CHARACTERIZATION OF PROTEIN-FLAVOR INTERACTIONS USING INVERSE GAS CHROMATOGRAPHY

By

OZLEM DOLU

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ABSTRACT OF THE THESIS

Characterization of Protein Flavor Interaction Using Inverse Gas Chromatography

By OZLEM DOLU

Thesis Director
Jozef L. Kokini

Flavor perception plays an important role in determining the consumer acceptability of a food product. Flavor perception is affected by the extent and strength of the interaction between the flavor and food matrices, which govern the retention/release mechanism of flavor from a food system. Protein-based foods constitute an important part of the diet as recommended by dietary guidelines. Therefore, a fundamental understanding of the protein-flavor interaction for different processing conditions would be useful for the improvement of such food products in terms of flavor quality.

In this research, we investigated the retention/release mechanism of selected flavor compounds on or from protein matrices by establishing quantitative design principles for these interactions. Thermodynamic parameters (partition coefficient Kp, free energy of adsorption ΔGs and the enthalpy of adsorption ΔHs) of the interaction between selected flavor compounds (hexane, hexanal, hexanol and d-limonene) and protein systems (soy

protein isolate and zein) were determined by using inverse gas chromatography under different temperatures and relatively humid conditions.

The inverse gas chromatography system was fitted with an additional humidification system that could maintain the relative humidity of the carrier gas, thus enabling the evaluation of the effect of relative humidity on the measured quantities. Increasing temperature and relative humidity led to less favorable interaction between selected flavors and proteins. Flavor retention at high relative humidity was less than at low relative humidity or at dry conditions. This suggests that flavor compounds and water molecules might be competing to bind to the available sides of the protein.

Quantitative characterization of the mechanism and thermodynamics of flavor binding and release in protein matrices will benefit the food industry to efficiently develop flavored foods.

ACKNOWLEDGEMENTS

I am extremely grateful to my advisor, Dr. Jozef L. Kokini, for his guidance, encouragement and support throughout this research. I would also like to thank Dr. Keith Cadwallader for his helpful advice and for allowing me to conduct experiments in his lab. I offer my regards and gratitude, as well, to many other people who supported me during the completion of this project: the professors, department staff, my lab classmates and friends at Rutgers Food Science. Lastly, I am heartily thankful to my family and friends for their invaluable moral support and endless love throughout my life.

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1 INTRODUCTION

The sense of taste affects the human dietary habits, nutritional and health status, and finally life quality (Smith & Margoslkee, 2001; Margolskee, 2002). People choose their food and decide how much they eat according to the appreciation they get when they eat the food (Sorenson, 2003). Flavor is one of the important sensory properties of food for consumer acceptance (Zhou and Cadwallader, 2004). A desired food flavor leads to satisfaction when a food is consumed (Heath and Pharm, 1978). A flavor compound is classified as a volatile organic compound that has a taste or smell. Flavor can be defined as a chemosensory impression of food molecules. This taste sensation affects the consumer impact of food material and determines the desire to eat that product again. The eater decides to eat this food material again if it gives a pleasurable sensation (Risch and Ho, 2000). Flavor perception depends on the composition of food product, processing conditions of the food product, the interaction between food components and flavor compounds, and human factors (Heath and Pharm, 1978; Reineccius, 2006). Flavors interact with the major food constituents, and their mobility changes as a result of those interactions, thus affects release of flavor compounds and finally perception changes (Reineccius, 2006). That is why interactions between flavor and food are important to improve the flavor quality of a food product.

Because of their multiple functional properties (emulsifying, stabilizing, foaming, water holding capacity, flavor binding, etc.), proteins are used as ingredients in food formulation. Another reason for using an isolated protein is to create the new food products rich in protein. While a water-binding capacity and gelation properties are desirable in a meat system (texture analogues meat), solubility is important for beverages

(protein-rich) (Kinsella, 1982; Guichard, 2006). It is well known that there is an interaction that takes place between flavor compounds (such as aldehydes, ketones, alcohols, ketones, and esters) and flavor compounds (Guichard, 2006, Bakker, 1995). Many types of protein have little characteristic flavor that does not affect the flavor profile of the food material. On the other hand, reversible or irreversible binding of flavor compounds to the protein content of the food material plays an important role on the overall flavor perception (O'Neill and Kinsella, 1987; Heng et.al., 2004; Guichard, 2006).

Inverse gas chromatography that was adapted from HP5890 GC was used in this research to investigate thermodynamic parameters of the protein-flavor interaction. The inverse gas chromatographic method is technically the same method as gas chromatography, except for the interesting phase. While GC is used to characterize the unknown volatile mobile phase by using a reference stationary phase, IGC is used to characterize the stationary phase (protein) by using a known volatile mobile phase (flavor compounds). After proteins are packed into the column and flavor injected into that column, the retention data is obtained from the IGC. By using retention data, valuable information on the surface properties of the protein can be obtained and thermodynamic parameters of flavor adsorption on protein can be interpreted (Zhou and Cadwallader, 2004).

Retention time and profile of the flavor compounds, free energy of adsorption, and enthalpy of adsorption were determined for proteins (zein and soy protein isolate) that have different physical properties at different conditions (temperature and relative humidity).

The focus of this research was the characterization of the fundamentals of flavor binding by proteins and the effect of the different types of protein on this binding. Furthermore, the effect of environmental conditions (temperature and relative humidity) on protein-flavor interactions was interpreted to be able to control possible changes in flavor from production to consumption.

2 OBJECTIVES

- Quantification of retention profile and thermodynamics of adsorption (the partition coefficient, free energy of adsorption, and enthalpy of adsorption) for flavor onto soy protein isolate and zein by using IGC.
- Analyze the influence of the physical properties of protein matrix and environmental factors (temperature, moisture) on the determined thermodynamic properties of adsorption.
- To determine the effect of heat treatment of protein on adsorption.
- Prediction and optimization of the food materials flavor quality during production and storage by appraisal of the nature and extent of the interaction between the flavor and protein system.

3 LITERATURE REVIEW

Intrinsic and extrinsic properties of the food material (shape, size, color, flavor, texture, shelf life, nutritional value, taste, safety, process, and environmental conditions) determine the food's quality and the consumer's decision to purchase and consume (Aramyan et.al, 2006). Flavor, which is an intrinsic property of food material, is explained by the perception of flavor and depends on the retention and release behavior from the food matrix during eating (Madane, 2006; Plug and Haring, 1994). One of the most important determinants for eating food is flavor, which stimulates the food cravings and assists in the completion of nutritional requirements (Jurdi-Haldeman, 1988).

Because of the importance of flavor in an individual's preference to buy, producers strive to develop food products with better flavors (Teff, 1996). Ability to control the flavors during processing and storage is not easy but necessary to get desirable flavors. During production and storage, flavors of food might be influenced by thermal processing, light, moisture, time, and ingredients that may result in less desirable flavors or more undesirable flavors (Heng et al., 2004; Parker, 2003). Firstly, retaining of flavors by food is desired until consumption. Later, a well-balanced release of flavors from food is wanted during food intake. The quantities of the binding mechanism between flavor and the food matrix shape both retention and the release behavior, and finally determine the flavor perception. Flavors are released from the food products during consumption, not according to a concentration ratio of flavors, but rather according to the strength and extent of interactions between flavor and food material. A better understanding of these interactions results in producing efficiently flavored food materials (Franzen and

Kinsella, 1974; Pelletier et al., 1998; Andriot et al., 2000; Delarue and Giampaoli, 2000; Fabre et al., 2002; Yildiz, 2003; Heng et al., 2004).

3.1 FLAVOR

Flavor compounds belong to organic volatile compounds group, which involves esters, terpenes, alcohols, aldehydes, and ketones (Hernandez-Muoz et al., 1998). The chemistry of volatile compounds controls their physical and chemical aspects (Reineccius, 1994). Those chemical compounds in food materials induce the flavor perception process by delivering the signals, which are generated by the receptors located in the mouth and nose, to the brain (Risch and Ho, 2000). Heymann and others defined flavor as: 'Flavor is the biological response to chemical compounds (the physical stimuli) by the sense, interpreted by the brain in the context of human experience.' (Heyman and et al., 1993)

In 1969, the U.S. Society of Flavor Chemists defined flavor as (Heath, 1978):

'Flavor is the sensation caused by those properties of any substance taken into the mouth which stimulates one or both of the senses of taste and smell and/or also the general pain, tactical and temperature receptors in the mouth.

A flavor is the substance which may be a single chemical entity or a blend of chemicals of natural or synthetic origin whose primary purpose is to provide all or part of the particular flavor effect to any food or other product taken into the mouth.'

The perception of flavor can be roughly formulized as (Risch and Ho, 2000):

Flavor perception= aroma+taste+mouthfeel+texture+pain/irritation

Aroma is the sensation of volatile compounds in the nose, and taste is the sensation of non-volatile compounds on the tongue in combination with the effects of mouth feel and texture (Ruth et al., 2008).

All the variables in this definition have to be evaluated in order to describe a flavor, but aroma is subjected to more extensive research since it is the ascendant constituent of flavor**.** Despite the fact that it is preferable to concentrate on aroma, the role of the other components should not be ignored since the interactions between them may be necessary when characterizing flavor perception (Risch and Ho, 2000).

3.1.1 FLAVOR COMPOUNDS IN FOOD

Food is a complex matrix composed of volatile and non-volatile constituents. Food materials carry a basic flavor profile generated by flavor compounds, which might exist in food naturally or is added to adjust the losses or modification in the flavor profile that appears during processing and storage (Phillippe, 2003). Initiation of the enzyme system and precursors during the production of processed food materials also plays an important role in the flavor profile of the end product. In some cases, initiation of enzyme systems and precursors may lead to unenviable results and removal of these effects may require further process. Therefore, additional components can be used to strengthen enviable flavor, mask unenviable flavor, or create a completely new flavor character (Heath and Reineccius, 1986).

3.1.1.1 Flavorings Naturally Present in Food (Plant Sources)

Fruits and vegetables, such as raspberries, grapes and apples, herbs, and spices can be used as food flavoring sources. Flavoring compounds obtained from natural sources are described as natural flavors. Insufficient intensity of these natural flavors is compensated

for by the addition of natural flavors from other sources to obtain an all-natural flavored food product (Boyacioglu et al., 2010). Due to their low intensity, fruits are not preferred as a major food flavoring if they are not the main component of the end product (for example, in fruit beverages or apple pie). Obtaining a concentrated form of these flavors from a plant source (fruits) is expensive since the raw materials are limited in flavor. Herbs, spices, citrus, vanilla, coffee, tea, and cocoa are more preferable sources from which to obtain natural flavoring substances (Reineccius, 2006).

3.1.1.2 Food Flavoring by Processing (Processed Flavor)

Description of "processed flavor" identifies various cases. The specific flavors of certain food products, such as meat, chocolate, coffee, French fries, and baked goods are generated from Maillard reactions, caramelization, and thermal oxidation in fats by the effect of heat treatment during thermal processing (Reineccius, 1994). Fermentation is another process that generates flavoring for food products, such as wine, yogurt, cheese, vinegar, and beer. In fermented foods, the starter cultures cause a series of chemical processes by either their microbial activity (flavor of alcoholic products) or enzymatic activity (flavor of aged cheese) (Smit et al., 2005). Occasionally, a mix of these processing techniques can be used to generate specific types of flavorings (Reineccius, 2006). For example, cocoa beans undergo in sequence: fermentation, drying, roasting, conching, and alkalization (Afoakwa et al., 2008). Tea may or may not require fermentation before roasting, and, in the case of vanilla, fermentation and curing is necessary (Reineccius, 2006).

3.1.1.3 Addition of Food Flavor

In the case of intrinsic flavor deficiency, food products might seem flavorless and unappealing, so that additional flavor compounds can be used to create a flavor profile, to suppress unenviable flavor characters, to change the present flavor profile, to improve inadequate intrinsic flavor, and to compensate for losses during processing. The combination of flavoring substances can be completely natural, or a mixture of natural and synthetic aromatic chemicals, or completely synthetic aromatic chemicals (Heath and Reineccius, 1986).

3.1.2 FLAVOR RETENTION/RELEASE

The sensory quality and strength of flavor perception depend on the flavor retention and release from a food matrix in the mouth. The binding of flavor compounds to the food matrix controls the flavor release and, consequently, perception (Preininger, 2006). The amount and rate of the flavor release from the food matrix are governed by several factors (Figure 3-1). These include: reversible and irreversible binding of flavor molecules to the food, temperature, food composition, structure, viscosity, chewing, and breathing process. (Reineccius, 2006; Seuvre, 2008).

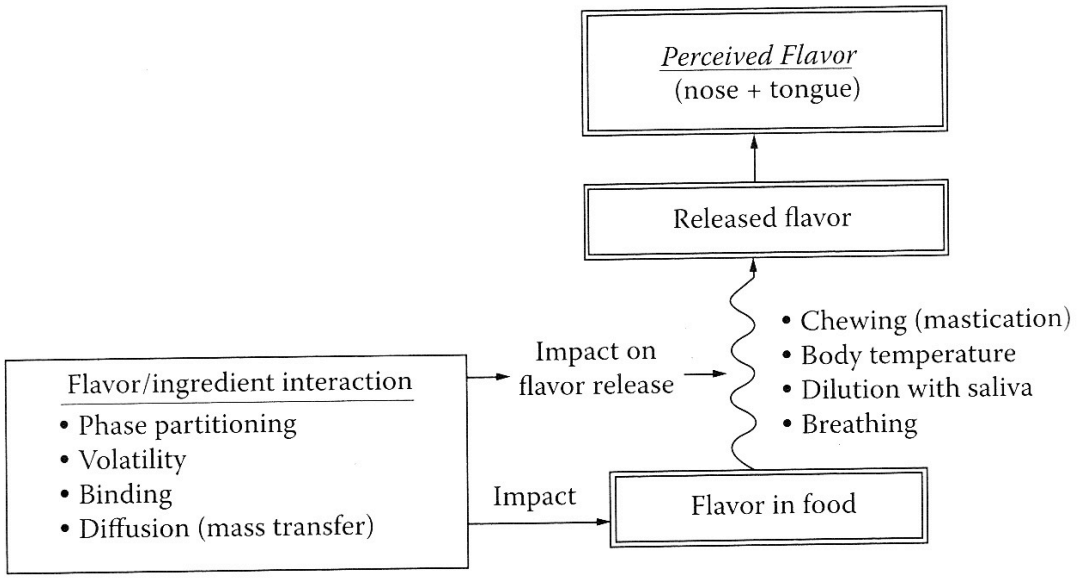


Figure 3-1: "Flavor food interaction diagram" (Preininger, 2006)

Thermodynamic and dynamic parameters are the main factors that affect the flavor release. (Preininger, 2006; Voilley, 2006);

If the flavor release occurs under static conditions (the established phase equilibrium in the food system), thermodynamic parameters control the release phenomena (Ruth, 2008), which depend on food composition and temperature (Roos, 2003). Under static conditions, partitioning of flavor molecules between the vapor phase (air) and the product phase (solid, hydrophilic liquid, or lipophilic liquid), according to the phase partition coefficient, defines the volatility of flavor molecules, which refers to the ratio of flavor concentration in the vapor phase to the product phase (Roos, 2003; Preininger, 2006).

If flavor release occurs under dynamic conditions (disturbed equilibrium in food system), kinetic parameters together with thermodynamics control the release phenomena (Ruth, 2008), which depend on food structure and texture (Roos, 2003). Under dynamic conditions, the phase equilibrium between the vapor phase and the product phase are

destroyed by the effect of mass transfer (diffusion) between phases. Mass transfer rate is a function of viscosity of a food product and determined by the mass transfer coefficient, which defines the velocity of flavor molecules as it moves through the food matrix (Roos, 2003; Preininger, 2006).

The equilibrium partition theory, which gives *maximum potential extent* of the flavor release (Philippe, 2003), is not enough to explain the whole release phenomena that take place during eating, which is a dynamic process (Leland, 1997). The release phenomena for liquid matrices (water or lipids) are relatively less complex and easy to explain by the partitioning theory (Voilley, 2006), which includes Henry law type principles. For example, when a liquid substrate, such as orange juice consumption, is a subject of matter, the flavor is sensed immediately, since the headspace is already saturated (Icoz and Kokini, 2006). On the other hand, the release phenomena for viscous or solid matrices require a more complex theory because it has more influences, such as diffusion (Voilley, 2006). For example, a complete flavor sensation from a strawberry cake (solid matrix) takes a couple of seconds to appear (Icoz and Kokini, 2006).

3.1.2.1 Flavor Retention

It is still not completely understood how flavor compounds come together with food components and create a specific flavor profile, although multiple studies continue to discover new facts about this process (Taylor and Linforth, 1996). When flavor substances are added to a food matrix, flavor molecules bind to the food matrix via reversible or irreversible interactions (Figure 3-2). In the case of reversible bindings, the sorption process involves both adsorption and absorption in which the flavor molecules

either bind to the outer surface of the food material and start to diffuse into the food matrix via the porous structure of the dry food material or dissolve in the food material according to the concentration or partial pressure gradient (Ruth, 2010). In this case, flavor molecules cannot build very strong interactions. On the other hand, if binding is irreversible, it means chemical bonding takes places between the flavor molecules and food material, which is referred to as chemisorption (Ruth, 2010). In the case of chemisorption, flavor molecules are fixed and cannot diffuse into the food matrix because of their bond strength.

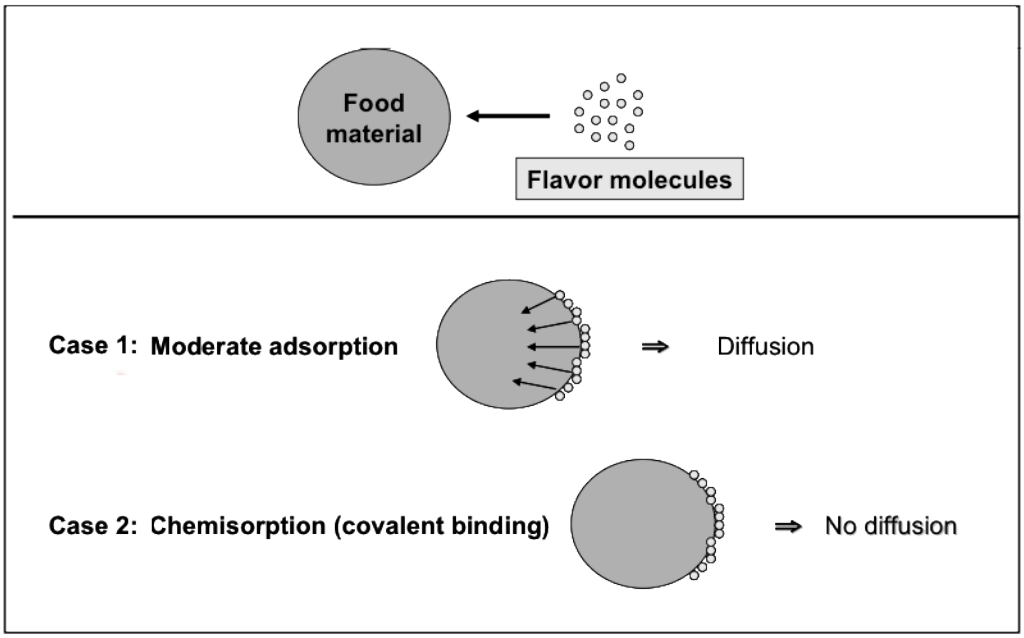


Figure 3-2: Schematics of binding possibilities during introduction of flavors into a solid food (Kokini, 2006)

3.1.2.2 Flavor Release

A flavor molecule can be perceived only if it is released from the food products while being eaten (Lubbers, 1998). During eating, complex oral processes affect the partition of flavor molecules and lead to repartition between the different phases and thus control the release phenomena. Because of reversible interaction (adsorption or absorption), release

(desorption) of flavor molecules takes place through mass transfer (Figure 3-3), which involves diffusion and migration of flavor molecules from the preliminary phase to the vapor phase (Kinsella, 1989; Kok and Smorenburg, 1999; Dimelow, 2005; Cayot, 2008). In the case of chemisorption, which is irreversible interaction, flavor molecules can no longer be diffused or moved, so there will be no flavor release (Lubbers, 1998).

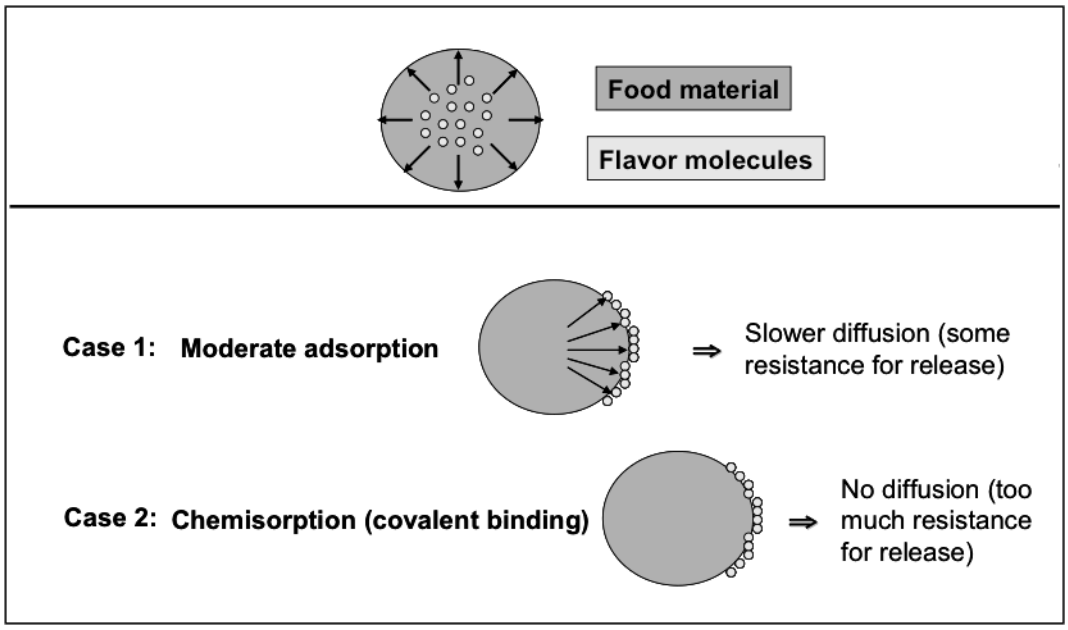


Figure 3-3: Schematics of possibilities during release of flavors from a solid food (Kokini, 2006).

3.1.2.3 Food in Mouth

As shown in Figure 3-4, digestion starts with a complex oral process that involves the first bite, chewing, mastication, transportation, formation of bolus, and swallowing (Salles et al., 2011; Chen, 2009). There are several factors that belong to food products that affect the oral process, like structure, phase, composition, texture, appearance, size, and shape (Foster, 2011). The purpose of in-mouth events is to make the food material a swallowable liquid by oral application, such as cutting, tearing, and grinding between the teeth, softening by absorption of saliva, melting or hardening caused by a phase change,

thinning by dilution with saliva, thinning by stirring with the tongue, and dissolving in saliva (Bourne, 2004).

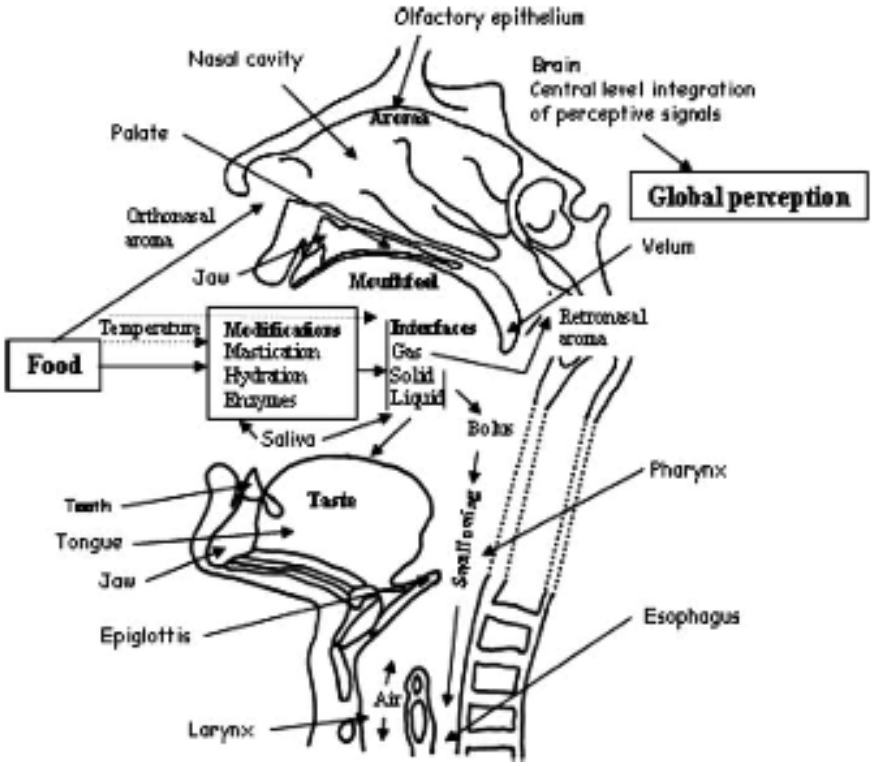


Figure 3-4: "Oral organ cross-section diagram: main events occurring in mouth" (Salles et.al, 2011).

The digestive function of the mouth process also controls the perception of flavor and texture (Figure 3-5) (Foster, 2011). The oral food process increases the surface area via splitting and increases the contact between saliva/air and food material, thus helping to taste compounds to dissolve in saliva and helping the flavor compounds to migrate from the food material into the in-mouth air phase (Salles et.al, 2011). Simultaneously, non-volatile taste compounds are being sensed on the tongue, which gives the impression of sweet, salty, bitter, sour, and umami, while volatile flavor compounds are sensed in the nose (Taylor and Linforth, 1996; Preininger, 2006).

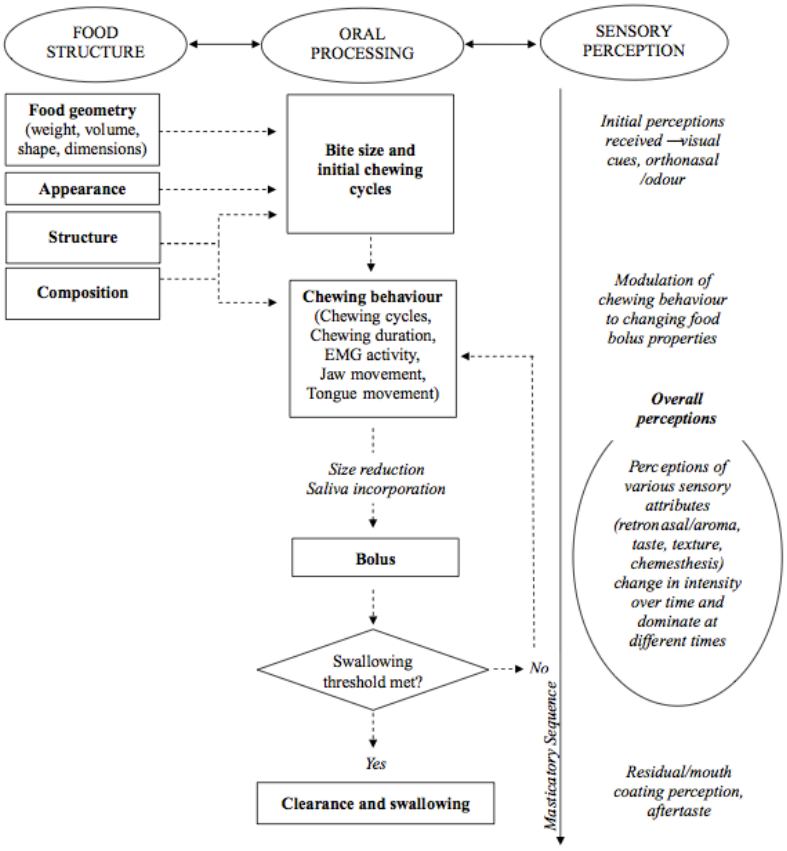


Figure 3-5: "Overview of interactions among food properties, oral processing, and sensory perception" (Foster, 2011).

3.2 FOOD/FLAVOR INTERACTION

Flavor compounds interact with various food matrix components, including proteins, polysaccharides, and lipids (Franzen and Kinsella, 1974; Guichard, 2002; Semenova 2002). The chemistry of flavor compounds (alcohols, aldehydes, ketones, and esters) and the position of the functional group in the biopolymer chain affect the extent of interaction between flavor and the food material (Semenova, 2002). As evidence, transferring of a carbonyl group from the end (2-nonanone) to the center of the hydrocarbon chain (5-nonanone) in ketones causes a drastic decrease in the binding constant (Damodran & Kinsella, 1980, Semenova, 2002). Flavor compounds may irreversibly bind to food components by covalent bonds, such as the interaction between

aldehyde and amino/sulfhydryl groups of protein. A flavor compound may also reversibly bind to food components by van der Waals interaction or hydrogen bond. A strong knowledge of atomic bonds and of the molecular interaction that takes place between flavor and food is necessary to formulize new products (Bakker, 2006).

3.2.1 BONDS AND INTERMOLECULAR FORCES

Sorption, which involves adsorption, absorption, and physical and chemical bonds, is used to describe the binding mechanism between a volatile compound and a food matrix (Taylor, 2007). There are two ways direction interactions take place between flavor compounds and a food matrix; holding of flavor compounds by attractive forces, and discharging of the flavor compounds by repulsive forces (Le Thanh, 1992).

The function of attractive forces between atoms at different strength levels is to form molecules or to connect molecules. These forces are classified in two major groups: covalent bonds due to the strong interactions between atoms, and non-covalent bonds due to the weak interactions between atoms. Non-covalent bonds are also divided into four groups based on their strength and formation (Lodish et.al, 2008).

3.2.1.1 Covalent Binding

The bonds that keep the very stable molecules together are called covalent bonds and they have a fixed bond direction and a specific bond length (Metzler, 2001). Covalent bonds form when two atoms share one or more electrons in their outer orbital (Lodish et al., 2008). Two atoms share the electrons because neither one of the two atoms is stronger than the other to break off the electrons (Freifelder, 1982). If the covalently bonded atoms have different electronegativity, the attraction forces of each atom on the electrons would be different and the bond would be called a polar covalent bond. In the case of similar or

equal electronegativity of atoms, the attraction forces on the electron would also be equal, and the bond would be called a nonpolar covalent bond (Lodish et al., 2008). Figure 3-6 shows that covalent bonds are stronger than non-covalent bonds.

