INVESTIGATION OF PHYSICO-CHEMICAL PROPERTIES OF SOY PROTEINS GLYCATED WITH D-PSICOSE

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submitted by **SERMET CAN BEYLIKÇI** in partial fulfillment of the requirements for the degree of **Master of Science in Food Engineering Department, Middle East Technical University** by,

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

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

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

Assist. Prof. Dr. Bekir Gökçen Mazı
Co-Supervisor, **Food Engineering, Ordu University** _____

Examining Committee Members:

Prof. Dr. Alev Bayındırlı
Food Engineering, METU _____

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

Prof. Dr. Gülüm Şumnu
Food Engineering, METU _____

Prof. Dr. Hami Alpas
Food Engineering, METU _____

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

Date: 13.12.2019

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Surname: Sermet Can Beylikçi

Signature:

ABSTRACT

INVESTIGATION OF PHYSICO-CHEMICAL PROPERTIES OF SOY PROTEINS GLYCATED WITH D-PSICOSE

Beylikçi, Sermet Can
Master of Science, Food Engineering
Supervisor: Assoc. Prof. Dr. Mecit Halil Öztop
Co-Supervisor: Assist. Prof. Dr. Bekir Gökçen Mazı

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Proteins are one of the most essential food components that is constantly used by food industry because of their functional properties as well as their nutritional value. Soy protein has become popular among the usable protein resources because of its various functional properties such as foaming, gelling, emulsifying and water holding capacities. However, certain drawbacks of soy protein like limited solubility especially in acidic environment, emerge the need for modification for further use in broader variety of products. Although there are several other modification techniques, glycation has recently drawn attention because of the safety of the method. Glycation is the first step of non-enzymatic browning reactions, also known as the Maillard reaction, that starts with the conjugation of sugar and protein molecules through their carbonyl and free amino groups respectively. Functional properties of proteins are found to be enhanced significantly by glycation. Rare sugar is a generic name of a sugar group consisting of monosaccharides that is not widely found in nature. The rare sugar D-Psicose has been investigated previously by many researches and drawn attention as it has been found to improve some physical and chemical properties of proteins, such as solubility, gelling, foaming and emulsifying abilities by increased Maillard reaction rates. In this study, glycation of soy protein and its effect on the

solubility of the proteins have been investigated. Three different parameters have been set as sugar type (Glucose, Fructose, D-Psicose), glycation pH (7, 10, 12) and protein-sugar ratio (1-0, 1-1, 2-1, 3-1, 5-1, 10-1). To explore the extent of the glycation and its effects on the solubility; degree of glycation (DoG), % reducing sugar (%RS) content, and free amino group (FAG) content experiments were performed. Results showed that each parameter had a significant effect ($p<0.05$) on the glycation of soy protein and, positive correlation was found between free amino group content and the solubility of the soy protein indicating that the glycation decreased the solubility of the soy protein under the present circumstances. Thus, it was concluded that glycation could not be offered as a modification strategy to improve the solubility of soy proteins.

Keywords: Glycation, Rare Sugar, Soy Protein, Characterization, Solubility

ÖZ

D-PSİKOZ İLE GLİKE OLMUŞ SOYA PROTEİNLERİNİN FİZİKO-KİMYASAL ÖZELLİKLERİNİN İNCELENMESİ

Beylikçi, Sermet Can
Yüksek Lisans, Gıda Mühendisliği
Tez Danışmanı: Doç. Dr. Mecit Halil Öztop
Ortak Tez Danışmanı: Dr. Öğr. Üyesi Bekir Gökçen Mazı

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Proteinler besleyici özelliklerinin yanı sıra fonksiyonel özellikleri sayesinde gıda endüstrisi tarafından sıkça kullanılan en temel gıda bileşenlerden biridir. Soya proteini, köpüklenme, jelleşme, emülsifikasyon, su tutma kapasitesi gibi çeşitli fonksiyonel özellikleri sayesinde kullanılabilir protein kaynakları arasında popüler hale gelmiştir. Ancak, soya proteininin özellikle asidik ortamlardaki kısıtlı çözünürlüğü gibi belirli eksiklikleri daha geniş çaptaki ürün çeşitlerinde kullanılabilmesi açısından modifikasyon gerekliliğini doğurmuştur. Diğer çeşitli modifikasyon tekniklerinin olmasına rağmen, güvenilirliği sebebiyle son zamanlarda glikasyon dikkat çekmektedir. Maillard reaksiyonu olarak da bilinen glikasyon, enzimatik olmayan bir reaksiyon olup sırası ile karbonil ve serbest amino grupları vasıtasıyla şeker ve protein moleküllerinin birleşmesi ile başlar. Glikasyon işlemi ile proteinlerin fonksiyonel özelliklerinde ciddi gelişmeler olduğu bulunmuştur. Nadir şeker, doğada yaygın olarak bulunmayan monosakkaritleri kapsayan şeker grubuna verilen genel addır. Nadir şeker D-Psikoz daha önce birçok araştırmacı tarafından incelenmiş ve Maillard reaksiyonu sayesinde proteinlerin çözünürlük, jelleşme, köpüklenme ve emülsifikasyon gibi birçok özelliğini geliştirdiğinin gözlenmesi üzerine dikkatleri üzerine çekmiştir. Bu çalışmada soya proteininin glikasyonu ve

çözünürlüğü üzerine etkileri incelenmiştir. Glikasyon koşullarını belirlemek üzere; şeker tipi (Glikoz, Fruktoz, D-Psikoz), Glikasyon pH'ı (7, 10, 12) ve protein-şeker oranı (1-0, 1-1, 2-1, 3-1, 5-1, 10-1) olmak üzere üç farklı parametre belirlenmiştir. Gliyasyon derecesinin belirlenmesi ve bunun çözünürlük üzerine etkilerinin keşfedilmesi amacıyla; glikasyon derecesi, indirgen şeker içeriği, serbest amino grubu içeriği ve çözünürlük deneyleri gerçekleştirilmiştir. Sonuçlar olarak, her bir parametrenin glikasyon derecesi üzerinde anlamlı etkisi ($p < 0.05$) olduğu ve serbest amino grubu miktarı ile soya proteini çözünürlüğü arasında pozitif bir korelasyon ($r=0.431$) olduğu gözlemlenmiştir. Bu durum, mevcut koşullar altındaki glikasyon işleminin soya proteini çözünürlüğünü azalttığını göstermektedir.

