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INVESTIGATING PHYSICOCHEMICAL PROPERTIES OF WET-GLYCATED SOY PROTEINS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
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BY

MUHAMMAD BIN ZIA

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FOR
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INVESTIGATING PHYSICOCHEMICAL PROPERTIES OF WET-GLYCATED SOY PROTEINS

submitted by **MUHAMMAD BIN ZIA** in partial fulfillment of the requirements for the degree of **Master of Science** i**n Food Engineering, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Serpil Şahin
Head of the Department, **Food Engineering** _____

Assoc. Prof. Dr. Halil Mecit Öztop
Supervisor, **Food Engineering, METU** _____

Prof. Dr. Behiç Mert
Co-Supervisor, **Food Engineering, METU** _____

Examining Committee Members:

Prof. Dr. S. Gülüm Şümnü
Food Engineering, METU _____

Assoc. Prof. Dr. Halil Mecit Öztop
Food Engineering, METU _____

Prof. Dr. Behiç Mert
Food Engineering, METU _____

Assist. Prof. Dr. E. Burçin Özvural
Food Engineering, Çankırı Karatekin University _____

Assist. Prof. Dr. Bekir Gökçen Mazı
Food Engineering, Ordu University _____

Date: 28.08.2020

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Name, Last name : Muhammad Bin Zia

Signature :

ABSTRACT

INVESTIGATING PHYSICOCHEMICAL PROPERTIES OF WET-GLYCATED SOY PROTEINS

Zia, Muhammad Bin
Master of Science, Food Engineering
Supervisor: Assoc. Prof. Dr. Halil Mecit Öztop
Co-Supervisor: Prof. Dr. Behiç Mert

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On a lab-scale, glycation is generally a lengthy process which if replicated on an industrial scale, would be costly. In this study, a simplified method for glycating soy protein isolate has been developed. Soy proteins were glycated with allulose, fructose and glucose in the presence of water. The protein-water ratio was changed from ½ to 1 (w/w) and glycation was conducted at 100°C and 130°C for 15 minutes, and samples were also dried at 50°C, 55% relative humidity for 1 day. The physicochemical properties of these proteins were analysed including free amino groups by OPA method, protein solubility by Lowry method, browning index, reducing sugar concentration using HPLC, structural changes via Fourier Transform Infrared (FT-IR) spectroscopy and hydration behavior using Time Domain Nuclear Magnetic Resonance (TD-NMR) relaxometry through T_2 relaxation time measurements. The results showed that proteins could be glycated under these simple conditions and with minimal control. It was found that high temperatures are not favorable for glycation and lower temperatures could be preffered. At high temperatures, proteins become more denatured and less soluble as shown through the Lowry method for protein solubility and TD-NMR results. Higher browning was seen at higher temperature, especially when allulose and fructose were used for

glycation. Proteins glycated with glucose were observed to be lighter in color. The highest glycation was obtained with glucose while allulose and fructose showed the lowest reactivity under the proposed conditions. Proteins with low solubility had longer T_2 relaxation times, indicating the presence of more free water and weaker water-binding capability.

Keywords: Glycation, Soy protein isolate, FT-IR, TD-NMR

ÖZ

SULU ORTAMDA GLİKE EDİLEN SOYA PROTEİNLERİNİN FİZİKOKİMYASAL ÖZELLİKLERİNİN İNCELENMESİ

Zia, Muhammad Bin
Yüksek Lisans, Gıda Mühendisliği
Tez Yöneticisi: Doç. Dr. Halil Mecit Öztop
Ortak Tez Yöneticisi: Doç. Dr. Behiç Mert

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Endüstriyel ölçekte çoğaltılması genellikle uzun bir süreç olan glikasyon, laboratuvar ölçeğinde maliyetlidir. Bu çalışmada, soya proteini izolatını glike etmek için basitleştirilmiş bir yöntem geliştirilmiştir. Soya proteinleri, su varlığında alüloz, fruktoz ve glikoz ile glike edilmiştir. 0.5-1.0 g/ml protein-su oranında 100-130°C'de 15 dakika ve 50°C ’de % 55 bağıl nemde bir gün boyunca olmak üzere glikasyon gerçekleştirilmiştir. Örneklerin fizikokimyasal özellikleri olan serbest amino grubu, protein çözünürlüğü, esmerleşme indeksi, indirgen şeker konsantrasyonu, yapısal değişiklikler ve hidrasyon davranışı sırasyıla OPA, Lowry, UV-VIS spektrofotometresi, HPLC, Fourier Transform Infrared (FT-IR) spektroskopisi ve Zamansal Alanda Nükleer Manyetik Rezonans (TD-NMR) ile T2 relaksasyon süresi ölçümü yöntemleri kullanılarak belirlenmiştir. Bu çalışmanın sonuçları, proteinlerin bu temel koşullar altında daha az kontrolle glike edilebileceğini göstermiştir. Yüksek sıcaklıkların glikasyon için uygun olmadığı ve daha düşük sıcaklıkların tercih edilebileceği bulunmuştur. Lowry yöntemiyle çözünür protein miktarı ve TD-NMR ile hidrasyon davranışı sonuçları yüksek sıcaklıkta protein denatürasyonun artığını ve proteinlerin daha az çözünür hale geldiğini göstermiştir. Yüksek sıcaklıkta, özellikle de glikasyon için alüloz ve früktoz kullanıldığında daha yüksek esmerleşme

görülmüştür. Glikoz ile glike edilen proteinlerin daha açık renkte olduğu gözlemlenmiştir. En yüksek glikasyon glikoz ile elde edilirken, önerilen koşullar altında alüloz ve fruktoz en düşük reaktiviteyi göstermiştir. Çözünürlüğü düşük proteinlerin daha uzun T2 relaksasyon sürelerine sahip olması daha fazla serbest su ve daha zayıf su bağlama kapasitesinin olduğunu göstermektedir.

Anahtar Kelimeler: Glikasyon, Soya protein izolatı, FT-IR, TD-NMR

To all those who are constantly learning

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INTRODUCTION

1.1 Soy Protein

1.1.1 Production of Soy Proteins

Soybean (Glycine max) is a type of legume which is well-known for its high protein and fat content. When compared to other legumes and cereals, it has the highest protein content and the second highest oil content (Thrane, Paulsen, Orcutt, & Krieger, 2016). Owing to its nutritional benefits, it has been cultivated in Asia for thousands of years and as the world deals with a growing population and as the need for alternate food sources grows, the soybean has become one of the most popular plant-based food sources.

Soybean is majorly produced in the western part of the world with United States, Brazil and Argentina being the main producers (United States Department of Agriculture, 2020), a big quantity of which is then exported to China, countries in European Union and in Southeast Asia. The total production of soybeans in the world is estimated to be around 358.28 million metric tons which is a 29% increase since 2013. 87% of these soybeans produced are crushed, 5% of which is used to produced defatted soybean meal which mainly serves as animal feed (98% of the soybean meal). Only a mere 2% is used to produce soy protein products (Potts et al., 2014).

In order to isolate proteins from soybeans, they go through a lengthy process which may vary from plant to plant and the technique applied directly impacts the

functional properties of these proteins obtained at the end (Alibhai, Mondor, Moresoli, Ippersiel, & Lamarche, 2006). The soybeans are cleaned, tempered, and then cracked and hulled. The hulls are used to produce animal feed whereas the remaining part is then turned into flakes. Oil is extracted through these flakes via solvent extraction and after the removal of the solvent, the flakes are dried and cooled. Following milling and classification, the resulting flour (~50% protein) is either sold as is or sent for further processing to produce soy protein concentrate and isolate (Johnson, White, & Galloway, 2008).

The production of soy protein isolate (SPI) differs from the production of soy protein concentrate (SPC) and the traditional method of producing SPI involves removal of the soluble carbohydrates using a mild alkali or water. Proteins are then separated from the insoluble carbohydrates using isoelectric precipitation, filtration, and centrifugation. The resulting protein curd is washed, neutralized, and dried to obtain SPI. More novel approaches to obtain these soy proteins involves membrane processing using reverse osmosis and ultrafiltration (Ma, 2015). Table 1.1 shows the composition of SPI (Endres, 2001).

Table 1.1. Percentage composition of soy protein isolate on wet and dry basis (Endres, 2001)

Constituent	As is	Moisture-free basis
Protein (N x 6.25)	90-92	90-92
Fat (per. ether)	0.5-1.0	0.5-1.0
Crude Fiber	0.1-0.2	0.1-0.2
Soluble Fiber	<0.2	<0.2
Insoluble Fiber	<0.2	<0.2
Ash	3.8-4.8	4.0-5.0
Moisture	4-6	
Carbohydrates (by difference)	3-4	3-4

1.1.2 Functional Properties of Soy Proteins and Their Usage in the Food Industry

Soy proteins mainly exist as globular storage proteins which mostly consist of two proteins know as glycinin and β-conglycinin (Catsimpoolas & Ekenstam, 1969; Koshiyama & Fukushima, 1976). Since they account for 90% of the total protein content, their functional properties are well researched and focused on. These properties include gelation, solubility, viscosity, emulsification, foaming, fat absorption, flavor-binding and thermal stability. Although soy proteins are well-known for having nutritional and health benefits including having high nutritional protein value which is equivalent to milk casein and egg proteins compared to other plant proteins (Hughes, Ryan, Mukherjea, & Schasteen, 2011), promoting muscle health (Candow, Burke, Smith-Palmer, & Burke, 2006; Deibert et al., 2004), controlling appetite (Neacsu, Fyfe, Horgan, & Johnstone, 2014), preventing cancer in certain cases (Badger, Ronis, Simmen, & Simmen, 2005) and improving cardiovascular health (Jenkins et al., 2010). They are mainly used in the food industry for its functional properties.

1.1.2.1 Solubility

Solubility is probably the most important functional property of soy proteins for which it is used in the food industry, and it also influences other properties such as gelation, emulsification, and foaming. Thus, soy protein quality can be directly described by its solubility. A protein's solubility can be described as the amount of protein in the liquid phase as compared to the total amount of protein in liquid and solid phase in equilibrium. Alternatively, it is defined as the amount of protein dissolved in a liquid that is collected after centrifugation of a protein solution (Pelegrine & Gasparetto, 2005). Solubility of soy proteins are affected by several factors including the production method of soy proteins and their storage, temperature, pH and ionic strength (Lee, Ryu, & Rhee, 2003; Shen, 1976).

Solubility of the soy proteins was found to be the lowest around its isoelectric point (pI) of pH 4.5. In a study it was found that a mixture of soy protein fractions showed the lowest solubility around pH 4-6 whereas certain fractions of proteins showed higher solubility compared to the native soy protein. Overall, away from the isoelectric point, the solubility of the soy proteins increased and was higher at pH 2-4 and even higher at pH 8-10, thus showing that a more alkaline pH favors the solubility of soy proteins. (Bian et al., 2003). At the pI, the proteins tend to have a zero net charge and there are strong attractive forces between which leads to insolubility. When a solubility vs. pH is plotted, it can be observed as a U-shaped curve (Zayas, 2001).

Solubility of proteins is affected by salt concentration in a solution with salt generally enhancing the solubility at low concentrations by increasing the activity of the solvent while simultaneously decreasing the free electrostatic energy of the proteins. This leads to an increase in the available water for proteins to bind with and is known as "salting-in". "Salting-out" refers to a phenomenon where salt interacts with water

at a higher rate, decreasing the water available for proteins (Mojica, Dia, & De Mejía, 2014a). With increasing concentration of a monovalent salt (NaCl), the soy proteins showed a salting out effect initially, which levelled off after a certain concentration of salt was reached. Unlike NaCl, increasing concentration of $CaCl_2$ had a salting out effect initially but after a certain concentration, it showed a salting-in effect (Lee et al., 2003).

Soy proteins are generally known to be more stable to heat compared to animal proteins. They have the ability to maintain their solubility up to 70-80°C. In certain cases, the solubility of proteins was observed to be increase. As temperature was increased and the proteins become denatured and were unfolded into a straight chain, allowing for interactions between protein and water to increase (Lee et al., 2003). It was also seen that increasing temperature to a high value of 120°C in an alkaline environment, increased protein solubility but this high temperature affected other functional properties due to disaggregation (Wang & Zayas, 1991).

1.1.2.2 Water Adsorption and Binding

The ability of proteins to interact with water can be expressed by different terms such as water hydration and holding, water binding, water absorption, water retention and other such similar terms. The terms are usually used in different context. Water holding capacity can be defined as ability of soy proteins to hold its own and added water after a certain force is applied to it. Retained water in the system can be described as water retention or absorption. However, all these terms are used to describe the interactions between water and proteins and the ability for these proteins to hold water in its three-dimensional structure. This ability is important for the food industry because the information regarding it, directly affects the formulation of

foods. It also allows for determining and developing the necessary packaging to maintain the moisture of the product (Zayas, 2001).

Soybean proteins have a good ability to bind to water and retain it. However, several factors affect this property including ionic strength, pH, temperature, type of salts and protein structure. It is also affected by processing technique and storage (Damodaran, 2007). It was reported that soy protein's water holding is directly linked with its denaturation as well. The more the protein unfolds, the more side chains are exposed to surface and the interactions with water increasing, thus increasing the water binding (Nguyen, Mounir, & Allaf, 2015).

1.1.2.3 Oil and Fat Binding

The importance of binding to fat depends on the type of food which directly affects the texture of the food. This property of proteins is important in many food formulations that consist of emulsions, fat entrapment in batters for sausages and cakes, preparation of dough, flavor absorption and fat emulsification in meats. This property of proteins is not as well studied as water-binding and is mainly explained by the physical entrapment of fats by proteins (Zayas, 2001).

Fat binding is affected by the size of protein powder particles. Powders with a lower density and smaller particles size manage to entrap more oil compared to high-density powder. Later it was explained by the ability of the nonpolar side chains on proteins molecules. More insoluble and hydrophobic proteins are known to have a high fat binding capacity (Kinsella, 1979).

Fat binding of soy proteins is known to be affected by factors such as processing conditions, concentration of protein, size of protein powder, their hydrophobic properties as well as the properties of oil. While denaturation may expose more nonpolar amino chains, hydrophobic domains may also get destroyed while proteins is being denatured (Zayas, 2001).

1.1.2.4 Gelation

Food gels are quite common in the food industry and can be produced in several different ways, including using plant and animal proteins. These gels involve either crosslinks between proteins or polysaccharides, forming strong gels or particulates aggregating together to form weaker gels (Nazir, Asghar, & Aslam Maan, 2017). Apart from water, gels may also be used to entrap essential oils, flavors and sugars.

Since soy proteins contain globular proteins, they form weaker gels and the gels are formed by a series of reactions involving denaturation, dissociation-association and finally aggregation (Hermansson, 1986). For the soy protein gel to be formed, the protein concentration needs to reach a critical value of around 6.6%. Below this concentration, the proteins tend to precipitate out (Bikbow, Grinberg, Antonov, Tolstoguzov, & Schmandke, 1979). Above this concentration, the gel strength increases, and texture improves. However, there are a few other factors that affect the gel structure including the method of gelation, ionic strength, pH, presence of other components such as sugars and lipids.