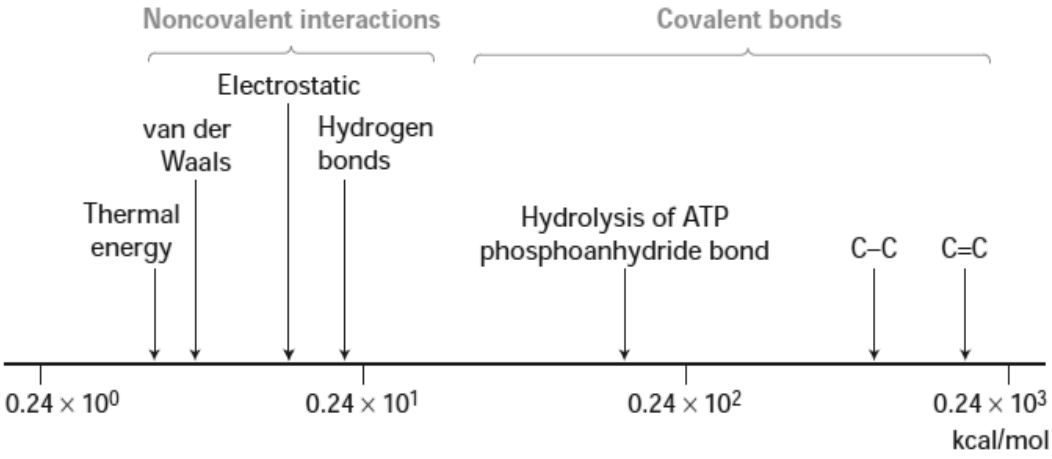


Figure 3-6: "Relative energies of covalent bonds and noncovalent interactions. energies are determined, as the energy required breaking a particular type of linkage. Covalent bonds are one to two powers stronger than noncovalent interactions. The latter are somewhat greater than the thermal energy of the environment at normal room temperature (25 C). Many biological processes are coupled the energy released during hydrolysis of a phosphoanhydride bond in ATP" (Lodish, 2008).

3.2.1.2 Non-covalent Interactions

Non-covalent interactions refer to the weak and non-permanent bonds that occur between molecules. They can be categorized in four major group: Ionic bonds, hydrogen bonds, van der Waals interactions, and hydrophobic effect (Lodish et al., 2008). These bonds are schematically shown in Figure 3-7.

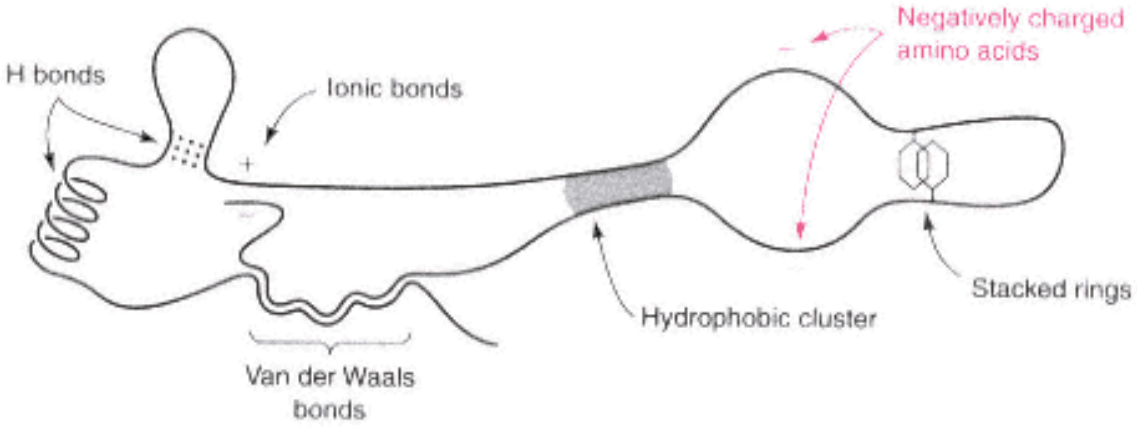


Figure 3-7: 'A hypothetical polypeptide chain showing attractive and repulsive interactions' (Malacinski, 1998).

3.2.1.2.1 Ionic bonds

An atom, which has a tendency to give electrons, transfers its excess electrons to a second atom, which has a tendency to accept the electron. The atom that accepts the electrons becomes a negatively charged particle (anion), and the atom that gives electrons becomes a positively charged particle (cation). The electrostatic interaction between these positively and negatively charged particles is called ionic interaction. In ionic bonding, the charged particles have the same attractive forces in all directions as distinct from the fixed direction of covalent bonding (Lodish et.al, 2008).

3.2.1.2.2 Hydrogen Bonds

Hydrogen bonding is an important type of interaction that takes place between polar molecules. Hydrogen bonds are weaker than covalent binding, but stronger than ionic bonds (Creighton, 1984). When a hydrogen atom connects to negatively charged atoms (oxygen, nitrogen, or fluorine) by covalent bonding, a dipole, which has the hydrogen on its positive side, occurs. When the dipole comes close to another dipole molecule, there is an electrostatic attraction that takes place between the partially positively charged

hydrogen atom and the negatively charged side of the second dipole. This attraction is known as hydrogen bonding (Metzler, 2001). Hydrogen bonds play an important role in protein structure. Table 3-2 shows some of the hydrogen bonds in proteins. Hydrogen bonds may occur between a carboxylic group of peptides and amides of peptides or hydroxyl groups of amino acids (Elgert, 2009).

Table 3-1: Hydrogen bonds in proteins

Type	Compounds	Optimal distance between Non-hydrogen atoms (A)
O-H-----O	Water	2.8
	Primary alcohols	2.7
	Carboxylic acids	2.6
N-H-----N	Ammonia, amines	3.1
N-H-----O	Urea, amides, peptides	2.9
(Creighton, 1984)		

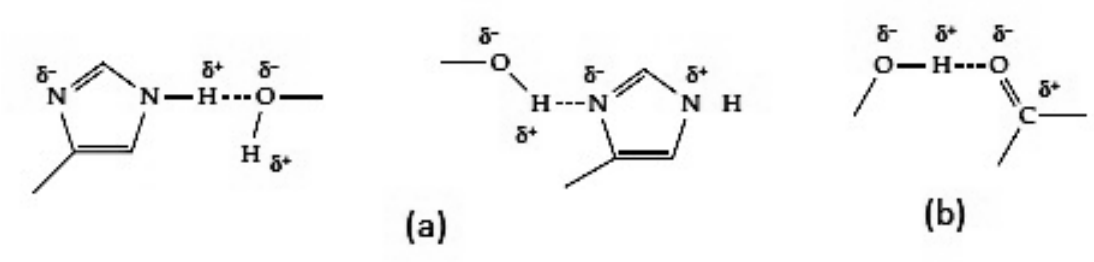


Figure 3-8: Example of H-bonding in proteins. (a) imidazole group of protein, and (b) carboxyl group with OH group (Metzler, 2001).

Hydrogen bonds between water molecules is the most remarkable example of hydrogen bonds that give unusual characteristics to water. Each hydrogen atom in a water molecule has a partially positive charge and each oxygen atom has a partially negative charge because of the higher electronegativity of an oxygen atom **(Figure 3-9)**. Each water molecule can form four hydrogen bonds with other water molecules. The attractive forces

between a hydrogen atom and an oxygen atom in two neighboring water molecules form water (Lehninger, 2005).

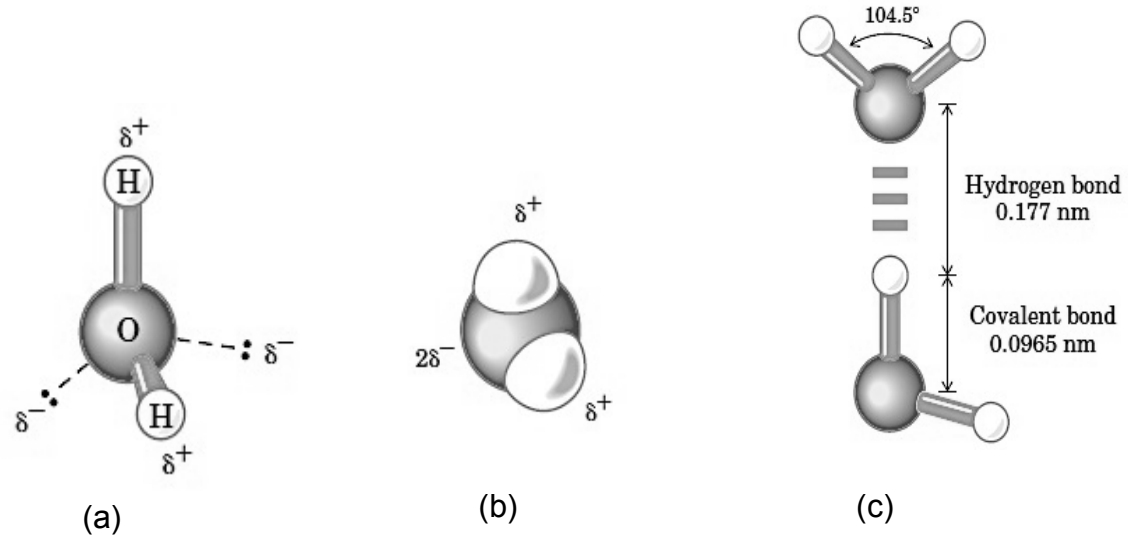


Figure 3-9: "Structure of the water molecule. The dipolar nature of the H2O molecule is shown by (a) ball-and-stick and (b) space-filling models. The dashed lines in (a) represent the nonbonding orbitals. There is a nearly tetrahedral arrangement of the outer-shell electron pairs around the oxygen atom; the two hydrogen atoms have localized partial positive charges and the oxygen atom has a partial negative charge. (c) Two H2O molecules are joined by a hydrogen bond between the oxygen atom of the upper molecule and a hydrogen atom of the lower one. Hydrogen bonds are longer and weaker than covalent O-H bonds" (Lehninger, 2005).

3.2.1.2.3 Van der Waals Forces

There is a weak attraction force between any two atoms or molecules (polar, nonpolar, and noble gases in the gas phase). These forces occur as a result of movement of the electrons in atoms or molecules. When two molecules come too close, the symmetric distribution of the electrons can be destroyed. Uneven configuration of electrons creates a nonpermanent dipole molecule or atom. This dipole affects the charge distribution of the neighboring atom, and creates a second nonpermanent dipole. The forces between these two dipoles caused by electrostatic attraction are called van der Waals forces (Creighton, 1984; Elgert, 2009; Lodish et al., 2008).

3.2.1.2.4 Hydrophobic bonds

A nonpolar molecule has no dipole moment that is why it cannot interact with polar molecules. Nonpolar groups are not soluble in polar solvent (i.e., water), but are soluble in nonpolar solvents (i.e., hexane). Nonpolar molecules in polar environments have a tendency to approach each other and this phenomena is known as hydrophobic bonding (Creighton, 1984; Walstra, 2003). When a nonpolar molecule is placed into water, water cannot bind to the molecule via the hydrogen bonds, so water starts to create a cage around the nonpolar molecules. Later, water molecules tend to push the nonpolar molecules to bring them closer to reduce the contact area with the nonpolar molecules (Lodish, 2008). Figure 3-10 shows how water molecules form a cage and nonpolar molecules aggregate in water.

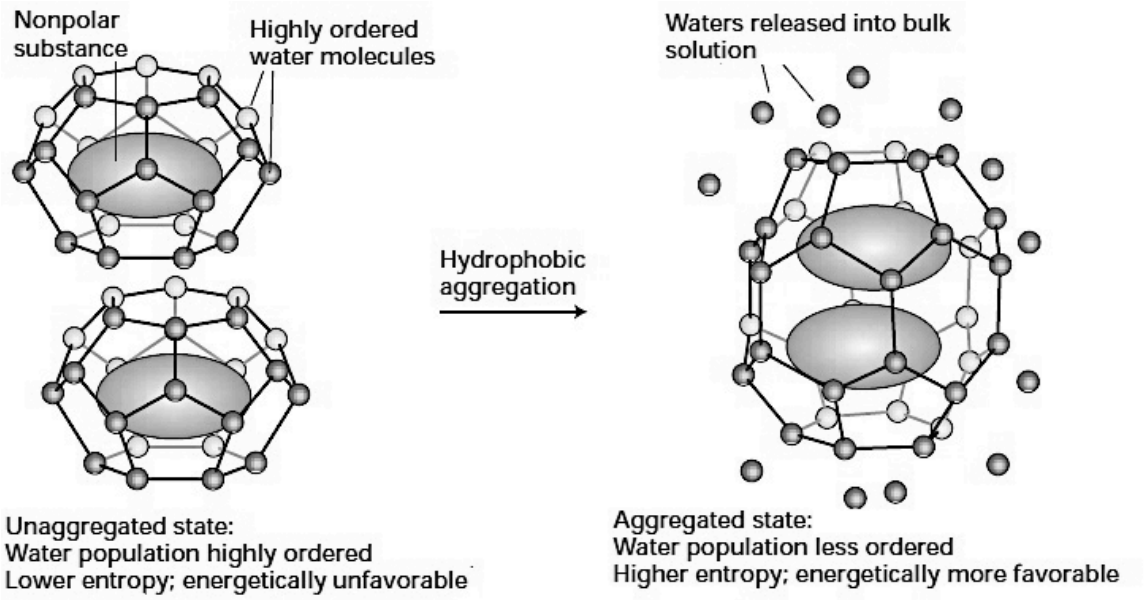


Figure 3-10: "Schematic depiction of the hydrophobic effect. Cages of water molecules that form around nonpolar molecules in a solution are more ordered than water molecules in the surrounding bulk liquid. Aggregation of nonpolar molecules reduces the number of water molecules involved in highly ordered cages, resulting in a higher entropic, more energetically favorable state (right) as compared with the unaggregated state (left)" (Lodish, 2008).

When a molecule, which includes both polar and nonpolar groups are placed into water, polar groups of the molecule form hydrogen bonds with water while nonpolar groups of protein tend to escape from water molecules. This behavior causes a new arrangement of molecules in which polar groups located in the outer region of the molecule and nonpolar groups stay in the interior region of the molecule. This is shown in **Figure 3.11** (Freifelder, 1982).

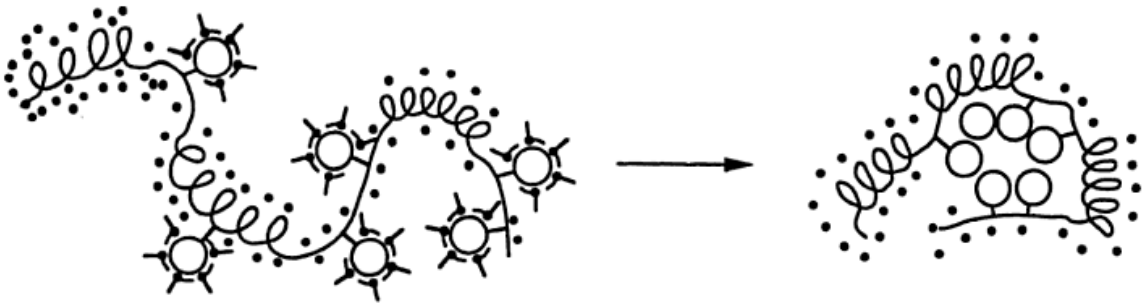


Figure 3-11: "Schematic illustration of a globular protein undergoing hydrophobic interaction. 'L-shaped' entities are water molecules oriented in accordance with proximity to a hydrophobic surface and dots represent water molecules associated with polar groups " (Fennema, 2008).

Hydrophobic interaction plays an important role in protein folding and sustaining the tertiary structure of proteins. Not all of the hydrophobic groups of globular proteins are located in the interior part because of the hydrophobic effect, and almost a half percent of the outer surface of the proteins are still composed of nonpolar groups (Fennema, 2008).

3.3 FLAVOR-PROTEIN INTERACTION

The use of proteins as an ingredient in food formulation is very common because of their nutritional and functional properties, such as emulsifying and stabilizing capacities (Guichard, 2002; Kuhn, 2006). While decreasing fat content makes the food product more popular because of health benefits, it dramatically affects the flavor profile of the food product because fats play a significant role in the binding of flavor molecules to the

food matrix (Kuhn, 2006). Increasing the number of conscious consumers who are looking for healthier diets, increases the preference for low-carb and low-fat foods (Reineccius, 2006). Proteins have a significant effect on sensory properties of food materials, such as appearance, color, texture, and flavor (Kinsella, 1979). Even though proteins have little or no flavor of their own, they play a key role in flavor perception because they affect the retention and release of flavor compounds (O'Neill, 1996). Proteins can react with different volatile chemical compounds, such as aldehydes, ketones, ionones, and esters (Guichard, 2006). The strength and type of those interactions dictate the release of flavor compounds during eating and thus determine the perceptive flavor of food material (Heng et al., 2004).

Interactions between flavor compounds and proteins have been studied many times to understand the binding mechanism and the effects of molecular structure of flavor compounds on binding (Plug and Haring, 1993). The results obtained from different studies were inconsistent and hard to compare even for same-flavor compounds because of a differentiation between protein batches and experimental conditions (Guichard, 2006). Thus, there is no well-defined understanding for the binding mechanism between proteins and flavor compounds. The interaction between flavor compounds and proteins might be reversible or irreversible, and the strength of this interaction depends on numerous factors, such as the chemical structure of flavor compounds, pH, water content, temperature, presence of ethanol, salt concentration, and structure of food protein (Guichard, 2002).

Chemical structure and composition, which dictates the physical and chemical characteristics of protein, determine the binding capacity of a protein. Therefore, the

amino acid composition and three-dimensional structure of proteins, which is formed via disulphide bridges and hydrogen bonds, are very important parameters in the binding of flavor compounds to proteins (Plug&Haring, 1993). The different amino acid content of each protein causes a different binding mechanism (Tan&Siebert, 2008). Reiners (2000) and Pelletier (1998) showed that while methoxypyrazines were binding to β-lactoglobulin, short acids and methylpyrazines did not bind to β-lactoglobulin. This suggests that the physical and chemical structure of flavor compounds is one of the decisive factors affecting the protein flavor interaction as well as the chemical structure and composition of protein (Andriot, 2000).

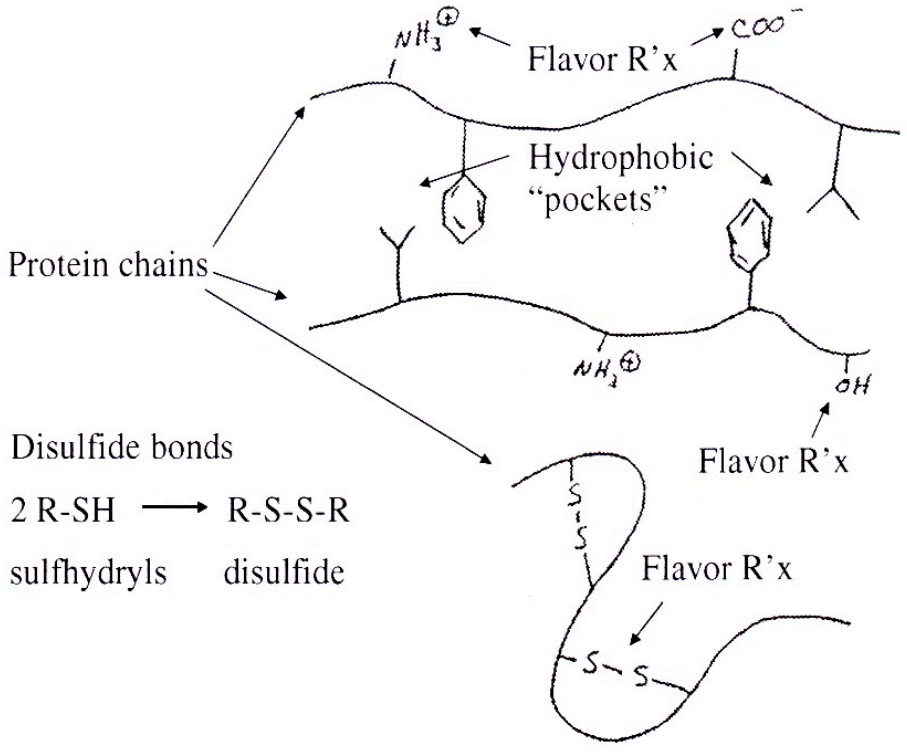


Figure 3-12: 'Opportunities for flavor to interact with protein molecules ' (Reineccius, 2006).

The binding of flavor molecules to proteins mostly occurs via reversible binding, such as hydrophobic, and hydrogen bonding (Figure 3-12)(Guichard, 2006). On the other hand,

an irreversible interaction can take place between flavor molecules and protein. An example of this is if the flavor compounds belong to the aldehyde group, a covalent bond might occur via the Schiff base formation (Tromelin, 2006).

3.3.1 SOY PROTEIN

Half a century ago, soy protein started to be used in all kinds of food product groups for many purposes because of its high nutritive value and functional properties. With the growing demand on food production that supplies the needs of today's world, agricultural science had to come up with enough grain yields to fulfill these needs. Soybean is one of the main crops in the world. In most cases, proteins are added to grains to extend the inadequate protein content and lack of essential amino acid content of grains. Soy protein is known as a great supplier of some essential amino acids; therefore, soy protein can be used as a supplement to strengthen grain's nutritional content. On the other hand, a large portion of protein requirements in the human diet is obtained from animal proteins. Because of the high cost of animal proteins and the negative effect on health, people turn toward plant source proteins. At this point, soy protein seems to be the best alternative since it is a renewable source within the plant source proteins due to its low-cost and well-qualified protein content. In addition to the economic benefits, soy protein products have many advantages because soy ingredients can have many functional roles in food products, such as emulsification, binding, and texture (Erickson, 1995; Hettiacchchy, 1998; Endres, 2001).

The use of soy protein in foods goes back to ancient times in eastern countries. Over the years, people had employed a lot of different methods for soybean processing, such as cooking, grinding, extracting, fermenting, and sprouting. As a result, traditional oriental

soybean foods were developed, for example tofu (soybean protein curd), soy sauce, miso (fermented soybean paste), natto (fermented whole soybeans), tempeh (fermented and deep-fried whole soybeans), sufu (fermented soybean protein curd), kinako (roasted soybean flour), soymilk, abura-age (deep-fried soybean protein curd), yuba (soybean protein film made from soy milk) (Wolf, 1970; Fukushima, 1991). These food products are one of the major sources of protein in the diet of many generations in the Orient. More recently, soybean and its products have become the center of attention all over the world due to their favorable economic and nutritional benefits, and a variety of soybean products are well positioned in the market (Fukushima, 1991).

The soybean known as Glycine max is from the legume family. It is the fruit of this legume that is the source of protein. The content of its seeds is: 40% protein, 18% oil, 15% insoluble carbohydrate (dietary fiber), 15% soluble carbohydrate (for example, sucrose and raffinose), and 14% moisture, ash, and others (Egbert, 2004; Fukushima, 1991). Soybean processing starts with cracking the seeds to get rid of the hulls, and then the oil is removed by solvent extraction. The derivative product is called defatted soy flakes, and is used to produce soy protein (soy protein concentrate and isolated soy protein) or other protein ingredients (soy flour and soy grits). The protein content of defatted soy flakes is around 50%) (Hettiaccachchy, 1998; Egbert, 2004).

3.3.1.1 Soy Protein Types

Based on their protein content, soy proteins are classified into three main groups: soy flours (min. 50% d.b.), soy protein concentrates (min 65% d.b.) and isolated soy protein (min 90% d.b.). Table 3.3 shows the composition of soy proteins (Endres, 2001).

Table 3-2: Composition of Soy proteins

	Defatted flours and grits		Concentrates		Isolates	
Constituent	As is	mfb[a]	As is	mfb	As is	mfb
Protein (N x 6.25)	52–54	56–59	62–69	65–72	86–87	90–92
Fat (pet. Ether)	0.5–1.0	0.5–1.1	0.5–1.0	0.5–1.0	0.5–1.0	0.5–1.0
Crude fiber	2.5–3.5	2.7–3.8	3.4–4.8	3.5–5.0	0.1–0.2	0.1–0.2
Soluble fiber	2	2.1–2.2	2–5	2.1–5.9	<0.2	<0.2
Insoluble fiber	16	17–17.6	13–18	13.5–20.2	<0.2	<0.2
Ash	5.0–6.0	5.4–6.5	3.8–6.2	4.0–6.5	3.8–4.8	4.0–5.0
Moisture	6–8	0	4–6	0	4–6	
Carbohydrates (by difference)	30–32	32–34	19–21	20–22	3–4	3–4

[a]mfb: moisture-free basis.

3.3.1.1.1 Soy Flours and Grits

Soy flour and grits are forms of soy protein that have the least protein content (between 40-50%). The contents of soy proteins are proteins, carbohydrates, ash, lipids, and other minor components (Wolf, 1970). They are obtained after the grinding and sizing process of defatted soy flakes, and mostly used in the bakery industry to improve nutritional value and product quality (Egbert, 2004).

3.3.1.1.2 Soy Protein Concentrates

There are three alternative ways of producing soy protein concentrates: aqueous alcohol leaching, acid leaching, and moist heat leaching to remove the soluble sugar, ash, and other minor components and off-flavor components (Figure 3-13). Nowadays, aqueous alcohol leaching is the favored procedure because of less off-flavor in end products (Lusas, 1995; Erickson, 1995; Endres, 2001)

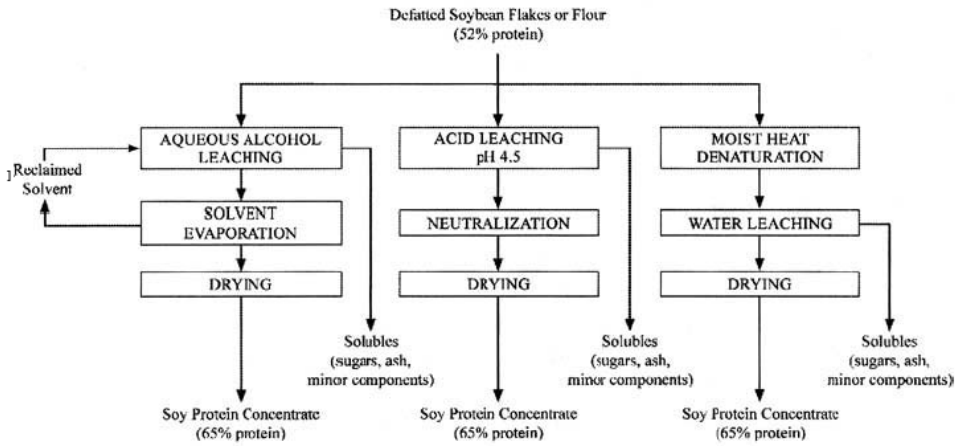


Figure 3-13: 'Production process of soy protein concentration' (Erickson, 1995).

As a result of its high water-binding capacity, soy protein concentrations are mostly utilized in meat products as an extender or replacer. They also used in the bakery and pasta industries to increase the protein content of food products. Another application is as a stabilizer in soup and sauce (Egbert, 2004; Stauffer, 2005).

3.3.1.1.3 Soy Protein Isolates

Soy protein isolate is one of the commercial types of soy protein that has the highest protein content (over 90%). The production process is based on the removal of insoluble carbohydrates (by alkaline extraction), soluble carbohydrates (by acid precipitation), and other components (Kolar, 1985; Hettiaccachchy, 1998). Figure 3-14 summarizes the general procedures of soy protein isolates production that follow two different ways that one can have an alcohol-wash step at first. The alcohol washing process plays an important role in removing soluble sugars and unwanted flavor and color components from defatted soybean flakes (Egbert, 2004).

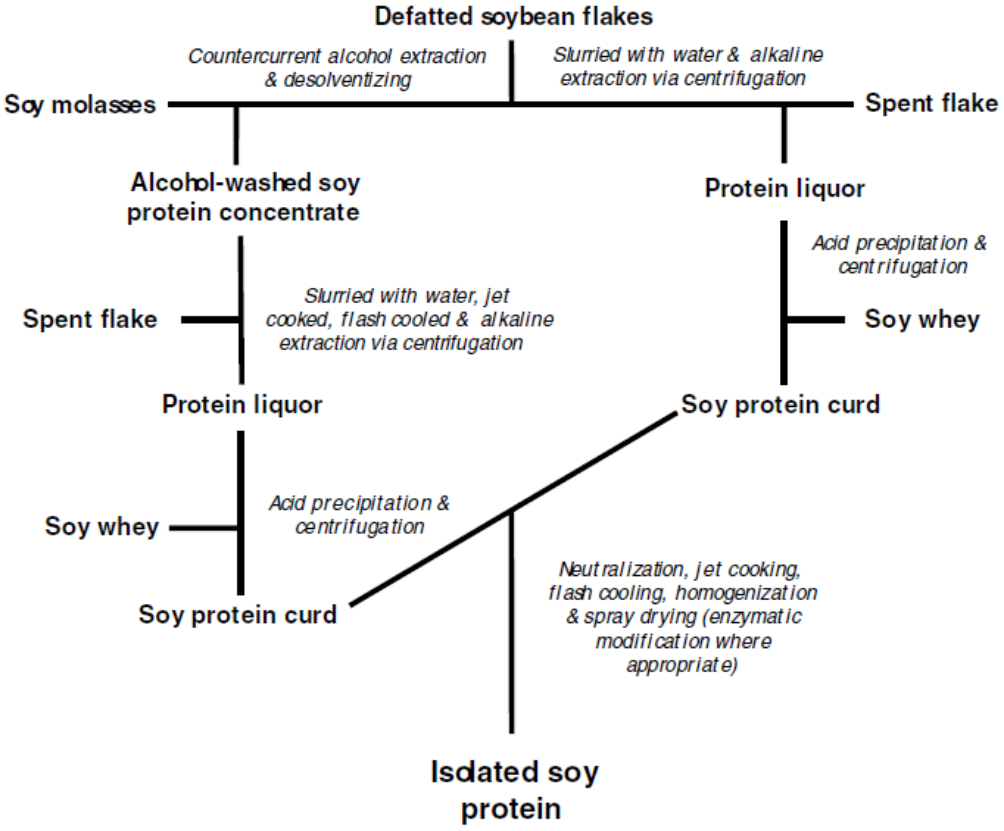


Figure 3-14: 'Production process for soy protein isolates' (Egbert, 2004).

Soy protein isolates are used in infant formulas (protein fortification, and fat emulsifier and stabilizer) and have meat (water-binding, texture enhancer, fat emulsifier and stabilizer), poultry (texture enhancer), seafood (water-binding, texture enhancer), dairy (structure and texture enhancers, emulsifier and stabilizer in coffee creamers, gelling agent in yogurt) and bakery (good moisture-retention, protein fortification) applications in industry (Kinsella, 1979; Kolar, 1985, Endres, 2001, Egbert, 2004).

3.3.1.1.4 Classification and Composition of Soy Protein

The obtained form of soy protein after the acid precipitation process is called soy globulins, which is a storage protein. Amino acids bind together by amide bonds that occur between an amino group of one amino acid and a carboxyl group of a second

amino acid to form polypeptide chains of proteins. Three-dimensional structures of the proteins occur as a result of the entangling and connection of polypeptide chains (Lusas, 2000; Sun, 2005).