Anahtar Kelimeler: Glikasyon, Nadir Şeker, Soya Proteini, Karakterizasyon, Çözünürlük

To my beloved family…

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INTRODUCTION

1.1. Soy Protein

Soybean, which is a legume species, is a good source of protein and edible oil. It consists of approximately 36% protein, 30% of carbohydrate, whose 50% is insoluble, and 18% of oil. By having the highest protein content among other legume and cereal species and with its high amount of high-quality nutritional components such as vitamins, minerals, polysaccharides, oligosaccharides, phospholipids and isoflavones, soybean is a valuable source for human diet (Thrane et al., 2017). Other than its nutritional benefits, Soy proteins provide excellent functional properties such as gelling, emulsifying abilities and oil and water holding capacities (Nishinari et al., 2014). The digestibility of the soy protein is also good in human body and its composition is similar to high quality animal source proteins (Singh et al., 2008; Wolf, 1969). Furthermore, the amino acid composition of soy protein is well balanced, as it contains all 9 essential amino acids (Nishinari, Fang, Guo, & Phillips, 2014).

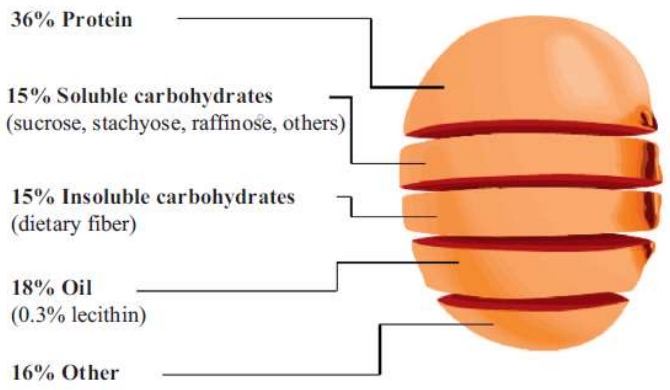

Figure 1.1. Typical soybean composition (Thrane et al., 2017)

While about 90% of soy protein is made up of globulins, the rest consists of albumins (Kunte, Gennadios, Cuppett, Hanna & Weller, 1997). Soy protein globulins are categorized under 4 subunits according to their sedimentation rates as 2S, 7S, 11S and 15S (Ciannamea *et al*., 2014). β-conglycinin (7S) and glycinin (11S) are the most important fractions among all since they are the two major components of soy protein (Kunte *et al*., 1997). The most used form of soy protein for scientific purposes is soy protein isolate since it is the purest form of soy protein with 90% protein content while another source, soy protein concentrate has only about 65% protein content (Singh et al., 2008; Koshy *et al*., 2015). Although soy protein has some drawback such as poor surface activity because of its high molecular weight (W. Li et al., 2016), and low solubility at acidic conditions, it was observed that these drawbacks can be eliminated and the functionality of the protein can be enhanced by modification since soy protein is chemically reactive because of its polar functional groups such as amine, hydroxyl and carboxyl groups (Tian *et al*., 2018).

1.2. Monosaccharides

Monosaccharides are the smallest unit of carbohydrate molecules. They made up of carbon atom chains with different configurations and primarily categorized by the number of carbon atoms they have and whether being an aldehyde (aldose) or ketone (ketose). They are also categorized according to the orientation of the furthest asymmetric carbon from the carbonyl group. The sugar named as D sugar if the hydroxyl group is on the right-hand side in a standard Fischer projection and as L sugar if otherwise. They are widely used in food industry because of their nutritional, organoleptic and functional values, accessibility and abundance. They are also required elements for Maillard reactions as carbonyl group source (Cheetangdee & Fukada, 2014).

1.2.1. Glucose

Glucose is the primary energy source of human metabolism and also the most abundant among all monosaccharides. Glucose is mostly found as D form in the nature and also called as dextrose (BeMiller, 2019b).

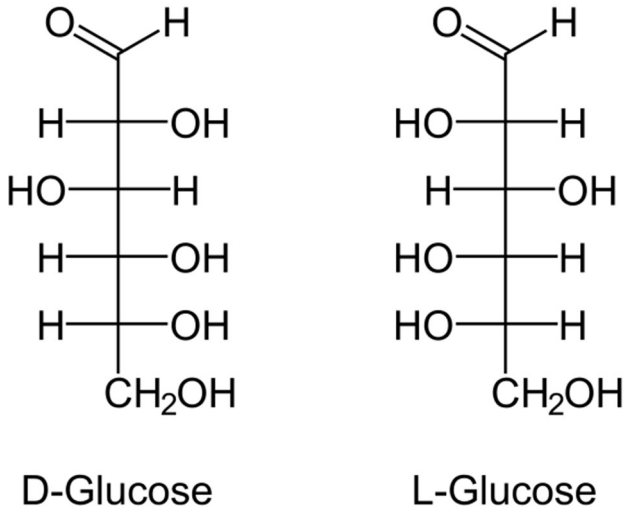

Figure 1.2. D- and L- forms of Glucose

Glucose (Fig. 1.2.), which is used as a common ingredient in various food industries, has a sweetness about 70% of the sweetness of sucrose (BeMiller, 2019a). Glucose can be used to improve the functional properties of proteins by Maillard reactions. It was observed to be effective on the improvement of soy protein functionality since the emulsification activity of soy protein was enhanced by glycation with glucose (Tian *et al*., 2011). For other protein sources or fractions, effectiveness of glycation with glucose has also been proven. In a study where glycation of β-lactoglobulin with glucose was compared with fructose and allulose, glucose samples resulted in higher degree of glycation than other monosaccharides (Cheetangdee & Fukada, 2014).

1.2.2. Fructose

Fructose, which is a typical ketohexose, is also abundant in nature and mostly found in fruits. Thus, it is also known as fruit sugar. The water absorption capacity or of

fructose is higher than glucose because of its better hydrogen bonding ability with water (BeMiller, 2019b). The sweetness of fructose is also higher than glucose, which is 1.2-1.7 times the sweetness of sucrose (BeMiller, 2019a). This higher sweetness makes fructose useful and economically profitable in food industry, especially in confectionary, bakery and soft drink products. Since fructose is more profitable than glucose, commercially produced glucose by starch hydrolysis has being converted to fructose or fructose-glucose mixtures by enzymatic isomerization rather than being used as itself.