As discussed earlier, globular proteins need to be denatured to form gels later. Different components of soy proteins, namely β-conglycinin and glycinin, gel at different temperatures. β-conglycinin forms a gel at a lower temperature of about

70°C whereas glycinin forms a gel at temperature of about 80-90°C (Nagano, Akasaka, & Nishinari, 1994).

Ionic strength and pH are other factors affecting the formation of gels. It was found that addition of salt in small amounts resulted in a gel with a fine stranded network of proteins. However, after a certain concentration of salt was crossed, the gel ended up being particulate and turbid (Puppo & Añón, 1998). Within a pH range of 3-6, soy protein gels were more coarse and stronger while gels with a pH from the isoelectric point of soy proteins (pH ~ 4.6), gels were finer-more stranded. A combined effect of all the factors was seen on gel strength (Shan et al., 2015).

1.1.2.5 Emulsification

Emulsification is another important property due to which proteins are utilized in the food industry. Food emulsions can be mostly described as macroemulsions with droplet size changing in the range of 0.2 -50 µm. Most food emulsions are either oil in water (O/W) or water in oil (W/O) emulsions, where O/W generally has a creamy texture while W/O has a greasy texture (Zayas, 2001). Proteins not only participate in emulsion formation but also aid in stabilizing the emulsions due to their amphiphilic nature. This property of proteins is affected by environmental factors such as pH, ionic strength, temperature and the presence of other compounds (Matsumura, Matsumiya, Kannan, Hettiarachchy, & Marshall, 2012).

Soy proteins are known to have high emulsifying properties compared to other plant proteins (Zayas, 2001). They mainly form emulsions by reducing the interfacial tensions between water and oil. Their emulsification activity is directly linked to their solubility and surface hydrophobicity. Furthermore, they also aid in stabilizing

the emulsion by creating a physical barrier at the interface. Emulsion stability is determined by the molecular flexibility of the proteins in the soy (Molina, Papadopoulou, & Ledward, 2001).

1.1.2.6 Foaming

Some proteins have good foaming properties. These properties are determined by protein's ability of rapid adsorption on the air-liquid interface during whipping, also by its capability to form a cohesive viscoelastic film through intermolecular interactions (Lomakina & Míková, 2006). Foams can be described as two-phase systems where air cells are divided by a continuous thin liquid layer known as the lamellar phase. The size of these air cells has a direct impact on the texture of food, more specifically the body, smoothness, and lightness of the food product. Good foaming agents have the ability to form stable foams quickly over a wide pH range and in the presence of foam inhibitors (Zayas, 2001).

Native soy protein has limited foaming ability, but modified SPI shows improved foaming ability due to increased surface activity after heat treatment. Thus, it can be said that denatured proteins show better foaming ability (Shao, Lin, & Kao, 2016).

1.1.2.7 Usage of Soy Proteins

Owing to their good functional properties, soy proteins are commonly used in the food industry. Usages of soy proteins according to the functional property and soy protein product is given in Table 1.2.

Table 1.2. Functional requirements of soy protein in different food products (Kinsella, 1979)

Functional Property	Mode of Action	Food System Used	Product*
Solubility	Protein solvation, pH dependent	Beverages	F,C,I,H
Water absorption and binding	Hydrogen-bonding of water, entrapment water (no drip)	Meats, sausages, breads, cakes	F,C
Viscosity	Thickening, water binding	Soups, gravies	F,C,I
Gelation	Protein matrix formation and setting	Meats, Curds, Cheeses	C,I
Cohesion-adhesion	Protein acts as an adhesion	Meats, sausages, baked, baked goods, pasta products	F,C,I
Elasticity	Disulfide links in deformable gels	Meats, bakery item	I
Emulsification	Formulation and stabilization of fat emulsions	Sausages, bologna, soups, cakes	F,C,I
Fat Absorption	Binding of free fat	Meats, sausages, doughnuts	F,C,I
Flavor-binding	Adsorption, entrapment	Simulated meats, bakery items	C,I,H
Foaming	Forms film to entrap gas	Whipped toppings, chiffon, desserts, angel cakes	I,W,H
Color Control	Bleaching (lipoxygenase)	Breads	F

*F, C, I, H and W are soy flour, concentrate, isolate, hydrolysate, and soy whey, respectively.

1.1.3 Modification of Soy Proteins

Modification of soy proteins is a growing research area and a lot of research has been carried out to modify the physiochemical properties of these proteins with application of processing technologies as well as enzymatic and chemical reactions. Although soy proteins exhibit excellent functional properties, these modification techniques have known to considerably improve them. These techniques include physical technologies like ultrasound, microfluidization, ultrafiltration and high hydrostatic pressure (HHP) and chemical enhancements by the means of acylation, acid treatment, phosphorylation, oxidation as well as enzymatic hydrolysis and Maillard reaction (Mojica, Dia, & De Mejía, 2014b).

Application of membrane technology and performing ultrafiltration to obtain SPI with low phytic content resulted in an enhancement of the solubility property (Lai et al., 2013) while the application of ultrasound also improved solubility of these proteins as well as gelling ability (Hu et al., 2013; Jambrak, Lelas, Mason, Krešić, & Badanjak, 2009). The emulsifying property was also found to improve after microfluidization was applied to emulsions made from native and preheated SPI (Peng, Xu, Liu, & Tang, 2018) and in another study by acylation of these proteins with saturated fatty acids (Matemu, Kayahara, Murasawa, Katayama, & Nakamura, 2011). Other chemical treatment such as using acid treatment improved surface and foaming properties (Ventureira, Martínez, & Añón, 2012) and phosphorylation also significantly improved functional properties such as solubility, emulsification and viscosity (Zhang, Li, & Ren, 2007). Enzymatic hydrolysis and reaction such as glycosylation by the use of microbial enzymes have also been used to improve these properties (L. Chen, Chen, Ren, & Zhao, 2011; Gan, Latiff, Cheng, & Easa, 2009; Song et al., 2018).

1.2 Glycation as a Method to Improve Functional Properties of Soy Proteins

1.2.1 Maillard Reaction

Maillard reaction's story goes back as far as 1912 where Louis-Camille Maillard made the astute observation that when sugars and proteins are heated, a yellow-brown color is developed (Maillard, 1912). Years on, Maillard reaction remains as one of the most well-researched topics in food science. Hundreds of thousands of research studies have been carried out on Maillard reaction and it is still widely focused on.

Maillard reaction can be described as a spontaneous reaction that takes place between carbonyl groups on reducing sugars and with the nucleophilic amino groups on amino acids, peptides, and proteins. The reaction takes different pathways and results in hundreds of compounds that provide different flavors and odors as well as different functional properties and change the nutritional value of food (Dworschák & Carpenter, 1980). The reaction has been described as very complex by Hodge who used the scheme given in Figure 1.1 to show the reaction pathways (Hodge, 1953).

The reaction is known to have three stages which are namely, early, intermediate and final stage (Mauron, 1981). In the early stage, carbonyl and amine group react and go through a condensation reaction followed by the formation of a Schiff base and then Amadori rearrangement products. In the intermediate stage, the Amadori products go under different process such as sugar dehydration and fragmentation, and amino acid degradation. The final stage is where the aldol and aldehyde-amine condensation happen which leads to formation of heterocyclic nitrogen compounds. Color development occurs in the final stage as well (Nursten, 2005).

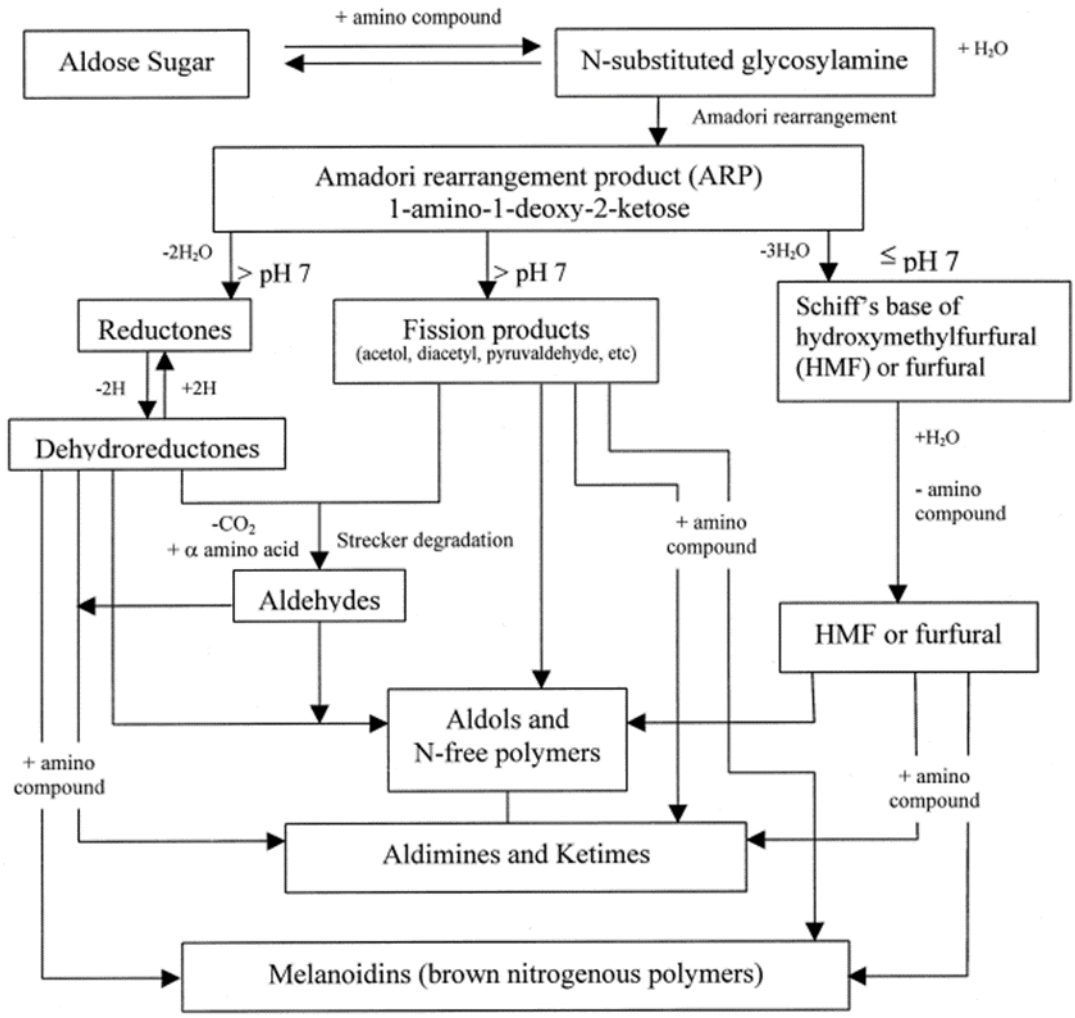

Figure 1.1. Hodge's Maillard Scheme (Hodge, 1953)

1.2.2 Glycation

One of the ways to modify proteins is to have them undergo the glycation reaction which is part of the early stage of Maillard reaction. Glycation is a nonenzymatic process which involves the covalent attachment of amine groups on amino acids to the reducing end on sugars. Initial glycation products include the Schiff base and

their consequent rearrangement to Amadori products. The crosslinks between these Amadori products to other proteins result in polymeric aggregates known as advanced glycation end (AGE) products (Friedman, 1996).

Changes in protein structure when combined with carbohydrates, have known to have desirable effects on the functional properties of these proteins. However, this is dependent on the extent of the reaction as well. If the reaction is not well-controlled, the formation of AGEs can take place which may have direct negative effects on the human health such as promoting diabetes and kidney-related diseases. Thus, it is important for the reaction to be well controlled. (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016; Uribarri et al., 2010).

The type of products obtained are not only influenced by the duration of the reaction but also pH, temperature, ratio of the protein-sugar, water activity and type of the reactants. (Oliver, Melton, & Stanley, 2006). Several studies have proven a significant improvement in the functional properties of properties such as increasing solubility (Katayama, Shima, & Saeki, 2002; Shepherd, Robertson, & Ofman, 2000), improved emulsifying properties (Li et al., 2013; Liu, Zhao, Zhao, Ren, & Yang, 2012), improved thermal stability (Álvarez, García, Rendueles, & Díaz, 2012; Zhu, Damodaran, & Lucey, 2010), enhanced foaming (Zhu, Liu, Fu, & Zhao, 2016) and gelling abilities (Spotti et al., 2013). Apart from improving these functional, many studies also reported benefits such as improving antioxidative, anti-hypertensive, antigenicity and antimicrobial properties as well (Gu et al., 2010; Guan et al., 2010; Huang et al., 2012; J. et al., 2011; Rufián-Henares & Morales, 2007).

1.2.3 Glycation Methods

Glycation is usually carried out on a lab scale under either dry or wet conditions using conventional heating. The dry method involves lyophilization of a mixture of protein and sugar and then keeping it under specific temperature and relative humidity (RH) for a prolonged period that may last days or weeks which may be considered as one of its biggest disadvantages. The glycation reaction is this case is limited by irregular contact between the reactants. In the case of rigid and folded proteins, it may end up in an uneven mixture of free proteins, carbohydrates and protein-sugar conjugates (Li et al., 2013; Zhuo, Qi, Yin, Yang, & Zhu, 2013). After drying the protein-sugar mixture, it is kept at temperature ranging between 40 to 80°C where 60°C is the most common setting. The RH is set at 65% to 79% and the reaction is allowed to proceed for hours or even days, after which the samples are collected and stored in a cool place for further characterization of the proteins (de Oliveira et al., 2016).

On a large scale, it is not very feasible to use the dry glycation method since the reaction time can reach a number of weeks and the experiment requires a lot of control, and thus from an industry point of view, it makes it a very costly method (D. Zhu, Damodaran, & Lucey, 2010). The wet glycation method involves forming an aqueous solution of proteins and sugars using a buffer solution. The solution is then heated at a certain temperature and the mixture is dried to collect the proteins. It is a relatively quicker process, however, it is difficult to dry the mixture as well as remove the buffer (Li et al., 2013; Zhuo et al., 2013). An optimum water activity of 0.5-0.8 is required for the reaction to happen in a desirable way. The reaction rate is slower in an aqueous solution since the concentrations of the reactants are low (Boekel, 2009). In order to achieve the dry mixture at the end, lyophilization may be applied. However, it is not an industrially practical process so processes such as roller-drying or spray-drying have also been suggested and used on a lab scale (Oliver et al., 2006; Tas, 2019). To heat the mixture, microwave heating has also

been used to obtain glycation of proteins. The method has been used to heat an aqueous solution of protein and sugars using microwaves, followed by lyophilization of the solution to obtain the glycated proteins (Bi et al., 2015; J. J. Guan, Qiu, Liu, Hua, & Ma, 2006; Namli, 2019).

Other novel glycation methods have also been used on a lab scale such as applying high hydrostatic pressure (C. H. Xu, Yu, Yang, Lin, & Zhao, 2010), electrospinning (Kutzli, Gibis, Baier, & Weiss, 2019; Turan, Gibis, Gunes, Baier, & Weiss, 2018), pulsed electric field (W. W. Sun, Yu, Zeng, Yang, & Jia, 2011; Z. Wang, Wang, Guo, Ma, & Yu, 2013) and ultrasound treatment (W. Chen et al., 2019; Fu et al., 2019; Perusko, Al-Hanish, Velickovic, & Stanic-Vucinic, 2015).