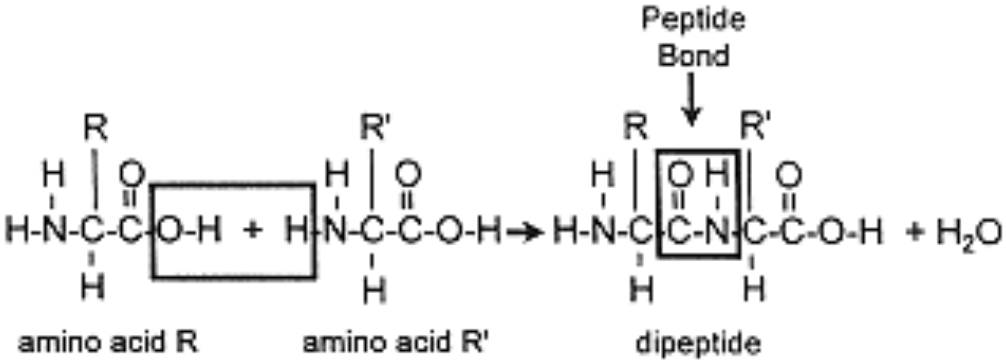


Figure 3-15: Formation of amide linkage in proteins (Lusas, 2000).

Soy proteins are composed of water-soluble albumins and mostly salt solution soluble globulins, which consist of 25% acidic amino acid, 20% basic amino acid, and 20% hydrophobic amino acid (Sun, 2005). Soy globulins have four main elements according to their sedimentation properties: 2S, 7S, 11S, and 15S (Table 3.4). The large portion of globulins is composed of 7S (conglycinin with a m.w. 100-200 kDa) and 11S (glycinin with m.w. 200-400 kDa) (Fukushima, 1991; Koshiyama, 1983; Brooks, 1985).

Table 3-3: Classification of soy protein components (Kinsella, 1979).

Fraction	Content %	Principal components
2S globulin	8	Trypsin inhibitor, cytochrome
7S globulin	35	Lipoxygenase, amylase, globulins
11S globulin	52	Globulins
15S globulin	5	Polymers

The amino acid composition of two major components (7S and 11S) is different. While both have high amounts of glutamic acid and aspartic acid, tryptophan and sulfur-containing amino acids are high in 11S globulin, and very low in 7S globulin. The –SH groups and S-S bonds 0 and 2 per molecule respectively in 7S globulin molecules, 2 and 20 per molecule respectively in 11S globulin molecules. The content of –SH groups and S-S bonds play an important role in the functional properties of soy globulins (Fukushima, 1991).

The 7S soy globulins consist of three main components: β-Conglycinin (β_1-β_6), ϒ-Conglycinin and basic 7S globulin (β_0) (Brooks, 1985). β-Conglycinin, which is the main form of 7S, gives 30-50% of total seed proteins. ϒ-Conglycinin and basic 7S globulin generate the minor portion of 7S (less than 3%) (Utsumi, 1997). 7S globulins have a trimeric structure of seven different combinations of three subunits: α (m.w. 57,000-68,000), α' (m.w. 57,000-72,000), and β (m.w. 42,000-52,000). These subunits are bound together via non-covalent binding to build up the trimer (Fukushima, 1991).

β-Conglycinin includes six different isomers (β_1 to β_6) that are created by different configurations (Figure 3.16) of α, α', and β subunits: β1- α'ββ, β_2- αββ, β_3- αα'β, β_4- ααβ, β_5- ααα', β_6- ααα (Koshiyama, 1983).

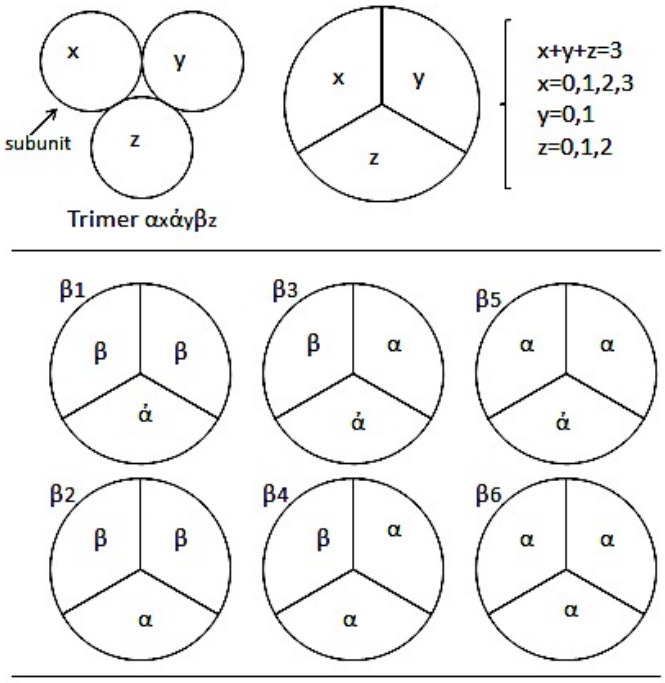


Figure 3-16: Schematic representation of 7S globulins (Koshiyama, 1983).

The 11S soy globulins are generally considered a simple homogeneous protein called glycinin (m.w. 320,000-360,000) and consist of two main subproteins: acidic poypeptide (A) and basic polypeptide (B), which hold together via disulfide bonds. 11S globulins build in a hexagonal structure of six subunits that are three acidic and three basic subunits. There are two hexagonal rings that hold them together, one placed on the second ring in the glycinin by electrostatic and/or hydrogen bonds (Fukushima, 1991; Brooks, 1985). The region that is formed by the two hexagonal rings is hydrophobic.

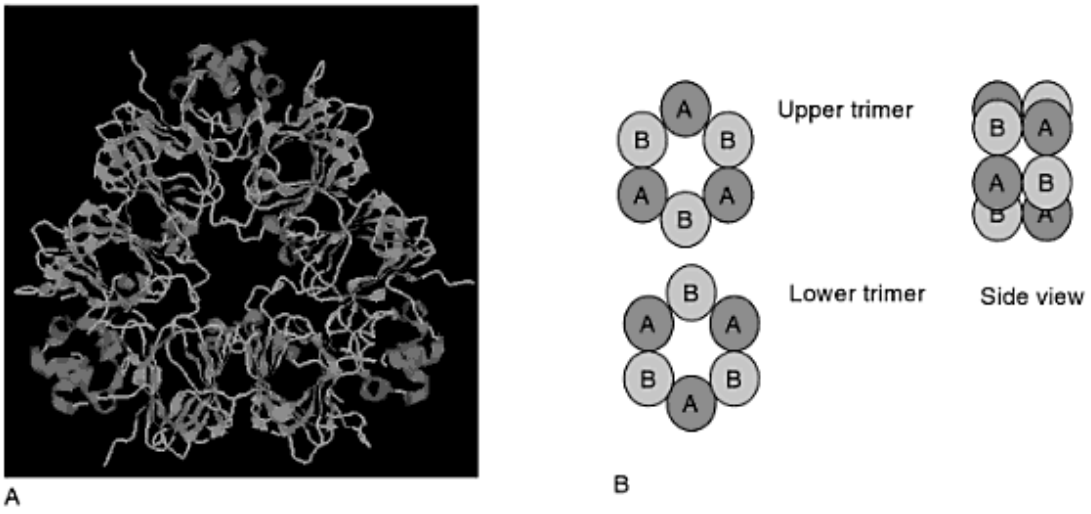


Figure 3-17: Ribbon diagram (A) and Schematic diagram (B) structure of 11S.

3.3.2 ZEIN

Corn or maize (Zea mays), which is an aboriginal crop of the U.S., is the most significant agricultural and economical produce in the U.S. (Lawton, 2002). Half of the annual production of corn, which is 560 million metric tons, is cultivated in the U.S. (Samarasinghe, 2007; Shukla and Cheryan, 2001). In many countries, corn is not an important part of the human diet; it is used as a snack (e.g. popcorn, sweet corn) or as a source for starches, oil, alcohol, and sweeteners. Farm animal feeding is the main usage for corn (Samarasinghe, 2007).

3.3.2.1 Structure and Composition of Corn Kernel

The corn kernel is a one-seeded fruit and includes three parts: pericarp (skin), germ (embryo), and endosperm, which consist of 10 to 12%, 6 to 8%, and 82 of dry kernel weight, respectively (Rooney et al, 2004).

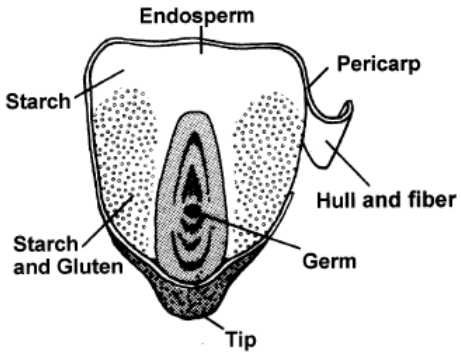


Figure 3-18: Structure of maize kernel (Shukla and Cheryan, 2001).

Seeds are covered by a protective layer called pericarp, which is composed of ash, fiber, oil, starch, and protein. The second part of grain is called embryo (germ), which is found in the lower part of the seed. The embryo consists mostly of oil, proteins, sugar, vitamins and minerals, and starch. The endosperm constitutes the largest portion of the seed's volume and weight and contains most of the starch, protein, and oil (Rooney et al, 2004). Table 3-5 shows the components of corn and their distribution.

Table 3-4: Compositions of corn and some corn processing by-products (Shukla and Cheryan, 2001).

Component	Whole kernel (wt%)	Components (dry weight, %)						
		Endosperm	Germ	Pericarp	Tip cap	CGF	CGM	DDGS
Starch	62.0	87.0	8.3	7.3	5.3	27.0	20	–
Protein	7.8	8.0	18.4	3.7	9.1	23.0	65	27
Oil	3.8	0.8	33.2	1.0	3.8	2.4	4	13
Ash	1.2	0.3	10.5	0.8	1.6	1.0	1	4
Others*	10.2	3.9	29.6	87.2	80.2	46.0	10	56**
Water	15.0	–	–	–	–	–	–	–

CGF – Corn gluten feed, CGM – corn gluten meal, DDGS – distillers dried grains with solubles
*By difference
**Includes glycerol, organic acids and other by-products

Two different methods are used to separate corn grain into its fractions. Dry milling process which, aims for physical fractionation is actually a basic grinding process and primary products of dry milling are corn grits, corn meal, and corn flour after separation

of corn germ. Wet milling process, which aims for fractionation of chemical constituents) is more complex than the dry milling process, and starts with the soaking of corn kernels in water. The main products of wet milling are germ (high in oil), fiber (mostly pericarps), protein (used in the production of corn gluten meal, CGM) and starch. Starch--the primary product of wet milling--is utilized as a raw material to produce glucose, high fructose corn syrup, and ethanol. The products of further processing of germ are corn oil, corn germ meal (CGM, 65-67% db protein content), and corn gluten feed (CGF). C Separated fiber stream (bran) undergoes a process to produce dietary fibers and CGF (Alexander, 1987; Inglett, 1970; Brekke, 1970; Ponte, 2000). There is another process called dry grind process (no fractionation) that is used to generate ethanol. Coproducts of dry grind process are wet grains, syrup, distillers dried grains (DDG), or DDG with soluble (DDGS) and carbon dioxide (Shukla and Cheryan, 2001).

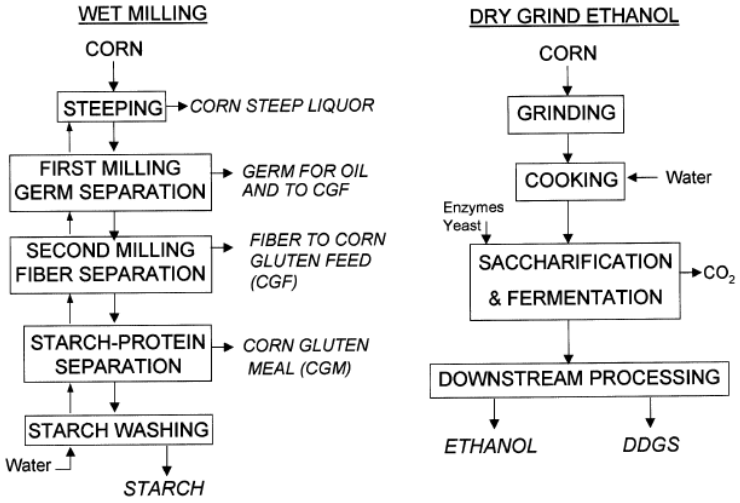


Figure 3-19: 'Process flow sheets for corn wet milling (left) and dry grind ethanol production from corn (right)' (Shukla and Cheryan, 2001).

Corn has a high content of starch and a low content of protein, but a large amount of processing results in counting corn as a protein source in farm animal feeding (Wright,

1987). The total protein content in dry seed is 10%, which composes the four major proteins: albumin, globulin, prolamine, and glutelin (Garratt, 1993).

Table 3-5: Distribution of major proteins in corn (Shukla and Cheryan, 2001).

Protein	Solubility	Whole kernel	Endosperm	Germ
Albumins	Water	8	4	30
Globulins	Salt	9	4	30
Glutelin	Alkali	40	39	25
Zein	Alcohol	39	47	5

3.3.2.2 Zein

Zein is the prolamine (alcohol-soluble protein) in corn and is mostly found in the endosperm of a kernel (Fu and Weller, 1999; Holding and Larkins, 2009). Zein's only role is to stock up the required nitrogen for seed growth (Tatham, 1993). There are two different commercial processes that are used to extract zein: Corn Product Corporation process (CPC process) and Nutrilite process. Corn gluten meal (CGM) is the raw material for both processes. Zein is produced with the CPC method and does not include oil. To remove the oil, the CPC method starts by adding isopropanol with ethanol in the first step. Zein is produced with the nutrilite process. It contains 2% oil and undergoes a further extracting process with 88% isopropanol to minimize the oil content (Shukla and Cheryan, 2001).

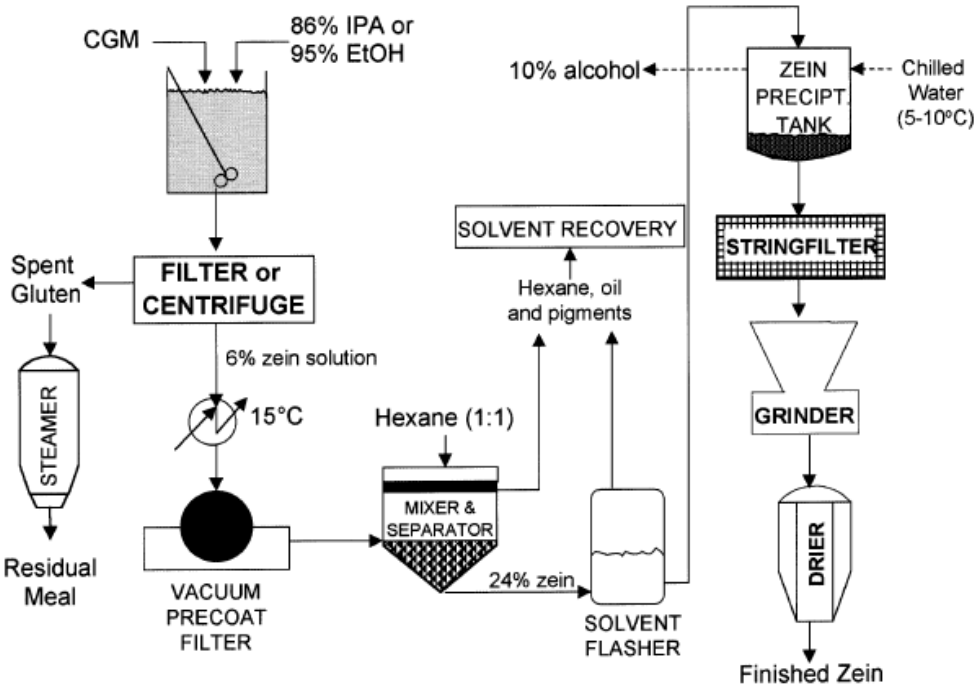


Figure 3-20: Zein production by CPC process (Shukla and Cheryan, 2001).

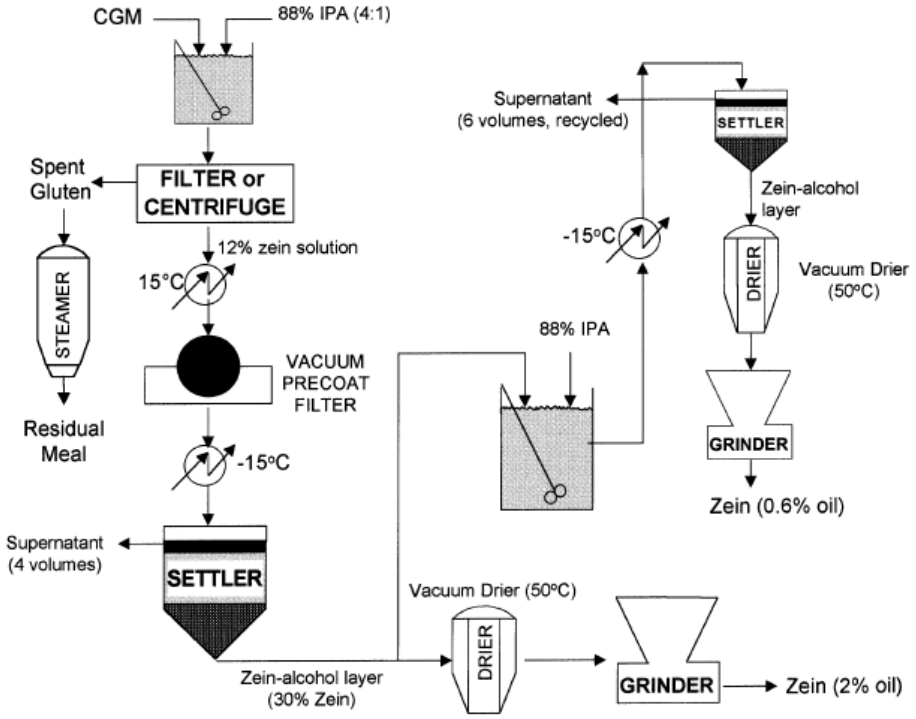


Figure 3-21: Zein production by Nutrilite process (Shukla and Cheryan, 2001).

Zein does not directly take place in the human diet. The most popular usage of zein in the food industry is for food coatings against oxygen, lipid, and moisture migration. It is also used in drug coatings to achieve the effective release of ingredients and mask the unpalatable taste (Padua and Wang, 2002). Zein has been under investigation because of its potential to construct edible and biodegradable zein-films (Shukla and Cheryan, 2001). Zein has nonfood applications, such as forming fibers for clothing and furniture and printing inks (Lawton, 2002).

3.3.2.2.1 Composition and Structure of Zein

Zein is a hydrophobic protein and carries exceptional features that make it different from other proteins. It is an aqueous-alcohol soluble, thus not soluble in only water or in only alcohol. Zein is also soluble in high concentrations of urea and high concentrations of alkali. The hydrophobic portion of the amino acid composition explains zein's solubility in binary solvents (Fu and Weller, 1999). It is a hydrophobic residue high protein that comprises leucine, proline, alanine, and phenylalanine. Zein lacks lysine and tryptophan (Shukla and Cheryan, 2001).

There are four different types of zein that show changes in their solubility, molecular size, and charge: α, β, γ, and δ (Esen, 1987). The largest portion of the prolamines in a kernel is the α-zeins (soluble in 95% ethanol), which represent 75-85% of the zein protein. It is categorized in two groups according to their molecular weight: 19kDa (Z19) and 22kDa (Z22) (Tatham, 1993). α-zeins indicate immensely hydrophobic features due to high contents of hydrophobic residue (Matsushima et al., 1997). The second plentiful portion is ϐ-zein, which represents the 10-15% of prolamines in maize. ϐ-zein is classified in two groups according to their molecular weight: 27kDa and 16 kDa. β-zein

(soluble in 86% ethanol) represents 10% of the total prolamines in maize and its molecular weight is 17kDa (Fu and Weller, 1999). β-zein is richer in cysteine and methionine content than α-zeins (Coleman and Larkins, 1999). δ –zein with 10 kDa molecular weight represents the minor part (~5%) of the prolamines in maize (Fu and Weller, 1999). δ –zein has a high methionine content (Coleman and Larkins, 1999).

The helical wheel model for zein was proposed by Argos et al. (1982). In this model, nine similar helical wheels are connected to each other via hydrogen bonds to form a stabilized structure of zein. Nine smaller cylinders (helical wheels) connect with each other to form an oval-cross-section cylinder Figure 3-22. Small circles represent the hydrogen bond between polar regions on each helical wheel (Argos, 1982).

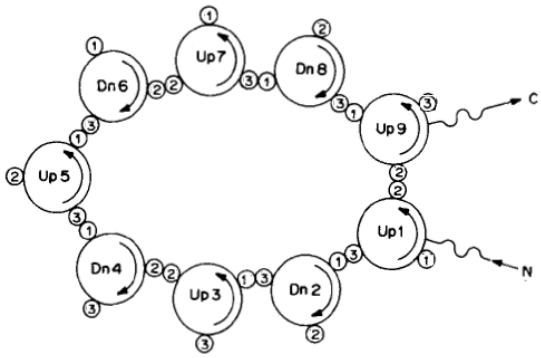


Figure 3-22: Representation of possible nine-helical zein protein structure (Argos, 1982).

One helix of the nine antiparallel helices can be seen as in Figure 3-23. A helical wheel consists of 18 amino acids and has three polar regions. Each polar region contains two polar amino acids. Three polar regions represent the three hydrogen bond capabilities, two of them for the connection with other helical wheels, and one of them for the neighboring zein molecules (Argos, 1982).

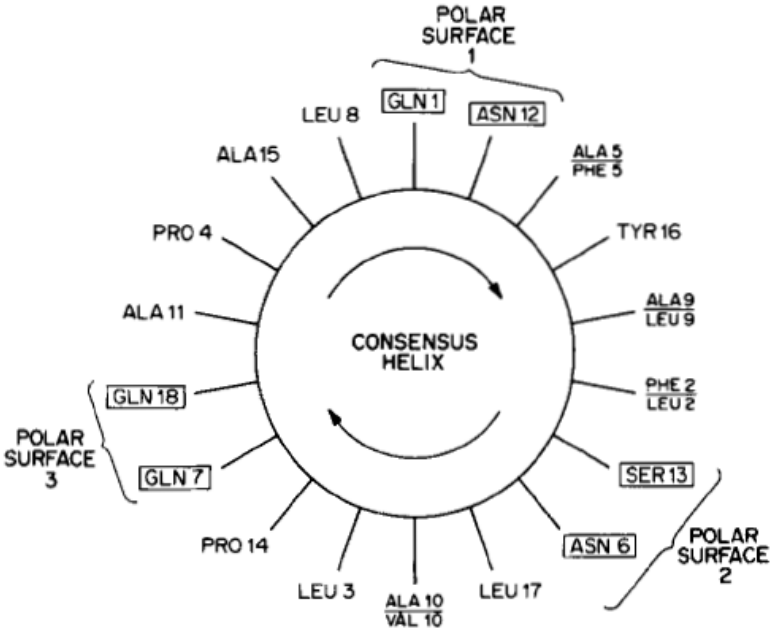


Figure 3-23: Helical wheel (Argos, 1982).

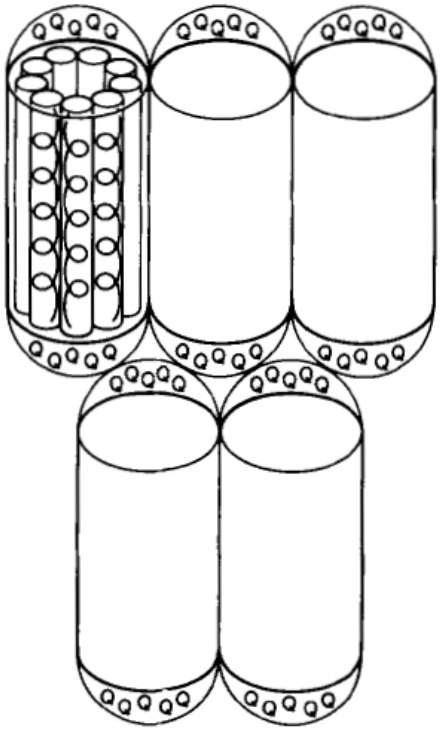


Figure 3-24: Possible arrangement of zein molecule (Argos, 1982).

Figure 3-24 shows the possible arrangement of zein molecular planes. Glutamine residues (Q) are positioned at the turn regions between the helix, and form hydrogen bonds between neighboring planes (Argos, 1982).

To develop three-dimensional models for α-prolamine, Garratt et al. (1993) used hydrophobic membrane propensities and helical wheels. The suggested structural model includes a helix that is enclosed by the other six helices so that each has three hydrophobic faces. The advantage of this hexagonal arrangement over the previous model is to give well-organized packaging and no large hole in the center. In the Matsushima et al. (1997) model, the researchers proposed that each helix is connected to the following helix by glutamine rich turns or loops to form a rectangular prism shape.

3.3.3 FLAVOR/SOY PROTEIN INTERACTIONS

The earliest soy protein–flavor binding studies were conducted by Arai et al. to understand the behavior of flavor compounds that inherently exist in soy-based products, such as n-hexanal, n-hexanol, n-heptanol, n-pentanol. The results demonstrated: (a) that binding affinity of those volatile flavor compounds to denatured soy protein is higher than native protein that causes an increase in the resistance of off-flavors to remove; and (b) also that protein-flavor binding capacity diminished with proteolytic enzyme treatment (Arai et al., 1970 a-b). Gremli examined the soy protein-flavor interaction to distinguish if the reaction was reversible or irreversible by using equilibrium headspace and high vacuum distillation–GC techniques. There was no interaction between soy protein and alcohols. While the interactions were partially irreversible between aldehydes and soy protein, interactions were reversible in the case of ketones. Increase in molecular weight of both aldehydes and ketones enhanced the retention. The molecular weight

dependent binding tendency of ketones changed to decrease after 2-decanone (Gremli, 1974). Damodaran and Kinsella studied the binding of carbonyls (2-nonanone, 2-octanone, nonanal, and 5-nonanone) to undenatured soy protein by using an equilibrium dialysis method. They examined the effect of the chain length of ketones, the position of keto group in the chain, temperature, and partial heat denaturation on binding. Results suggested that the binding of ketones increases with increasing chain length. A decrease in the binding affinity was observed as the keto group moved towards the chain center (nonanal> 2-nonanone> 5-nonanone) because of an increase in steric hindrance. This occurs when keto groups move towards the center and destabilize hydrophobic interaction between the volatile compounds and protein. There was no difference in binding behavior at 25 C and 45 C, but a decrease in the binding affinity at 5 C, which might be the result of a modification that occurs in tertiary and quaternary structures of soy protein at low temperatures. The binding of 2-nonanone to partially denatured soy protein was greater than native soy proteins suggested that heat induced modification of soy protein leads to an increase in hydrophobicity and thus increases the binding affinity (Damodaran and Kinsella, 1981). Later Damodaran and Kinsella continued to study 2-nonanone and different soy protein fractions (7S and 11S fractions) to understand the effect of the structural states of soy protein on interactions by using the equilibrium dialysis method. 7S fraction almost had the same binding affinity with whole soy protein, but 11S fraction had very little binding affinity compared to whole soy protein. This suggested that the carbonyls mostly bound to the 7S fraction of soy protein because it might have more available hydrophobic binding site at the contact area for flavor compounds. A decrease in the binding affinity at the presence of urea and succinylation,

which cause conformational modification, pointed to the possibility that protein structure plays an important role in the interaction with the carbonyls (Damadoran and Kinsella, 1981). Chung and Villota focused on interactions between alcohols and soy protein isolate. Their results demonstrated that a weak interaction takes place between the soy protein isolate and n-butanol and n-hexanol, and that there might be hydrogen bonding in addition to hydrophobic interaction. It was also suggested that heat denaturation of soy protein decreases the interaction between alcohols and the soy protein isolate because heat denaturation increases the density and decreases water solubility that, in turn, decreases the accessibility of alcohols to the binding sites of protein (Chung&Villota, 1989). O'Keefe et al. studied two major storage proteins found in soybeans, glycinin and β-conglycinin, and determined the thermodynamics of their interaction with volatile flavor compounds and soy protein by using the headspace technique. Hexane interacted with both glycinin and β-conglycinin only at 5 C; at higher temperatures there was no interaction. The interaction of volatile flavor compounds with glycinin was greater than with β-conglycinin. Hexanol had less affinity for glycinin at all three temperatures compared to hexanal and 2-hexanone. Glycinin had a higher binding affinity for all volatile compounds than β-conglycinin at all temperatures (O'Keefe et al., 1991). Li et al. studied three different proteins (casein, whey protein, and soy protein) and vanillin to compare the binding of flavor compounds to different proteins under the same experimental conditions. This study showed that the binding site of soy protein was higher than whey protein and casein, and also that binding sites were increasing with the decreasing temperature for all three proteins. The researchers demonstrated that each protein interacted with flavor in a different way, such as the interaction between vanillin

and whey protein was greater than that of soy protein and casein. It was also concluded that an enthalpy-driven reaction took place for whey protein and for casein with vanillin. In the case of soy protein, an entropy-driven reaction was observed that suggested that any structural alterations, such as heat-induced denaturation of soy protein, play an important role in the interaction with vanillin (Li et al., 2000). Zhou et al. examined the binding of 2-pentyl pyridine to commercial SPI and purified β-conglycinin and glycinin of soy proteins to understand the influence of different experimental conditions, such as temperature, pH and salt concentration. Results indicated that an increase in temperature (from 25 to 74 C) increased the interaction of 2-pentyl pyridine with protein and its fractions, while the binding of 2-pentyl pyridine at 4 C was greater than at 25 C. According to that study, pH was another factor that affected the interaction: the binding affinity at alkaline conditions > neutral conditions > acidic conditions. It was concluded that increasing salt concentration decreased the interaction between 2-pentyl pyridine and soy proteins and its fractions because of increased vapor pressure and destabilized electrostatic interaction (Zhou et al., 2002). Gkionakis et al. studied different proteins and amino acids, including soy protein, to analyze the interactions between lactones γ-9, γ-10, δ-10, δ-11. Lactones flavor showed similar binding behavior with soy protein isolate. In the case of the addition of lactones flavors together to the system, a difference (decrease) in binding behavior occurs, which is explained by a competition between lactones to bind the functional group of the SPI (Gkionakis et al., 2007).