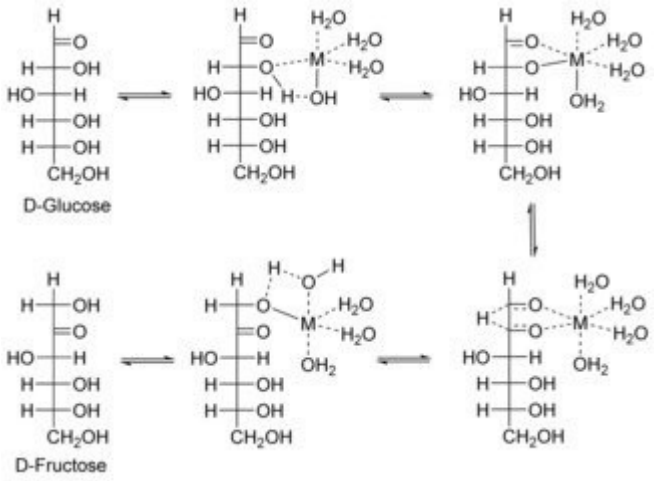

Figure 1.3. Isomerization of D-Glucose to D-Fructose (Delidovich & Palkovits, 2016)

Isomerization (Fig. 1.3.) may occur spontaneously and irreversibly or may be directed with the help of an enzyme and certain set of conditions. Alkaline conditions and increased temperature generally lead to spontaneous or non-enzymatic isomerization (Akram & Hamid, 2013; Oshima *et al*., 2014). In this regard, the molecular structure of a sugar may be affected by Maillard reaction or vice versa (Cheetangdee & Fukada, 2014).

1.2.3. D-Psicose

Rare sugars are the generic name of monosaccharides that are rarely found in the nature. Since rare sugars are partially digested in human body and thus have low

caloric value, they have drawn attention of food industry which aims to meet the needs of ever-growing society. D-Psicose is one of these rare sugars and it is C-3 epimer of fructose. The sweetness of D-Psicose is equivalent to 70% of the sweetness of sucrose. As confirming the general characteristics of rare sugars, D-Psicose has a significantly low caloric value, 0.39 kcal/g (Oh, 2007). With the 2012 dated announcement of U.S. Food and Drug Administration (FDE) where D-Psicose considered as GRAS (Generally Recognised as Safe), further scientific and commercialization studies on D-Psicose has gained momentum.

The pioneer study regarding the enzymatic production of D-Psicose was done by Ken Izumori in Kagawa University, 1994. For the production of D-Psicose, D-Tagatose 3 epimerase (DTE) enzyme was used. The limitations in the amount of enzyme and product yield had been causing very high production cost. Later on, with the immobilization of DTE enzyme enabled the reuse of the enzyme and significantly reduced the production cost. Moreover, natural sources of D-Psicose such as Zuina tree has been investigated and propagated as an alternative production method (Ogawa *et al*., 2017).

It is shown that D-Psicose has presented great organoleptic properties (Chattopadhyay *et al*., 2014) and has been used in various commercial products such as ice cream, beverages, bakery products and yogurt (Parkway and Estates, 2015) . Furthermore, the usage of D-Psicose reported to contribute not only to the organoleptic properties such as sweetness, smoothness and desirable mouthfeel but also to the functional enhancements of the products that they used in. These enhancements are high solubility, enhanced antioxidant activity and gelling property, low glycemic response and calorie (Best, 2010; Mu *et al*., 2012).

In the studies conducted with egg white protein and albumin, D-Psicose has been used in Maillard reaction. Results showed that, glycation with D-Psicose provides enhanced flavor and antioxidant activity (Sun *et al*., 2006). Another study, where foaming ability of glycated egg white protein was investigated in, showed that the effect of D-Psicose

on the foaming ability of glycated egg white protein was higher than the effects of glucose and fructose. Same study showed that when sucrose was replaced with D-Psicose in a cookie formulation, the crust color and the antioxidant activity of the products are observed to be better (Sun *et al*., 2008).

1.3. Modification of soy protein

Soy protein has become one of the major proteins that is demanded by food industry. Its high nutritional value, good functional properties and low production cost are some of the main reasons that makes it quite popular. On the other hand, some certain drawbacks of soy protein such as allergenicity, limited utilization and limited solubility prevent the full potential of its utilisation. In order to eliminate the drawbacks, several modification techniques have been applied on soy protein. These modifications help to improve the functional properties of the protein. The modifications are categorized by the used scientific approach such as physical, chemical and enzymatic modifications (Barac *et al.*, 2007; Schmohl and Schwarzer, 2014).

Physical modification is mostly achieved by thermal treatment. While there are various ways to apply thermal treatment such as wet heating (O'Mahony *et al*., 2017), dry heating (Sedaghat Doost *et al*., 2019), microwave heating (Guan *et al*., 2006), steam infusion (Wang & Johnson, 2001), the effect on the soy protein also varies depending on the conditions. It was observed that elimination of undesired volatile compounds, reducing of lipoxygenase activity and protease inhibitor activity can be achieved by thermal processing (Csapó and Albert, 2019). From functionality point of view; solubility, foaming and emulsification activities can be improved by heat treatment (Wang & Johnson, 2001), as well as gelation, water binding capacities and adhesive bonding strength (Vnučec *et al*., 2015). Furthermore, the digestibility can also be improved by thermal treatment (Kumar *et al.*, 2002).

There are several ways to chemically modify the soy protein. The aim behind chemical soy protein modification can also vary such as, protease activity reduction, phytic acid

reduction, increase or decrease of the solubility, elimination of undesirable flavor and odor compounds (Barac *et al.*, 2007).

Setting the pH value of soy protein mixtures to 4.0-4.2, which is known as acidification, in known to decrease the solubility of soy protein up to 90% (Wolf, 1969). On the other hand, setting the pH value at high extremes such as 11-12, significantly increases the solubility of soy protein but also causing formation of some toxic products such as lysinoalanine. Thus, alkaline modifications are preferred to be conducted at mild alkaline conditions such as pH 8, and with a combination of mild thermal treatment (50-60°C). These conditions are observed to increase the solubility of soy proteins up to 56.15% with reduced risk of toxicity (Barać, 2002).

Amidation and esterification are some of the other chemical modification methods. These methods are used to block available carboxyl groups resulting slightly altered isoelectric points. While amidation leading to formation of a modified soy protein with isoelectric point of 4.2 and 78% reduced available carboxyl groups, esterification with ethanol and methanol reduces carboxyl group availability by 55 and 83% respectively and set isoelectric point to 5.2. Although these modifications result in slightly lower emulsification activity compared to native soy protein, the stabilities of the emulsions prepared with modified proteins are observed to be increased significantly compared to native protein for both methods (Muhammad *et al*., 2012).

