD = 0.26. The mean error is 0.0439 for the temperature experiment (Table 6.1). For the four temperature runs a mean error of 6.36% for 70 °C, 4.19% for 80°C and 3.82% for 90°C was calculated for 20% egg albumin solutions.. Variability in error could be due to sample preparation and handling of protein gels and/or to instrumental analysis. It has been reported by Woodward et al., (1986) that gel hardness rose with increased in temperature, time, pH, and protein concentration level and decreased with added salt. Significant pH-temperature and pH-heating dependence was observed in obtaining hard gels. Gels generally increased in hardness with increased temperature and time, but the rate of changes varied with pH.

Other researchers (Watanabe et al., 1985, Montejano et al., 1984) have obtained the same results in which egg albumin showed a rapid increase in rigidity starting at 71°C and leveling off at 83°C. Kato et al. (1990), Hisham et al. (1990), and Matsudomi et al. (1991) reported that gel intensity of egg albumin increases as heating time increases in dry state. A good correlation was found by Yoshinori et al. (1990) between surface sulfydryl (-SH) group of egg albumin and gel strength as a function of temperature. As temperature was increased sulfydryl groups of protein was increased while total sulfydryl groups decreased. Margoshes (1990) reported that when the surface (-SH) groups concentration was measured, the gel strength increased with more surface (-SH) groups. The data indicates that the surface (-SH) groups plays important role in strong heat induced gel formation in

egg albumin. It is possible that by increasing temperature above 70°C (-SH) groups can enhance gel network formation by forming S-S bonds between other protein molecules; however internal (-SH) groups may form internal S-S bonds that locks the protein molecules into a conformation that makes poor gel formers (Margoshes, 1990). Opening the structure of egg albumin by drying (perhaps to a limited extend) apparently might promote stronger gel formation due to higher concentration of surface (-SH) groups. pH of protein solution has pronounced effect on the denaturation temperature of protein in acid media. Heat induced aggregation and denaturation of albumin are shifted to lower temperature, when the pH was decreased from 5.5 to 2.5, the denaturation decreased from 76°C to 65°C (Barbute et al., 1986), while Woodward et al. (1986) reported that pH-temperature interactions occurred for gel hardness. Gels generally increased in hardness with increasing temperature and time, but the rate of changes varied with pH. These findings along with the result of our work suggest that factors influencing the rheological properties of protein gels are interconnected and opens a new opportunity for a better understanding of protein gelation mechanism.

6.7 Conclusion

It seems reasonable to conclude that a) egg albumin gels are built from partially unfolded molecules with a significant amount of secondary structure and cross-linked with the network of hydrogen bonded intermolecular β-sheet structure strengthened by exposed hydrophobic residues and b) PLS calibration models can be developed successfully for prediction of gel strength from the infrared spectra of the gels.

Chapter 7 Conclusion

The feasibility of using FTIR spectroscopy coupled with an ATR accessory for studying protein secondary structure has been demonstrated to reveal valuable information about structure-functionality relationships of food proteins, particularly, the nature and the mechanism of protein gelation. These results demonstrate that FTIR spectrometer, along with PLS, once properly calibrated, can be used to predict the rheological properties of protein gels without performing the actual compression test.

Variable-temperature studies of protein particularly, two protein mixtures provided information on the unfolding and aggregation process that accompany the thermal denaturation of the proteins. Variable temperature FTIR spectroscopy also evidence of cooperativity of protein denaturation in a two protein system. Furthermore, for the first time a direct correlation between gel strength and peak height of the aggregation band of protein has been established. The successful demonstration of the applicability of FTIR/ATR and variable temperature FTIR techniques combined with resolution enhanced and multivariate analysis method for correlating physical gel strength to the molecular structure of the protein opens a new opportunity to examine correlation of other rheological properties, such as, elasticity, water holding capacity, foaming, stress and strain of the gel to the molecular composition of the system employing FTIR spectroscopy. Other industrial advantages of this method could lies in the area of versatility of this technique that can be fully automated for processing purposes, its speed of analysis and the accuracy of the results that are comparable to official methods used in industry.

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