The research regarding the interaction between flavor compounds and soy protein at dry conditions was performed by Crowther et al., to understand the binding mechanism and to determine the effect of temperature and moisture on binding. The heat of adsorption

and adsorption coefficient were calculated for the interaction between soy protein and alcohols, aldehydes, and ketones by using gas chromatography. The results suggested that there was a decrease in the interaction between soy protein and volatile compounds in the case of protein denaturation (Crowther et al., 1980). Later, Aspelund and Wilson also employed the gas chromatographic technique to study the interaction between flavor compounds and soy protein at dry conditions to understand the adsorption phenomena and strength of the adsorption by using calculated thermodynamic values, such as heats of adsorption, free energy of adsorption, enthalpy of adsorption, and entropy of adsorption. Their results demonstrated that the weakest interaction took place between soy protein and hydrocarbons via van der Waals forces. Ketones, aldehydes, and methyl ester interacted with soy protein via hydrogen bonding in addition to van der Waals forces. The oxygen atom of ketones, aldehydes, and methyl ester might form a hydrogen bond by interacting with the functional group of the soy protein. The strongest adsorption occurred between the soy protein and alcohols via forming two hydrogen bonds, besides van der Waals forces. This research suggested that the functional group of volatile flavor compounds had a significant effect on the interaction with the soy protein (Aspelund and Wilson, 1983). To examine the interaction of flavor compounds with soy protein isolate under controlled relative humidity, Zhou at al., used inverse gas chromatography, thus thermodynamic values were calculated to interpret the interaction with flavor compounds (hexane, hexanal, 1-hexanol). Even though each flavor compound had the same carbon number, their binding behavior was not similar: 1-hexanol had the highest binding affinity to soy protein isolate, which might include two hydrogen bond and van der Waals forces; followed by hexanal, which included one hydrogen bond in addition to van der

Waals forces; and the lowest binding affinity was observed for hexane, which included only weak van der Waals forces. These results suggested that the functional group of volatile compounds played an important role in the interaction between flavor compounds and soy protein isolate. If increasing the RH from 0% to 30% decreases the binding affinity of all volatile flavor compounds, it is suggested that water molecules compete with the flavor molecules to bind the functional group of soy protein isolate (Zhou at al., 2004). Zhou et al. performed an additional study to evaluate the effect of the chemical structure and relative humidity on the interaction of flavor compounds with soy protein isolate by using inverse gas chromatography. Results were in a good agreement with the previous study which was demonstrated significant effect of the functional group of flavor compounds on binding and suggested that alcohol formed weak interaction with SPI via van der Waals forces, ester, ketone, aldehyde formed hydrogen bonding or dipole forces in addition to van der Waals forces and alcohols formed strongest binding via high-energy hydrogen bonding or two hydrogen bonds in addition to van der Waals forces. Increasing the relative humidity from 0% to 30% RH; didn't influence the binding of hexane and limonene to SPI, but decreased the binding of ketone, aldehyde, ester, and alcohols because of competition between water molecules and flavor to bind the soy protein isolate. Additional increases of relative humidity from 30% to 40% and 50%; didn't affect the binding of aldehydes, ketones, and esters, but increased the binding of alcohols due to the interaction between water molecules and alcohols via hydrogen bonding or dipole forces (Zhou et al., 2006). In a comparative study, Zhou et al. examined the interactions of flavor compounds (γ-butyrolactone, butyric acid, diacetyl, hexanal, 2-ethyl butyric acid, pentane-2, 3-dione) with wheat versus soy-containing

crackers. The binding behavior of hexanal and diacetyl, demonstrated a weak bonding because wheat and soy-containing wheat crackers were similar. The interaction of butyric acid and γ-butyrolactone with a soy protein-containing cracker was greater than with a wheat cracker because of an enhanced polarity as a result of adding SPI to the cracker (Zhou et al., 2006).

3.4 EFFECT OF WATER

Water can be considered as the source of all life. Interactivity of the biological molecules, such as proteins, nucleic acids, and membranes, with water forms the basis of their structures and functions (Raschke, 2006). Water is the main component of food. It has important effects on food safety, quality, and physical properties. The presence of water in a food product has a very extensive interval starting from extremely low values and reaching up to 98%. It exists in large amounts in fresh products and beverages while this amount reduces to very low values in baked and dried products. Most food products include water as an integral element. On the other hand, some products have water supplemented in the production stage (Lewicki, 2004).

The water component influences physical structure, such as electrical and heat conductivity, density, and rheological behavior of the product. In this context, it plays an important role on the technological design of the food product. Water existence determines corrosiveness and impurity of materials. Stability and shelf life of the food products are affected by water, since it forms the basis for microbiological life and enzymatic interactions. The water quantity in a product also determines the storage volume and mass, thus transport costs are closely related to this component. In the

context of commercialism, water quantity in expensive food products are legally regulated, since water is cheaper when compared to other materials (Isengard, 2001).

The water molecular structure and the interactivity between the water molecule and the remainder of food components determine the state of water in a food product. Hydration refers to the reaction between solute molecules and water molecules. In the case of polar molecular existence, the interactions are controlled by hydrogen bonding (Lewicki, 2004). The binding behavior of water molecules is different for polar and nonpolar groups. Polar groups interact directly with water molecules, while interactions among the water molecules are magnified by nonpolar groups (Raschke, 2006). Figure 3-25 refers to two states of water in macromolecules. In the case of structure water, water molecules stay stationary in a macromolecule structure, while in the case of hydration water, mobility of the water molecule is not strictly limited. In accordance with hydrogen and/or ionic bond, relocation of the water molecule occurs. Thus, it can be considered that the interactivity between water and macromolecules build and stabilize the spatial shape of the biopolymers (Lewicki, 2004).

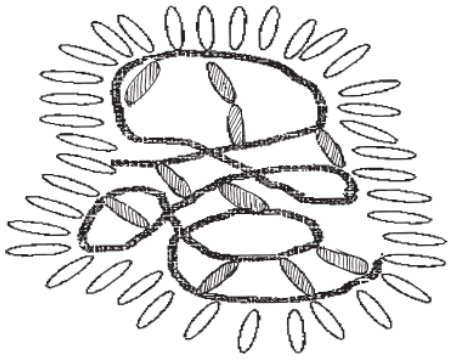


Figure 3-25: 'Hydration of macromolecule shaded area- structure water, plain area- hydration water' (Lewicki, 2004).

Hydrogen bonds and hydrophobic actions are closely related to the interactivity between water and food components. The H-bond system of water sets a basis for both types of interactions (Meste, 2006).

Table 3-6: '*Classification of types of water –solute interactions'* (Fennema, 2008).

Type	Example	Strength of interaction compared to water–water hydrogen bond[a]
Dipole–ion	Water-free ion Water-charged group on organic molecule	Greater[b]
Dipole–dipole	Water–protein NH Water–protein CO Water–sidechain OH	Approx. equal
Hydrophobic hydration	Water + $R^c \rightarrow$ R(hydrated)	Much less ($\Delta G > 0$)
Hydrophobic interaction	R(hydrated) + R(hydrated) $\rightarrow R_2$ (hydrated) + H_2O	Not comparable[d] (> hydrophobic interaction; $\Delta G < 0$)

[a]About 12–25 kJ/mol.
[b]But much weaker than strength of single covalent bond.
[c]R is alkyl group.
[d]Hydrophobic interactions are entropy driven, whereas dipole–ion and dipole–dipole interactions are enthalpy driven.

3.4.1 BINDING MECHANISM BETWEEN WATER AND PROTEIN

Spatial structure of a protein influences the protein's physicochemical and operational features and behavior in the processing, storage, preparation, and consumption stages. This spatial conformation of the protein is significantly influenced by the interactions of protein with water in the sense of building and preserving the structure (Zhong and Sun, 2000). It is proven that water is a key component of enzyme catalysis, protein folding, protein architecture, structural stability, protein dynamics, protein plasticity, ligand binding, and a variety of specific reactions (Mattos, 2002). Thus, the interaction of food proteins with water provides the vital functional application to them (Table 3-8).

Wettability, dispersability, viscosity, thickening, dough formation, gelation, solubility, and surfactant properties are shaped by these interactions (Kinsella, 1982).

Table 3-7: Classification of water thermodynamically associated with proteins at increasing water activities (Kinsella, 1982).

Structural Water:	Water H-bonded to specific groups, participates in stabilisation of structure, unavailable for chemical reaction.
Hydrophobic Hydration Water:	Structured cage-like water surrounding apolar residues; like structural water, is very much involved in stabilising protein structure.
Monolayer Water:	The first adsorbed water monolayer, H-bonded; unavailable as solvent, may be available for chemical reactions; ranges from 4–9 g/100 g protein.
Unfreezable Water:	This roughly includes all water (structural monolayer, and perhaps some adsorbed multilayer water) that does not freeze at normal temperature—amounts to 0·3–0·5 g/g protein and corresponds to water up to $A_w = 0{\cdot}9$. The amount varies with polar amino acid content and includes some water available for chemical reactions.
Capillary Water:	Water held physically in clefts or by surface forces in the protein molecule (e.g. water entrapped in gels, cheese curd); physical properties similar to those of bulk water.
Hydrodynamic Hydration Water:	This represents the water 'loosely' surrounding the protein that is transported with the protein during diffusion (centrifugation); it has properties typical of normal water.

To designate the function of globular proteins, water is considered as a reference point since it has a significant effect on structure and dynamics of proteins. There are three main classes identified in order to sort the water molecules in protein solutions: internal, surface, and bulk water. Internal water molecules, which densely contain hydrogen bonds and include the main portion of protein structure, populate the internal parts and deep splits. On the other hand, surface water molecules are not specified as well as internal

water molecules. They react with the protein surface and are thus more active than internal water molecules (Tarek and Tobias, 2000).

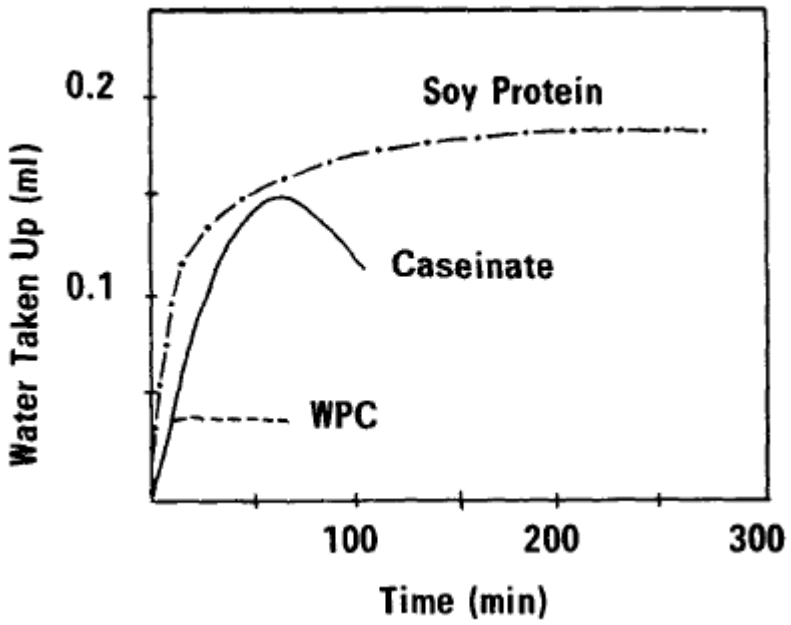


Figure 3-26: 'Swelling (water uptake) as a function of time for 20 mg sample '(Chou and Morr, 1979)

When a dehydrated protein is the subject of matter, such as for a spray-dried protein isolate, water absorption should be taken into account in the case of water vapor or liquid exposure (Figure 2). In figure 3, the sequence of protein-water interaction for dry protein is demonstrated. If water vapor is applied to a dry protein powder, monolayer coverage is constituted as all available surface polar sites take up the water molecules. Additional layers of water are generated as a result of further water absorption. Thus, liquid-water condensation occurs due to the water-water interaction after the multilayered stage and the protein fragments are expanded. Swelling of these fragments continues until enough water saturates every single protein fragment to enable the solution of the fragment. Water absorption is a solid process having no precise boundaries between the individual

phases and, at any time during the water absorption process, protein swelling can happen (Chou and Morr, 1979).

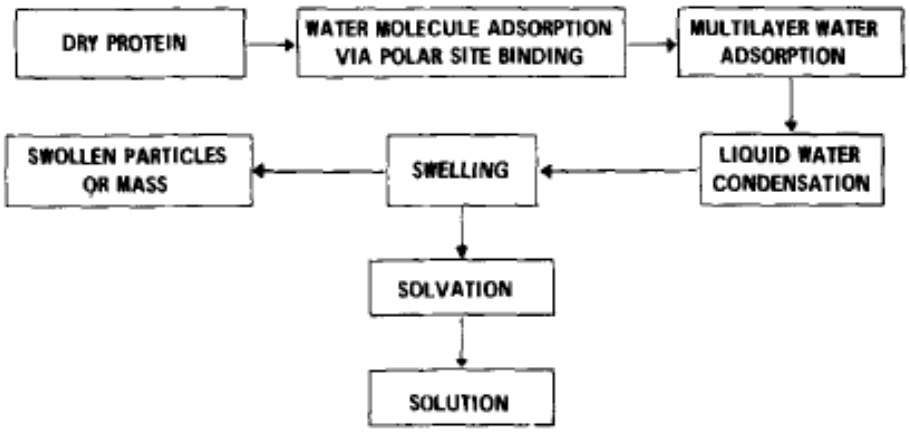


Figure 3-27: 'Sequence of protein-water interaction for dry protein' (Chou and Morr, 1979).

Coulombic, hydrogen bonding, van der Waals, and hydrophobic interactivities occur between water and particular structural groups of the protein culminate in the hydration of proteins. Water absorption and preponderant interactions in which charged groups are involved are required in the early phases of hydration. Since the remains of amino acids introduce broad distinctions in ionic, polar, and nonpolar sites, these types of interactions differ in strength (Towns, 1995).

According to the structure of the amino acid side chain, three surface group types of protein molecules are introduced in aqueous media. These surface types are: polar-charged groups, such as those of glutamic acid (-CH2CH2COO-) or lysine [-(CH2)4NH3+], polar-neutral groups, such as those of serine (-CH2OH) or glutamic acid at low pH (-CH2CH2COOH), and nonpolar groups, such as those of valone [-CH(CH3)2] or methionine (-CH2CH2-s-CH3) (Coultate, 2002). A water molecule that is near a

protein totally differs in the interaction process when compared with a water molecule in bulk water. Hydrogen bonds are produced when polar groups on the protein surface react with water and, as the amino acids are charged (as well as the N- and C- termini), electrostatic interactions with water occur. On the other hand, 'hydrophobic interactions' take place as a result of nonpolar atoms on the protein surface. These hydrophobic interactions improve the local structure of neighboring water molecules (Raschke, 2006).

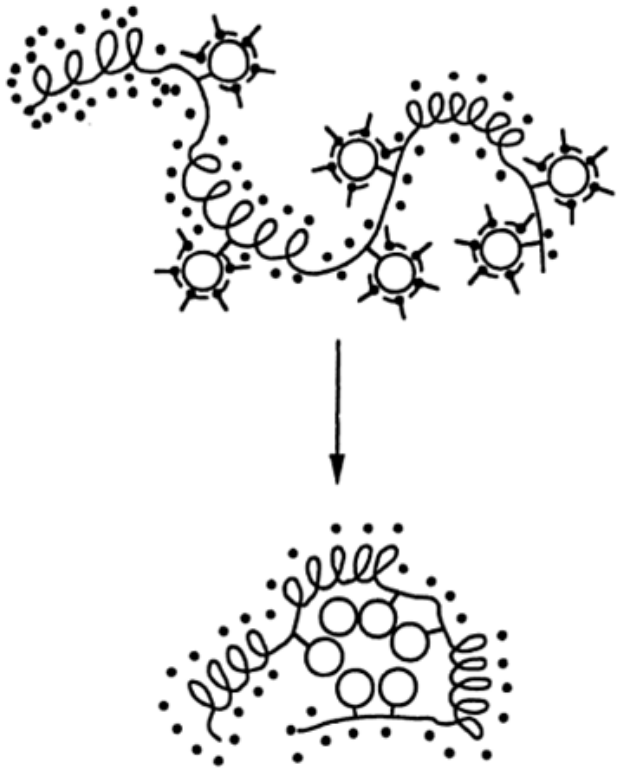


Figure 3-28: "Schematic illustration of a globular protein undergoing hydrophobic interaction. Open circles are hydrophobic groups. 'L-shaped' entitles are water molecules oriented in accordance with proximity to a hydrophobic surface, and dots represent water molecules associated with polar groups" (Fennema, 2008).

3.4.2 EFFECT OF WATER ON RELEASE/RETENTION

A plasticizing or antiplasticizing effect results when the amount of water component is augmented. Since water has the characteristics of a solvent and supports the mobility of polymer chains, the plasticization effect causes deformation. Materials become soft, extensible and flowing, and loses crispiness, hardness, or toughness (Lewicki, 2004).

Flavor components can be stuck in the hydrophilic or the lipid phase in solid and partly solid products. Compounds trapped in a dry hydrophilic phase stay stationary and can only become remobilized if the matrix is softening, swelling, or dissolving (Plug & Haring, 1994). Water activity on the compound itself influences solid-state stability of proteins in two ways: (1) altering the dynamic activity or structural stability of protein; (2) involving the reactions or forming an environment that enables mobilization of reactants. Augmented suppleness of the protein due to water existence in the solid state yields to impairment in structural stability (Towns, 1995).

Figure 3-29 illustrates results of an experiment, which shows the impact of texture on the dispatching of unstable flavor compounds. By inserting 1% carboxymethylcelulose (CMC), the texture of the aqueous solutions is changed, thus viscosity of the media is enhanced while keeping the phase partitioning unchanged. The graphs in Figure 3-29 indicate that plain water always has higher release rates when compared with the CMC solutions (de Roos, 2003).

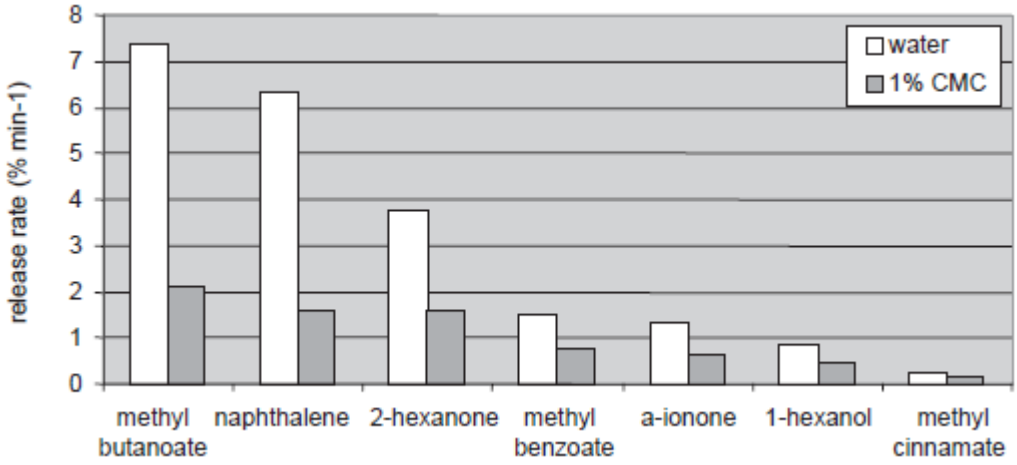


Figure 3-29: Dynamic flavor release from stirred aqueous solutions with and without carboxymethylcellulose (CMC) at 37C (de Roos, 2003).

Since the interactions of volatiles are closely related to the shape and water content of the protein, the influence of soy protein can be complex. It is known that being subjected to a small amount of water can cause flavor loss since the solubility of compounds are boosted (Plug & Haring, 1994). The shelf life of low moisture foods is significantly influenced by storage level humidity (RH) level. As a result, due to the modification of flavor retention/release properties of the food by moisture barter with the surroundings, the flavor quality of the product deteriorates (Zhou, 2006).

3.5 HEAT TREATMENT

Heating is only one of the protein structure modification techniques, but it is utilized most frequently in order to enhance operational characteristics of the protein structure (Sorgentini et al., 1995). Without changing the amino acid sequence, the structure of the protein can be modified through protein denaturation, which might be created by pH, detergents, urea, guanidine hydrochloride, or by heat treatment (Zhong and Sun, 2000). As denaturation takes place, unfolded molecules populate the protein structure and a decrease in solubility occurs. Capabilities associated with the protein structure, such as

gelification, foaming, and emulsification, are also affected as a consequence of denaturation (Petruccelli and Anon, 1995). Applying temperatures more than 70C while heating soy proteins causes dissociation in their quaternary structures, denaturation in their subunits, and enhancement in the constitution of protein accumulations by the way of electrostatic, hydrophobic, and disulfide interchange bonding mechanisms. After hydrophobic groups of proteins are uncovered by heat denaturation, these groups interact and decrease in solubility, and accumulation behaviors are observed as a result (Morr, 1990). Heating caused modifications in the structure of soy globulins, and these were reviewed by Saio et al. (Kinsella, 1979).

Effect of Heat on Some Physical Properties of Soy Protein

Property	Heating temperature (C) 80	100	120	140	160
Subunit structure	dissociation-unfolding			degradation	
Solubility	decrease - precipitation			increase in solubility	
Viscosity	increase—decrease			decrease	
Hydration	increase		decrease		
Gelation (following heating)	regular	hard-fragil		soft elastic	sol

Figure 3-30: Effect of heat on physical properties of soy protein.

As it is widely known, denaturation causes the unfolding of firm protein structure and, in turn, unfolding causes disruption and restoration of intermolecular and intramolecular interactivities (Zhong and Sun, 2000). Since broadly hydrophobic medium of the native protein molecule conceals a small part of the polar groups, it is generally accepted that these groups cannot bind water. It is considered that thermal denaturation causes polypeptide chain to be unfolded. This results in exposure of the remaining few polar groups and, in turn, some extra water binding is revealed. Because of the hydrophilic

nature of the peptide bond parts of the frame, constricted capability in water binding is observed on the recently revealed hydrophobic parts of the polypeptide chain. Hence, moderate augmentation of water binding is encountered–between approximately 30% and 45% in most of the proteins during denaturation. Nevertheless, a reduction in water-binding capability can be seen due to an occasional supersession of protein-to-protein interactions over protein-to-water interactions, which is caused by the entanglement of unfolded polypeptide chains around each other in the case of aggregation and results from denaturation. Dissolution of bound water caused by roasting of meat can be demonstrated as a well-known example of this phenomenon (Coultate, 2002).

A change in flavor perception will occur when the formulation of a food product is changed since attributes of interactions might vary. It is considered that thermal denaturation causes the polypeptide chain to unfold resulting in the exposure of the remaining few polar groups, and, in turn, some extra water binding is revealed. Because of the hydrophilic nature of the peptide bond parts of the frame, constricted capability in water binding is observed on lately revealed hydrophobic parts of the polypeptide chain. Hence, moderate augmentation of water binding is encountered in most of the proteins during denaturation--between 30% and 45% (Coultate, 2002). There are studies for various systems, which investigate the effects of heat treatment on the flavor-protein interactions. Damadoran and Kinsella (1981) considered interactions as hydrophobic in nature, and resolved that the partial denaturation of soy protein resulted in an increment of 2-nonanone binding when compared to native soy protein. The researchers proposed that subunits of a denatured protein might have been exposed to reformation, which would lead to the improvement of hydrophobicity of current hydrophobic sites. Crowther

et al. (1980) stated the importance of H-bonding interactions. According to that study, unfolding of the protein causes a reduction in the binding of alcohols and ketones in denatured soy protein. As a result, more nonpolar regions of protein are revealed and the presence of polar H-bonding sites for alcohols and ketones decreases. According to Chung and Villota (1989), because of potential protein aggregation, the binding of alcohol to the protein is reduced by the thermal treatment of soy protein, which, in turn, causes the surface area of the protein to shrink and results in infrequent binding sites. Heng et al. (2004) analyzed interactions between flavor volatiles and pea proteins and how heat influenced these interactions. Heating of vicilin reduces the binding of aldehydes and ketones to the protein. The surface area of the protein shrinks as a result of heating, which is also a result of protein aggregation (Heng et al., 2004).

3.6 INVERSE GAS CHROMATOGRAPHY

In the past 50 years, Inverse Gas Chromatography (IGC) became a very effective technique to determine the physicochemical data for various nonvolatile materials (Voelkel, 2009). Gas chromatography is an analytical technique that is used to examine the unknown substances injected (mobile phase) into the chromatographic system that include well-known substances (stationary phase) packaged into the column and placed into the chromatographic oven, thus obtained results give information about the injected unknown substances. Inverse gas chromatography has the same chromatographic system as gas chromatography, basically including a mobile phase, a stationary phase packed into the column, an oven, and inert gas. The difference between GC and IGC is that while GC focuses on the mobile phase, IGC focuses on the stationary phase (Boutboul et al., 2002; Zhou and Cadwallader 2004; Al Ghamdi, 2002).

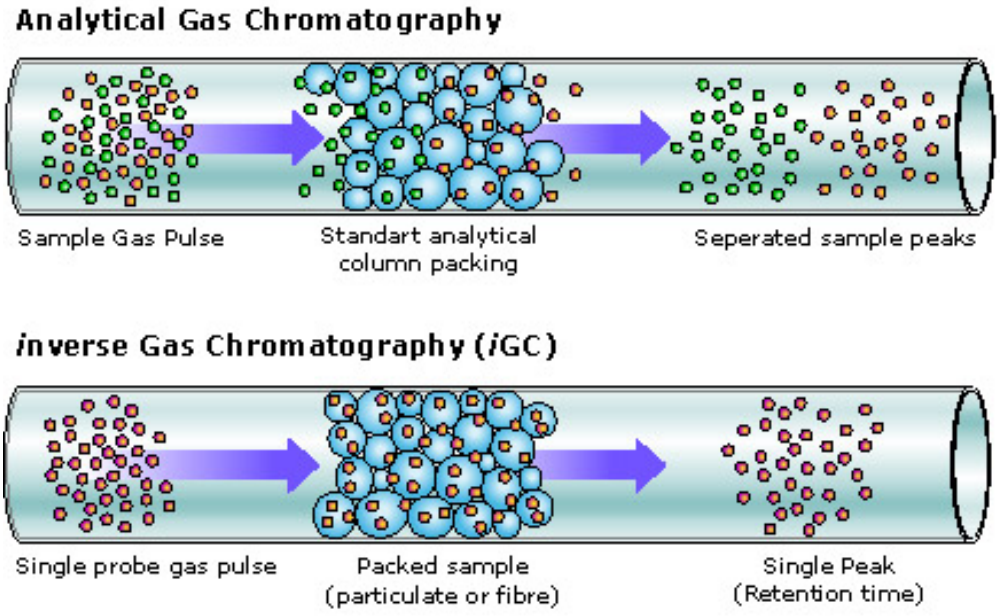


Figure 3-31: Difference between GC and IGC (adapted from www.porotec.de).

The stationary phase might be a solid or a liquid material, which is packaged into a column by either coating on an inert substrate (packed column) or on the column wall (capillary column). Inert gas helps the mobile phase to pass through the stationary phase and to reach the detector (Von Meien, 1997). The retention time and elution curve are obtained from the system and are used to determine the thermodynamic parameters (Von Meien, 1997) that are partition coefficient, solubility coefficients, and also enthalpies of adsorption, free energies of adsorption as well as kinetic parameters and physic-chemical properties, such as diffusion coefficients, surface energy, phase transitions, crystallinity, and specific area (Boutboul et al. 2002). Inverse gas chromatography has been used in a broad range of applications; for example, pharmaceuticals, natural and synthetic polymers, food products and ingredients, flavoring and perfume, and minerals Boutboul and et al., 2002).

IGC has a wide range of application in the food industry, which is used to measure water sorption in dry food products, or the sorption of aroma compounds on food packaging polymers, and to analyze the interaction between flavor compounds and food materials (Boutboul et al., 2002). IGC was used to determine the retention of flavor compounds on carbohydrate matrices (starch and maltodextrins) and it was concluded that it was a useful technique for obtaining the thermodynamic parameters (Delarue, 2000). Boutboul et al. used IGC to observe the effect of relative humidity on the aroma-starch interaction (Boutboul et al., 2000) and later to obtain thermodynamic properties and sorption isotherm for the interaction between flavors and high amylose cornstarch (Boutboul et al., 2002). Partition coefficients, the activity coefficient, and Henry's constants were calculated by injecting flavor compounds into the inverse gas chromatographic system to examine the physic-chemical characterization of two different chewing gum bases and their binding with the flavors (Niederer, 2003). Zhou et al. used the IGC technique for a series of studies: to determine the effect of relative humidity on interactions of flavor compounds with soy protein isolate (Zhou et al., 2004); to interpret the effect of the chemical structure of flavor compounds, the functional group, and relative humidity on the interactions between the flavor compound and SPI (Zhou et al., 2006); to describe the effect of the presence of soy protein in wheat soda crackers on interactions of flavors with the crackers (Zhou et al., 2006).

The great benefit of inverse gas chromatography makes it suitable for studying its use with dry and semi-dry food materials. Additionally, it is a simple, fast, and accurate technique when compared with traditional equilibrium methods due to the high

sensitivity of the detector, which is capable of measuring the very low level of flavor (Nano grams per gram) (Zhou et al., 2004).

4 MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 STATIONARY PHASE/ PROTEINS

4.1.1.1 Soy Protein Isolate

One of the stationary phases was soy protein isolate (PRO-FAM 974), which was obtained by Archer Daniels Midland Co., (ADM, Decatur, IL). The protein content of SPI was more than 90% and maximum fat content was 4%. 152-178μm (80/100 U,S. standard mesh) particle size of soy protein isolate was obtained by sieving. Soybean globulins (name of the proteins that are generated from soybeans) have four components after ultracentrifugation: 2S, 7S, 11S, and 15S and their percentages are 15, 34, 41.9, and 9.1 respectively. 7S and 11S are the two major components, and their amino acid compositions are shown in Table 4-1 (Fukushima, 1991).