Other methods that are mostly used for chemical modification of soy protein isolate are acetylation and succinylation (Lundblad, 2010). Amino groups of soy protein bind with acetyl anhydrates in acetylation process, resulting the protein to unfold partially. By acetylation modification, while solubility of the soy protein increases slightly, gelation abilities decreases and isoelectric point lowers (Kester and Richardson, 2010). Succinylation is a more effective method of modification since the unfolding of soy protein occurs more extensively. Succinylation of soy proteins result in higher solubility and better hydration and emulsification properties compared to the native protein (Franzen and Kinsella, 1976).

The enzymatic approach to protein modification is also another method that has been used to improve mostly organoleptic properties of soy proteins. One of the major problems of soy protein is the undesirable flavors caused by medium-chain aldehydes (MacLeod & Ames, 1988). Separating the volatile compounds from soy protein by breaking the bonds and irreversibly oxidizing and removing the aldehyde compounds by using proteases and oxidases are common solutions to this problem (Abdo & King, 1967; Takahashi *et al*., 1980). However, it was observed that using proteases may still lead to other drawbacks such as bitter taste caused by the formation of low-molecular peptides (Fujimaki *et al*., 1968). Thus, as other modification methods mentioned previously enzymatic approach is also a limited soy protein modification technique.

1.4. Glycation

Food proteins are one of the essential parts of human diet. As a nutritional point of view while proteins supply amino acids as basic building blocks for organisms (Friedman, 1996a), they also provide functional properties to the food systems such as solubility, gelling, emulsifying, foaming, etc. (Zayas, 1997). These properties are known to depend on both intrinsic and extrinsic factors (Damodaran, 1996). Since the functional properties can easily be altered by these factors, food processing becomes a delicate work where controlling especially the extrinsic factors is vital to achieve the desired final product.

Glycation plays an important role in the improvement of the functional properties of proteins (Oliver *et al*., 2006). Among others, Maillard (Maillard, 1912) reaction has been put forward as a method of glycoprotein production since it requires no harsh conditions and chemicals. Maillard reaction can be controlled by altering the environmental factors such as pH, temperature, water activity, and intrinsic factors and ratio of the reactants (Labuza & Baisier, 1992; Van Boekel, 2001). By this way, producing modified proteins with improved functionality becomes possible.

The scheme created by Hodge (1953) shows the pathway of the Maillard reaction (Fig. 1.4.). The reaction takes places in three stages; early, advanced and final. The reaction

starts with the condensation of the carbonyl groups of a reducing sugar with the amino groups of reacting protein or amino acid in the early stage. This condensation leads to formation of a Schiff base and releases water. The condensation is followed by an irreversible reaction called Amadori rearrangement to form 1-amimo-deoxy-2-ketose which is called Amadori rearrangement product (ARP) (Ames, 1992).

The ARP's then degrade depending on the pH of the system. If the pH is 7 or below 1-2-enolization occurs forming mainly furfural from pentoses of HMF from hexoses. If the pH is higher than 7, 2,3-enolization is thought to occur to form reductones and fission products such as diacetyl, acetol and pyruvaldehyde. The reaction continues with a stage called Strecker degradation where products of the previous stage reacts with amino acids to form aldehydes and α-aminoketones.

In the advanced stage many different reactions occur, such as oxidation, cyclization, isomerization, dehydration, fragmentation and so on, leading different pathways and a vast mixture of compounds (Friedman, 1996b; Ledl & Schleicher, 1990; Martins, Jongen, & Van Boekel, 2001). Final stage is where the color of the product occurs with the formation of melanoidins, which are nitrogen-containing, water insoluble, colored polymers (Friedman, 1996b; Ledl & Schleicher, 1990; Martins, Jongen, & Van Boekel, 2001).

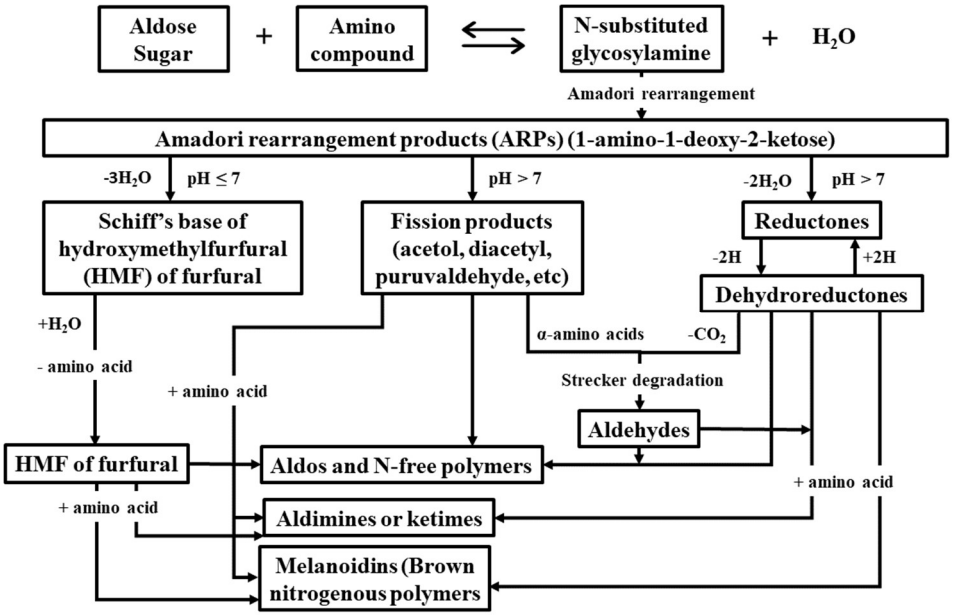

Figure 1.4. Maillard reaction scheme (Hodge, 1953; Zhang & Zhang, 2007 and Liu et al. 2012)

Proteins from different food sources have their own unique reaction characteristics during glycation. While the main source of amino groups are mostly ε-amino groups originated from lysine residues, there are other reacting groups although to a lesser extent; guanidino group from arginine, imidazole group from histidine, indole group from tryptophan and so on (Ames, 1992).

Water activity is an important parameter for glycation. Although glycation can occur in both dry and wet conditions, the optimum a_w for glycation was found to be between 0.5 and 0.8 (Liu, Ru and Ding, 2012). Increasing the reaction time and temperature were also found to increase the reaction rate. pH of the environment, ratio of the carbonyl and amino groups, and molecular properties of the carbohydrate and protein sources are also important parameters of the reaction (Sanmartín, Arboleya, & Moreno, 2009).