1.3 Characterization of Glycated Soy Proteins

1.3.1 Degree of Glycation

A conventional approach to find out the extent of the glycation reaction or to find the degree of glycation, is to check the amount of free amino groups present in the food system. In other words, it is the free amino groups present in the lysine that react with reactive carbohydrates that result in the formation of glycated proteins (Aalaei, Rayner, & Sjöholm, 2019).

There are several methods that may be used to find out these free amino groups. One of the more common and reliable methods is known as the OPA (O-phthalaldehyde) method. Developed in 1981 at University of Carolina, it is a method used to measure the availability of lysine. It is based on the reaction of free amino groups on proteins with the OPA compound in the presence of reduced sulfhydryl groups such as those

in β-mercaptoethanol. The reaction resulted in the production of a fluorescent substance (Figure 1.2), the fluorescence intensity of which was then measured. A linear relationship between the fluorescence intensity and the lysine content, among different proteins including Lysozyme, Ovalbumin, κ-Casein, Bovine Serum Albumin, β-Lactoglobulin and α-Lactalbumin, was established (Goodno, Swaisgood, & Catignani, 1981).

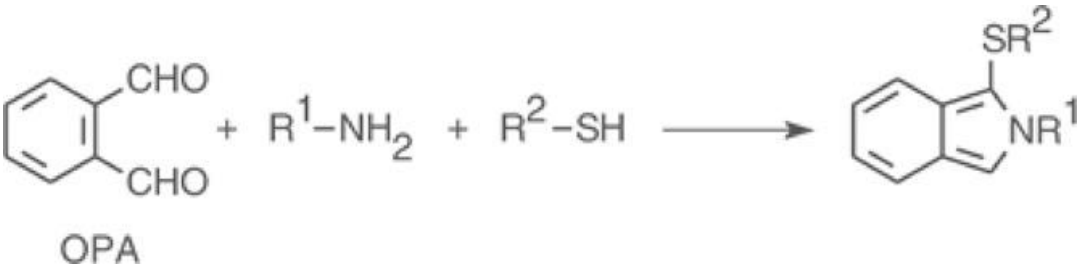

Figure 1.2. Reaction of O-phthalaldehyde with SH-group containing compound and free amino groups on proteins (de Oliveira et al., 2016)

The method has been developed further by different scientists over the years to improve its reliability by checking food products containing both animal and plant proteins. Since fluorometer is not easily available in all labs, work was done on the method so that it may also be used with a spectrophotometer (Vigo, Malec, Gomez, & Llosa, 1992).

The OPA method is affected by factors such as pH and the accessibility and availability of lysine residues in native proteins. It works well around the pH of 6-9 in buffer solutions; however, they should not contain any amines. (Held, 2006). The reaction is very quick and sensitive, even down to nanograms and it is also more convenient to use over other methods that involve the use of fluorescamine or ninhydrin, 2,4,6-trinitrobenzenesulfonic acid (Church, Swaisgood, Porter, & Catignani, 1983).

1.3.2 Protein Solubility

As discussed earlier, protein solubility is an important functional property of proteins and directly affects their performance in improving food systems. While most methods are more sensitive to the amino acid composition in different proteins, Lowry Method is fairly constant from protein to protein, and thus used more commonly (Waterborg & Matthews, 1996).

Lowry Method was developed in 1951 and involves the usage of Folin phenol reagent (Folin-Ciocalteau reagent) to find out the amount of soluble proteins in a solution (Lowry, Rosebrough, Farr, & Randall, 1951). It is a two-step process which is based on the biuret reaction which involves formation of a complex of cupric ions and proteins, and the subsequent amplification of this reaction with the Folin phenol reagent (Folin & Ciocalteu, 1927). This reaction results in the formation of a strong blue color, the strength of which is directly dependent on the tyrosine and tryptophan content of the proteins (Sapan et al., 1999). The absorbance of the sample is then read via a spectrophotometer. The method is sensitive down to 0.01 mg protein/ml and is appropriate for solutions containing 0.01-1.0 mg protein/ml solution (Waterborg & Matthews, 1996).

Although the reaction is simple and the method is easy to perform, it is affected by several factors. Complex food matrices contain several compounds other than the aromatic amino acid that react with the Folin phenol reagent, giving an overestimate of the protein content (Everette et al., 2010). Furthermore, it is also affected by presence of salt, which result in giving an overestimate of both animal and plant proteins (Mæhre, Dalheim, Edvinsen, Elvevoll, & Jensen, 2018). The method is also very photosensitive and requires to be performed in the dark (Dawson & Heatlie, 1984).

1.3.3 Sugar Analysis by High Performance Liquid Chromatography

Since glycation is a process that involves a reaction between the reducing sugars and proteins, an analysis on the composition of the initial and final reducing sugar amount can be considered as an indicator for the extent of glycation. The conventional method for determining the reducing sugar content are the DNS method (Miller, 1959) or the Somogyi-Nelson method (Smogyi, 1952). Both methods require considerable time and are prone to empirical errors. Other analysis methods to find the reducing sugar composition include gas chromatography (Ruiz-Matute, Montilla, del Castillo, Martinez-Castro, & Sanz, 2007), near-infrared spectroscopy (He, Wu, Feng, & Li, 2007), high performance liquid chromatography (HPLC) (Kakita, Kamishima, Komiya, & Kato, 2002; Ouchemoukh, Schweitzer, Bachir Bey, Djoudad-Kadji, & Louaileche, 2010; Sims, 1995) and thin layer chromatography method (Baron & Economidis, 1963).

Initially known as high-pressure liquid chromatography, HPLC techniques have been considerably developed over the years, and thus the abbreviation remains the same, but the name has been changed to high-performance liquid chromatography. It is an instrumental method that is used to identify a certain unique compound from a mixture of compounds (Crowley, 2020). HPLC is considered as one of the most promising methods in determining the sugar composition due to its efficiency, availability, and selectivity. It requires easy sample preparation and provides accurate results (W. Xu, Liang, & Zhu, 2015). Although there are a variety of separation mechanisms, HPLC measurement for carbohydrates is further divided into high performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD), high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and high-performance liquid chromatography-refractive index detector (HPLC-RID). HPLC-ELSD is known to have high sensitivity but works well at only lower concentration of sugars (Bhandari, Kumar, Singh, & Kaul, 2008). HPAEC-PAD is a technique that can be used for

measuring oligosaccharides (Morales, Corzo, & Sanz, 2008) but has poor detection stability and may require frequent change of electrodes used in the equipment. HPLC-RID is a reliable method to measure the amount of reducing sugars present and produces accurate results. Thus, it is widely used for detection of carbohydrates (Barreira, Pereira, Oliveira, & Ferreira, 2010).

1.3.4 Time Domain Nuclear Magnetic Resonance (TD-NMR) Relaxometry

TD-NMR relaxometry is a noninvasive technique which is being increasingly used on a lab and industrial scale using low-field NMR systems, due to their cost-effectiveness, convenience, and robustness. Apart from research and development on a lab scale, it is also used in food supply chain for process and quality control purpose. It allows for testing and hypothesis generation to be done in a short time (van Duynhoven, Voda, Witek, & Van As, 2010).

TD-NMR is dependent on the innate ability of charged particle i.e. free protons, in a nucleus to spin in the direction of a magnetic field when placed in it; in other words, nuclear magnetism. In a bench-top NMR system, which is usually low-field (magnetic field ranging between 10 mT to 1 T) NMR instrument, a magnetic field is generated between a coil by applying a radio frequency (RF) pulse. Most of these instruments focus on the ^{1}H (proton) spins since they are available in an abundant amount in most food systems and their large gyromagnetic ratio results in provision of a detectable magnetization (Mitchell, Gladden, Chandrasekera, & Fordham, 2014). Initially, the sample is placed in a static magnetic field where most of these protons align themselves in the direction of the magnetic field (along the longitudinal (z)-axis) which results in a net magnetization. By using the RF pulse, the proton spins are flipped into a newly introduced magnetic field in a x-y plane which depends on

the angle of flip. When the pulse is removed, the protons relax back to their original position in the external static magnetic field. This results in two types of signal to be generated, one is the T_1 relaxation time of the spin-lattice relaxation time and the second is the T_2 relaxation time, also known as the transverse relaxation time or the spin-spin relaxation time. T_1 relaxation time is the time required for the spins to return to their original position whereas T_2 relaxation time is the time it takes for the magnetization in the x-y plane to decay (Brown & Semelka, 2003).

These relaxation times are highly dependent on the structure and composition of food, and thus vary according to food type. Their relaxation differences are mainly due to the differing molecular mobility (Guthausen, 2016). NMR relaxometry and spectroscopy has been used for analysis of food components including water, fats and proteins, during processing and packaging, for quality control purposes, to perform conformation analysis in polysaccharides and to understand the nutritional aspects in certain foods (Marcone et al., 2013).

TD-NMR is a fast and accessible tool to analyze the water populations present in food. Water may exist in foods in various physical forms, but chemical differences are hardly seen, and these differences are not noticeable in spectral data. T_2 relaxation times of water in a food system depends on its concentration and the rotational mobility of the protons in them. This information can be used to identify different water populations within a proteinaceous sample, and thus giving an idea about the water binding ability of proteins (Alonso, 2018). Several studies have managed to correctly identify the hydration behavior of different proteins including SPI and glycated SPI and relate them to T_2 relaxation time. These times were shown to decrease for a protein with better water-binding ability (Dekkers et al., 2016; Namli, 2019; Peters et al., 2016; Peters, Vergeldt, Boom, Jan, & Goot, 2017; Tas, 2019).

1.3.5 Fourier Transform Infrared Spectroscopy

Fourier-transform infrared spectroscopy (FTIR) is a method used to obtain an infrared emission or absorption spectrum of a solid, liquid or gas. FTIR spectrometer collects data of high spectral resolution over a wide spectral range (Faix, 1992). Infrared radiation can be largely regarded as thermal energy which induces stronger molecular vibrations in covalent bonds. The fundamental principle is that the bonds between different components absorb light at different frequencies (Rahmah et al., 2016). The light is measured using an infrared spectrometer which produces the output of an infrared spectrum. The output signal is converted by using 'Fourier transform' to map the information of the spectrum to the computer (Oyerinde & Bello, 2016).

Many organic compounds such as proteins, carbohydrates, lipids, glycolipids, and nucleic acids structures can be analyzed by using this tool. Furthermore, FTIR is an accurate, non-destructive, sensitive, and rapid method that detects the functional groups of a sample to define its chemical composition. FTIR is also one of the most effective techniques for obtaining information on especially protein conformation due to the unique hydrogen bonding of C = O and N-H groups for each type of structure (Long et al., 2015). Moreover, Infrared (IR) spectroscopy is a potential tool to study glycated proteins (Huang, Liao, Wang, & Lin, 2016). The spectrum can be interpreted by dividing it into 3 different regions which are near infrared region (IR) (12800~4000cm^{-1}), mid-IR (4000~200cm^{-1}) and far-IR (50~1000cm^{-1}) (Kulea, 2014). The sugar residue, amide protein group and water all produce distinguishable bands of absorption in the mid-IR range and carbohydrates can be characterized by using IR spectroscopy.

As can be noted, the FTIR spectroscopy analysis provides detailed information about whether the sample includes a specific functional group by simply looking at the

peak within the broad range of spectrum graph. Therefore, it is commonly used for chemical analysis with high accuracy and reliability (Naczk & Shahidi, 2004).

1.4 Objectives

As discussed earlier, the soy protein is commonly being used for several purposes in the food industry. However, the use of glycated soy proteins or any other glycated proteins is not common due to lack of proper production methods. Both the conventional and novel methods rely on instruments that are costly, and the processes are lengthy and time consuming. Thus, these methods are not industrially feasible for mass production. Furthermore, there is no study in the literature that focuses on a study to simplify the glycation method.

The primary objective of this study is to develop a method to glycate soy protein isolate with different monosaccharides, that tries to overcome limitations of time and cost of the methods that already exist. To measure the method's efficiency, these glycated proteins are to be characterized physiochemically by observing their solubility, browning index and free amino group amount, sugar amount, hydration behavior and structural bonds.

Allulose was used in addition to glucose and fructose to compare their effects as allulose is classified as a rare sugar with several potential health benefits in addition to improving glycated proteins' properties (Tas, 2019).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Commercial soy protein isolate (90% protein content in dry weight) was purchased from Alfasol, Turkey. Dextrose was purchased from Smart Kimya Tic., D-fructose was purchased from Merck KGaA and D-allulose was purchased from Santiva Inc. Downers Grove, IL, USA. All chemicals and reagents were purchased from Sigma-Aldrich and were of analytical grade.

2.2 Methods

2.2.1 Preparation of Glycated Proteins

2 ml and 4 ml of distilled water (pH made up to 10 using 0.1N NaOH) were added to 0.5g of sugar (dry solids) respectively. 2 g of soy protein isolate (SPI) was added to this solution in a petri dish and well mixed. The samples were covered and left in an incubator at 25°C for 24hrs to ensure hydration of the proteins. Following day, each type of sample went under three different glycation methods. They were heated at 100°C using a water bath and 130°C using an oil bath for 15 minutes each and then cooled immediately. Samples were also kept in an incubator set at 50°C and 55 %RH (relative humidity) for 24hrs to allow glycation to occur. The schematics given in Figure 2.1 represents the preparation of glycated proteins.

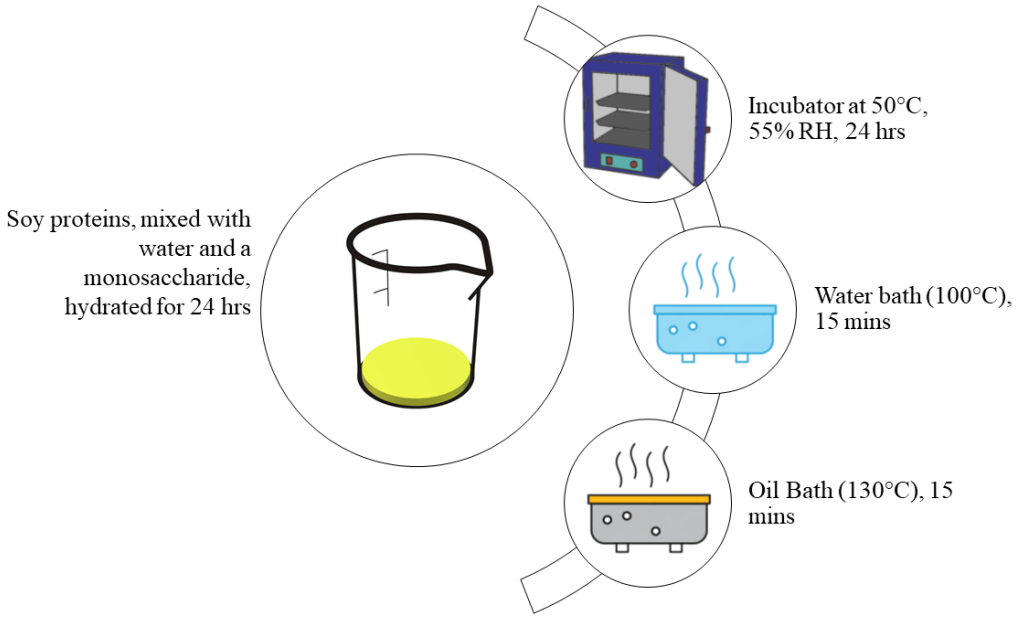

Figure 2.1. Preparation of glycated soy proteins

Following glycation, the samples were grinded using a grinder and used for analysis. 0.1g of glycated samples was added to 10 ml of distilled water and mixed using a shaker (Daihan Scientific Co., Ltd., Korea) for 24hrs. Following day, the sample solutions were further mixed by using a high shear homogenizer (UltraTurrax, WiseTis Homogenizer, Wieteg Labortechnik GmbH, Germany) at an rpm of 16,000 for 2.5 minutes. The solutions were then centrifuged (MF-80, Hanil Science Industrial Co. Ltd., South Korea) at 4000 RPM for 5 Minutes. The supernatant was collected and used for further analysis in determining degree of glycation, browning index and protein solubility.