Table 4-1: Amino acid composition of soybean globulins (Fukushima, 1991)

	Amino Acid Composition (a.a. % per 100g protein)					Amino Acid Composition (a.a. % per 100g protein)			
Amino Acids	**11S (glycinin)**	**7S (β-con glycinin)**	**7S (γ-con glycinin)**	**[a]ΔG_t (kJ/mol)**	**Amino Acids**	**11S (glycinin)**	**7S (β-con glycinin)**	**7S (γ-con glycinin)**	**[a]ΔG_t (kJ/mol)**
Tryptophan	1.5	0.3	0.7	14.21	Alanine	4.0	3.7	4.7	2.09
Lysine	5.7	7.0	6.8	--	Half-cystine	1.7	0.3	1.1	4.18
Histidine	2.6	1.7	2.8	2.09	Valine	4.9	5.1	6.4	6.27
Arginine	8.9	8.8	6.3	--	Methionine	1.3	0.3	1.4	5.43
Aspartic Acid	13.9	14.1	10.0	2.09	Isoleucine	4.9	6.4	4.4	12.54
Threonine	4.1	2.8	4.2	1.67	Leucine	8.1	10.3	7.6	9.61
Serine	6.5	6.8	6.5	-1.25	Tyrocine	4.5	3.6	2.1	9.61
Glutamic Acid	25.1	20.5	17.5	2.09	Phenyl alanine	5.5	7.4	5.5	10.45
Proline	6.9	4.3	5.9	10.87	Amide ammonia	1.6	1.7	--	
Glycine	5.0	2.9	6.1	0	Sugar	0.8	4.9	2.9	

[a] Reference (Damodaran, 1996)

4.1.1.2 Zein

The other stationary phase, zein, (F4000) was purchased from Freeman Industries, Tuckahoe, NY. The protein content of zein was 88-96%. Fat content was reported as between 1.18-5.26% (Wang et al., 2003; Sessa and Palmquist, 2009). 180-208μm (65/80

U.S. standard mesh) particle size of zein was obtained by sieving. Table 4-2 shows amino acid compositions of zein fractions.

Table 4-2: Amino acid composition of zein fractions (Larkins et al., 1993)

Number of amino acid residues in zein fractions						Number of amino acid residues in zein fractions					
Amino Acids	g-zein	a-zein	b-zein	d-zein	[a]ΔG_t (kJ/mol)	Amino Acids	g-zein	a-zein	b-zein	d-zein	[a]ΔG_t (kJ/mol)
Leu	19	42	15	15	9.61	Gly	13	2	12	4	0.00
Gln	30	31	28	15	-0.42	Thr	9	7	5	5	1.67
Ala	10	34	18	7	2.09	Arg	5	4	7	0	0.00
Pro	51	22	13	20	10.87	His	16	3	4	3	2.09
Ser	4	18	11	8	-1.25	Cys	14	1	6	5	4.18
Phe	2	8	0	5	10.45	Glu	2	1	5	0	2.09
Asn	0	13	2	3	0.00	Met	2	5	11	29	5.43
Ile	4	11	1	3	12.54	Asp	0	0	4	1	2.09
Tyr	4	6	16	1	9.61	Lys	0	0	0	0	0.00
Val	15	17	4	5	6.27	Trp	0	0	1	0	14.21

[a] Reference (Damodaran, 1996)

Meager solubility in water (polar solvents) of nonpolar molecules, which are easily soluble in nonpolar solvents, is called hydrophobicity (Koehl and Delarue 1994; Rose et al., 1985). The hydrophobicity is described in terms of free energy of transfer ($\Delta G_{transfer}$) of 1 mol of solute between an aqueous phase (water) to an organic phase (ethanol) (Lesser and Rose, 1990; Damodaran, 1996). Hydrophilic residues have negative values of free energy of transfer, while hydrophobic residues have positive values of free energy of transfer. Zein is considered more hydrophobic than soy protein because of its higher content of hydrophobic amino acid.

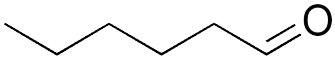
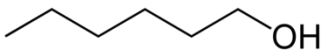
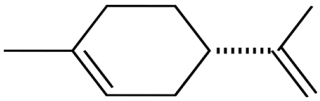
4.1.1.3 Application of Heat Treatment: Cooked Proteins

Soy protein isolate and zein were cooked at 375°F for 30 minutes in a conventional oven. The temperatures of the cooked proteins were 120-125°C and 110-115°C for soy protein and cooked zein, respectively.

4.1.1.4 Volatile Compounds

Hexane, hexanal, hexanol, and d-limonene (Sigma-Aldrich, MO) were the flavor compounds selected based on their molecular structure to show the effect on flavor-protein interaction.

Table 4-3: Properties of Flavor Compounds

Flavor Compound	Structure	Molar Mass	logP	Purity
Hexane		**86.18**	**3.90-4.11**	**95%**
Hexanal		**100.15**	**1.78**	**98%**
Hexanol		**102.17**	**1.82**	**98%**
D-limonene		**136.24**	**4.83**	**98%**

4.2 METHODS

4.2.1 COLUMN PREPARATION

After equilibration of all the samples to 0.00 aw over P_2O_5, the glass column was filled with stationary phase that 1.00g of raw soy protein; 0.8g of raw zein; 1.00g cooked soy protein; 0.86g cooked zein, individually. A glass column (17.8 cm × 4 mm ID; Supelco, Bellefonte, PA) was used to pack the stationary protein matrix by tapping onto a hard

surface and a mechanical vibrator was used to pack the column. The packaging process was ended when the column totally filled with powder and appeared to be entirely smooth. Glass wool and stainless steel screens were used to plug both ends of the column. Each column was placed into the IGC system (Hewlett Packard 5890, Series II Gas Chromatograph; Agilent Technologies, Wilmington, DE) and conditioned for 24 hours to remove foreign volatiles and to reach the targeted RH%. The chromatographic system was operated isothermally at 38, 44, and 50°C in order to interpret the effects of temperature on the measured thermodynamic quantities and interaction between flavor compounds and proteins. Whenever the temperature was increased, the column needed six hours to reach equilibrium conditions.

4.2.2 INVERSE GAS CHROMATOGRAPHY

Inverse Gas Chromatography (IGC) is a dynamic method based on similar working principles of gas-solid chromatography, but instead of it being used as a separation process, it was employed to study the surface energies of solid samples and to characterize the thermodynamic interactions between selected volatile compounds and a food matrix (Zhou et al., 2004; Boutboul et al., 2002; Newell et al., 2001; Boutboul et al., 2000; Ostroff et al., 1988; Rebar et al., 1984). The schematic diagram of the experimental set-up is illustrated in Figure 4-1.

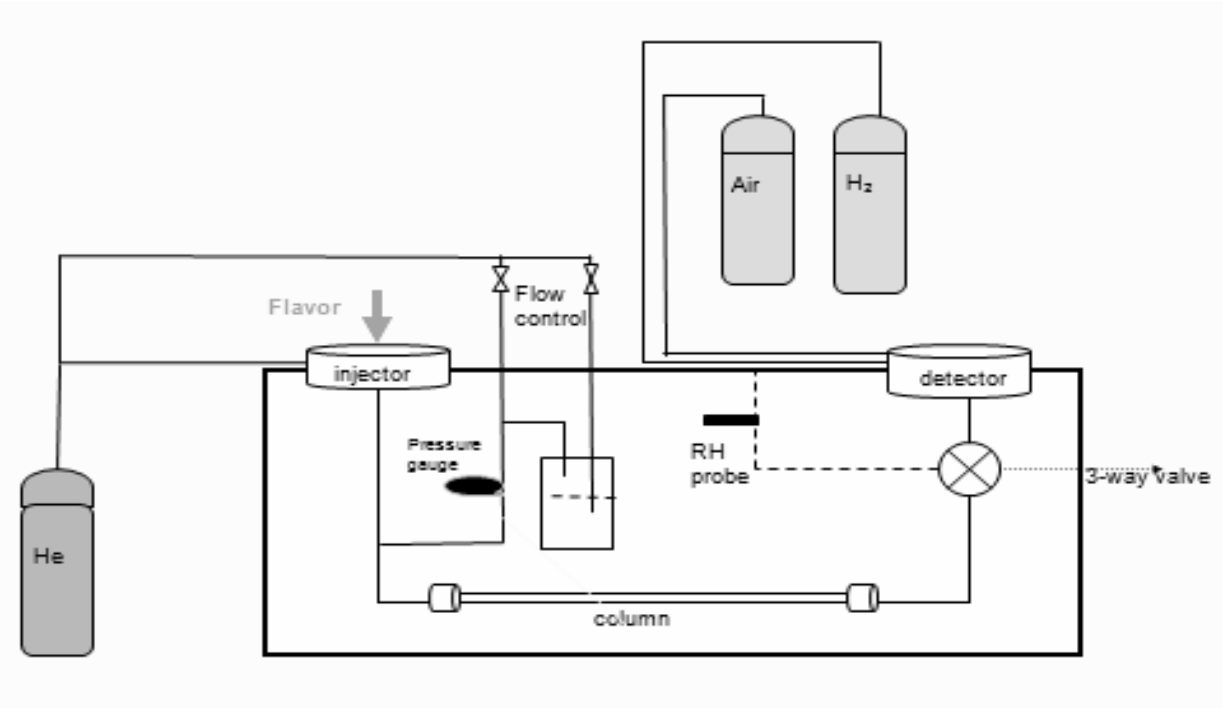


Figure 4-1: Experimental set-up of the Inverse Gas Chromatography

A conventional type of gas chromatography (Hewlett Packard 5890, Series II Gas Chromatograph; Agilent Technologies, Wilmington, DE) was converted to an Inverse Gas Chromatography (Figure 4.2). The glass column (17.8cm x 4mm I.D.; Supelco, Bellefonte,PA) was packed with proteins and placed into the oven. Deactivated stainless steel tubing (0.32 mm I.D.; Valco Instruments Co., Inc; Houston, TX) was used to connect the glass column to the injector and detector. The gas chromatography had a Flame Ionization Detector (FID) with a temperature set at 250°C, and the injector temperature was set at 250°C. The carrier gas was dry helium gas, and its flow rate was adjusted to 20 mL/min. In order to interpret the influence of water on flavor-protein interactions, a humidifier was set up as part of the chromatographic system. Carrier gas was divided into two streams: dry and humidified (dry helium was bubbled in a bottle filled with distilled water). The percent relative humidity was adjusted by mixing dry and

humidified carrier gas. The flow direction of the carrier gas was oriented either to the detector or to the relative humidity/temperature probe by installing a three-way valve into the system.

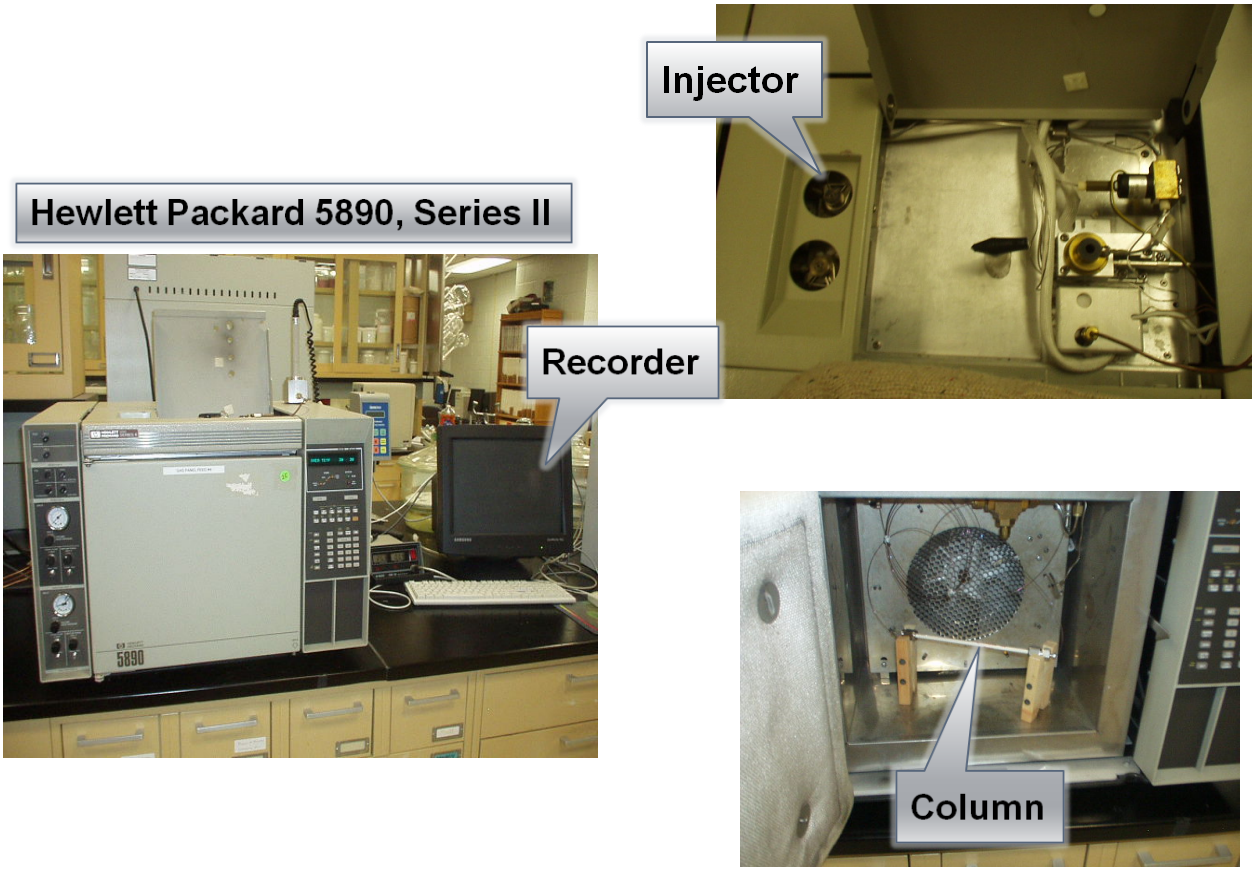


Figure 4-2: Experimental set-up of the Inverse Gas Chromatography

The variation between retention times for replicate injections was less than 7%. The thermodynamic parameters were calculated after averaging triplicate retention times of each flavor compound on different matrix materials.

4.2.3 Determination of Thermodynamics of Interactions

Splitless injections of 0.005 µl of the volatile compounds were performed by using a 0.5µl syringe (Supelco, Belefonte, PA) in order to calculate the thermodynamics of adsorption. After obtaining the retention time from the inverse gas chromatographic

system, the net retention volume can be calculated. The volume of carrier gas that is enough to wash the probe away from the stationary phase under temperature and pressure of column is called net retention volume (V_N, mL) and defined as the following equation (Zhou et al., 2004; Rebar et al., 1984; Ostroff et al., 1988; Boutboul et al., 2002a).

$$V_N = \frac{3}{2}\cdot\left(\frac{\left(P_i/P_o\right)^2-1}{\left(P_i/P_o\right)^3-1}\right)\cdot\frac{T_{ov}}{T_{fl}}\cdot\frac{P_{fl}}{P_o}.F.\left(t_R-t_A\right) \qquad \text{Equation 1}$$

Factor because of pressure gradient in the column

Corrected flow

In this equations; t_R (min): retention time of probe; t_A (min): retention time of unretained probe (butane); P_o (psia): column outlet pressure; P_i (psia): column inlet pressure; P_{fl} (psia): flow meter pressure; T_{ov} (K): oven temperature; T_{fl} (K): flow meter temperature; F (mL/min): carrier gas flow rate.

The specific retention volume (V_g, mL/g) is then defined as (Zhou et al., 2004; Rebar et al., 1984; Ostroff et al., 1988; Boutboul et al., 2002a);

$$V_g = \frac{V_N}{m_S}\cdot\frac{273.15}{T_{ov}} \qquad \text{Equation 2}$$

In this equation, m_S (g): weight of stationary phase. K_P, which is the partition coefficient of the volatile probe between the stationary phase and the mobile phase, is defined as (Zhou et al., 2004; Rebar et al., 1984; Ostroff et al., 1988; Boutboul et al., 2002a);

$$K_P = \frac{V_g \cdot \frac{m_S}{V_S} . T_{ov}}{273.15}$$ Equation 3

where V_S (mL): volume of the stationary phase.

By using the following equation, the free energy of adsorption (ΔG_S) of each flavor compound on different protein samples can be calculated at three different temperatures (Zhou et al., 2004; Rebar et al., 1984; Ostroff et al., 1988; Boutboul et al., 2002a);

$$\Delta G_S = -R.T_{ov} . \ln K_P$$ Equation 4

where R: universal gas constant.

To calculate the enthalpy of adsorption (ΔH_S) of each flavor compound on protein, the specific retention volume is plotted against ln (1/T), which yields ($-\Delta H_S/R$). Enthalpy of adsorption is defined as (Zhou et al., 2004; Rebar et al., 1984; Ostroff et al., 1988; Boutboul et al., 2002a);

$$-\frac{\Delta H_S}{R} = \frac{d \ln V_g}{d(1/T_{ov})}$$ Equation 5

5 RESULTS AND DISCUSSION

5.1 EVALUATIONS AND VALIDATION OF SYSTEM PERFORMANCE

Reproducibility of the replications for the same column and different columns is very good. Overall, variability between two replications for the same columns was less than 4% and for different columns was less than 7%. Figure 5-1 shows as an example the retention times obtained by injection of d-limonene on a raw soy protein isolate at 38°, 44° and 50°C under 20% RH. Figure 5-2 shows the retention times obtained by injections of hexanol on cooked zein at 38°, 44° and 50°C under 20% RH. All of the raw data are shown in Appendix A-1.

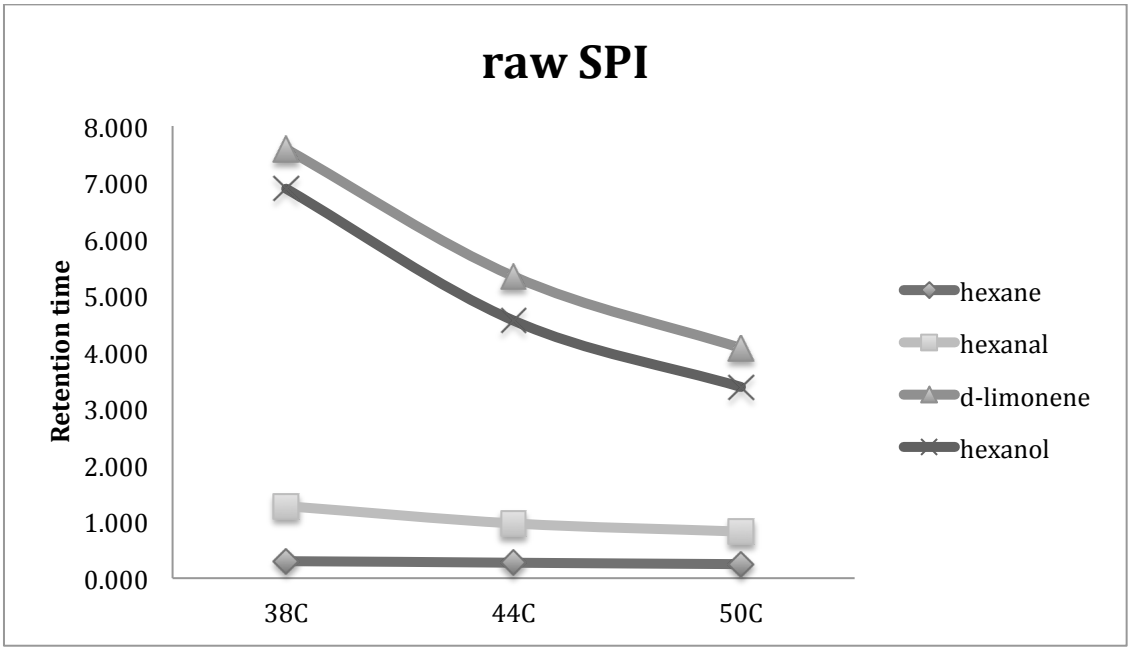


Figure 5-1: Retention times for d-limonene on raw soy protein isolate at 38°, 44°, and 50°C.

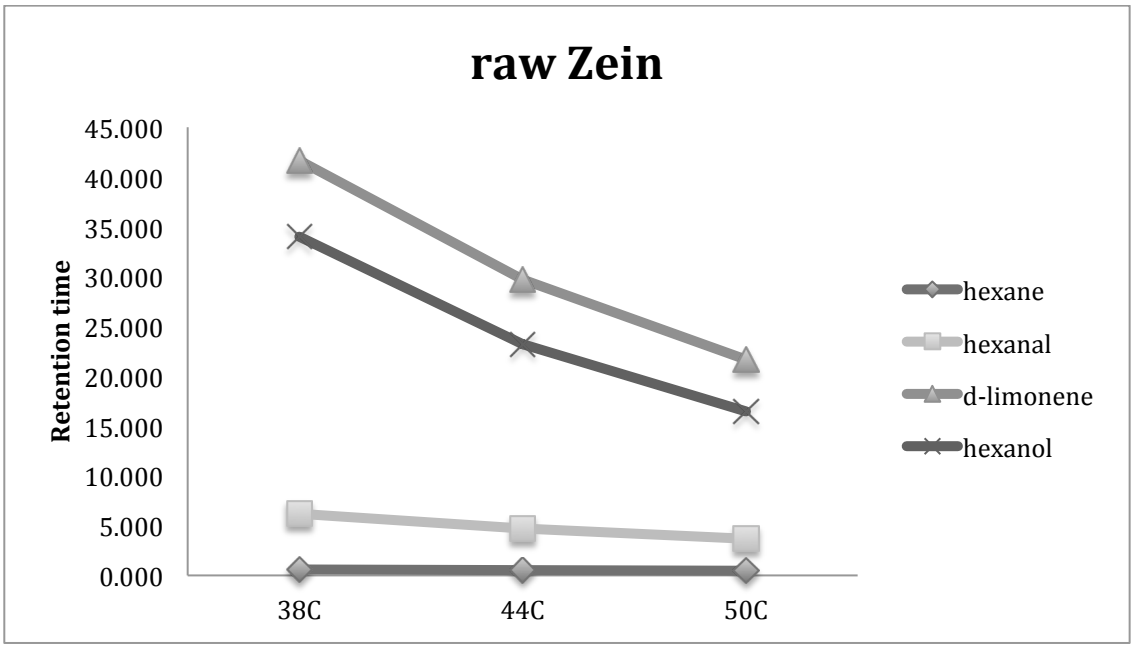


Figure 5-2: Retention times for hexanol on cooked zein at 38°, 44°, and 50°C.

5.2 EFFECT OF PROTEIN STRUCTURE AND NATURE ON BINDING

Soy protein is a mixture of hydrophilic and hydrophobic amino acids. Zein is a relatively, more hydrophobic protein than soy protein. The average hydrophobicity was determined by using amino acid compositions of soy protein isolate and zein: 4.1 kJ/mol and 4.9 kJ/mol, respectively. Specific retention volumes (Vg, mL/g) of each flavor compound were calculated at different temperatures (38°, 44°, and 50°C) for soy protein isolate and zein. Specific retention volumes (V_g, mL/g) of four flavor compounds (hexane, hexanal, d-limonene, and hexanol) on raw soy protein isolate and raw zein at 38°C are shown in Figure 5-3. The mean squared error for these data is shown as an error bar and the raw data leading to the calculation of the error are shown in Appendix A-1 as well. In addition to insure that the changes in magnitude are significant we have also added ANOVA results in Appendix A-1

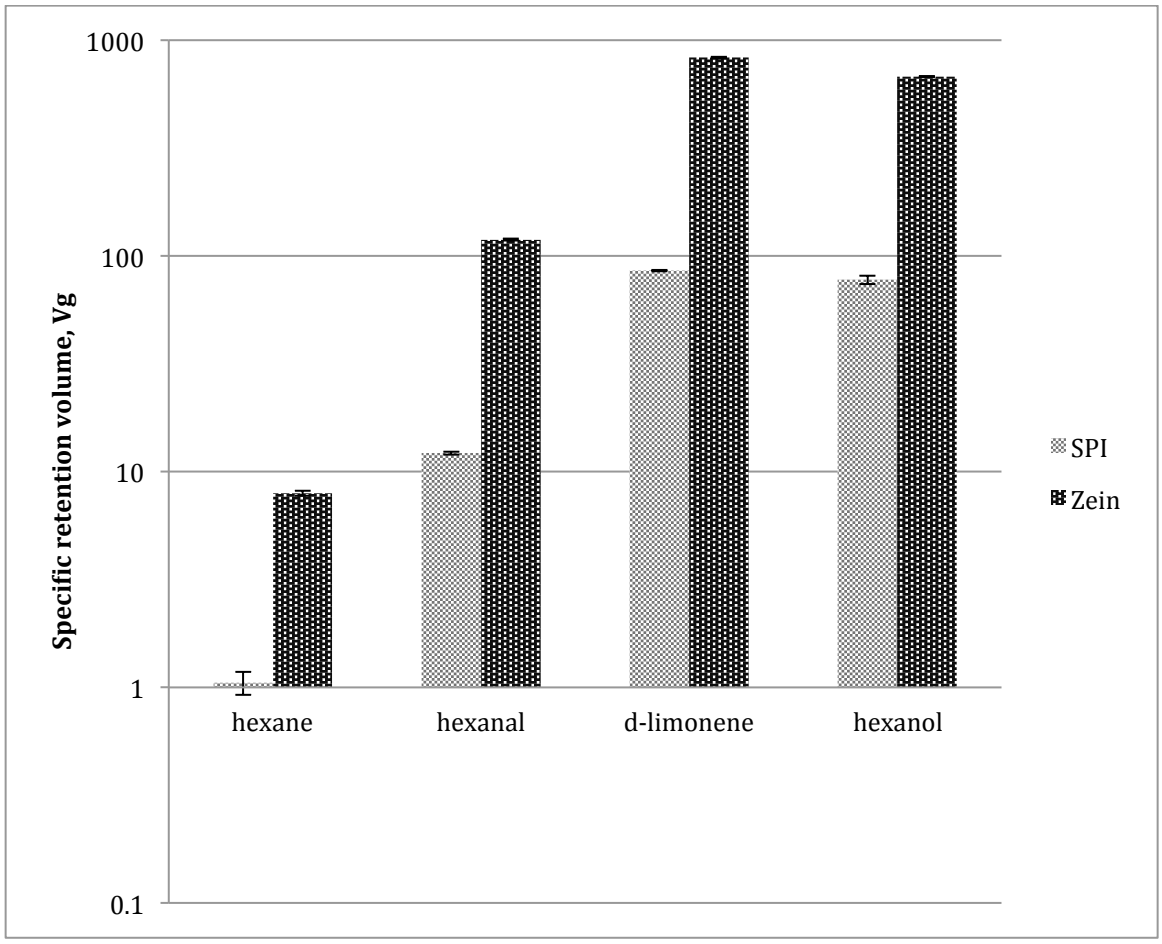


Figure 5-3: Specific retention volume of flavors on raw soy protein and raw zein at 38° C.

Specific retention volume (Vg) is defined as the net retention volume corrected to standard temperature and pressure and it is a measure of how much the absorbed molecule is retained on the solid matrix. Clearly, the specific retention volume of each injected flavor compound on zein is much higher than that of soy protein isolate. This outcome suggest that the relative hydrophobicity among other factors might be quite important in terms of affecting the retention/adsorption of these flavors. This result suggests that food formulated with different proteins will have considerably different flavor profiles when different proteins are used.

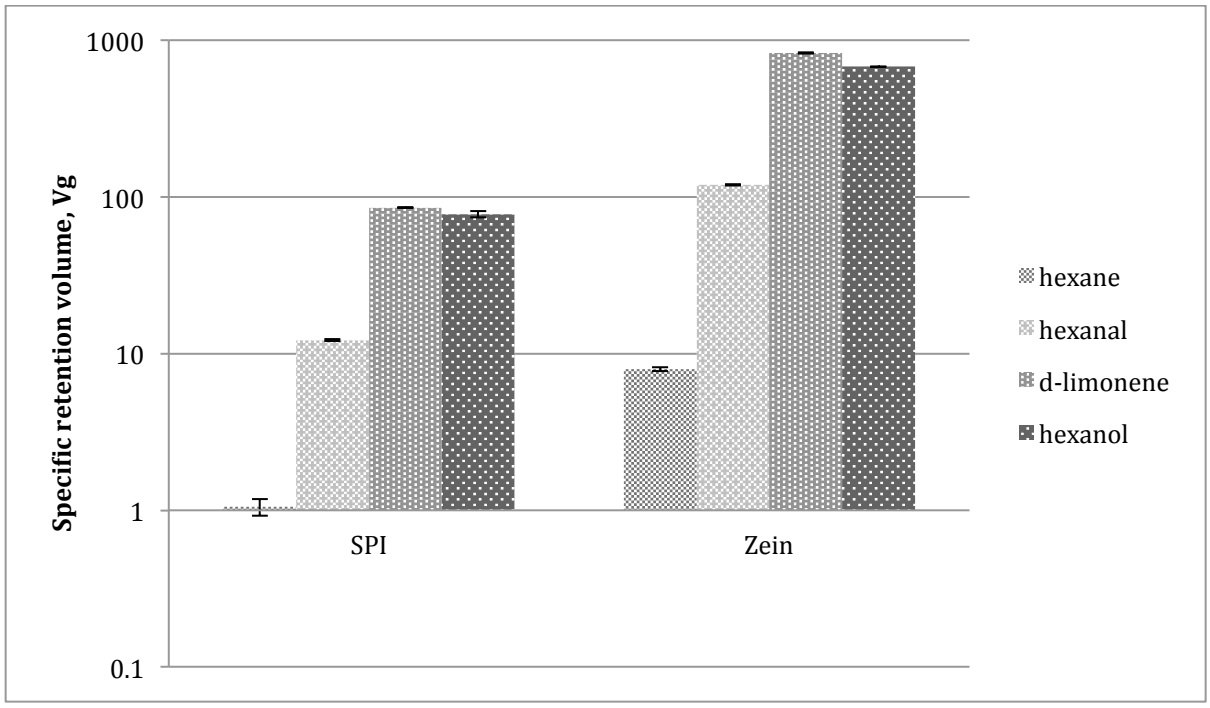


Figure 5-4: Specific retention volume of flavors on raw soy protein and zein at 38°C.

The higher retention volumes on the more hydrophobic but not exclusively hydrophobic protein zein also pointed out that the hydrophobic groups were more effective in binding the flavor compounds to the surface of proteins. However, hexane (alkane), one of the most hydrophobic flavor compounds, is not retained well on both protein matrixes. This appears to be because there needs to be an adequate hydrophobic hydrophilic balance in the flavor molecule to be retained well. On the other hand, d-limonene is while being a highly hydrophobic flavor compound, also has hydrophilic segments, so hydrophobic interactions of limonene would form with the hydrophobic regions of zein and the hydrophobic regions in the soy protein isolate coupled with softer significant hydrophilic interactions with the hydrophilic amiono acids of both proteins. It can be noted that the specific retention volumes of soy protein isolate and zein are ranked in the same order (Figure 5-4); d-limonene > hexanol > hexanal > hexane. Highly hydrophobic d-limonene

had higher retention volumes than hexanol and hexanal (both have hydrophobic and hydrophilic groups) or hexane (highly hydrophobic flavor).

In inverse gas chromatography, while injected flavor compounds were passing through the protein matrix, separation takes place based on the differential partitioning between the mobile phase and the protein matrix. The partition coefficient (Kp), which is calculated by using Equation 3, is the equilibrium constant that demonstrates the equilibrium between adsorption and desorption. Figure 5-5 shows the partition coefficient of four different flavor compounds on soy protein isolate and zein at 38°C. Increasing Kp values indicate that there is more adsorption of flavor compounds onto protein matrix. Partition coefficient of flavor compounds while binding to zein was notably higher than binding to soy protein (Figure 5-5), validating the results obtained for specific retention volumes (Figure 5-3).