1.5. Objective of The Study

Soy proteins are important vegetable proteins due to their high essential amino acid content. Their gelling, emulsifying and foaming ability makes them perfect vegetable protein sources that can be used in many formulations. However, soy protein is a challenging protein. Its solubility is not that high and that could cause problems in its utilization.

Rare sugars are low calorie monosaccharides that are not digested in the body completely thus do not gave high caloric values. In that regard, they can be good alternatives to artificial sweeteners. D-Psicose is one of the rare sugars and it has started to be used in many formulations. Its brand name is *Allulose* and its commercial production has already started. One of the advantages of D-Psicose is its ability to participate in Maillard reactions more compared to the other monosaccharides. Since its tendency to react with proteins is high; it could be a good strategy to modify the properties of soy proteins using D-Psicose through glycation.

The objective of this study is to glycate soy protein with D-Psicose and with glucose and fructose as the control and investigate the extent of glycation through some measurements. Glycation was performed at 3 pH values (pH 7,10 and 12) and for 5 different protein:sugar ratios (1:1, 2:1, 3:1, 5:1 and 10:1) for all sugar types. To understand the extent of the glycation degree *of glycation* (DoG), *free amino groups* (FAG), *%reducing sugar* (RS) and *solubility* of the proteins were measured by different experiments.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

The materials used in preparation and analysis of glycated samples are shown as follows. D-Psicose (D-allulose, Sanitava Inc. Downers Groove, IL, USA), glucose (Dextrose, Tito, Turkey), D-fructose (Merck, KGaA, Darmstadt, Germany) were used as sugar sources. Soy protein Isolate (90% protein content) was purchased from Alfsasol, Turkey. Sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$), potassium phosphate monobasic anhydrous (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH) and potassium chloride (KCl), which used for buffer solutions, were purchased from Sigma-Aldrich Chemical Co. (Saint Louis, MO, USA). 3,5-Dinitrosalicylic acid (DNS) reagent, ortho-phthalaldehyde (OPA) reagent, bovine serum albumin (BSA), glycine, sodium deodecyl sulfate (SDS), ethanol (C_2H_5OH), sodium potassium tartrate tetrahydrate ($KNaC_4H_4O_6.4H_2O$), β-mercaptoethanol (2-mercaptoethanol) were purchased from also from Sigma-Aldrich Chemical Co. (Saint Louis, MO, USA) and di-sodium tetraborate decahydrate ($Na_2B_4O_7.10H_2O$) were purchased from Merck KGaA (Darmstadt, Germany) which used for analyses.

2.2. Methods

2.2.1. Preparation of The Samples

The preparation of the glycated proteins was done according to the study of van de Lagemaat *et al.* (2007) with slight modifications. Soy protein powder was mixed with three different sugar types (Glucose, Fructose and D-Psicose) at a protein:sugar ratio (w/w) of 1:1, 2:1, 3:1, 5:1 and 10:1. The protein - sugar mixtures were mixed with buffer solutions at different pH values namely Potassium phosphate monobasic

anhydrous (9.36 g/L) – Sodium phosphate dibasic heptahydrate (32.73 g/L) buffer for pH 7, Sodium bicarbonate (3.46 g/L) – Sodium hydroxide (0.71 g/L) buffer for pH 10, and Potassium chloride (12.1 g/L) – Sodium hydroxide (1.55 g/L) buffer for pH 12 as the final volumes and total concentration of soy protein - sugar mix in each sample was kept at 5% (w/w). The mixtures were hydrated completely with a magnetic stirrer and transferred to petri dishes before being frozen in a freezer (Arçelik, Turkey) and lyophilized (Beijing Songyuan Huaxing Technology Development Co., Ltd., China). Lyophilization lasted for 48 hours. The powdered samples were kept in a climacteric chamber at 50°C and 50% RH for 24 hours for glycation. As the control sample, soy protein was exposed to same conditions without sugar addition. The glycated samples were sealed and kept under room temperature (25°C) in dark until being used for analyses.

2.2.2. Characterization of The Glycated Products

2.2.2.1. Determination of the Degree of Glycation

The degree of glycation assay was conducted as described in a study (Zeng *et al.*, 2013) . 1% sample mixtures prepared at pH 7 buffer were homogenized (10 minutes with magnetic stirrer) and centrifuged (15 minutes at 4000 g). The absorbance values (nm) of the supernatant were measured at 420 nm with a UV-VIS spectrophotometer (Optizen Pop Nano Bio, Mecasys Co., Ltd., Korea).

2.2.2.2. Determination of the Free Amino Groups

The OPA reagent was prepared according to the procedure described in a previous study (Zhang *et al.*, 2012) . 80 mg of ortho-phthalaldehyde (OPA) was dissolved in 2 mL of 95% ethanol and mixed with 50 mL of 100mM sodium tetraborate buffer (pH 9.75), 5 mL of 20% (w/v) SDS solution and 200 μL of β-mercaptoethanol and then the solution was diluted to 100 mL with distilled water. The prepared ortho-phthalaldehyde (OPA) reagent was used within 2 hours to preserve the effectiveness of the reaction.

The free amino group assay was conducted as described in a previous study (Nooshkam and Madadlou, 2016) with slight modifications. 1.5mL of OPA solution was added to 0.5 mL of sample solution taken from the supernatant of the previously mixed (10 minutes with magnetic stirrer) and centrifuged (15 minutes at 4000 g) 1% mixtures of glycated proteins in pH 7 buffer. After 2 minutes of incubation in dark at room temperature, the absorbance values of the samples were measured at 340 nm with spectrophotometer. The amounts of free amino groups were calculated with a standard curve prepared with glycine for 6 different concentrations, 0.0017, 0.0033, 0.0066, 0.013, 0.027, 0.033 g/L. The standard curve was obtained on the experiment day. Standard curve is given in the appendix B (Figure B.2.). Since the total amount of samples by weight kept constant, the amount of soy protein used for each protein:sugar ratio differs. In order to get comparable results, the units of all results obtained from the standard curve was converted to g FAG/ g soy protein with the below formulation:

$$FAG\ \left(\frac{g}{g} protein\right) = \frac{FAG\ obtained\ from\ calibration\ curve\ \left(\frac{g}{100} ml\right)}{Initial\ protein\ amount\ of\ the\ sample\ \left(\frac{g}{100} ml\right)}$$

2.2.2.3. Determination of the % Reducing Sugar (%RS) Content

DNS reagent was prepared according to the method of Coughlan & Moloney (1988). Three hundred g of sodium potassium tartrate (Rochelle salt) and 10 g of dinitrosalicylic acid (DNS) were added to 800 ml of 0.5 N NaOH and the mixture was heated gently until all reagents were dissolved. Finally, the volume of the solution was completed to 1L with distilled water.