2.2.2 Measurement of Reducing Sugar Composition

In order to measure the sugar composition in the glycated proteins, 0.5 g of solid sample was dissolved in 9 ml of double distilled water (DDW) (Milli-Q Water System, Millipore S.A., France). The resulting solution was kept in a shaker (Daihan Scientific Co. Ltd., Korea) overnight to hydrate the proteins properly. Two solutions, Carrez I and Carrez II were prepared to add to the protein solutions the following day. Carrez I was prepared by adding 15 g of potassium hexacyanoferrate (II) trihydrate to 100 ml of DDW while Carrez II was prepared by mixing 30 g of zinc sulfate heptahydrate in 100 ml of DDW. 0.5 ml of each Carrez solution was added to the protein solutions. Following centrifugation of these protein solutions, the supernatant was transferred to small vials after filtering through 0.45 µm nylon filters to be further used for HPLC analysis using an HPLC system (Shimadzu Scientific Industrial Co. Ltd., South Korea). The mobile phase was initially filtered under vacuum using a 0.2 µm membrane. Flow rate was set at 1 ml/min whereas injection volume and oven temperature were set at 20 µl and 40°C respectively.

A calibration curve was also prepared using sugar solutions prepared with concentrations including 5, 8, 10, 15 and 20 g/l. The calibration curves are given in the appendix A (Figure A.3., 4., 5.)

2.2.3 Measurement of Soluble Proteins

Modified Lowry method (Sun, Hayakawa, & Izumori, 2004) was used to measure protein solubility in the supernatant collected earlier. Initially three different reagents, Regent 1, Reagent 2 and Reagent A were prepared which were then mixed at a ratio of 1:1:100 to prepare Lowry Reagent. A Folin-Ciocalteu phenol reagent

was also prepared by mixing 2N commercial stock solution with distilled water in a 1:1 ratio. The contents of all the reagents used are given in Table 2.1.

Table 2.1. Reagents of Lowry Method

Reagent A:	2% Na_2CO_3 dissolved in 0.1 N NaOH
Reagent 1:	2% $CuSO_4.5H_2O$, Copper source
Reagent 2:	2% Na-K Tartarate
Lowry Reagent:	Mix of Reagent A:1:2 with a ratio of 100:1:1
Folin and Ciocalteu's Phenol Reagent:	Diluted 2N stock solution as a ratio of 1:1 with distilled water
Bovine Serum Albumin:	20 ml 1 mg/ml BSA stock solution

0.5 ml of supernatant was added to 2.5 ml of Lowry reagent in test tubes and kept in dark at room temperature for ten minutes after mixing. 0.25 ml of Folin-Ciocalteu phenol reagent was then added to these tubes and following mixing, stored in the dark at room temperature for 30 mins. The absorbance of the samples was measured at 750 nm using a UV-VIS spectrophotometer (Optizen POP Nano Bio, South Korea). The results were compared with a calibration curve prepared earlier.

The calibration curve was prepared using bovine serum albumin with varying concentrations of 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1.0 g/l. The curve is given in appendix A (figure A.1).

2.2.4 Determination of Degree of Glycation

A modified OPA Method (Zhuo et al., 2013) was used to measure free amino group content. 80 mg of OPA (ortho-phtalaldehyde) was mixed with 2 ml of 95% Ethanol Solution. The resulting solution was added to 50 ml of a 0.1 M borax buffer, 200 µL β-mercaptoethanol and 5 ml of 20% (w/v) sodium dodecyl sulphate (SDS) solution. The solution was then replenished to 100 ml by using distilled water. 0.5 ml of the supernatant collected earlier was then mixed with 1.5 ml of the prepared OPA reagent and left in the dark for two minutes. Absorbance of the final mixture was measured at 340 nm using a UV-VIS spectrophotometer (Optizen POP Nano Bio, Mecasys Co. Ltd., South Korea). The results were compared to a calibration curve prepared using glycine at varying concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 g/l. The curve is given in the appendix A (figure A.2).

2.2.5 Determination of Browning Index

To determine the degree of browning, absorbance of 2 ml of the supernatant collected earlier was measured at 420 nm using a UV-VIS spectrophotometer (Optizen POP Nano Bio, Mecasys Co. Ltd., South Korea).

2.2.6 Fourier Transform Infrared (FT-IR) Spectroscopy Analysis

The structural bonds in the glycated protein samples were analyzed by using IR Affinity-1 Spectrometer with Attenuated Total Reflectance (ATR) attachment (Shimadzu Corporation, Kyoto, Japan). The absorbance of the samples was measured between 4000-500 cm^{-1} at a resolution of 4 cm^{-1} by applying 32 scans.

2.2.7 Determination of Hydration Behavior using Nuclear Magnetic Resonance (NMR) relaxometry

0.15g of solid sample was taken in small tubes and 0.45ml of distilled water was added to it and well mixed. Spin-spin relaxation times (T_2) were observed for these samples by using a CPMG sequence on a 0.32 T (20.34 MHz) NMR instrument (Spin Track GmbH, Kirchheim/Teck, Germany). T_2 times were measured by CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence. Relaxation period was set to 1100 ms while number of echoes were fixed at 1200 with an echo time of 500 µs. The data was plotted using MATLAB (The MathWorks Inc., USA) considering a mono-exponential behavior.

2.2.8 Statistical Analysis

Statistical analysis on all the results were carried out by using analysis of variance (ANOVA) on the Minitab software (Minitab Inc., Coventry, UK). Tukey's comparison test was used to compare the results at confidence interval of 95%. All data were represented as mean ± standard deviation of three replicates for each experiment. Assumptions of ANOVA (Normality and Test of Equal Variances) were checked and outliers were excluded before further analysis.

2.3 Experimental Design

Table 2.2. Parameters of the experimental design

Factors	Levels	Responses
Glycation Temperature	50°C, 100°C, 130°C	1. Reducing Sugar Composition 2. Soluble Protein Content 3. Degree of Glycation 4. Browning Index 5. Structural changes using Four Transform Infrared (FTIR) Spectroscopy Analysis 6. Hydration Behavior using Nuclear Magnetic Resonance (NMR) Relaxometry
Sugar Type	Allulose Fructose Glucose	
Protein-water ratio (w/w)	1:1, 1:2	

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Analysis of Reducing Sugar Composition

HPLC-RID method was used to measure the remaining reducing sugar amount after glycation of proteins due to its reliability and accuracy. Reducing sugar amount was also measured for native soy protein and it was seen that no sugars were present. Standard sugar solutions were used to determine the concentration of these sugars in glycated protein sample and their retention times were used to identify them. It was observed that after the heat treatment was applied to protein-sugar mixtures, the sugars remained in their original form and did not isomerize. This was confirmed by the HPLC experiment which did not show peaks for any other form of the monosaccharides.

The amount of remaining reducing sugar was given as a percentage of the initial sugar added to the mixture. Figure 3.1 and 3.2 show the percentages of reducing sugar which remained in the sample after reaction was over for three different glycation temperatures with two different protein-water ratios. Statistical results (Table C.1., 2.) are given in Appendix C.

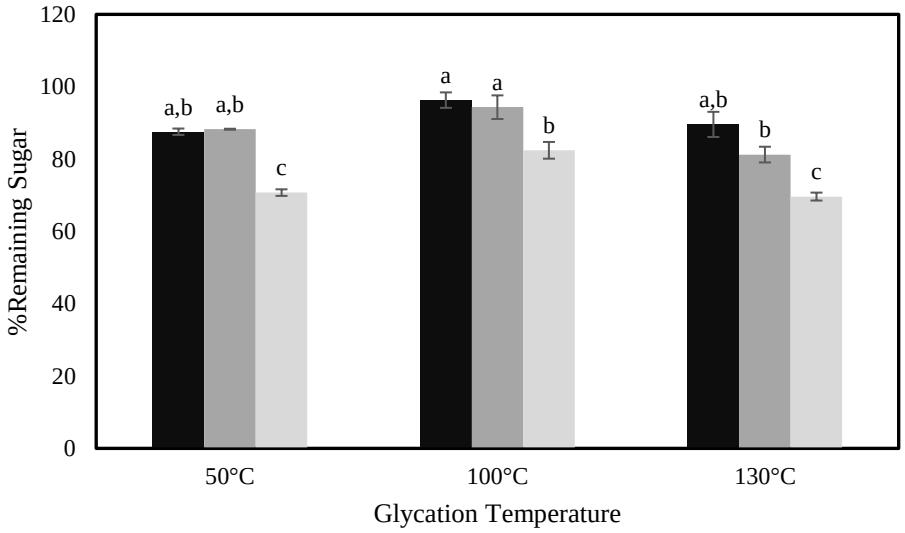

Figure 3.1. % remaining sugar in proteins glycated at different temperatures with allulose (■), fructose (▣) and glucose (□) at a protein- water ratio of 1:1 (w/w)

Figure 3.1 represents results for reaction mixture containing a protein-water ratio of 1:1 (w/w). The amount of remaining sugar changes significantly from sugar to sugar as well as with the glycation temperature overall ($p<0.05$). After incubation of the proteins-sugar mixture at 50°C for one day, proteins with fructose and allulose had significantly higher remaining sugar compared to those glycated with glucose, and similar trend was seen over glycation temperatures of 100°C and 130°C, where allulose- and fructose-glycated proteins had higher amount of remaining sugar ($p<0.05$). Previous studies have reported no significant difference between initial rates of fructose and allulose due to similarity in structure (both are ketohexoses) (Zeng, Zhang, Guan, Zhang, & Sun, 2013), however the glucose has been to known to have a higher reaction rate in the initial stages of Maillard reaction, and thus is consumed faster (Sun et al., 2004). The mean remaining sugar amount is the similar for both glycation with incubation and at 130°C, and lower compared to glycation at 100°C. This is expected as extended time for glycation to occur and higher temperature are factors that influence the reaction, and thus more sugar was depleted (Oliver et al., 2006).

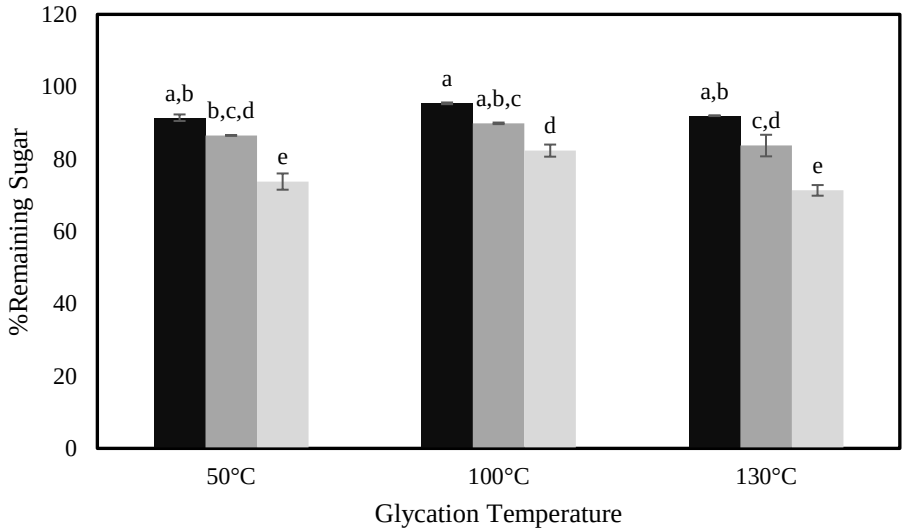

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Figure 3.2. % remaining sugar in proteins glycated at different temperatures with allulose (■), fructose (▩) and glucose (□) at a protein- water ratio of 1:2 (w/w)

The results for reaction mixture containing a protein-water ratio of 1:2 (w/w) are given in figure 3.2. The trend is similar to that seen in Figure 3.1 with proteins glycated with allulose and fructose having significantly higher remaining sugar compared to those with glucose ($p<0.05$). Overall, the mean remaining sugar for different protein-water ratio was not different from each other.

The reaction involving sugars and proteins is a complicated and complex reaction. It can potentially take several different pathways as well as sugar may react on its own and go under reactions such as isomerization and caramelization. The amount of remaining sugar provides necessary, however incomplete information on the amount of proteins that are glycated, and thus results need to be discussed further with other experimental results.

3.2 Measurement of Soluble Proteins

Protein solubility is considered as a necessary property upon which soy protein usage is based on. It was measured using the Lowry method and given as the number of grams of soluble proteins per liter of a glycated protein solution. The amount of soluble proteins was always significantly lower for all glycated proteins when compared with native control protein ($p<0.05$). When hydrated overnight, soy protein's solubility increased and was as high as 3.81 g/l. As they undergo significant changes while glycation, their solubility decreases. However, since this value was significantly very high ($p<0.05$), it was decided not to include the data along with the other experimental data. Figure 3.3 and 3.4 show the protein amount which remained in the supernatant after glycated protein solutions were centrifuged. The results are separated according to three glycation temperatures with two different protein-water ratios. Statistical results (Table C.3., 4.) are given in Appendix C.

The results for reaction mixture containing a protein-water ratio of 1:1 (w/w) are given in Figure 3.3. Both the temperature and the sugar type affected the results significantly ($p<0.05$) where proteins had the highest mean solubility after they were glycated at a 100°C and lowest after the treatment at 130°C. Proteins experience denaturation at both high and low temperatures and the amount of denaturation depends on the conditions. The denaturation temperature for the subunits of soy proteins range between 60.5°C to 96.5°C (Wang, Liu, Ma, & Zhao, 2019). At 130°C, proteins are denatured more due to it being a higher temperature than 100°C. For the incubation method, it is possible that a more unfolded state is thermodynamically favorable after proteins are kept at 50°C for an extended period of time (Mojica et al., 2014a). At this protein-water ratio, it was noticed that proteins glycated with glucose were generally more soluble than proteins with fructose and allulose.