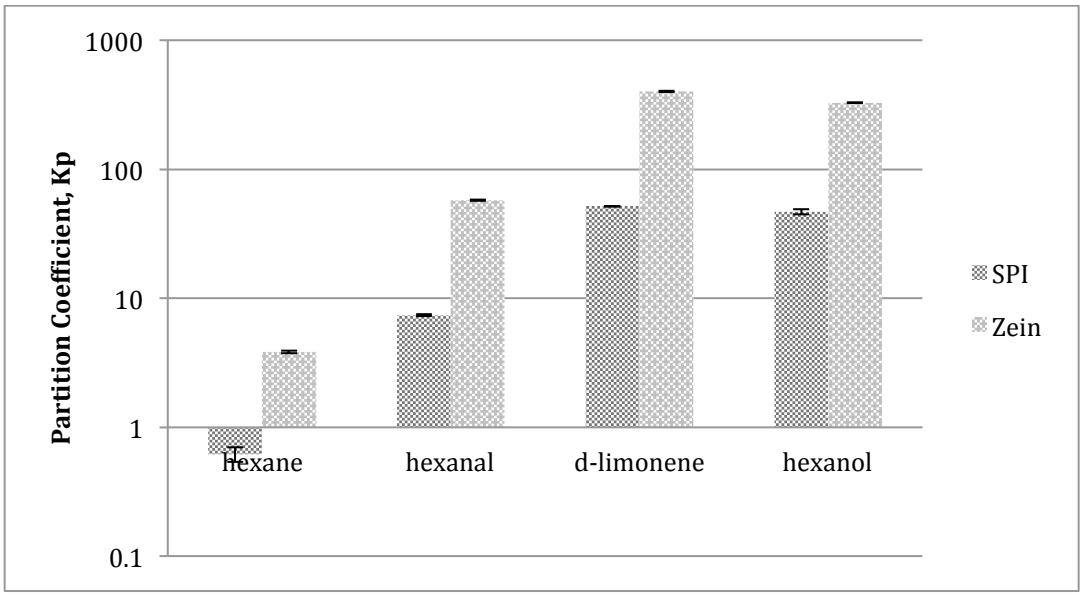


Figure 5-5: Partition coefficient of flavor compounds on soy protein isolate and zein.

5.3 EFFECT OF TEMPERATURE ON BINDING

Temperature is an important environmental parameter that affects adsorption. To analyze the influence of temperature on adsorption, thermodynamic properties are determined at three different temperatures. Figure 5-6 and Figure 5-7 show Gibbs free energies of adsorption (ΔG_S, kJ/mol) of each flavor compound on soy protein isolate and zein at different temperatures (38°, 44°, and 50° C). ΔG_S relates to the spontaneity of interactions that take place between the flavor compounds and the food matrix (Cadwallader, 2004). The positive values of Gibbs free energy indicate that adsorption is thermodynamically unfavorable and the negative values of Gibbs free energy indicate that adsorption is thermodynamically favorable.

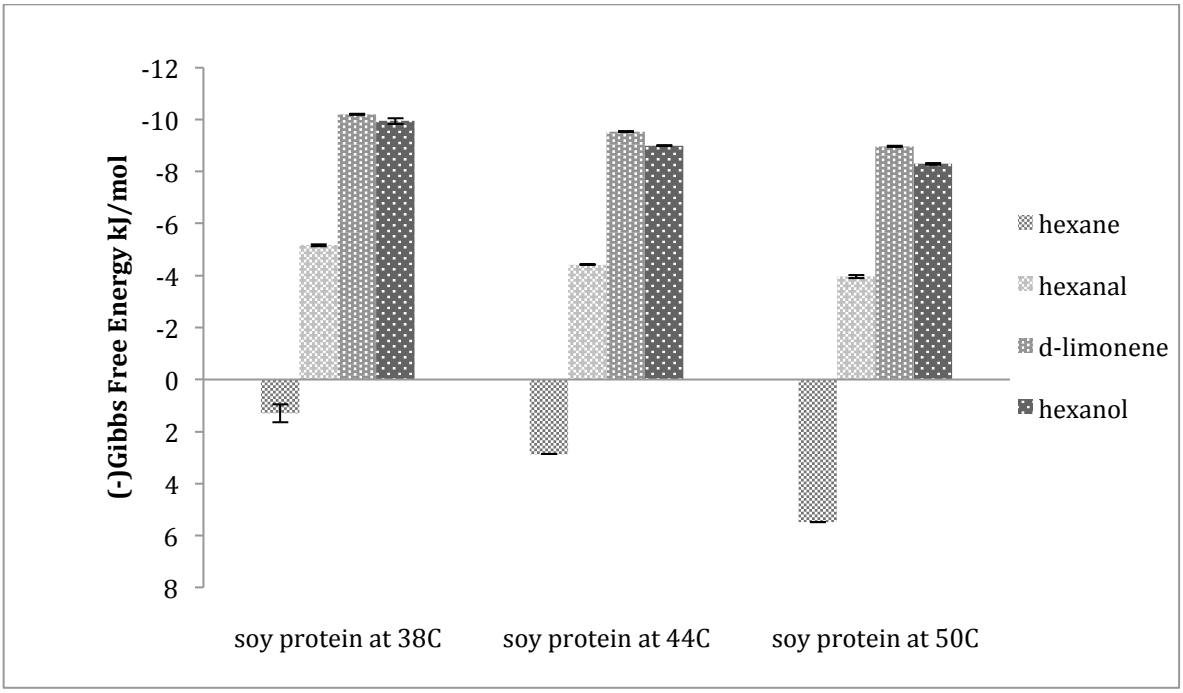


Figure 5-6: Free energy of adsorption of four different flavors on soy protein isolate at three different temperatures.

According to Figure 5-6, ΔG_S values were negative for hexanal, hexanol and d-limonene on soy protein isolate that points to a favorable adsorption, but ΔG_S values were negative for hexane that points to an unfavorable adsorption. By comparing the (-)ΔG_S values calculated for each flavor compound at different temperatures, it was found that an increase in temperature causes a decrease in the magnitude of the (-)ΔG_S values, suggesting that adsorption is more favorable at lower temperatures and conversely less favorable at high temperatures.

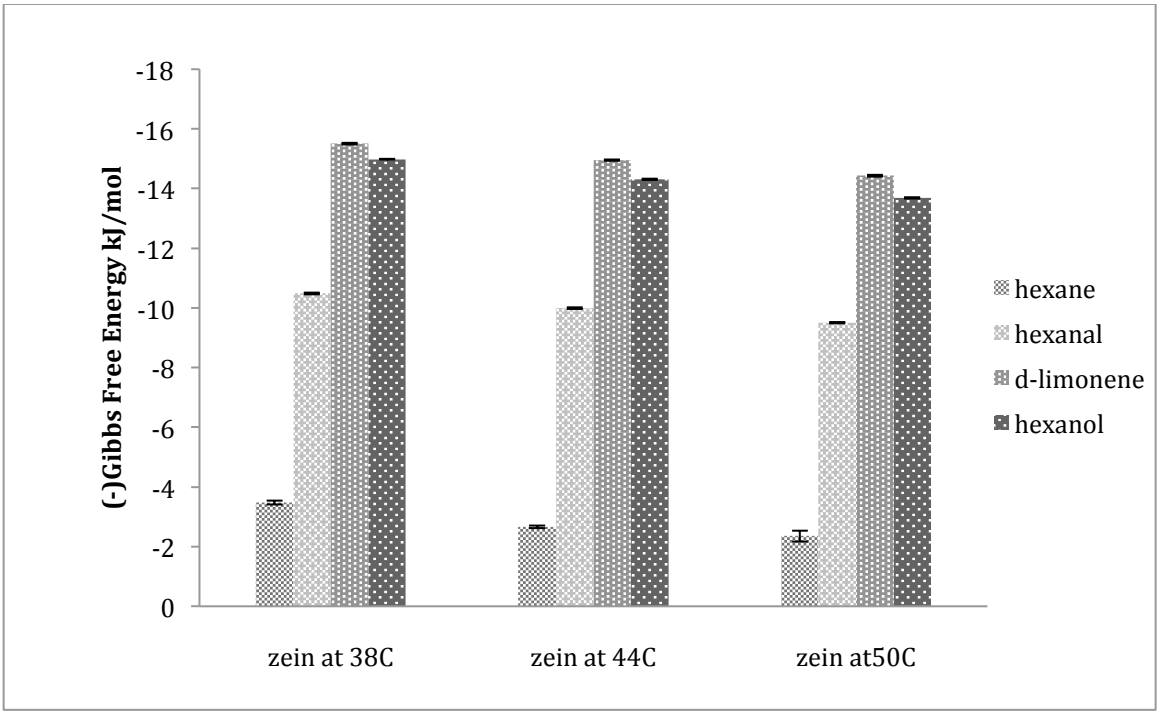


Figure 5-7: Free energy of adsorption of four different flavors on zein at three different temperatures.

As shown in Figure 5-7, in the case of zein, all ΔG_S values were negative for each flavor compound, suggesting spontaneous adsorption for all compounds. The same trend was

observed for zein since (-)ΔG_S values decreased with the increasing temperature, suggesting that adsorption is more favorable at lower temperatures.

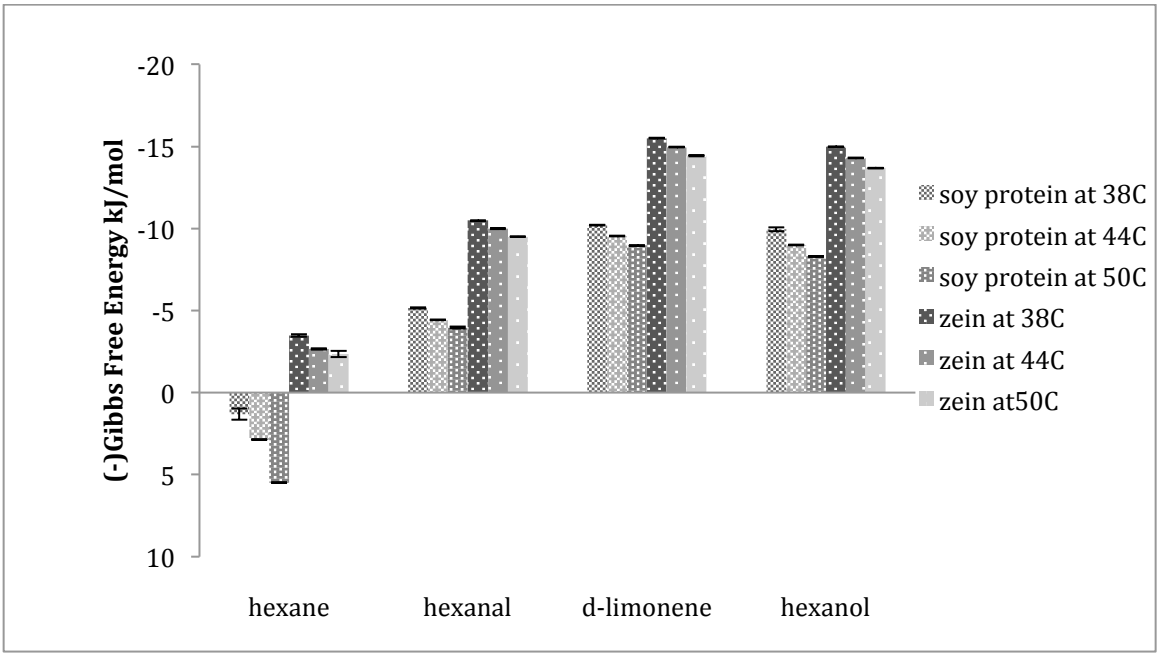


Figure 5-8: Free energy of adsorption of four different flavors on soy protein isolate and zein at three different temperatures.

The Gibbs free energies of adsorption for each flavor compound on both soy protein and zein at different temperatures were plotted together in Figure 5-8. As shown in the graph, all four flavors had notably different retention/release profiles on each protein where consistent with the discussion above, zein had higher (-)ΔG_S than soy protein at all temperatures studied. This suggests a higher affinity of all flavors towards being adsorbed on the surface of zein. A similar pattern was observed in terms of the strength of the binding with the volatile molecules to both zein and soy protein isolates and the strength of binding followed the following order for both d-limonene > hexanol > hexanal > hexane (Figure 5-9). In all cases we observe that flavor molecules that have a

strong hydrophobic component with some hydrophilic character showed the highest affinity to both zein and soy protein surfaces.

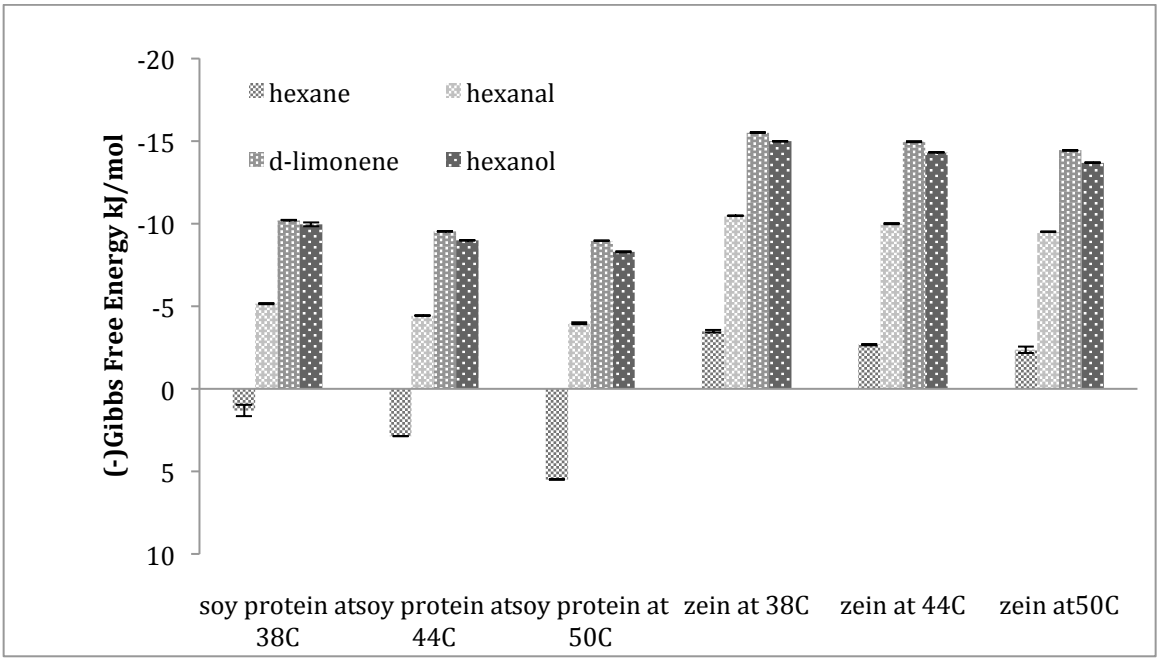


Figure 5-9: Free energy of adsorption of four different flavors on soy protein isolate and zein at three different temperatures.

The same data was plotted in a different way, this time as Gibbs free energy on each protein matrix versus temperature for different flavor compounds (Figure 5-9). Figure 5-9 shows that d-limonene had the highest negative Gibbs free energies, indicating better adsorption on both soy protein and zein at each temperature. Hexanol also had high values of negative Gibbs free energy, indicating that a thermodynamically favorable adsorption occurs between the protein matrix and hexanol. Only hexane Gibb's free energy of adsorption on soy protein has positive values, indicating there was little or no binding between hexane and soy protein. When the oven temperature was 38°C, which is the lowest temperature in this study, higher (-) ΔG_S values were obtained as evidence that more spontaneous adsorption occurs at lower temperatures.

5.4 EFFECT OF CHEMICAL STRUCTURE OF FLAVOR COMPOUND ON BINDING

Results showed that the chemical classes of flavor compounds determined the binding strength of selected flavors (d-limonene, hexanol, hexanal, and hexane) to proteins (soy protein and zein). As was discussed above, the binding strength of volatile probes to both zein and soy protein isolates that were observed in the following order for both proteins: d-limonene & hexanol > hexanal > hexane. The differences between the binding strength of each flavor compound can be explained by different functional groups on flavor compounds, depending which chemical families they belong to.

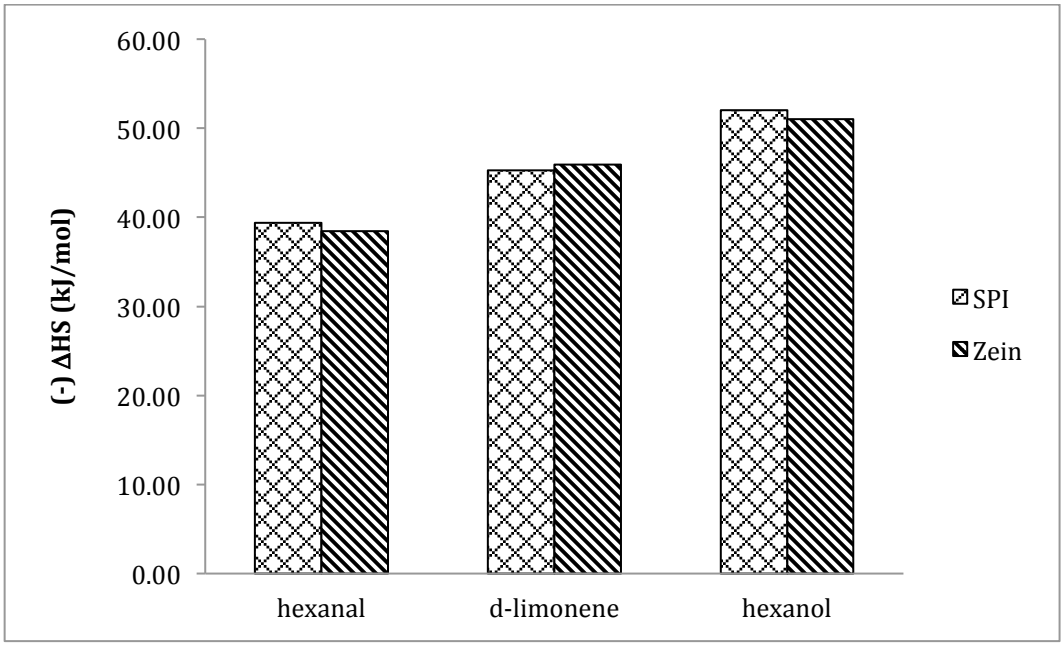


Figure 5-10: Enthalpy of adsorption of volatile probes on soy protein and zein.

Figure 5-10 shows the enthalpies of adsorption (ΔH_S, kJ/mol) of hexanal, hexanol, and d-limonene on soy protein and zein. Hexane was not included in the figure because of its very low retention time and unfavorable interaction. The unfavorable adsorption of hexane, the only flavor compound that had Gibbs free energy values very close to zero or were positive, indicates its weak binding ability. This was most likely caused by very

weak nonspecific interactions known as van der Waals dispersion forces. The favorable adsorption of both hexanal and hexanol suggested the possibility of the existence of specific interactions (hydrogen bonding, dipole forces) as well as nonspecific interactions. The strength of binding between hexanol and proteins pointed to either the formation of a high-energy hydrogen bond or the formation of two hydrogen bonds. Hexanol contains a hydroxyl group that has the capability of forming two hydrogen bonds because hydrogen of a hydroxyl group may act as both electron acceptor and electron donor. Hydrogen of a hydroxyl group on hexanol might be bonded to negatively charged functional groups of proteins and form its first hydrogen bond. The second hydrogen bond might be formed by the binding between the oxygen of a hydroxyl group on hexanol and positively charged functional groups of protein. In the case of hexanal, the functional group is a carbonyl group, which contains negatively charged oxygen atoms that might form a hydrogen bond as a result of interacting with positively charged groups of proteins.

Table 5-1: Comparison of heat of adsorption, ΔH_S, of flavor compounds at dry conditions for both soy protein isolate and zein.

ΔH_S	Hexanal	d-limonene	Hexanol
SPI	39.17kJ/mol	44.85 kJ/mol	55.45 kJ/mol
Zein	39.07 kJ/mol	45.57 kJ/mol	49.87 kJ/mol

The energy needed to form a hydrogen bond is between 12-40 kJ/mol (Boutboul, 2002; McMullin, 1975). The data shows that enthalpy of adsorption is higher for hexanol (55.45 kJ/mol for SPI, 49.87 kJ/mol for zein), which implies either the formation of a high-

energy hydrogen bond or the formation of two hydrogen bonds. When enthalpy of adsorption of flavor compounds was compared, it was found that the ΔH_S values of hexanal, and hexanol on zein ($\Delta H_{S\ hexanal}$=-39.07 kJ/mol, $\Delta H_{S\ hexanol}$=-49.87 kJ/mol) was close to those on soy protein ($\Delta H_{S\ hexanal}$=-39.17 kJ/mol, $\Delta H_{S\ hexanol}$=-55.45 kJ/mol) (Table 5-1), indicates the stronger possibility of hydrogen bonding for both soy protein and zein. There is no distinctive difference between the enthalpy of adsorption of hexanal, limonene, and hexanol for both proteins.

5.5 Effect of Heat Treatment on Binding

Thermal processes are some of the most common treatments in the production of processed foods. The conformational state of proteins shapes the flavor binding, so it means that the factors that affect the protein conformation also affect the flavor binding. Temperature is one of the factors that changes the protein conformation (Kuhn, 2006). Determining flavor retention after heat treatment may help to understand if the flavor retention on the individual proteins is affected by the changes in the conformation of proteins caused by heat treatment, characteristics of processed food products. In this part of the study, soy protein isolate and zein were baked at 375 degrees F for 30 minutes. The result showed that there is a decrease in the value of the thermodynamic binding energy of the interaction between the flavor compounds and proteins after heat treatment.

The binding of the four flavor compounds to cooked soy protein isolate and cooked zein is shown in terms of specific retention volumes (Vg, mL/g), and free energies of adsorption (ΔGS, kJ/mol) in Figure 5-11

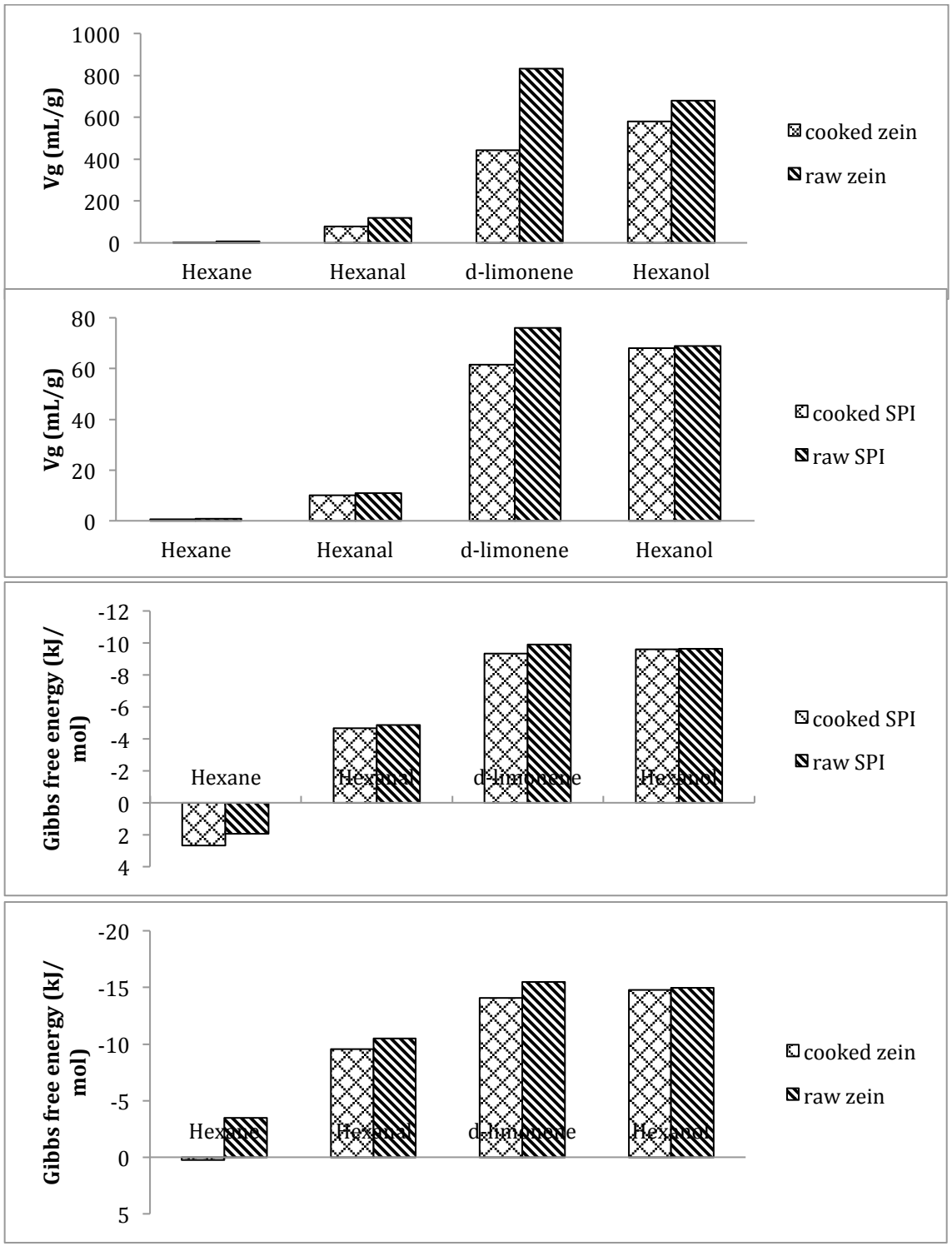


Figure 5-11: Comparison of Vg (a,b) and Gibbs free energy (c,d) of adsorption of volatile probes on cooked and raw proteins.

A similar pattern was observed with raw soy protein isolate and raw zein in terms of strength of the binding with the flavor compounds to both cooked zein and cooked soy protein isolates that were observed in the following order for both d-limonene&hexanol> hexanal> hexane. Higher values of Gibbs free energies for d-limonene and hexanol point out more favorable adsorption of these flavor compounds than hexane and hexanal on both protein matrix. When the results are compared, it is clearly seen that there is a decrease in binding of all flavor compounds after heat treatment. Lower Gibbs free energies show that adsorption is less favorable and less spontaneous after heat treatment. Cooking procedure in current study cause denaturation of both Soy protein (above 73.5 for 7S and 88.9 C for 11S), and zein (above 100C)(Hua, 2005; Wu, 1997). So cooking process in current study cause denaturation and aggregation, which decrease the surface area and cause less favorable binding in result.

5.6 EFFECT OF WATER ON BINDING

Retention of flavor compounds on two different proteins (soy protein isolate and zein) was studied under dry and humid conditions. The inverse gas chromatographic system was developed by adapting a regular gas chromatograph into a packed column configuration with a humidification system for the carrier gas in order to determine the effect of water activity on the measured quantities. Retention times and thermodynamic properties of flavor adsorption on different matrices were measured under different conditions (temperature and relative humidity). Dry conditions corresponded to 0% RH level and humid conditions corresponded to 20% RH level. For two different RH levels (0% and 20% RH), the binding affinity of the four volatile probes to soy protein isolate and zein showed similar trends.

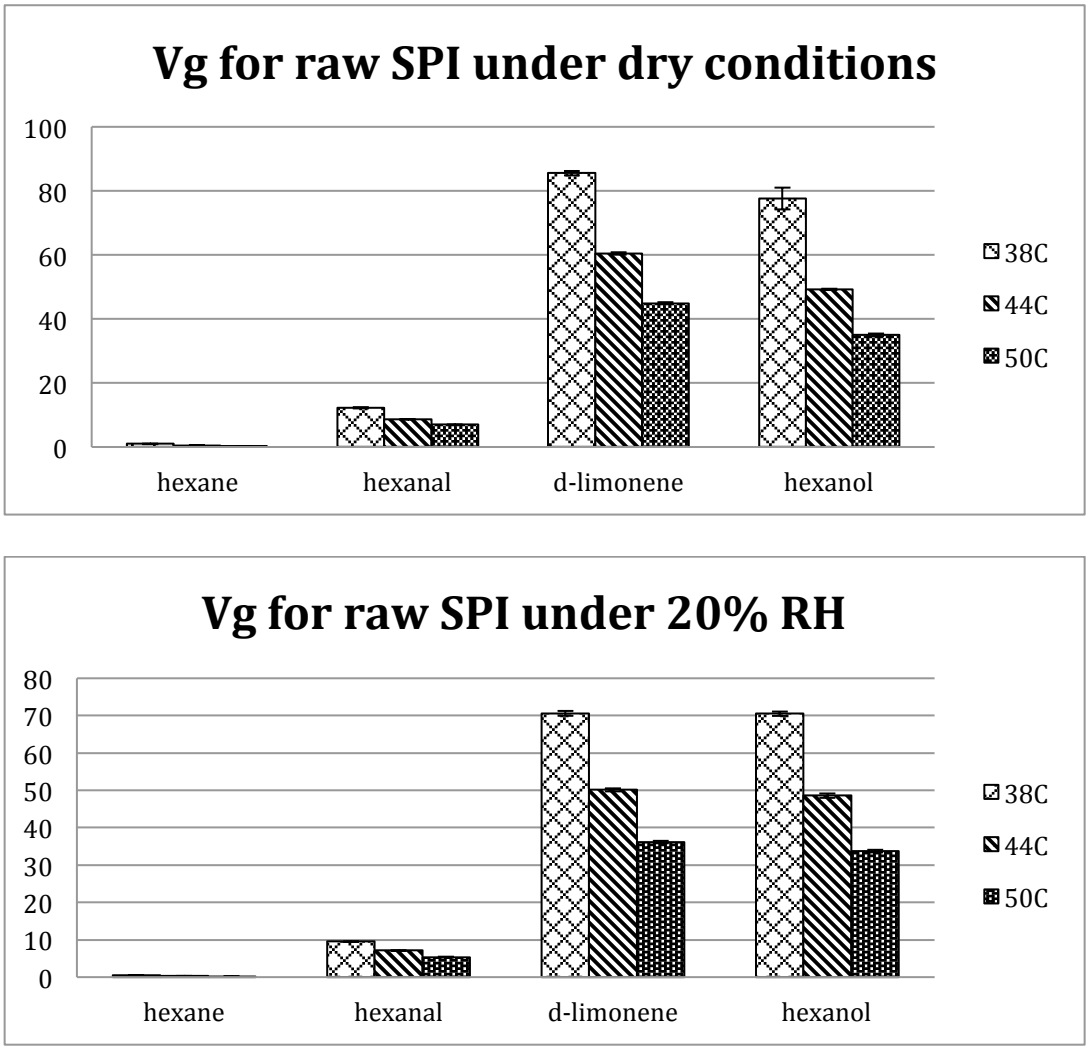


Figure 5-12: Comparison of Vg for raw SPI under (a) dry conditions and (b) 20% RH.