Reducing sugars in the solution were assayed as described in Saqib and Whitney (2011) with slight modifications. Samples were prepared from glycated proteins as 1% mixtures at pH 7 buffer. After mixing with magnetic stirrer for 5 minutes, the samples were centrifuged at 4,000 g for 15 minutes. 3ml of DNS reagent were added

to 1ml of sample solution taken from the supernatant of the centrifuged sample mixtures and kept for 10 minutes at 90°C water bath before they were cooled to room temperature with cold water and their absorbance values were measured at 540 nm with the spectrophotometer. The reducing sugar contents of the samples were calculated from the standard curve prepared daily by glucose solutions with 6 different concentrations; 0.06, 0.09, 0.12, 0.15, 0.18, 0.21 g/L. An example of the standard curve in given in Appendix B (Figure B.1.). To remain in the measurable absorbance range, some of the samples were diluted with an appropriate rate. Thus, obtained results from the calibration curve were multiplied by the relevant correction factor. The % reducing sugar (%RS) content (reducing sugar content that was bound to the soy protein via glycation) were calculated by subtracting the reducing sugar content of the solution from the initial sugar amount of the pre-glycation sample mixtures. Thus, the attached sugar percentages were calculated by dividing the attached reducing sugar amount to the initial sugar amount of the pre-glycation sample mixtures. The formulation of %RS content is given below:

$$RS\ (\%) = \frac{Initial\ sugar\ content\ of\ the\ sample\ \left(^{g}/_{L}\right) - Reducing\ sugar\ content\ of\ supernatant\ \left(^{g}/_{L}\right)}{Initial\ sugar\ content\ of\ the\ sample\ \left(^{g}/_{L}\right)} \times 100$$

2.2.2.4. Determination of the Solubility

The solubilities of glycated proteins were determined by the ultraviolet transmittance method as described in a previous study (Tian, Chen and Small, 2011) with slight modifications. 1% mixtures of glycated protein samples in pH 7 phosphate buffer were mixed with a magnetic stirrer for 15 minutes and then centrifuged for 15 minutes at 4,000 g. Samples taken from the supernatant of the centrifuged mixtures were separated and the transmittance of the samples were measured at 280 nm against pH 7 buffer. The concentrations of the samples were calculated by the standard curve prepared on the analysis day by bovine serum albumin (BSA) with 5 different concentrations; 0.002, 0.004, 0.006, 0.008, 0.01 g/L. The standard curve is given in Appendix B (Figure B. 3). To remain in the measurable absorbance range, some of

the samples were diluted with an appropriate rate. Thus, obtained results from the calibration curve were multiplied by the relevant correction factor. The solubilities of the glycated protein samples were determined as the percent ratio of the proteins in supernatant to proteins in the initial mixture. The formulation is given below:

$$Solubility\ (\%) = \frac{Protein\ content\ of\ the\ supernatant\ \left(\frac{g}{L}\right)}{Initial\ Protein\ Content\ \left(\frac{g}{L}\right)} \times 100$$

2.2.2.5. Experimental Design

In this study sugar type, glycation pH and protein-sugar ratio were determined as experimental parameters for all responses. Glucose, Fructose and D-Psicose were chosen as the sugar types. Considering the solubility of soy protein and Maillard reaction rates, pH 7, pH 10 and pH 12 were chosen as glycation pH values. From previously conducted studies, Protein-sugar ratios were chosen as 1:0 (Control group), 1:1, 2:1, 3:1, 5:1 and 10:1.

Table 2.1. A summary of experimental design

Factors	Levels	Responses
Sugar Type	D-Psicose Glucose Fructose	Degree of Glycation (DoG)
pH	7 10 12	Free Amino Groups (FAG)
Protein:sugar Ratio	1 2 3 5 10	% Reducing Sugar Content (%RS) Solubility

2.2.2.6. Statistical Analysis

Statistical analysis was done for all the experimental data to check the significant difference of factors used. All measurements were performed as duplicates or triplicates. Analysis of variance (ANOVA) was performed using the general linear model (GLM) by using Minitab V17 (Minitab Inc., Coventry, UK) at 5% significance level. For the comparisons, Tukey's comparison test was performed at 95% confidence interval. All the assumptions of ANOVA (Normality of the residuals and test for equal variances) were checked prior to analysis and irrelevant data were removed if necessary. If assumptions failed Box-Cox transformation was applied. The letters on the tables indicate significant difference among the samples ($p<0.05$).

CHAPTER 3

RESULTS AND DISCUSSION

As explained in the previous chapters, in this thesis, effect of 3 different parameters were investigated on the physiochemical properties of glycated soy proteins. *pH*, *protein:sugar ratio* and *sugar type* were the factors examined. Degree of glycation, free amino group and % reducing sugar contents and solubility of the proteins were determined for the glycated soy proteins. The detailed experimental design was given at the end of Chapter 2.

3.1. Degree of Glycation (DoG)

The browning of the glycated products was investigated by obtaining the absorbance values of the glycated samples at 420 nm. Although the rate of browning, thus, the degree of glycation does not solely depend on Maillard reactions, it was found in a previous study where they investigated the glycation of amino acids alanine, glycine and lysine, the effect of caramelization on the browning of the glycated samples was insignificant compared to the effect of Maillard reaction (Morales and Jiménez-Pérez, 2001). Thus, for this study it was also hypothesized that this effect could also be insignificant for soy proteins. Degree of glycation results are given in Table 3.1., Multiple factor ANOVA was conducted, and the data satisfied the assumptions of ANOVA (Normality and Equality of Variances) thus no transformation was applied on the data set. Coefficient of variance was set to 10% for all analyzed data. Since there were 3 replicates, at least 2 data were considered for each treatment. ANOVA and multiple comparison results obtained by MINITAB for the degree of glycation are provided in the Appendix (Table A.1. and A.2.1. - A.2.5.).