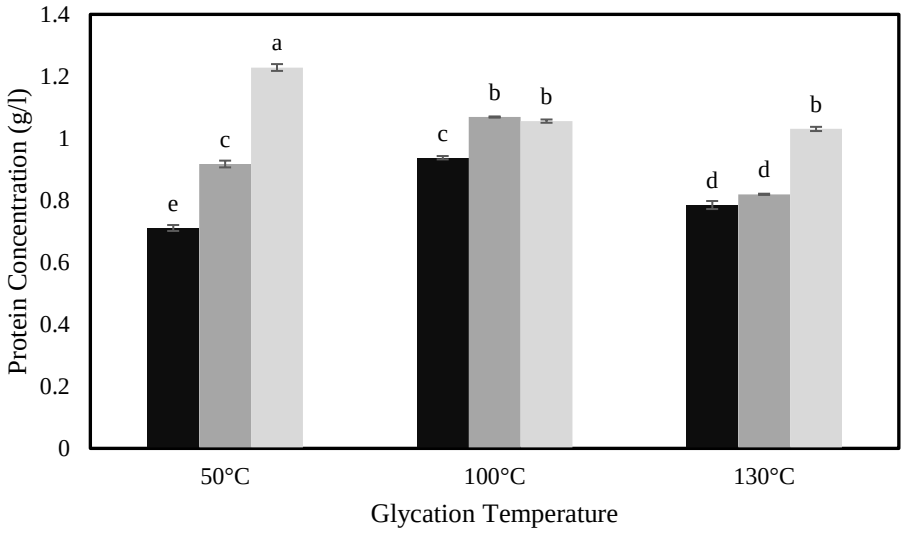

Figure 3.3. Soluble protein concentration in solutions prepared with proteins glycated at different temperatures with allulose (■), fructose (▣) and glucose (□) at a protein-water ratio of 1:1 (w/w)

Figure 3.4. shows results for a protein-water ratio of 2:1 (w/w). It was observed that both temperature and sugar type had a significant effect on the protein solubility ($p<0.05$). Proteins glycated with fructose were seen to have the highest solubility, followed by glucose and then allulose. Proteins glycated at 100°C were more soluble compared to those at 50°C and 130°C, which were significantly not different from each other ($p>0.05$) as seen in lower protein-water ratio as well.

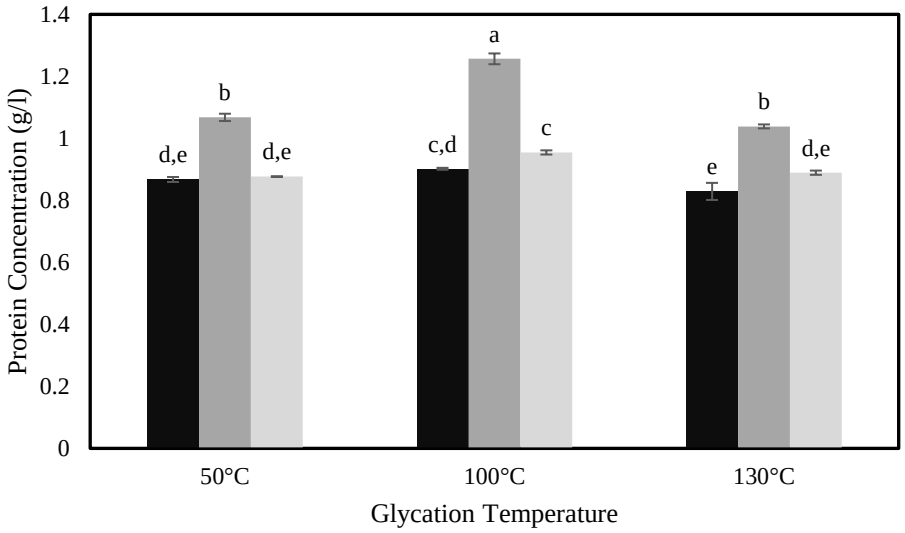

Figure 3.4. Soluble protein concentration in solutions prepared with proteins glycated at different temperatures with allulose (■), fructose (▩) and glucose (□) at a protein-water ratio of 1:2 (w/w)

It was noticed that there was no specific trend seen in the sugar type and the solubility. This could be related to different reaction pathways that changed drastically due to the conditions provide. No specific relation between the amount of soluble proteins and the type of monosaccharides used was seen in the previous studies on glycation of proteins with monosaccharides (Namli, 2019; Sun et al., 2004; Tas, 2019).

3.3 Determination of Degree of Glycation

Degree of glycation was measured by using the OPA method which measures the amount of free amino acid content. Glycation results in the reaction between amine groups on the proteins and carbonyl group on the sugar. Thus, the free amino acid content is expected to decrease as the glycation proceeds. Furthermore, the lower the amount of free amino acid groups, the more the amount of protein which is glycated.

The results were divided according to the protein-water ratio used and are shown in Figure 3.5 and 3.6. They are represented as free amino group in proteins glycated using different sugar types at three different temperatures. Statistical results (Table C.5., 6.) are given in Appendix C

Figure 3.5 shows results for proteins glycated with protein-water ratio of 1:1. Both temperature and sugar type had a significant effect on the amount of free amino groups. It was seen that proteins were utilized more at 50°C, followed by 130 °C and the proteins were least glycated at 100°C. Proteins glycated with glucose generally had significantly lower amount of free amino groups compared to proteins with allulose and fructose. This may be related to glucose having a higher initial rate of reaction compared to fructose and allulose (Cheetangdee & Fukada, 2014). Proteins glycated at this protein-water ratio at 100°C showed a good positive correlation ($R=0.806$) ($p<0.05$) between the amount of soluble proteins and free amino groups, so higher free amino groups may also be associated with a higher amount of soluble proteins. On the other hand, with the incubation method and glycation at 130°C, proteins had a negative correlation ($p<0.05$) between soluble proteins and free amino groups ($R = -0.852$ and $R = -0.971$ respectively). This can be explained by a higher degree of unfolding in proteins and a change in structure can cause the free amino groups to increase while hydrophobic interactions between the proteins and water also increase, resulting in a decrease in solubility (Damodaran, 2007). Good correlation ($p<0.05$) was also seen between the amount of remaining sugar and free amino groups at 50°C and 130°C which indicated that the glycation was occurring as sugar was being used up ($R = 0.984$ and $R = 0.905$ respectively).

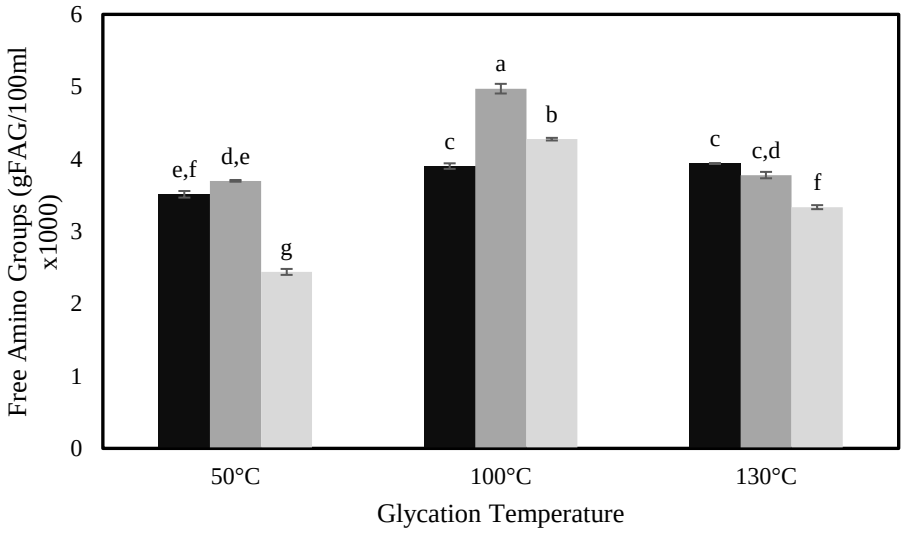

Figure 3.5. Free amino groups in soluble proteins in solutions prepared with proteins glycated at different temperatures with allulose (■), fructose (▣) and glucose (□) at a protein- water ratio of 1:1 (w/w)

The results for amount of free amino groups for proteins glycated with a protein-water ratio of 1:2 are given in Figure 3.6. Temperature and sugar type, both, had an overall significant effect on the amount of free amino groups ($p<0.05$). Similar to the results for a protein-water ratio of 1:1, the proteins glycated with glucose had the lowest amount of free amino groups. The proteins glycated with the incubation method had the lowest amount of free amino groups, followed by 130°C and then 100°C. However, it was noticed that individual differences between temperature and sugar types brought down the mean free amino groups amount for the incubation method due to glucose having the lowest amount of free amino groups. A positive correlation ($p<0.05$) was also seen between the amount of soluble proteins and free amino groups for proteins glycated at 100°C which again indicates that high free amino groups may be due to high amount of soluble proteins ($R = 0.806$).

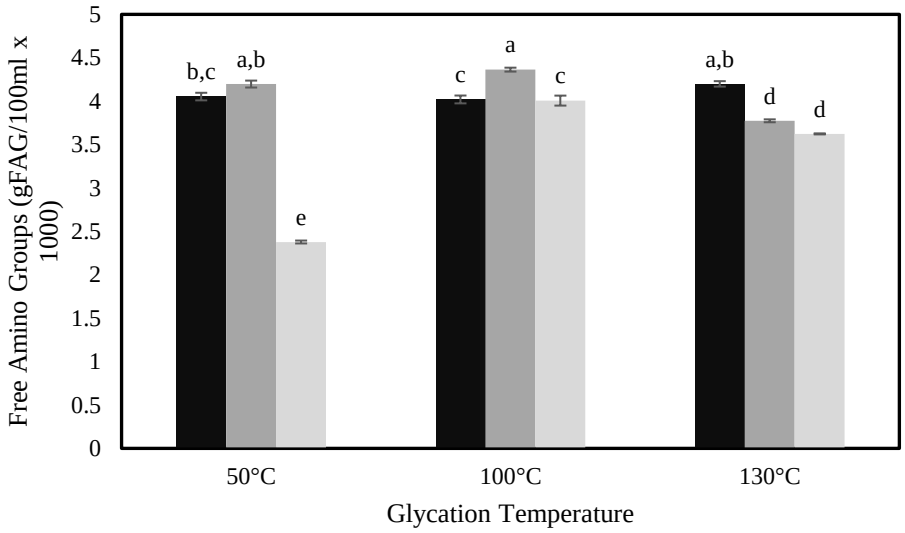

Figure 3.6. Free amino groups in soluble proteins in solutions prepared with proteins glycated at different temperatures with allulose (■), fructose (▣) and glucose (□) at a protein- water ratio of 1:2 (w/w)

The amount of free amino groups for native soy proteins was 14.11 gFAG/100ml which is again significantly higher compared to FAGs in glycated proteins ($p<0.05$). This could also be due to the higher solubility of soy proteins.

3.4 Determination of Browning Index

Formation of melanoidins (brown nitrogenous polymers) occurs during the final stage of the Maillard reaction (Nursten, 2005). Formation of these compounds and subsequent measurement of their absorbance at 420 nm is a method to measure the extent of the reaction. High absorbance value is indicative of the reaction going in its final stages and thus defeats the purpose of glycation (Yu, Zhao, Hu, Zeng, & Bai, 2012). However, this method can be used as an indicator for the extent of the reaction and thus can be used as justification for the method's success.

Figure 3.7 and 3.8 show the results for absorbance of glycated protein solutions at 420 nm, divided wrt to the protein-water ratio. Within each figure, the results are divided wrt to the glycation temperature and the sugar type. Absorbance value is directly related to the brownness of the solution. Statistical results (Table C.7., 8.) are given in Appendix C.

Figure 3.7 represents results for reaction mixture containing a protein-water ratio of 1:1 (w/w). Both the temperature and the sugar type had a significant effect on the browning intensity ($p<0.05$). Contrary to expectations, proteins glycated at 130°C showed the lowest browning with this method while proteins glycated during incubation had the highest mean absorbance. Among sugars, allulose had the highest mean absorbance value while fructose had the lowest mean absorbance.

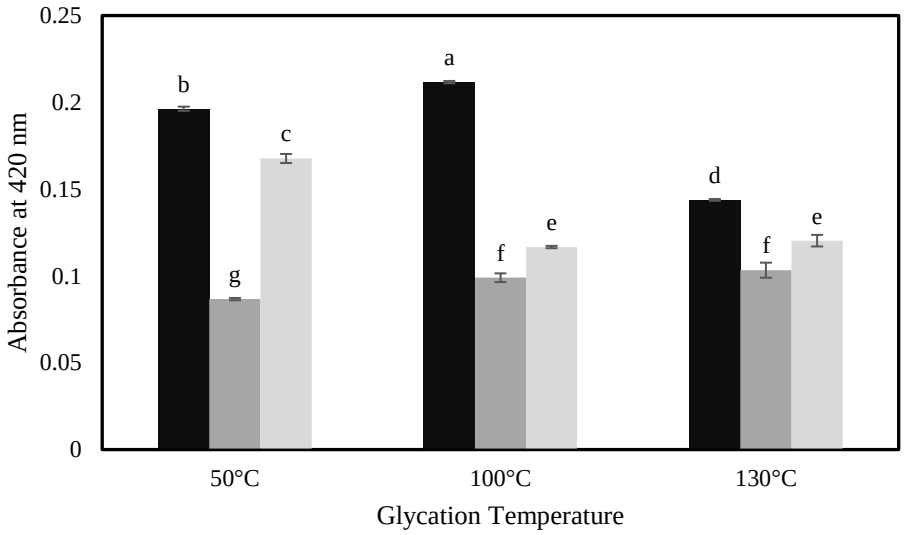

Figure 3.7. Absorbance of solutions prepared with proteins glycated at different temperatures with allulose (■), fructose (▣) and glucose (□) at a protein- water ratio of 1:1 (w/w)

Figure 3.8. shows results for browning intensity for proteins glycated with a protein-water ratio of 1:2. Unlike proteins glycated with a protein-water ratio of 1:1, these proteins had a higher browning intensity at 100°C compared to at 130°C and 50°C. However, allulose had the highest mean browning intensity, indicating that browning proceeds faster with allulose, even when a small amount of this sugar is consumed. The results are similar to previous studies where globular proteins glycated with allulose generally showed a higher browning, indicating an advanced Maillard reaction, although glucose has been known show a higher initial rate of reaction (Cheetangdee & Fukada, 2014). It is generally believed that the carbonyl groups of aldohexoses (glucose) being more electrophilic compared to that in the ketohexoses (allulose and fructose). This has been the case in this study as well with proteins glycated with glucose showing lower free amino groups. However, as the advanced stage approaches for some proteins in the mixture, the reactivity of the hexoses and the intermediates formed during the initial stages, become more prominent. The reactivity of these molecules was higher in the case where allulose was involved in the reaction (Sun et al., 2004; Zeng et al., 2013).

The results at 130°C do not necessarily represent the actual browning which was apparent in the sample. Melanoidins, formed in the later stages of the reaction, are not known to be soluble (Langner, Rzeski, Langner, & Rzeski, 2014), and thus would not impart any color to the supernatant. Another method is better be used to accurately identify browning and relate it to the degree of glycation in proteins.