Specific retention volumes of adsorption for each flavor compound on raw soy protein isolate both for 0% (a) and 20% RH (b) at different temperatures were plotted in Figure 5-12. As shown in the graphs, retention volumes of flavor compounds decreased with the 20% increase in the relative humidity. This indicates that a competition exists between flavor compounds and water molecules to bind the protein. Water molecules possess some of the available binding sides of the protein, so it decreases the flavor binding.

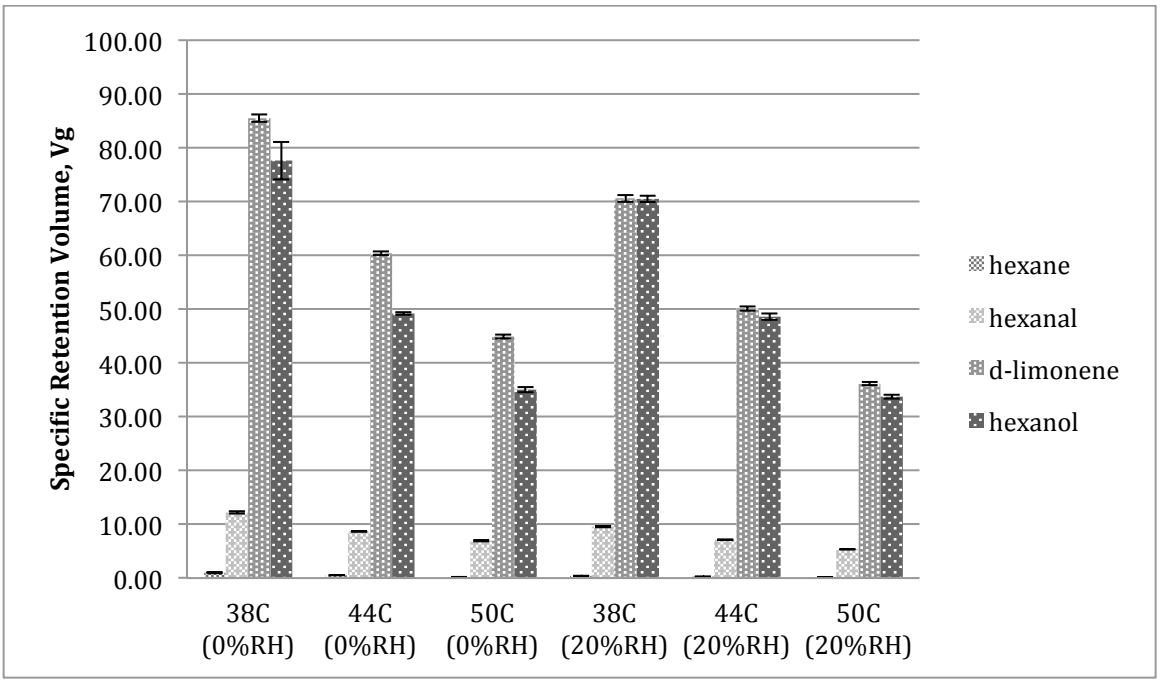


Figure 5-13: Comparison of Vg of adsorption of volatile probes on raw proteins under dry condition and 20% RH.

When the same data was plotted together (Figure 5-13), d-limonene had the highest specific retention volumes on SPI for both 0% and 20% RH at all temperatures. This suggests that d-limonene has better adsorption than any other flavor compounds. Hexanol also had high values of specific retention volume, indicating that good adsorption occurs between SPI and hexanol on both for 0% and 20% RH. Specific retention volumes of each flavor decreases with an increasing RH level.

Gibbs Free Energy of adsorption for each flavor compound on soy protein isolate under both dry and humid conditions is indicated in Figure 5-14. The graph shows that a parallel shift is observed in Gibbs free energy of adsorption between dry conditions and with humid conditions.

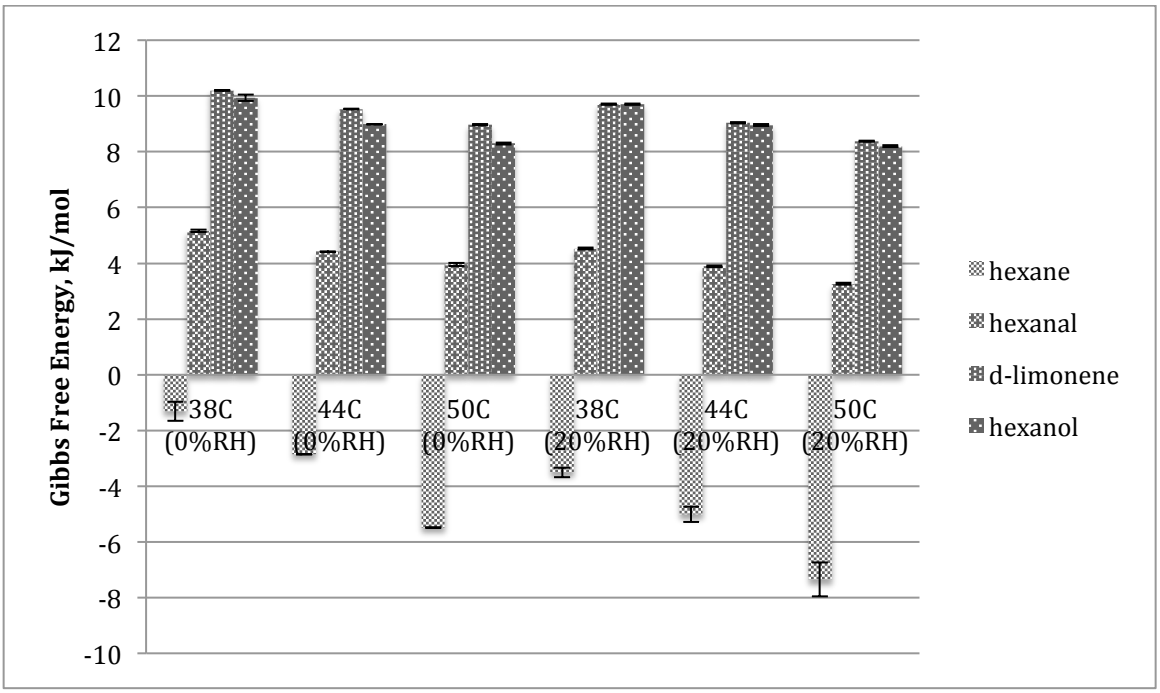


Figure 5-14: Comparison of Gibbs Free Energies of adsorption of volatile probes on raw proteins under dry condition and 20% RH.

Retention of hexane was very low when compared with the other flavor compounds, suggesting that adsorption is thermodynamically less favorable. The higher negative Gibbs Free energy of d-limonene and hexanol suggests that adsorption is thermodynamically favorable for those flavor compounds under both dry and humid conditions. When the relative humidity increased from 0% to 20%, the retention of all flavor compounds decreased. This led to a decrease in the negative Gibbs free energy of adsorption of each flavor compound on soy protein isolate. Similar to the results under dry conditions, a more favorable adsorption for d-limonene and hexanol and a less favorable adsorption for hexane were observed at humid conditions.

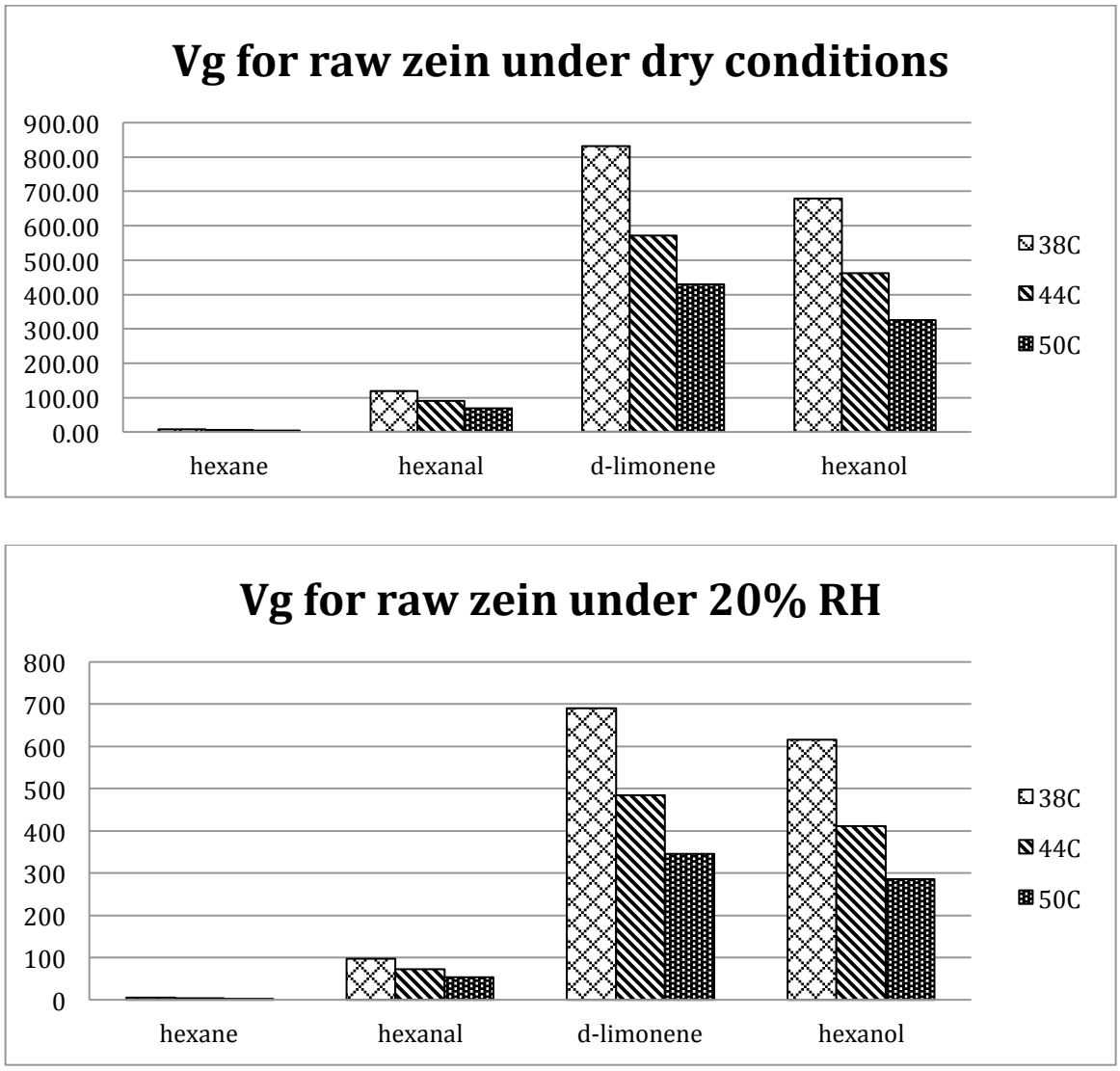


Figure 5-15: Comparison of Vg for raw zein under (a) dry conditions and (b) 20% RH.

Specific retention volumes of adsorption for each flavor compound on raw zein for both 0% (a) and 20% RH (b) at different temperatures were plotted in Figure 5-15. As shown in the graphs, specific retention volumes of each flavor compound exhibit the same trends with the presence of water; d-limonene had the highest retention, followed next by hexanol and then by hexanal, and hexane had little or no retention.

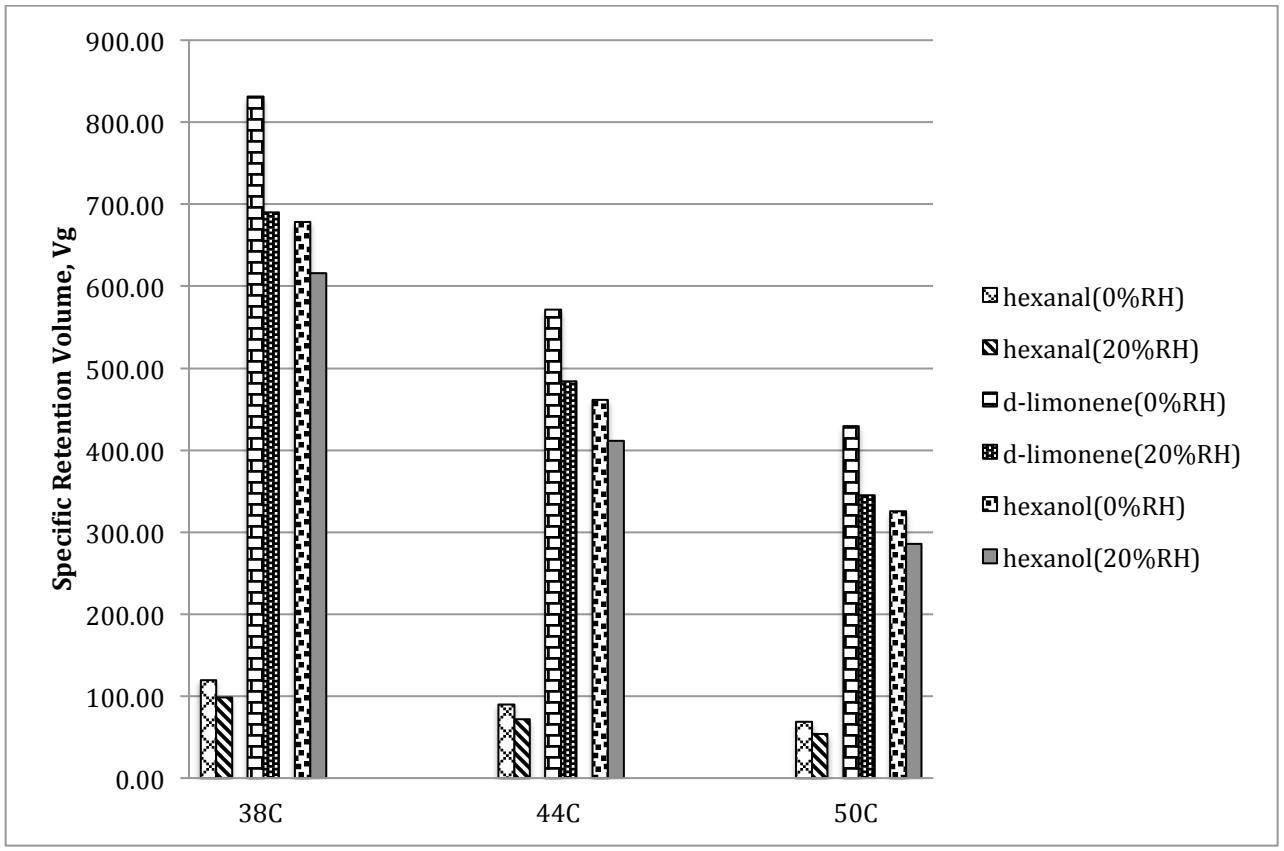


Figure 5-16: Effect of increasing %RH on Vg for raw zein at three different temperatures.

Figure 5-16 clearly shows the decrease in the specific retention volume of each flavor compound with increasing relative humidity at three different temperatures when the food matrix was raw zein. At dry and humid conditions, the tendency of the flavor compounds to adsorption on zein is similar to that of the soy protein isolate: d-limonene > hexanol > hexanal > hexane. As explained for the soy protein isolate, flavor compounds and water molecules share the available binding side of protein, resulting in a decrease in flavor adsorption with the presence of water in the environment.

Gibbs Free Energy of adsorption for each flavor compound on raw zein under both dry and humid conditions is indicated at three different temperatures in Figure 5-17. The graphs show that a parallel shift is observed in Gibbs free energy of adsorption between

dry conditions and humid conditions. Free energies of adsorption of hexanol and d-limonene on zein were lower when relative humidity increased, indicating that adsorption is less spontaneous.

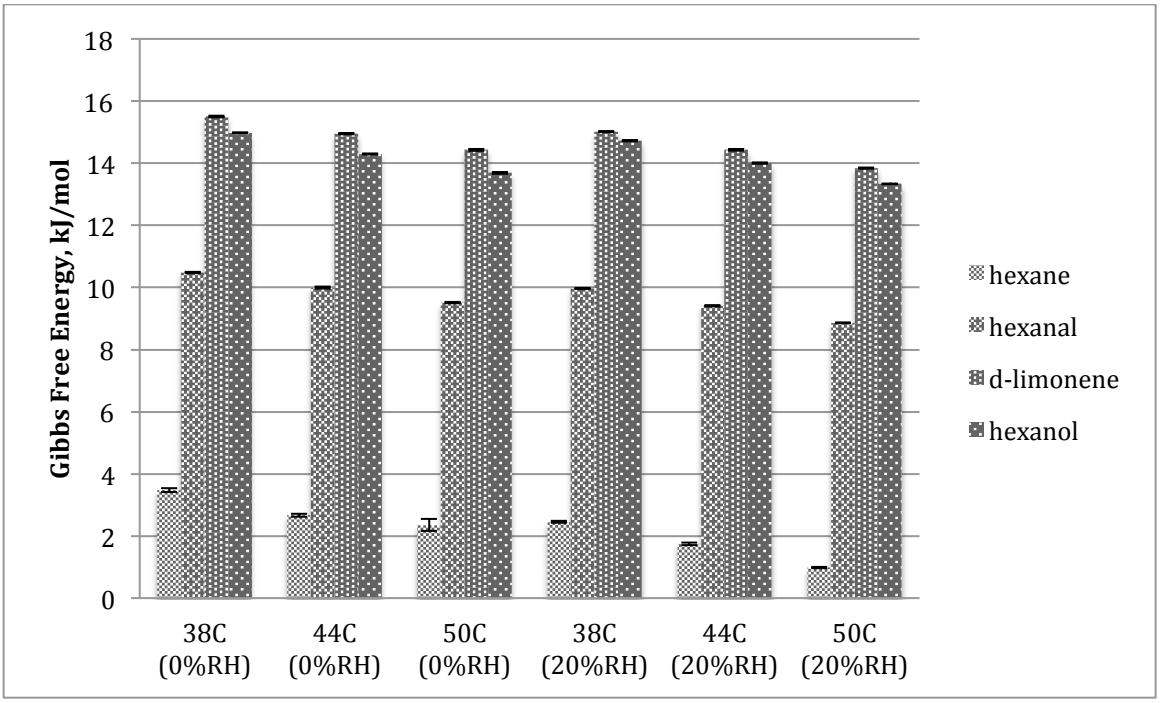


Figure 5-17: Comparison of Gibbs Free Energies for raw zein under (a) dry conditions and (b) 20% RH.

The lower specific retention volumes and lower Gibbs free energy values of each flavor compound on soy protein isolate and zein under humid conditions, compared to under dry conditions, suggested a decrease in the affinity of flavors to adsorption under humidified conditions. This was probably due to the affinity of water molecules binding to available sides of protein. Zhou and Cadwallader (2006) also reported that the presence of water decreases the adsorption of flavors due to the competition between flavor compounds and water molecules.

Figure 5-18 shows the binding affinities of flavor compounds onto raw and heat-treated soy protein isolates at dry conditions (a) and with the presence of water (b). Increasing the relative humidity results in a decrease in retention for each flavor compound at dry conditions in the case of using thermally processed soy protein isolate as a food matrix. As previously discussed, even though there is a decrease in the specific retention volumes for each flavor compound, we can observe a different outcome, which is that hexanol had higher retention volumes than d-limonene after heat treatment at dry conditions. Later on in the study, when the relative humidity increased from 0% to 20% (Figure 30-b), the results will vary for hexanol that in the case of raw proteins, d-limonene at all conditions always demonstrate the higher retention, and flavor retention decreases with increasing relative humidity. This graph shows that increasing relative humidity results in an increase in specific retention volumes of hexanol after heat treatment. Similar results were found for zein (Figure 5-19 –(a)&(b)).

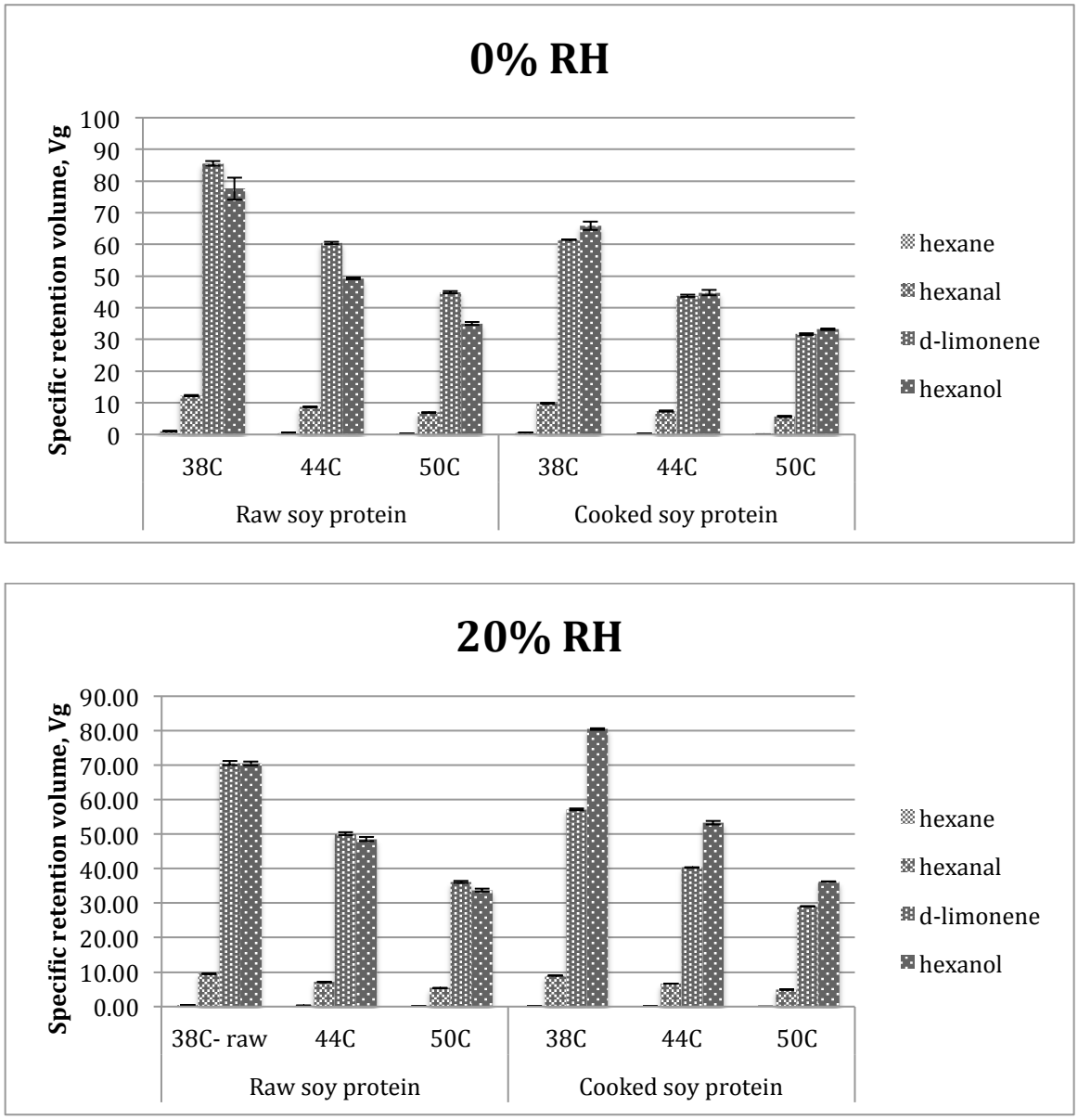


Figure 5-18: Comparison of Vg for raw SPI and cooked SPI under (a) dry conditions and (b) 20% RH.

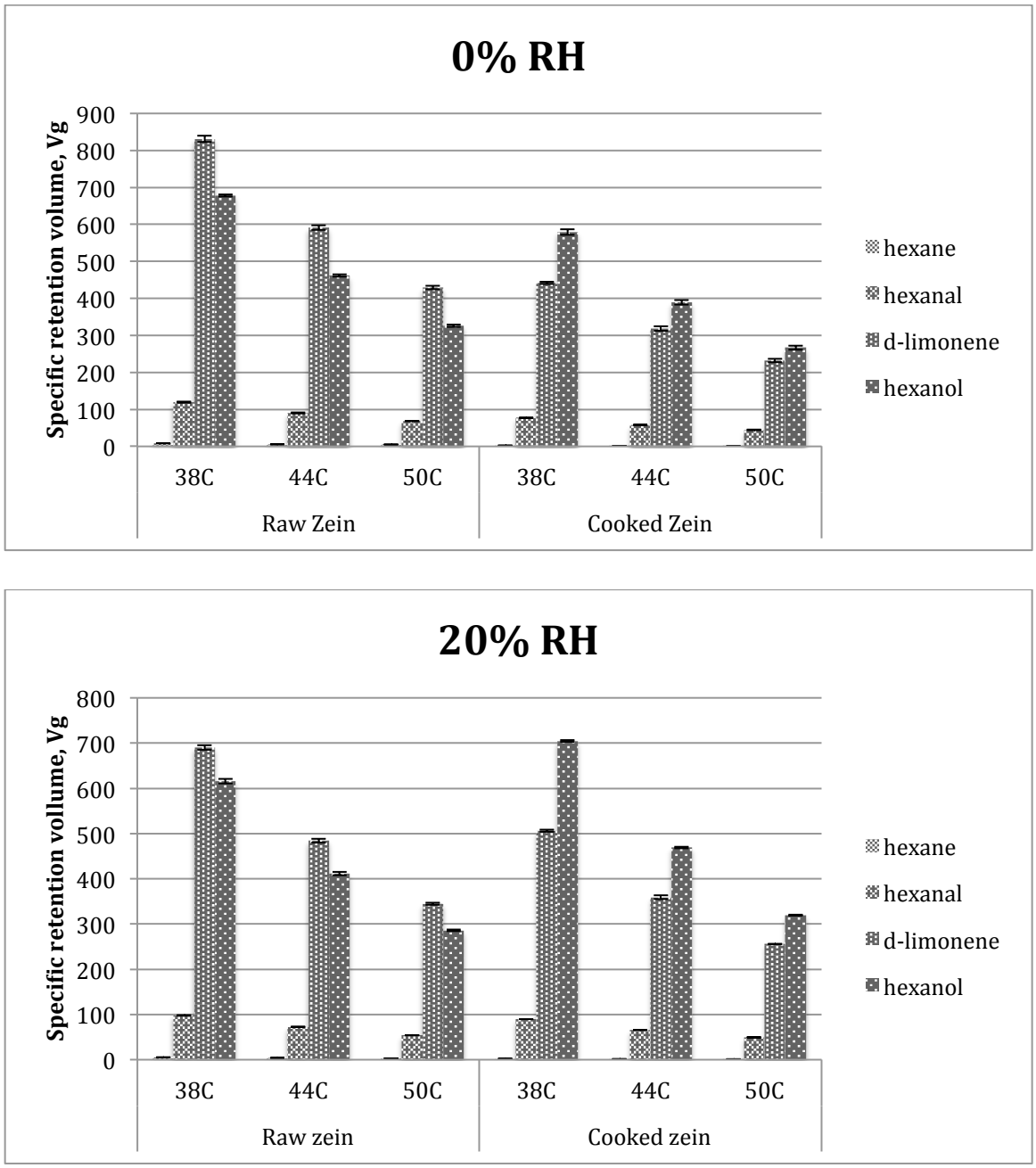


Figure 5-19: Comparison of Vg for raw zein and cooked zein under (a) dry conditions and (b) 20% RH.

Free energies of adsorption of each flavor compound on raw and cooked soy proteins under dry conditions and with the presence of water are indicated in Figure 5-20. Gibbs

free energy of each flavor on raw and cooked proteins had similar trends at dry conditions. A decrease in Gibbs free energies of all flavor compounds was observed after the cooking process (Figure 5-20 – a). If the soy protein isolate was exposed to heat treatment, adsorption was less favorable and less spontaneous for each flavor compound at dry conditions. In the case of the presence of water, lower Gibbs free energies of flavor compounds on cooked soy protein isolate, compared to raw soy protein isolate, showed a decrease in the affinity of flavors to adsorption on cooked SPI except hexanol (Figure 5-20- b). Higher Gibbs free energies of hexanol on cooked SPI were observed with the presence of water when compared to raw SPI. Similar findings were found for zein (Figure 5-21 –(a)&(b)).

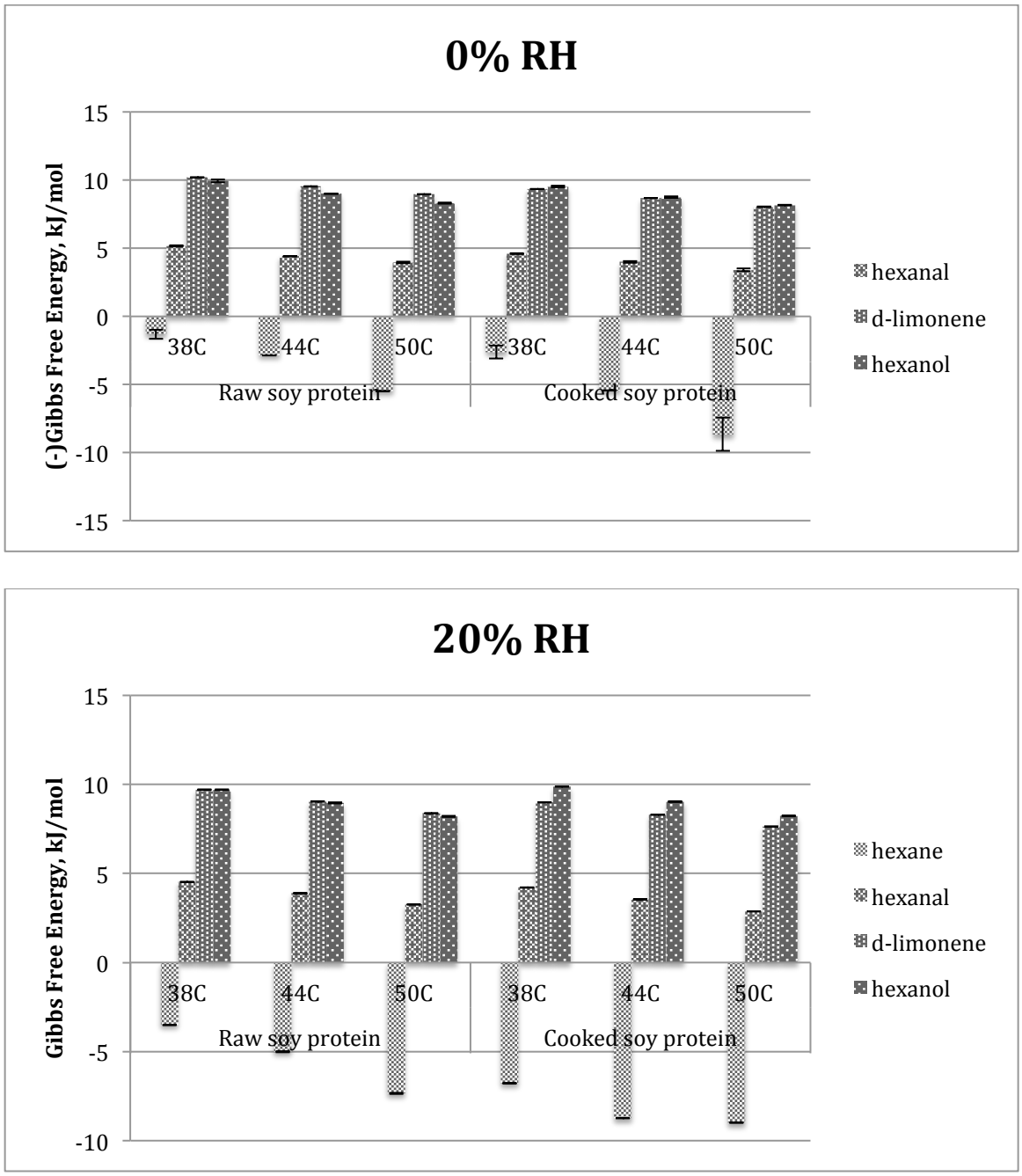


Figure 5-20: Comparison of Gibbs free energy for raw SPI and cooked SPI under (a) dry conditions and (b) 20% RH.