When the way ANOVA results were examined it was observed that all factors , 2 and 3 way interactions (sugar type, protein:sugar ratio and pH) were statistically

significant on the degree of glycation ($p<0.05$) (Appendix *Table* A.1.). In Table 3.1., lettering for ANOVA was conducted for each sugar ratio.

Table 3.1. Degree of Glycation of the glycated samples

Protein:Sugar Rate	Absorbance (nm)								
	Glucose			Fructose			D-Psicose		
	pH 7	pH 10	pH 12	pH 7	pH 10	pH 12	pH 7	pH 10	pH 12
1:1	0.033 ± 0.002^{e}	0.101 ± 0.004^{c}	0.032 ± 0.002^{e}	0.033 ± 0.001^{e}	0.116 ± 0.006^{b}	0.017 ± 0.001^{f}	0.080 ± 0.002^{d}	0.139 ± 0.006^{a}	0.038 ± 0.001^{e}
2:1	0.032 ± 0.007^{bc}	0.040 ± 0.005^{ab}	0.045 ± 0.001^{ab}	0.023 ± 0.004^{cd}	0.043 ± 0.001^{ab}	0.017 ± 0.003^{d}	0.053 ± 0.003^{a}	0.049 ± 0.003^{a}	0.025 ± 0.002^{cd}
3:1	0.033 ± 0.002^{ab}	0.036 ± 0.002^{ab}	0.026 ± 0.002^{c}	0.022 ± 0.001^{c}	0.032 ± 0.002^{b}	0.016 ± 0.001^{d}	0.037 ± 0.001^{a}	0.035 ± 0.002^{ab}	0.027 ± 0.002^{c}
5:1	0.024 ± 0.002^{cd}	0.034 ± 0.003^{ab}	0.035 ± 0.000^{ab}	0.018 ± 0.001^{de}	0.029 ± 0.001^{bc}	0.016 ± 0.001^{e}	0.032 ± 0.001^{ab}	0.038 ± 0.004^{a}	0.022 ± 0.001^{cde}
10:1	0.023 ± 0.001^{def}	0.030 ± 0.002^{bc}	0.036 ± 0.003^{a}	0.018 ± 0.001^{fg}	0.020 ± 0.001^{ef}	0.012 ± 0.001^{g}	0.026 ± 0.001^{cde}	0.032 ± 0.002^{ab}	0.028 ± 0.005^{bcd}

*Lettering in the tables indicate the significance differences for the same protein:sugar ratio at 95% significance level (Appendix Table A.2.1.-A.2.5.: Two Way ANOVA)

3.1.1. Effect of Sugar Type

The type or characteristics of the reactants are known to be one of the many parameters that are effective on the Maillard reaction (Morales and Jiménez-Pérez, 2001; Zeng *et al.*, 2011). Different types of sugars are expected to cause a variety in the rates of Maillard reaction under same conditions.

According to an early study conducted by Lewis and Lea (1950) the reactivity order (% amino nitrogen drop/day) of the reducing sugars, which were stored with casein at 37°C and 70% RH, was *xylose* > *arabinose* > *glucose* > *lactose* > *maltose* > *fructose* where glucose was found ten times more reactive than fructose. Although the reactivities of sugars were not the sole parameter effecting the degree of glycation since the order may be different for other proteins and polymerization and browning steps might occur at different rates for each sugar, they still have a great effect on the degree of glycation.

A study conducted by Kato et al (1986), where the reactivities of a number of aldohexoses were investigated at 50°C and 65% RH towards ovalbumin, suggested that glucose gave lower degrees of polymerization then mannose and galactose. Although increasing storage time and sugar ratio resulted in color development in each system, galactose was found to be the most effective aldohexose on color development. However, the results of the study suggested that protein polymerization and color development did not have to be coexisting and might have occurred independently during Maillard reaction. A supporting later study (Kato *et al.*, 1989) showed that removing excess glucose from the system of ovalbumin-glucose at an early stage of the Maillard reaction, resulted in a strong suppression of color development, whereas protein polymerization continued. The greater reaction rate of galactose used system was thought to be related with the C_4 hydroxyl group of galactose since it was confirmed with another aldohexose, talose, having the C_4 hydroxyl group as galactose in same configuration. While the polymerization and

color development behaviors of galactose and talose systems were similar, glucose and mannose systems showed different results (Kato *et al.*, 1989).

In this study, three-way ANOVA results showed that sugar type was significant, and the highest DoG was observed on *D-psicose* samples followed by *glucose* and *fructose* respectively ($p<0.05$). According 2-way ANOVA results (Appendix Table A.2.1. - A.2.5.), except protein:sugar ratio of 1, glucose and D-psicose samples showed the highest DoG values and fructose had the least ($p<0.05$). In a study comparing the browning degrees of D-psicose and fructose which were glycated with lysine, (Li, Luo and Feng, 2011) it was shown that the browning of the D-psicose samples was higher than the fructose samples after 4 h of incubation which indicated that D-psicose tended to be more reactive in advanced Maillard reactions than fructose under the specified conditions. In our case, 3-way ANOVA results confirmed this finding ($p<0.05$). However, when the protein:sugar ratio was examined independently it was observed that psicose and glucose samples had similar DoG values except the ratio of 1:1. At 1:1 ratio D-psicose samples still gave the highest values. The study was designed such that protein concentation in the glycated mixture was increased to see if the used sugar concentrations were sufficient or not. At 1:1 ratio although caramelization was found insignificant in previous studies, D-Psicose is known to caramelize quite easily at the glycation condition. It is possible that the unused D-psicose could have caramelized more at that ratio and contributed more to DoG. For other ratios, Maillard could be the controlling mechanism and thus D-Psicose and glucose had similar values ($p>0.05$). A representative figure has been shown below (Fig. 3.1.).