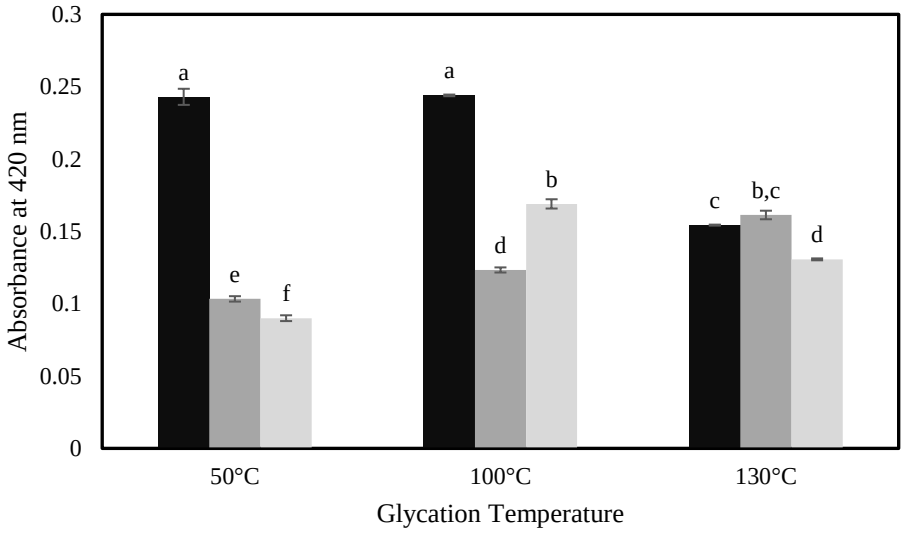

Figure 3.8. Absorbance of solutions prepared with proteins glycated at different temperatures with allulose (■), fructose (▣) and glucose (□) at a protein- water ratio of 1:2 (w/w)

3.5 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

Glycated proteins were compared with control SPI to observe the structural changes that occurred during the reaction by using FT-IR. Changes in the intensity of the existing peaks and formation of new peaks indicated how the reaction has proceeded.

Figure 3.9 shows the FT-IR spectra for proteins glycated at 50°C with a protein-water ratio of 1:2 compared with native control SPI. This serves as a good example of the trend seen in the other results. Spectra for the other glycated proteins are given in appendix section.

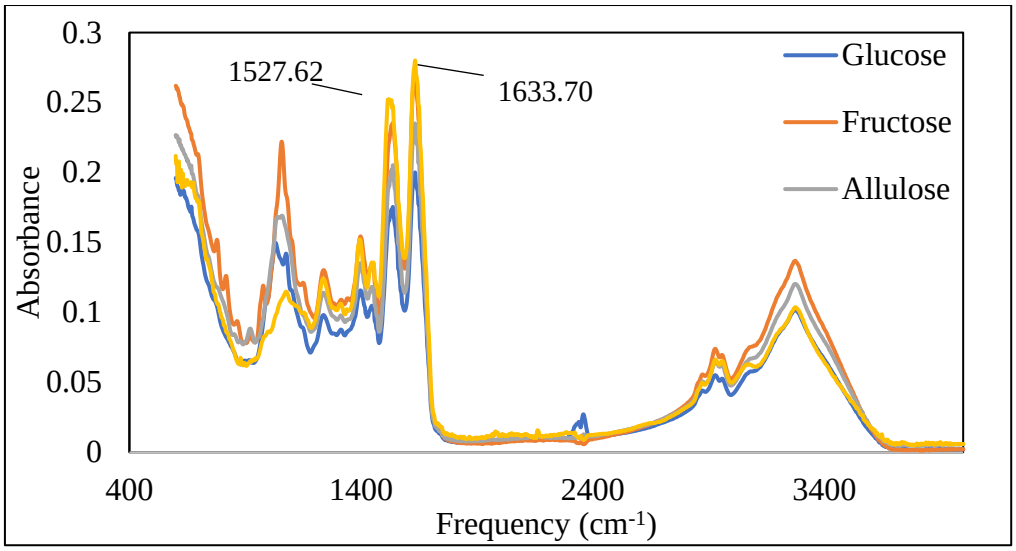

Figure 3.9. FT-IR spectra of native SPI and proteins glycated with glucose, fructose and allulose at a protein-water ratio of 1:2 (w/w) under incubation conditions

The peaks between 900 and 1400 are representative of C-O and C-C groups stretching present in carbohydrates and the peaks are distinctive for each type of sugar due to characteristic bands (Ibrahim, Alaam, El-Haes, Jalbout, & De Leon, 2006). These peaks are evidently missing for control SPI. The peaks observed at 1527.62 and 1633.70 are representative of Amide II and Amide I respectively. C-N stretching and N-H bending is linked with the amide II band while amide I consists of C=O stretching in the peptide bonds of proteins (Oliver, Kher, McNaughton, & Augustin, 2009). When compared with control SPI, the peaks of glycated proteins decrease with different sugar types, with proteins glycated with glucose having the lowest peaks. This is due to high amount of glycation observed in glucose. Peaks of proteins with fructose are seen to be closer to the peaks observed in control SPI (Mao, Pan, Hou, Yuan, & Gao, 2018). Results were similar for other glycation methods and matched with their OPA and Lowry method results.

3.6 Determination of Hydration Behavior by Nuclear Magnetic Resonance (NMR) Relaxometry

NMR relaxometry was a method used to check the hydration behavior of the glycated proteins. As discussed earlier, T_2 relaxation times were measured to obtain information about the interaction of water molecules with proteins. Although remaining sugar amount was high in the sample after glycation, its effect on the T_2 values was not considered as important due to being in a much smaller quantity compared to the proteins. It has been well established that different proton populations have different T_2 relaxation times in any specific food sample (Kirtil, Cikrikci, McCarthy, & Oztop, 2017). Although a multicomponent model can be obtained via TD-NMR relaxometry data from a hydrated proteinaceous sample, majority of the signal obtained is from the free water which directly influences the T_2 relation time. Increase in protein concentration or an increase in the water-binding capability of proteins resulted in a decrease in the free water, and thus decreasing the relaxation time (Dekkers et al., 2016).

Table 3.1 and 3.2 show the T_2 values for proteins glycated at two different protein-water ratios. The results are given for different glycation temperatures separately. ANOVA was also carried out in these results. Statistical results (Table C.9., 10.) are given in Appendix C.

T_2 time for native control soy protein was found as 33.16 ± 0.50 ms which is significantly lower compared to T_2 relaxation times of all the glycated proteins. Thus, it can be deduced that glycation increased the T_2 times. This can also be confirmed by the decrease in solubility of glycated proteins compared to native soy proteins, representing weaker water-binding behavior. Furthermore, higher temperatures led to protein denaturation and decreased the solubility.

T_2 values for proteins glycated with a protein-water ratio of 1:1 are given in table 3.1 Proteins glycated at 100°C and 130°C were similar in terms of T_2 values, and had higher and significantly different ($p<0.05$) values from proteins glycated with incubation method. When comparing sugars, proteins glycated with allulose had the highest T_2 values, followed by fructose and then glucose. If just glycation had to be considered, it would show that samples with glucose should have the highest T_2 based on the overall generalization that glycation reduces solubility since the proteins glycated with glucose have the lowest free amino groups. However, that is not the case and glucose show the lowest T_2 among the sugar types. This also implied that proteins which were glycated with glucose, had a stronger interaction with water. When correlations with other parameters were compared, it was observed that at all temperatures, T_2 had negative relationship with amount of soluble proteins glycated at 50°C, 100°C and 130°C (R = -0.979, R = -0.702, R = -0.957 respectively) ($p<0.05$). This is an expected result since more soluble proteins are better at binding with water.

Table 3.1. T_2 values for proteins glycated at different temperatures with allulose, fructose and glucose at a protein-water ratio of 1:1 (w/w)

Sugar Type	**T_2 (ms) 50°C**	**T_2 (ms) 100°C**	**T_2 (ms) 130°C**
Allulose	$89.27 \pm 0.07^{b,c}$	106.09 ± 2.02^{a}	96.51 ± 1.48^{b}
Fructose	$69.42 \pm 0.36^{d,e}$	$89.64 \pm 2.72^{b,c}$	87.77 ± 1.36^{c}
Glucose	53.63 ± 0.74^{f}	$61.38 \pm 2.86^{e,f}$	71.85 ± 0.95^{d}

Table 3.2 includes the results for proteins glycated at a protein-water ratio of 1:2. Both temperature and sugar-type had a significant effect on the T2 values of these proteins. The mean T2 values were higher for this protein-water ratio of 1:2 compared to 1:1 with proteins glycated at 100°C having the highest T2 values

followed by 130°C and 50°C. The sugars seemed to follow a similar trend as well with proteins with allulose having the highest T2 and glucose having the lowest T2 values.

Table 3.2. T_2 values for proteins glycated at different temperatures with allulose, fructose and glucose at a protein-water ratio of 1:2 (w/w)

Sugar Type	**T_2 (ms) 50°C**	**T_2 (ms) 100°C**	**T_2 (ms) 130°C**
Allulose	140.56 ± 2.79^{f}	363.72 ± 16.58^{b}	$252.55 \pm 9.83^{c,d}$
Fructose	$205.31 \pm 3.50^{d,e}$	280.01 ± 7.84^{c}	$193.73 \pm 2.50^{e,f}$
Glucose	80.44 ± 2.06^{g}	472.13 ± 26.23^{a}	$163.13 \pm 3.74^{e,f}$

Overall, it may not be enough to say that glycation directly influences T_2 relaxation times. Since the complex compounds that are formed because of glycation show different behavior with water, and these compounds vary due to sugar type. Thus, it is important to look at individual properties of proteins to make a conclusion.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In the presented study, soy proteins were glycated with allulose, fructose and glucose in the presence of minimal amount of water, an amount enough to just hydrate the proteins at two different protein-water ratios. These proteins were glycated at two different temperatures for 15 minutes as well as glycated while in controlled incubation conditions for a day. The results were compared with native SPI.

Proteins glycated at a 130°C were less soluble and were darker in color compared to proteins glycated at lower temperatures, and thus indicating that the proteins had reached later stages of Maillard browning, defeating the purpose of glycation. It can be concluded that proteins in this case were more denatured. Furthermore, this browning is not best recorded by using the method used. Rather than this method, L* a* b* values may be recorded using a colorimeter as the compounds formed at higher temperatures were not very soluble.

It was found that proteins had higher glycation with glucose compared to fructose and allulose. This was confirmed by both the HPLC analysis and free amino group determination. The result is also same as previous studies which report glucose as having higher initial rates of reaction, and thus being used up in a higher amount. While reaction rate is higher for glucose, protein reaction with allulose and fructose result in darker compounds even if a small amount of these sugars is used.

The study was successful in proving that glycation is occurred in the given condition which are easy to replicate on an industrial scale. However, there is a need to further develop this study to optimize the conditions used. These may involve factors that affect glycation including changing the time, temperature, pH, sugar type or the protein-sugar ratio. There is also a need to apply further analyses on the proteins such as checking their emulsification behavior and fat binding, amino acid analysis by using HPLC and using SDS-PAGE analysis to understand the molecular weight of the compounds formed. Further study of the quality of these proteins will help in understanding where and how they can be used so that their use in the industry can be promoted.

Moreover, this study simplifies the glycation process and reduces the time required to obtain products. If replicated on an industrial scale, it may prove to be a cost-effective and efficient method.

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APPENDICES

A. CALIBRATION CURVES

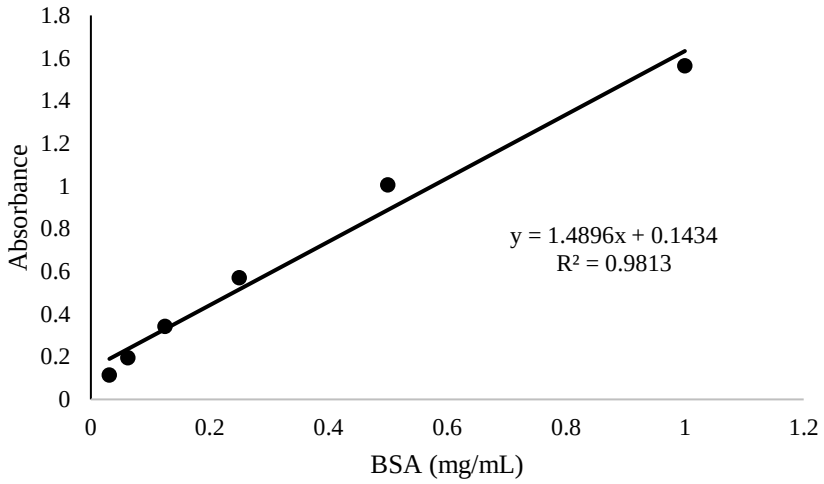

Figure A.1. Calibration curve for Lowry Method prepared by Bovine Serum Albumin (BSA) to measure protein concentration

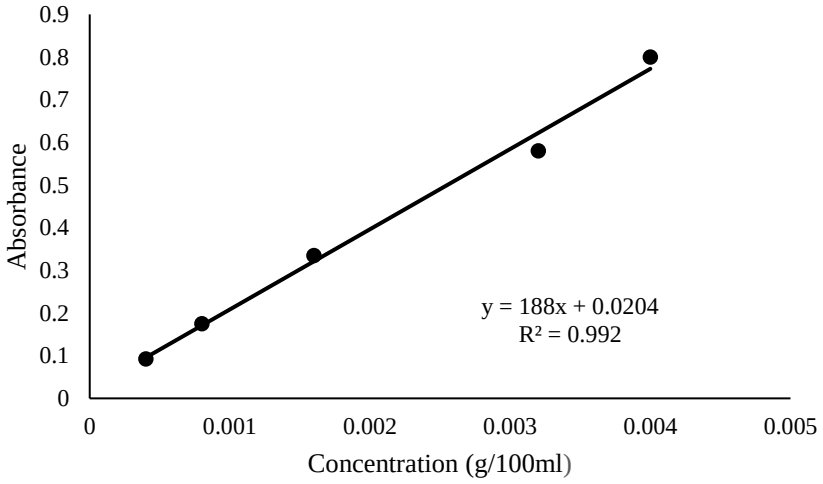

Figure A.2. Calibration curve for OPA method prepared by glycine for free amino group determination