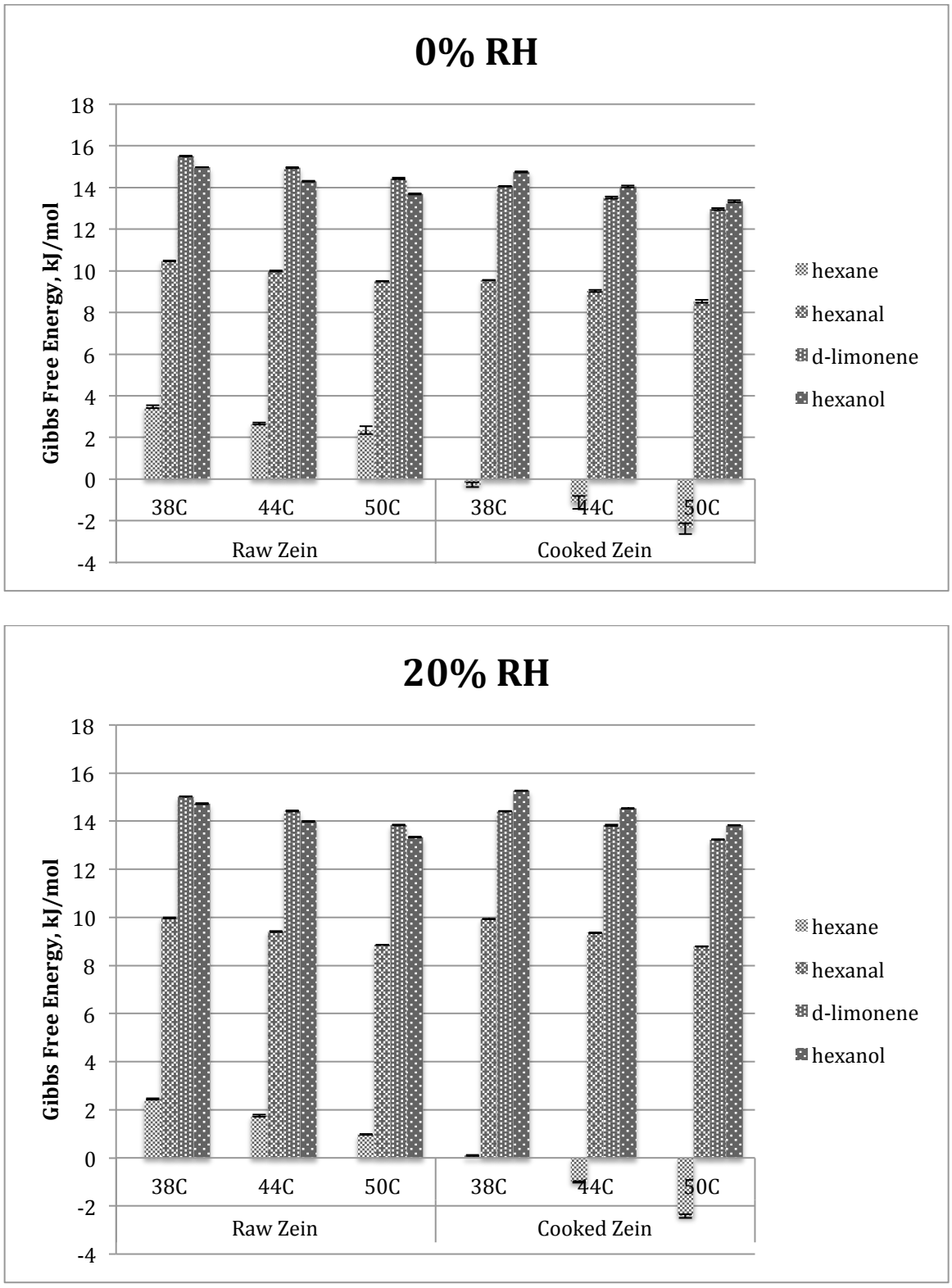


Figure 5-21: Comparison of Vg for raw zein and cooked zein under (a) dry conditions and (b) 20% RH.

6 CONCLUSION

The results indicated that adsorption of different flavor compounds onto different protein matrices might be a result of the mixture of various binding interactions, both hydrophobic, hydrogen bonds, van der Vaals, etc. Thermodynamics data of adsorption provide valuable knowledge about the flavor-protein interaction. Results clearly showed that the chemical structure of flavor compounds is one of the major factors that affects the interaction between protein and flavor molecules.

Heat treatment causes protein denaturation and thus formation of aggregation if the temperature is higher than the denaturation temperature. Binding of flavor compounds to raw and heat-treated proteins indicated similar trends, but the binding capacity is smaller for heat-treated protein, suggesting that aggregation of protein decreases the surface area and thus available binding sites of protein. This might play an important role in the flavor quality of the end product because of decreases in the retention of flavors.

Increasing relative humidity results in a decrease in flavor binding because of the competition between flavor molecules and water molecules to bind the active sites of the protein. In the presence of water, the interaction between flavor compounds and water molecules should be taken into account to interpret the flavor adsorption onto the proteins.

A clear understanding of the adsorption of flavors onto proteins under various conditions, such as temperature, and RH differences would help the food industry for development of more acceptable food products.

7 APPENDIX – 1

Raw soy protein
RH=0%
F=20 ml/min

Po (psia)	Tfl (K)	Pfl (psia)	ms (g)	Vc (mL)	F (mL/min)	Pi (psig)	Tov (K)	R (m3.Pa/mol.K)
14.7	298	14.7	1	1.885	20	20	*	8.314

T(oven)=38°C=311 K

hexane	0.350	0.350	0.316	0.300
Hexanal	1.360	1.433	1.400	1.450
d-limonene	8.466	8.383	8.466	8.700
Hexanol	6.900	7.516	8.100	8.433

T(oven)=44°C=317 K

hexane	0.283	0.283	0.283	0.283
Hexanal	1.056	1.065	1.083	1.075
d-limonene	6.050	5.990	6.133	6.116
Hexanol	4.946	4.950	5.050	5.016

T(oven)=50°C=323 K

hexane	0.250	0.250	0.250	0.250
Hexanal	0.916	0.930	0.866	0.900
d-limonene	4.616	4.650	4.516	4.516
Hexanol	3.650	3.533	3.733	3.550

Cooked soy protein
RH=0%
F=20 ml/min

Po (psia)	Tfl (K)	Pfl (psia)	ms (g)	Vc (mL)	F (mL/min)	Pi (psig)	Tov (K)	R (m3.Pa/mol.K)
14.7	298	14.7	1	1.885	20	20	*	8.314

T(oven)=38°C=311 K

hexane	0.283	0.266	0.300	0.316
Hexanal	1.150	1.216	1.200	1.166
d-limonene	6.183	6.183	6.166	6.166
Hexanol	6.816	6.733	6.233	6.633

T(oven)=44°C=317 K

hexane	0.250	0.250	0.250	0.250
Hexanal	0.900	0.933	1.000	0.950
d-limonene	4.383	4.533	4.466	4.466
Hexanol	4.766	4.633	4.400	4.450

T(oven)=50°C=323 K

hexane	0.233	0.233	0.250	0.238
Hexanal	0.750	0.733	0.816	0.816
d-limonene	3.383	3.266	3.233	3.300
Hexanol	3.433	3.383	3.483	3.466

Raw soy protein

RH=20%

F=20 ml/min

Po (psia)	**Tfl (K)**	**Pfl (psia)**	**ms (g)**	**Vc (mL)**	**F (mL/min)**	**Pi (psig)**	**Tov (K)**	**R (m3.Pa/mol.K)**
14.7	298	14.7	1	1.885	20	17.01	*	8.314

T(oven)=38°C=311 K

hexane	0.277	0.273	0.273	0.264	0.262	0.264
Hexanal	1.11	1.107	1.112	1.058	1.067	1.079
d-limonene	6.424	6.465	6.5	6.67	6.691	6.747
Hexanol	6.409	6.495	6.5	6.662	6.662	6.729

T(oven)=44°C=317 K

hexane	0.258	0.257	0.258	0.247	0.248	0.247
Hexanal	0.885	0.887	0.891	0.849	0.854	0.858
d-limonene	4.795	4.81	4.844	4.637	4.671	4.688
Hexanol	4.694	4.694	4.761	4.453	4.499	4.515

T(oven)=50°C=323 K

hexane	0.245	0.245	0.246	0.236	0.235	0.236
Hexanal	0.724	0.728	0.73	0.697	0.701	0.702
d-limonene	3.525	3.549	3.557	3.415	3.414	3.44
Hexanol	3.337	3.342	3.354	3.19	3.159	3.225

Cooked soy protein
RH=20%
F=20 ml/min

Po (psia)	Tfl (K)	Pfl (psia)	ms (g)	Vc (mL)	F (mL/min)	Pi (psig)	Tov (K)	R (m3.Pa/mol.K)
14.7	298	14.7	0.94	1.885	20	17.61	*	8.314

T(oven)=38°C=311 K

hexane	0.242	0.240	0.234	0.242	0.247	0.248
Hexanal	1.015	1.001	0.992	0.992	1.001	1.003
d-limonene	5.194	5.179	5.175	5.089	5.062	5.110
Hexanol	7.146	7.137	7.059	7.222	7.131	7.151

T(oven)=44°C=317 K

hexane	0.229	0.234	0.224	0.231	0.241	0.249
Hexanal	0.801	0.802	0.797	0.797	0.799	0.801
d-limonene	3.720	3.719	3.706	3.663	3.694	3.652
Hexanol	4.804	4.760	4.797	4.970	4.626	4.874

T(oven)=50°C=323 K

hexane	0.221	0.221	0.220	0.224	0.225	0.224
Hexanal	0.654	0.640	0.647	0.656	0.659	0.653
d-limonene	2.727	2.716	2.727	2.703	2.676	2.688
Hexanol	3.346	3.347	3.331	3.277	3.438	3.417

Raw zein
RH=0%
F=20 ml/min

Po (psia)	Tfl (K)	Pfl (psia)	ms (g)	Vc (mL)	F (mL/min)	Pi (psig)	Tov (K)	R (m3.Pa/mol.K)
14.7	298	14.7	0.8	1.885	20	4	311	8.314

T(oven)=38°C=311 K

hexane	0.600	0.650	0.632	0.622
Hexanal	6.283	6.266	6.066	6.066
d-limonene	42.416	42.283	40.916	40.983
Hexanol	34.216	34.300	33.983	33.666

T(oven)=44°C=317 K

hexane	0.516	0.516	0.500	0.500
Hexanal	4.766	4.850	4.650	4.616
d-limonene	30.116	30.333	29.200	29.150
Hexanol	23.433	23.566	22.983	22.933

T(oven)=50°C=323 K

hexane	0.466	0.466	0.516	0.433
Hexanal	3.683	3.700	3.583	3.633
d-limonene	22.150	22.033	21.216	21.216
Hexanol	16.833	16.666	16.266	16.116

Cooked zein

RH=0%

F=20 ml/min

Po (psia)	Tfl (K)	Pfl (psia)	ms (g)	Vc (mL)	F (mL/min)	Pi (psig)	Tov (K)	R (m3.Pa/mol.K)
14.7	298	14.7	0.86	1.885	20	3	*	8.314

T(oven)=38°C=311 K

hexane	0.316	0.333	0.316	0.316
Hexanal	4.266	4.316	4.183	4.200
d-limonene	23.416	23.500	22.883	22.916
Hexanol	30.330	31.316	29.800	29.650

T(oven)=44°C=317 K

hexane	0.316	0.300	0.283	0.283
Hexanal	3.383	3.316	3.133	3.150
d-limonene	17.250	17.266	16.250	16.183
Hexanol	20.950	21.016	19.900	19.866

T(oven)=50°C=323 K

hexane	0.266	0.283	0.266	0.266
Hexanal	2.650	2.666	2.433	2.433
d-limonene	12.666	12.633	11.816	11.933
Hexanol	14.550	14.633	13.533	13.583

Raw zein

RH=20%

F=20 ml/min

Po (psia)	Tfl (K)	Pfl (psia)	ms (g)	Vc (mL)	F (mL/min)	Pi (psig)	Tov (K)	R (m3.Pa/mol.K)
14.7	298	14.7	0.8	1.885	20	2.7	311	8.314

T(oven)=38°C=311 K

hexane	0.492	0.492	0.493	0.478	0.478	0.478
Hexanal	4.991	4.999	4.994	4.821	4.831	4.826
d-limonene	33.651	33.808	33.816	32.642	32.692	32.616
Hexanol	30.07	30.165	30.152	28.618	29.688	29.212

T(oven)=44°C=317 K

hexane	0.425	0.425	0.425	0.413	0.412	0.41
Hexanal	3.742	3.747	3.753	3.615	3.609	3.596
d-limonene	23.837	23.847	23.841	22.95	22.913	22.867
Hexanol	20.113	19.989	20.478	19.716	19.671	19.302

T(oven)=50°C=323 K

hexane	0.369	0.369	0.37	0.366	0.364	0.364
Hexanal	2.837	2.839	2.837	2.782	2.782	2.78
d-limonene	16.99	16.985	16.959	16.412	16.358	16.59
Hexanol	13.792	14.133	14.12	13.797	13.751	13.75

Cooked zein

RH=20%

F=20 ml/min

Po (psia)	Tfl (K)	Pfl (psia)	ms (g)	Vc (mL)	F (mL/min)	Pi (psig)	Tov (K)	R (m3.Pa/mol.K)
14.7	298	14.7	0.86	1.885	20	1.94	*	8.314

T(oven)=38°C=311 K

hexane	0.332	0.332	0.330	0.329	0.328	0.328
Hexanal	4.690	4.690	4.734	4.747	4.731	4.741
d-limonene	25.282	25.349	25.132	25.760	25.812	25.761
Hexanol	35.171	35.838	35.247	35.553	35.554	35.556

T(oven)=44°C=317 K

hexane	0.293	0.293	0.294	0.296	0.297	0.295
Hexanal	3.491	3.499	3.497	3.557	3.557	3.551
d-limonene	17.996	17.988	17.986	18.813	18.835	17.689
Hexanol	23.469	23.585	23.628	23.877	23.936	23.827

T(oven)=50°C=323 K

hexane	0.267	0.266	0.274	0.267	0.267	0.266
Hexanal	2.659	2.664	2.675	2.695	2.688	2.686
d-limonene	12.976	12.959	12.992	13.165	13.147	13.084
Hexanol	16.119	16.152	16.174	16.346	16.355	16.214

GIBBS FREE ENERGY

raw soy protein, 0%
RH

						average	se
38C	hexane	0.74	0.74	1.61	2.14	1.31	0.34
	hexanal	-5.05	-5.22	-5.14	-5.25	-5.17	0.04
	d-limonene	-10.19	-10.16	-10.19	-10.26	-10.20	0.02
	hexanol	-9.64	-9.87	-10.07	-10.18	-9.94	0.12
44C	hexane	2.86	2.86	2.86	2.86	2.86	0.00
	hexanal	-4.38	-4.40	-4.46	-4.44	-4.42	0.02
	d-limonene	-9.52	-9.49	-9.56	-9.55	-9.53	0.01
	hexanol	-8.97	-8.97	-9.02	-9.01	-8.99	0.01
50C	hexane	5.48	5.48	5.48	5.48	5.48	0.00
	hexanal	-4.01	-4.06	-3.81	-3.95	-3.96	0.06
	d-limonene	-8.99	-9.01	-8.93	-8.93	-8.97	0.02
	hexanol	-8.32	-8.23	-8.39	-8.24	-8.30	0.04

cooked soy protein, 0% RH

						average	Se
38C	hexane	2.86	3.86	2.14	1.61	2.61	0.49
	hexanal	-4.52	-4.70	-4.66	-4.57	-4.61	0.04
	d-limonene	-9.35	-9.35	-9.34	-9.34	-9.35	0.00
	hexanol	-9.61	-9.58	-9.37	-9.54	-9.53	0.05
44C	hexane	5.43	5.43	5.43	5.43	5.43	0.00
	hexanal	-3.82	-3.95	-4.19	-4.01	-3.99	0.08
	d-limonene	-8.63	-8.73	-8.68	-8.68	-8.68	0.02
	hexanol	-8.86	-8.79	-8.64	-8.67	-8.74	0.05
50C	hexane	10.58	10.58	5.48	7.94	8.65	1.22
	hexanal	-3.27	-3.18	-3.59	-3.59	-3.40	0.11
	d-limonene	-8.11	-8.00	-7.98	-8.03	-8.03	0.03
	hexanol	-8.15	-8.11	-8.19	-8.18	-8.16	0.02

raw soy protein, 20% RH

								average	se
38C	hexane	2.98	3.21	3.21	3.82	3.98	3.82	3.50	0.17
	hexanal	-4.59	-4.58	-4.60	-4.44	-4.46	-4.50	-4.53	0.03
	d-limonene	-9.64	-9.66	-9.67	-9.74	-9.75	-9.77	-9.70	0.02
	hexanol	-9.63	-9.67	-9.67	-9.74	-9.74	-9.76	-9.70	0.02
44C	hexane	4.35	4.45	4.35	5.67	5.52	5.67	5.00	0.28
	hexanal	-3.95	-3.96	-3.98	-3.80	-3.83	-3.84	-3.89	0.03
	d-limonene	-9.07	-9.08	-9.10	-8.98	-9.00	-9.01	-9.04	0.02
	hexanol	-9.01	-9.01	-9.05	-8.87	-8.89	-8.90	-8.96	0.03
50C	hexane	6.06	6.06	5.89	8.52	9.01	8.52	7.35	0.60
	hexanal	-3.32	-3.34	-3.35	-3.17	-3.19	-3.20	-3.26	0.03
	d-limonene	-8.42	-8.44	-8.44	-8.33	-8.33	-8.35	-8.38	0.02
	hexanol	-8.26	-8.26	-8.27	-8.13	-8.10	-8.16	-8.20	0.03

cooked soy protein, 20% RH

								average	se
38C	hexane	6.55	7.02	9.39	6.55	5.65	5.50	6.78	0.57
	hexanal	-4.26	-4.21	-4.18	-4.18	-4.21	-4.22	-4.21	0.01
	d-limonene	-9.03	-9.02	-9.02	-8.97	-8.96	-8.98	-9.00	0.01
	hexanol	-9.89	-9.88	-9.85	-9.91	-9.88	-9.89	-9.88	0.01
44C	hexane	#NUM!	#NUM!	9.52	13.18	6.86	5.42	8.74	1.71
	hexanal	-3.55	-3.53	-3.56	-3.53	-3.54	-3.55	-3.55	0.00
	d-limonene	-8.32	-8.31	-8.32	-8.28	-8.30	-8.27	-8.30	0.01
	hexanol	-9.04	-9.03	-9.01	-9.13	-8.93	-9.08	-9.04	0.03
50C	hexane	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
	hexanal	-2.87	-2.78	-2.83	-2.88	-2.90	-2.87	-2.86	0.02
	d-limonene	-7.63	-7.62	-7.63	-7.61	-7.58	-7.59	-7.61	0.01
	hexanol	-8.23	-8.23	-8.21	-8.17	-8.31	-8.29	-8.24	0.02

raw zein, 0% RH

						average	se
38C	hexane	-3.30	-3.63	-3.52	-3.45	-3.48	0.07
	hexanal	-10.53	-10.52	-10.44	-10.44	-10.48	0.03
	d-limonene	-15.55	-15.54	-15.46	-15.46	-15.50	0.03
	hexanol	-14.99	-15.00	-14.97	-14.95	-14.98	0.01
44C	hexane	-2.74	-2.74	-2.59	-2.59	-2.66	0.04
	hexanal	-10.02	-10.07	-9.96	-9.94	-10.00	0.03
	d-limonene	-14.99	-15.01	-14.91	-14.91	-14.96	0.03
	hexanol	-14.33	-14.34	-14.27	-14.27	-14.30	0.02
50C	hexane	-2.33	-2.33	-2.84	-1.92	-2.35	0.19
	hexanal	-9.53	-9.54	-9.45	-9.49	-9.51	0.02
	d-limonene	-14.49	-14.48	-14.38	-14.38	-14.43	0.03
	hexanol	-13.75	-13.72	-13.66	-13.63	-13.69	0.03

cooked zein, 0% RH

						average	se
38C	hexane	0.39	-0.08	0.39	0.39	0.27	0.12
	hexanal	-9.57	-9.60	-9.51	-9.52	-9.55	0.02
	d-limonene	-14.09	-14.10	-14.03	-14.03	-14.06	0.02
	hexanol	-14.76	-14.84	-14.72	-14.70	-14.76	0.03
44C	hexane	0.34	0.88	1.62	1.62	1.12	0.31
	hexanal	-9.15	-9.09	-8.93	-8.95	-9.03	0.05
	d-limonene	-13.59	-13.60	-13.43	-13.42	-13.51	0.05
	hexanol	-14.11	-14.12	-13.98	-13.97	-14.04	0.04
50C	hexane	2.64	1.60	2.64	2.64	2.38	0.26
	hexanal	-8.66	-8.68	-8.41	-8.41	-8.54	0.08
	d-limonene	-13.06	-13.05	-12.87	-12.90	-12.97	0.05
	hexanol	-13.44	-13.45	-13.24	-13.25	-13.35	0.06

raw zein, 20% RH

								Average	se
38C	hexane	-2.52	-2.52	-2.53	-2.38	-2.38	-2.38	-2.45	0.03
	hexanal	-10.02	-10.02	-10.02	-9.92	-9.93	-9.93	-9.97	0.02
	d-limonene	-15.06	-15.07	-15.07	-14.98	-14.98	-14.98	-15.02	0.02
	hexanol	-14.76	-14.77	-14.77	-14.64	-14.73	-14.69	-14.73	0.02
44C	hexane	-1.84	-1.84	-1.84	-1.67	-1.66	-1.63	-1.75	0.04
	hexanal	-9.46	-9.46	-9.47	-9.36	-9.36	-9.35	-9.41	0.02
	d-limonene	-14.48	-14.48	-14.48	-14.38	-14.38	-14.37	-14.43	0.02
	hexanol	-14.03	-14.01	-14.08	-13.98	-13.97	-13.92	-14.00	0.02
50C	hexane	-1.02	-1.02	-1.04	-0.96	-0.92	-0.92	-0.98	0.02
	hexanal	-8.89	-8.89	-8.89	-8.83	-8.83	-8.83	-8.86	0.01
	d-limonene	-13.89	-13.89	-13.88	-13.79	-13.78	-13.82	-13.84	0.02
	hexanol	-13.32	-13.38	-13.38	-13.32	-13.31	-13.31	-13.34	0.01

cooked zein, 20% RH

								Average	se
38C	hexane	-0.15	-0.15	-0.07	-0.04	-0.04	-0.09	-0.09	0.02
	hexanal	-9.91	-9.91	-9.95	-9.94	-9.94	-9.93	-9.93	0.01
	d-limonene	-14.38	-14.38	-14.43	-14.43	-14.43	-14.41	-14.41	0.01
	hexanol	-15.24	-15.29	-15.27	-15.27	-15.27	-15.26	-15.26	0.01
44C	hexane	1.07	1.07	1.03	0.95	0.91	0.99	1.00	0.03
	hexanal	-9.33	-9.34	-9.34	-9.38	-9.38	-9.38	-9.36	0.01
	d-limonene	-13.80	-13.80	-13.80	-13.92	-13.92	-13.75	-13.83	0.03
	hexanol	-14.51	-14.52	-14.52	-14.55	-14.56	-14.55	-14.53	0.01
50C	hexane	2.47	2.54	2.00	2.47	2.47	2.54	2.42	0.08
	hexanal	-8.77	-8.77	-8.78	-8.81	-8.80	-8.80	-8.79	0.01
	d-limonene	-13.22	-13.22	-13.22	-13.26	-13.25	-13.24	-13.24	0.01
	hexanol	-13.81	-13.82	-13.82	-13.85	-13.85	-13.83	-13.83	0.01

	Raw Zein protein RH=0%			Cooked Zein protein RH=0%			Raw zein protein RH=20%			Cooked Zein protein RH=20%		
Enthaply												
Hexane	-31.32			-60.22			-44.09			-70.55		
Hexanal	-39.07			-35.59			41.91			-42.29		
Limonene	-45.57			-43.35			-48.03			-47.03		
Hexanol	-49.87			-51.70			-54.89			-54.85		
Gibbs												
	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C
Hexane	-3.48	-2.66	-2.35	0.27	1.12	2.38	-2.45	-1.75	-0.98	-0.09	1.00	2.42
Hexanal	-10.48	-10.00	-9.51	-9.55	-9.03	-8.54	-9.97	-9.41	-8.86	-9.93	-9.36	-8.79
Limonene	-15.50	-14.96	-14.43	-14.06	-13.51	-12.97	-15.02	-14.43	-13.84	-14.41	-13.83	-13.24
Hexanol	-14.98	-14.30	-13.69	-14.76	-14.04	-13.35	-14.73	-14.00	-13.34	-15.26	-14.53	-13.83
Entropy												
	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C
Hexane	-0.09	-0.09	-0.09	-0.19	-0.19	-0.19	-0.13	-0.13	-0.13	-0.23	-0.23	-0.23
Hexanal	-0.09	-0.09	-0.09	-0.08	-0.08	-0.08	0.17	0.16	0.16	-0.10	-0.10	-0.10
Limonene	-0.10	-0.10	-0.10	-0.09	-0.09	-0.09	-0.11	-0.11	-0.11	-0.10	-0.10	-0.10
Hexanol	-0.11	-0.11	-0.11	-0.12	-0.12	-0.12	-0.13	-0.13	-0.13	-0.13	-0.13	-0.13

	Raw soy protein RH=0%			Cooked soy protein RH=0%			Raw soy protein RH=20%			Cooked soy protein RH=20%		
Enthalpy												
Hexane	-111.40			-199.43			-79.43			-137.81		
Hexanal	-39.17			-39.73			-40.19			-42.87		
Limonene	-44.85			-44.26			-43.91			-47.82		
Hexanol	55.45			-50.17			-47.83			-55.50		
Gibbs												
	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C
Hexane	1.31	2.86	5.48	2.61	5.43	8.65	3.50	5.00	7.35	5.50	5.42	#NUM!
Hexanal	-5.17	-4.42	-3.96	-4.61	-3.99	-3.40	-4.53	-3.89	-3.26	-4.22	-3.55	-2.87
Limonene	-10.20	-9.53	-8.97	-9.35	-8.68	-8.03	-9.70	-9.04	-8.38	-8.98	-8.27	-7.59
Hexanol	-9.94	-8.99	-8.30	-9.53	-8.74	-8.16	-9.70	-8.96	-8.20	-9.89	-9.08	-8.29
Entropy												
	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C	T=38°C	T=44°C	T=50°C
Hexane	-0.36	-0.36	-0.36	-0.65	-0.65	-0.64	-0.27	-0.27	-0.27	-0.46	-0.45	#NUM!
Hexanal	-0.11	-0.11	-0.11	-0.11	-0.11	-0.11	-0.11	-0.11	-0.11	-0.12	-0.12	-0.12
Limonene	-0.11	-0.11	-0.11	-0.11	-0.11	-0.11	-0.11	-0.11	-0.11	-0.12	-0.12	-0.12
Hexanol	0.21	0.20	0.20	-0.13	-0.13	-0.13	-0.12	-0.12	-0.12	-0.15	-0.15	-0.15

Soy Protein (x1, x2, x3: temp, humid, heat)

1. Hexanal

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
X1	16.0026	2	8.00131	804.31	1.90977e-041
X2	4.2207	1	4.22067	424.27	1.58218e-027
X3	2.5958	1	2.59584	260.94	1.55088e-022
Error	0.5471	55	0.00995		
Total	23.3663	59			

2. D-limonene

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
X1	17.3226	2	8.66132	2673.16	1.64516e-055
X2	2.9016	1	2.90162	895.53	1.01891e-035
X3	9.4248	1	9.42481	2908.8	2.6172e-049
Error	0.1782	55	0.00324		
Total	29.8273	59			

3. Hexanol

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
X1	24.0577	2	12.0288	660.86	0
X2	0.0499	1	0.0499	2.74	0.1033
X3	0.0346	1	0.0346	1.9	0.1738
Error	1.0011	55	0.0182		
Total	25.1433	59			

Zein (x1, x2, x3: temp, humid, heat)

1. **Hexanal**

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
X1	11.5574	2	5.77872	101.61	0
X2	0.2438	1	0.24379	4.29	0.0431
X3	2.5789	1	2.57888	45.34	0
Error	3.128	55	0.05687		
Total	17.5081	59			

2. **D-limonene**

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
X1	12.976	2	6.488	130.11	0
X2	0.1762	1	0.1762	3.53	0.0654
X3	13.3459	1	13.3459	267.63	0
Error	2.7427	55	0.0499		
Total	29.2409	59			

3. **Hexanol**

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
X1	19.2345	2	9.61725	216.44	0
X2	0.1325	1	0.13252	2.98	0.0898
X3	0.6209	1	0.62088	13.97	0.0004
Error	2.4439	55	0.04443		
Total	22.4318	59			

Soy Protein (x1, x2, x3: temp, heat)

3. Hexanol (0% humid)

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
X1	9.14341	2	4.5717	271.88	3.15808e-015
X2	0.43202	1	0.43202	25.69	5.87022e-005
Error	0.33631	20	0.01682		
Total	9.91173	23			

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