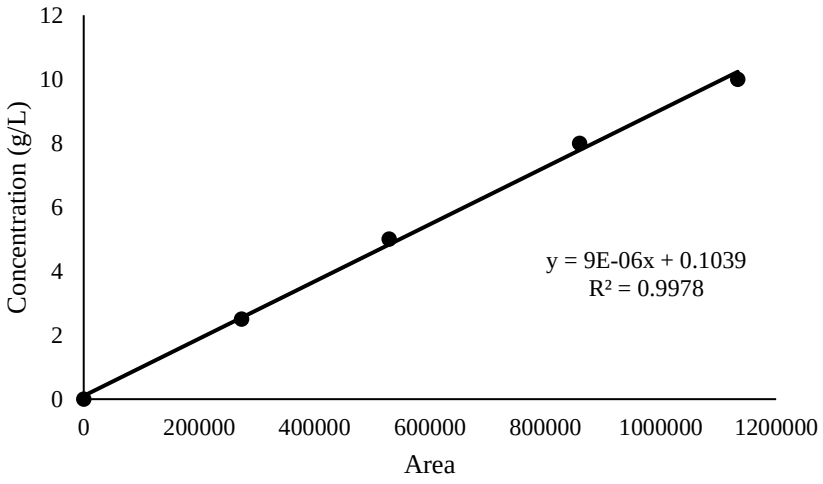

Figure A.3. HPLC calibration curve of glucose

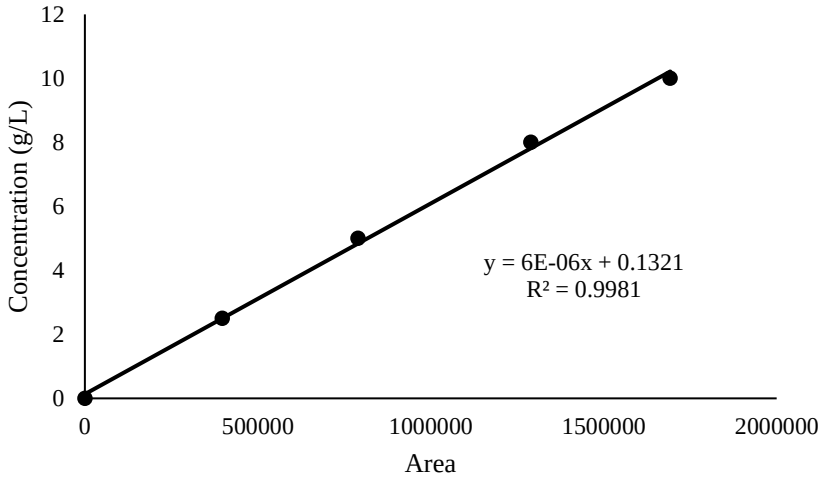

Figure A.4. HPLC calibration curve of fructose

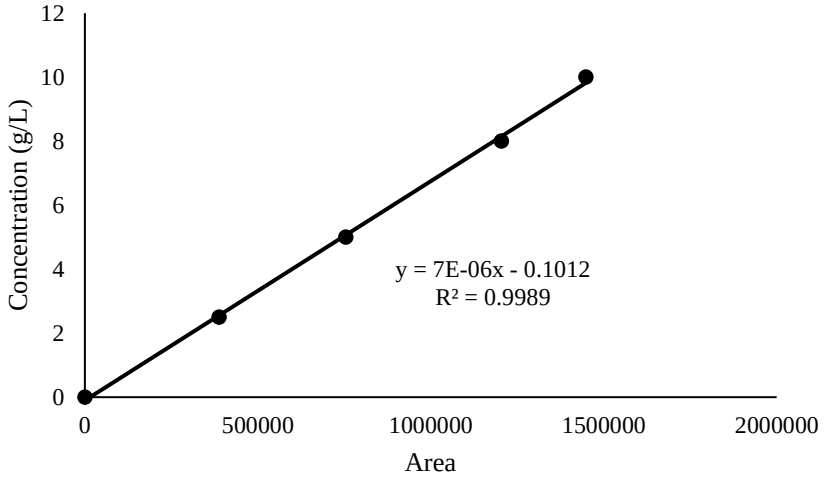

Figure A.5. HPLC calibration curve of allulose

B. COMPARATIVE FIGURES

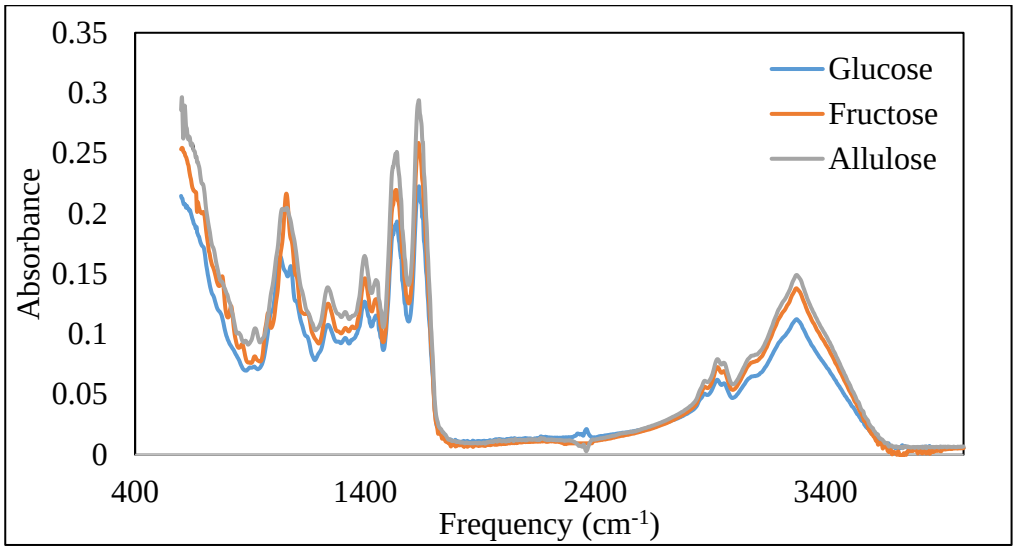

Figure B.1. FT-IR spectra of proteins glycated with glucose, fructose and allulose at a protein-water ratio of 1:1 (w/w) under incubation conditions

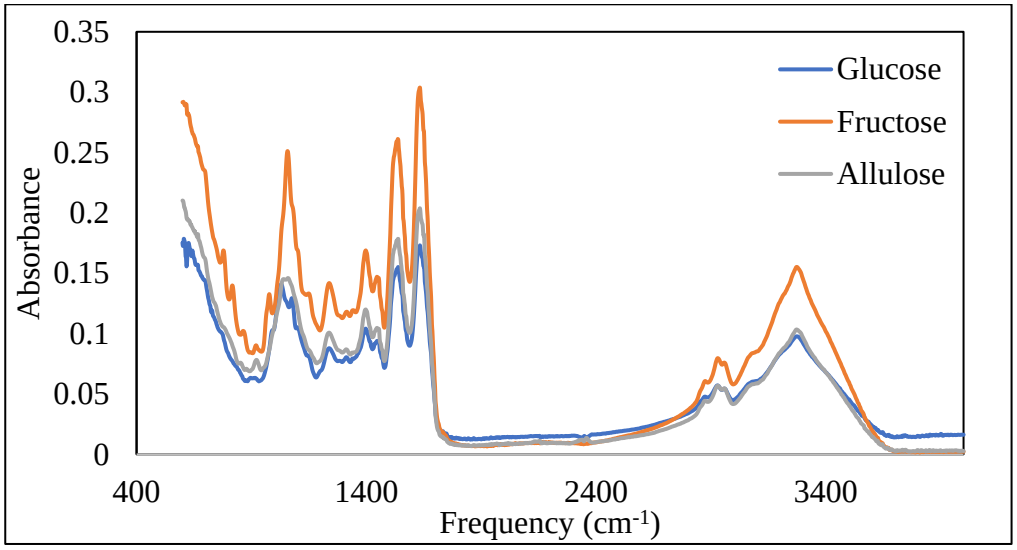

Figure B.2. FT-IR spectra of proteins glycated with glucose, fructose and allulose at a protein-water ratio of 1:1 (w/w) at 100°C

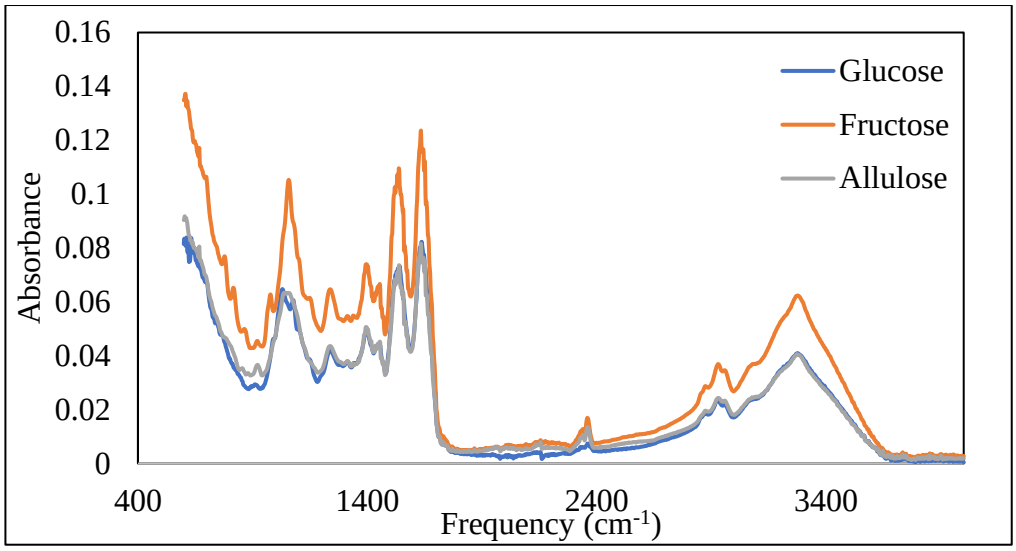

Figure B.3. FT-IR spectra of proteins glycated with glucose, fructose and allulose at a protein-water ratio of 1:2 (w/w) at 100°C

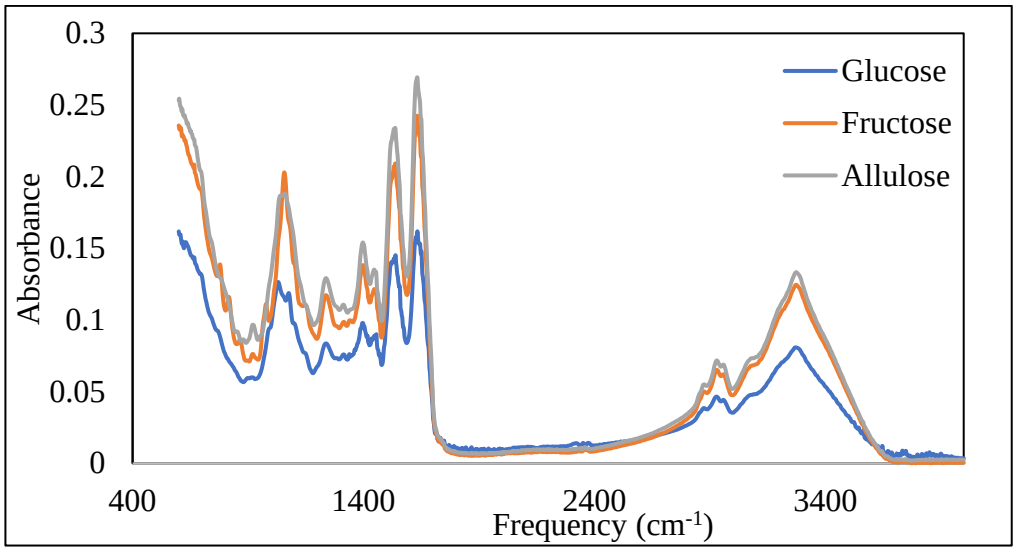

Figure B.4. FT-IR spectra of proteins glycated with glucose, fructose and allulose at a protein-water ratio of 1:1 (w/w) at 130°C

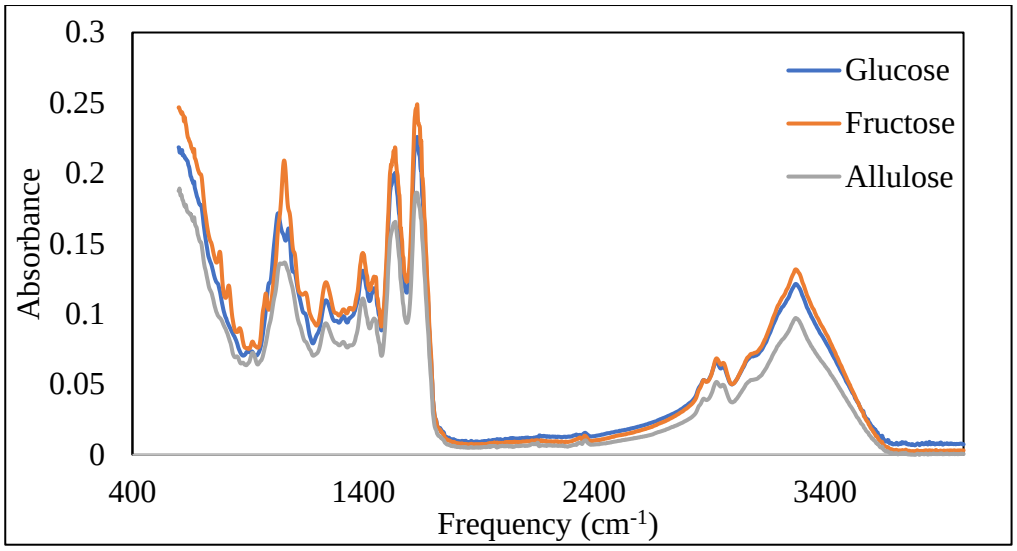

Figure B.5. FT-IR spectra of proteins glycated with glucose, fructose and allulose at a protein-water ratio of 1:2 (w/w) at 130°C

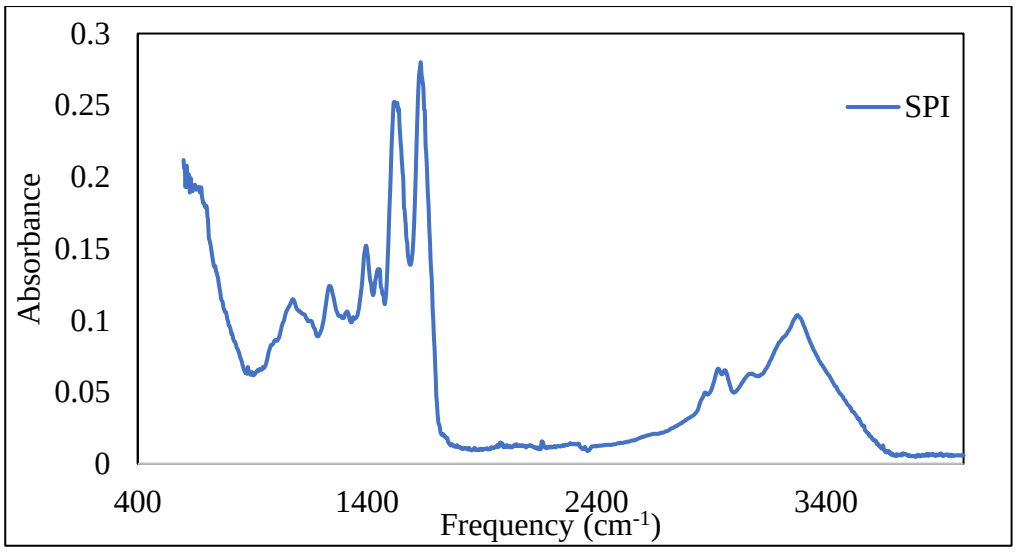

Figure B.6. FT-IR spectra of native control SPI

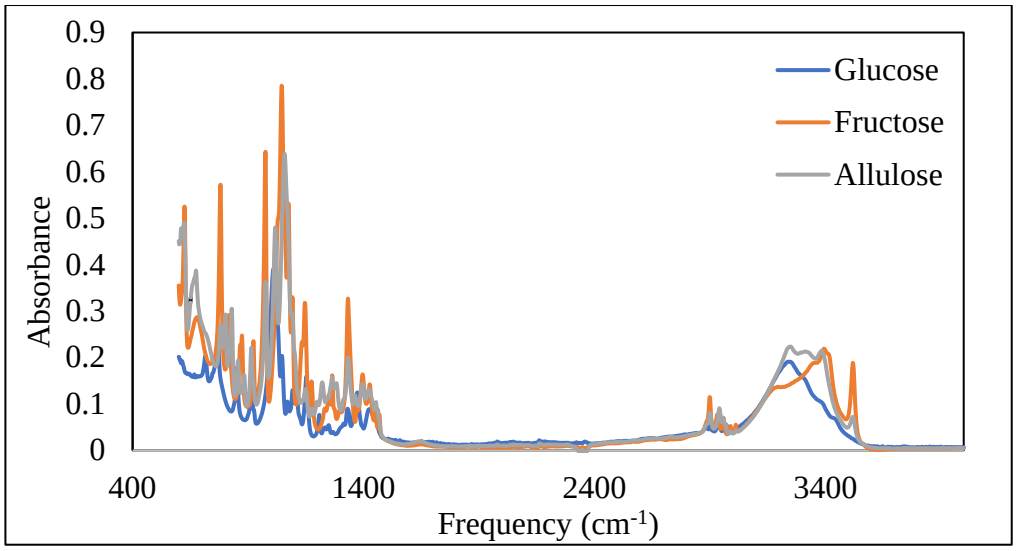

Figure B.7. FT-IR spectra of glucose, fructose and allulose

C. STATISTICAL ANALYSES

Table C.1. ANOVA and Tukey's Comparison Test for remaining sugar (%) in proteins glycated at different temperatures with allulose, fructose and glucose at a protein- water ratio of 1:1 (w/w)

General Linear Model: %Remaining Sugar versus Temperature, Sugar Type

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Temperature	Fixed	3	50, 100, 130
Sugar Type	Fixed	3	Allulose, Fructose, Glucose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	601.40	300.70	22.42	0.000
Sugar Type	2	1448.31	724.16	54.00	0.000
Temperature*Sugar Type	4	83.83	20.96	1.56	0.227
Error	18	241.39	13.41		
Total	26	2374.93			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3.66203	89.84%	85.32%	77.13%

Comparisons for %Remaining Sugar

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping
100	9	91.0177	A
50	9	82.1750	B
130	9	80.1473	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping	
Allulose	9	91.1436	A	
Fructose	9	87.9411	A	
Glucose	9	74.2553		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Sugar Type	N	Mean	Grouping		
100 Allulose	3	96.2962	A		
100 Fructose	3	94.3413	A		
130 Allulose	3	89.5824	A	B	
50 Fructose	3	88.2479	A	B	
50 Allulose	3	87.5521	A	B	
100 Glucose	3	82.4155		B	
130 Fructose	3	81.2343		B	
50 Glucose	3	70.7251			C
130 Glucose	3	69.6253			C

Means that do not share a letter are significantly different.

Table C.2. ANOVA and Tukey's Comparison Test for remaining sugar (%) in proteins glycated at different temperatures with allulose, fructose and glucose at a protein- water ratio of 1:2 (w/w)

General Linear Model: %Remaining Sugar versus Temperature, Sugar Type

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Temperature	Fixed	3	50, 100, 130
Sugar Type	Fixed	3	Allulose, Fructose, Glucose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	232.31	116.157	17.54	0.000
Sugar Type	2	1353.13	676.563	102.17	0.000
Temperature*Sugar Type	4	52.43	13.109	1.98	0.141
Error	18	119.19	6.622		
Total	26	1757.07			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.57328	93.22%	90.20%	84.74%

Comparisons for %Remaining Sugar

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping
100	9	89.2044	A
50	9	83.9079	B
130	9	82.3515	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping

Allulose	9	92.9424	A		
Fructose	9	86.7092		B	
Glucose	9	75.8122			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Sugar Type	N	Mean	Grouping			
100 Allulose	3	95.4107	A			
130 Allulose	3	91.9907	A	B		
50 Allulose	3	91.4260	A	B		
100 Fructose	3	89.8722	A	B	C	
50 Fructose	3	86.5275		B	C	D
130 Fructose	3	83.7277			C	D
100 Glucose	3	82.3303				D
50 Glucose	3	73.7703				E
130 Glucose	3	71.3360				E

Means that do not share a letter are significantly different.

Table C.3. ANOVA and Tukey's Comparison Test for soluble protein concentration in solutions prepared with proteins glycated at different temperatures with allulose, fructose and glucose at a protein- water ratio of 1:1 (w/w)

General Linear Model: Soluble Proteins (g/L) versus Temperature, Sugar Type

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Temperature	Fixed	3	50, 100, 130
Sugar Type	Fixed	3	Allulose, Fructose, Glucose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	0.091262	0.045631	224.40	0.000
Sugar Type	2	0.391323	0.195661	962.21	0.000
Temperature*Sugar Type	4	0.153390	0.038348	188.58	0.000
Error	18	0.003660	0.000203		
Total	26	0.639635			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0142599	99.43%	99.17%	98.71%

Comparisons for Soluble Proteins (g/L)

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping
100	9	1.02072	A
50	9	0.95228	B
130	9	0.87835	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping

Glucose	9	1.10490	A		
Fructose	9	0.93526		B	
Allulose	9	0.81119			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Sugar Type	N	Mean	Grouping				
50 Glucose	3	1.22861	A				
100 Fructose	3	1.06885		B			
100 Glucose	3	1.05567		B			
130 Glucose	3	1.03042		B			
100 Allulose	3	0.93764			C		
50 Fructose	3	0.91733			C		
130 Fructose	3	0.81960				D	
130 Allulose	3	0.78502				D	
50 Allulose	3	0.71090					E

Means that do not share a letter are significantly different.

Table C.4. ANOVA and Tukey's Comparison Test for soluble protein concentration in solutions prepared with proteins glycated at different temperatures with allulose, fructose and glucose at a protein- water ratio of 1:2 (w/w)

General Linear Model: Soluble Proteins (g/L) versus Temperature, Sugar Type

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Temperature	Fixed	3	50, 100, 130
Sugar Type	Fixed	3	Allulose, Fructose, Glucose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	0.073602	0.036801	78.20	0.000
Sugar Type	2	0.337476	0.168738	358.57	0.000
Temperature*Sugar Type	4	0.028924	0.007231	15.37	0.000
Error	18	0.008471	0.000471		
Total	26	0.448473			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0216930	98.11%	97.27%	95.75%

Comparisons for Soluble Proteins (g/L)

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping
100	9	1.03774	A
50	9	0.93727	B
130	9	0.91897	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping

Fructose	9	1.12101	A		
Glucose	9	0.90690		B	
Allulose	9	0.86609			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Sugar Type	N	Mean	Grouping			
100 Fructose	3	1.25661	A			
50 Fructose	3	1.06775		B		
130 Fructose	3	1.03866		B		
100 Glucose	3	0.95466			C	
100 Allulose	3	0.90196			C	D
130 Glucose	3	0.88933				D E
50 Glucose	3	0.87670				D E
50 Allulose	3	0.86737				D E
130 Allulose	3	0.82894				E

Means that do not share a letter are significantly different.

Table C.5. ANOVA and Tukey's Comparison Test for free amino groups in soluble proteins in solutions prepared with proteins glycated at different temperatures with allulose, fructose and glucose at a protein- water ratio of 1:1 (w/w)

General Linear Model: FAG (g/100ml)x1000 versus Temperature, Sugar Type

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Temperature	Fixed	3	50, 100, 130
Sugar Type	Fixed	3	Allulose, Fructose, Glucose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	6.2047	3.10234	719.61	0.000
Sugar Type	2	2.8877	1.44386	334.91	0.000
Temperature*Sugar Type	4	2.2397	0.55992	129.88	0.000
Error	18	0.0776	0.00431		
Total	26	11.4097			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0656592	99.32%	99.02%	98.47%

Comparisons for FAG (g/100ml)x1000

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping
100	9	4.38342	A
130	9	3.68381	B
50	9	3.21690	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping

Fructose	9	4.14997	A		
Allulose	9	3.78428		B	
Glucose	9	3.34988			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Sugar Type	N	Mean	Grouping					
100 Fructose	3	4.97296	A					
100 Glucose	3	4.27482		B				
130 Allulose	3	3.93794			C			
100 Allulose	3	3.90248			C			
130 Fructose	3	3.77837			C	D		
50 Fructose	3	3.69858				D	E	
50 Allulose	3	3.51241					E	F
130 Glucose	3	3.33511						F
50 Glucose	3	2.43972						G

Means that do not share a letter are significantly different.

Table C.6. ANOVA and Tukey's Comparison Test for free amino groups in soluble proteins in solutions prepared with proteins glycated at different temperatures with allulose, fructose and glucose at a protein- water ratio of 1:2 (w/w)

General Linear Model: FAG (g/100ml)x1000 versus Temperature, Sugar Type

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Temperature	Fixed	3	50, 100, 130
Sugar Type	Fixed	3	Allulose, Fructose, Glucose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	1.55738	0.77869	213.36	0.000
Sugar Type	2	3.51664	1.75832	481.78	0.000
Temperature*Sugar Type	4	3.40379	0.85095	233.16	0.000
Error	18	0.06569	0.00365		
Total	26	8.54350			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0604123	99.23%	98.89%	98.27%

Comparisons for FAG (g/100ml)x1000

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping
100	9	4.13002	A
130	9	3.86554	B
50	9	3.54270	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping

Fructose	9	4.11155	A	
Allulose	9	4.09087	A	
Glucose	9	3.33585		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Sugar Type	N	Mean	Grouping			
100 Fructose	3	4.36348	A			
130 Allulose	3	4.19947	A	B		
50 Fructose	3	4.19725	A	B		
50 Allulose	3	4.05319		B	C	
100 Allulose	3	4.01995			C	
100 Glucose	3	4.00665			C	
130 Fructose	3	3.77394				D
130 Glucose	3	3.62323				D
50 Glucose	3	2.37766				E

Means that do not share a letter are significantly different.

Table C.7. ANOVA and Tukey's Comparison Test for Absorbance of solutions prepared with proteins glycated at different temperatures with allulose, fructose and glucose at a protein- water ratio of 1:1 (w/w)

General Linear Model: Browning Absorbance versus Temperature, Sugar Type

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Temperature	Fixed	3	50, 100, 130
Sugar Type	Fixed	3	Allulose, Fructose, Glucose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	0.003696	0.001848	120.82	0.000
Sugar Type	2	0.034661	0.017330	1132.97	0.000
Temperature*Sugar Type	4	0.009240	0.002310	151.02	0.000
Error	18	0.000275	0.000015		
Total	26	0.047872			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0039110	99.42%	99.17%	98.71%

Comparisons for Browning Absorbance

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping		
50	9	0.150222	A		
100	9	0.142444		B	
130	9	0.122444			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Allulose	9	0.183889	A

Glucose	9	0.134889	B	
Fructose	9	0.096333		C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Sugar Type	N	Mean	Grouping					
100 Allulose	3	0.211667	A					
50 Allulose	3	0.196333		B				
50 Glucose	3	0.167667			C			
130 Allulose	3	0.143667				D		
130 Glucose	3	0.120333					E	
100 Glucose	3	0.116667					E	
130 Fructose	3	0.103333						F
100 Fructose	3	0.099000						F
50 Fructose	3	0.086667						G

Means that do not share a letter are significantly different.

Table C.8. ANOVA and Tukey's Comparison Test for Absorbance of solutions prepared with proteins glycated at different temperatures with allulose, fructose and glucose at a protein- water ratio of 1:2 (w/w)

General Linear Model: Browning Absorbance versus Temperature, Sugar Type

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Temperature	Fixed	3	50, 100, 130
Sugar Type	Fixed	3	Allulose, Fructose, Glucose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	0.006067	0.003033	147.83	0.000
Sugar Type	2	0.042506	0.021253	1035.79	0.000
Temperature*Sugar Type	4	0.024408	0.006102	297.40	0.000
Error	18	0.000369	0.000021		
Total	26	0.073350			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0045297	99.50%	99.27%	98.87%

Comparisons for Browning Absorbance

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping
100	9	0.178778	A
130	9	0.148778	B
50	9	0.145444	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping

Allulose	9	0.213778	A	
Glucose	9	0.129889		B
Fructose	9	0.129333		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Sugar Type	N	Mean	Grouping				
100 Allulose	3	0.244000	A				
50 Allulose	3	0.243000	A				
100 Glucose	3	0.169000		B			
130 Fructose	3	0.161333		B	C		
130 Allulose	3	0.154333			C		
130 Glucose	3	0.130667				D	
100 Fructose	3	0.123333				D	
50 Fructose	3	0.103333					E
50 Glucose	3	0.090000					F

Means that do not share a letter are significantly different.

Table C.9. ANOVA and Tukey's Comparison Test for T_2 results for proteins glycated at different temperatures with allulose, fructose and glucose at a protein-water ratio of 1:1 (w/w)

General Linear Model: T_2 versus Temperature, Sugar Type

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Temperature	Fixed	3	50, 100, 130
Sugar Type	Fixed	3	Allulose, Fructose, Glucose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	1308.5	654.26	77.80	0.000
Sugar Type	2	5551.4	2775.70	330.06	0.000
Temperature*Sugar Type	4	369.0	92.26	10.97	0.000
Error	18	151.4	8.41		
Total	26	7380.3			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.89994	97.95%	97.04%	95.39%

Comparisons for T_2

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping
100	9	85.7056	A
130	9	85.3778	A
50	9	70.7767	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
Allulose	9	97.2933	A		
Fructose	9	82.2789		B	
Glucose	9	62.2878			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Sugar Type	N	Mean	Grouping				
100 Allulose	3	106.093	A				
130 Allulose	3	96.513		B			
100 Fructose	3	89.643		B	C		
50 Allulose	3	89.273		B	C		
130 Fructose	3	87.770			C		
130 Glucose	3	71.850				D	
50 Fructose	3	69.423				D	E
100 Glucose	3	61.380					E F
50 Glucose	3	53.633					F

Means that do not share a letter are significantly different.

Table C.10. ANOVA and Tukey's Comparison Test for T_2 results for proteins glycated at different temperatures with allulose, fructose and glucose at a protein-water ratio of 1:2 (w/w)

General Linear Model: T_2 versus Temperature, Sugar Type

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Temperature	Fixed	3	50, 100, 130
Sugar Type	Fixed	3	Allulose, Fructose, Glucose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	255167	127584	328.39	0.000
Sugar Type	2	3029	1514	3.90	0.039
Temperature*Sugar Type	4	88433	22108	56.90	0.000
Error	18	6993	389		
Total	26	353622			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
19.7107	98.02%	97.14%	95.55%

Comparisons for T_2

Tukey Pairwise Comparisons: Temperature

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping		
100	9	371.954	A		
130	9	203.138		B	
50	9	142.103			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping	
Allulose	9	252.278	A	
Glucose	9	238.570	A	B

Fructose	9	226.348	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Temperature*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Sugar Type	N	Mean	Grouping				
100 Glucose	3	472.133	A				
100 Allulose	3	363.720		B			
100 Fructose	3	280.010			C		
130 Allulose	3	252.553			C	D	
50 Fructose	3	205.307				D	E
130 Fructose	3	193.727					E F
130 Glucose	3	163.133					E F
50 Allulose	3	140.560					F
50 Glucose	3	80.443					G

Means that do not share a letter are significantly different.