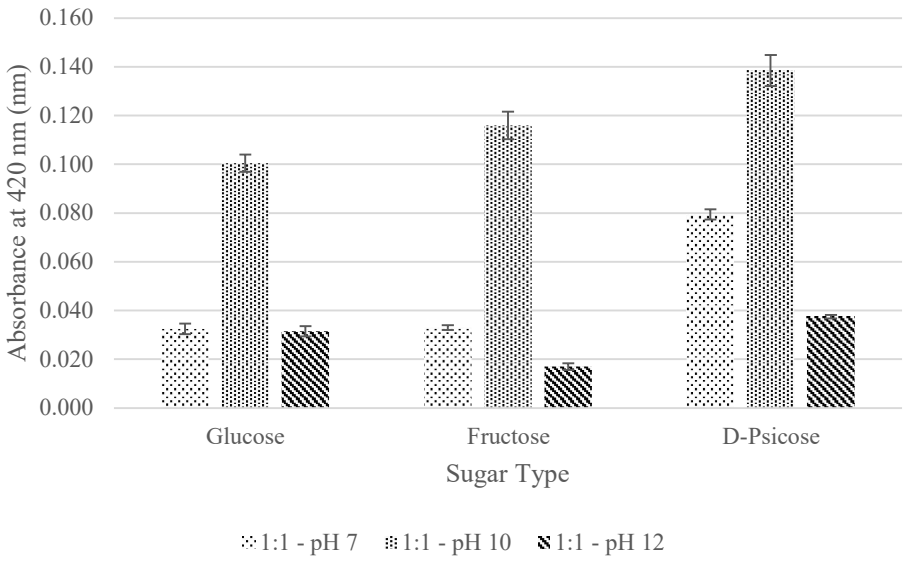

Figure 3.1. Effect of sugar type on the Degree of Glycation of the glycated samples at 1:1 protein:sugar ratio.

In general, ketoses are considered to be less reactive than aldoses due to their relatively less electrophilic carbonyl groups (Yeboah *et al*., 1999). It has been found in another study that the development rate of fluorescence in glycated α-lactalbumin samples has been observed to be higher with glucose than fructose (Sun *et al.*, 2006).

In another study conducted by reacting hemoglobin with several different monosaccharides in aqueous solution it was shown that the formation rate of Schiff base was proportional with the amount of the sugar that existed in the carbonyl form in the solution. Among the tested aldohexoses the lowest reaction rate was observed in the glucose system (Bunn and Higgins, 1981). With the knowledge of galactose and talose existing in higher proportions of carbonyl form in aqueous solutions than glucose and mannose (Hayward and Angyal, 1977) it could be expected higher sugar – free amino group initial binding rates for galactose and talose systems. However, a later study (Kato et al, 1986) investigating ovalbumin – aldohexose systems showed that there was no significant difference in free amino group decrease among any of the glucose, galactose and mannose systems, proving hemiacetal:carbonyl ratio of

sugar in aqueous solution and could not directly associated with the reaction rate in lower moisture systems. Instead, the reaction rate and thus the degree of glycation or browning more likely to depend on the formation rate of the further stage products formed by the degradation of Amadori Rearrangement Products (ARP) and subsequent reactions. It was suggested in the study of Kato et al. (1986) that the reactivity difference between mannose, glucose group and galactose, talose group might have been caused by the fact that while galactose and talose formed intermediate Maillard products having stable trans chair configuration with ovalbumin, mannose and glucose from intermediate products having energetically unfavorable cis chair configuration.

3.1.2. Effect of pH

It is known that pH is one of the many factors effecting the rate of Maillard reaction (Ashoor and Zent, 1984; Morales and Jiménez-Pérez, 2001; Belton, 2003).

When 3-Way ANOVA results were examined it was seen that pH was a significant factor ($p<0.05$). pH 10 samples showed the higher degree of glycation whereas pH 12 samples showed the lowest. According to these results, the intensity of brown color formation did not show a constant increase or decrease pattern with the increase of pH. On the contrary, an optimum pH value existed for the highest degree of glycation value. A representative figure has been shown below (Fig. 3.2.). As the sugar content of the samples decreased, the significant difference between different pH value samples faded and the results became closer to the results of non-glycated samples.

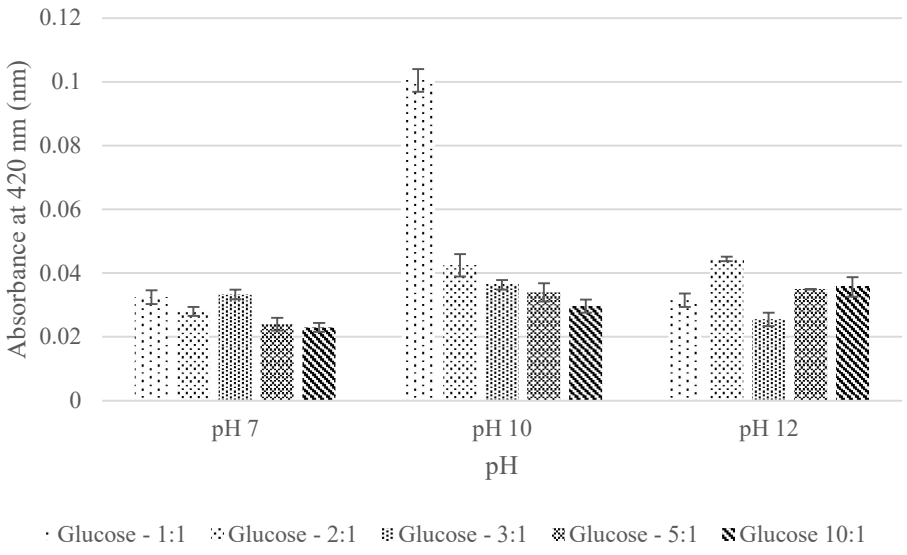

Figure 3.2. Effect of pH on the Degree of Glycation of the samples glycated with glucose.

DoG values for the non-glycated samples were also measured and pH was also found significant for those samples ($p<0.05$) (Appendix A.2.6.). pH 7 samples had the highest value wherease as pH increased DoG decreased. The decrease on the pH of the non-glycated samples could be explained by the bleaching the inherent color pigments in soy proteins.

There are numerous studies suggesting that the degree of glycation increases with the increase of pH (Ashoor and Zent, 1984). However, most of them did not exceed pH 9 during their studies. In a study (Ajandouz and Puigserver, 1999) where the effect of pH on Maillard reaction kinetics of Fructose – Lysine complex was investigated between pH 4 and pH 12, it was also observed that the degree of glycation increased with the increase of pH. On the other hand, another previous study which observed the degree of glycation values of several sugar – amino acid complexes between pH 6 and pH 12, showed that there existed an optimum pH value for browning since all sugar – amino acid complexes had the highest degree of glycation value around pH 10. After pH 10 the degree of browning seem to slightly decrease up to pH 12 (Ashoor

and Zent, 1984). It is known that pH is effective on the Maillard reaction especially on the degradation pathway of Amadori Rearrangement products since reductones that formed at higher pH values had higher browning potential than furfurals that form as pH decreases (Belton, 2003). It is also important to point out that, in this study it is not a simple amino acid such as lysine that is being glycated. We talk about a complex protein like soy protein whose solubility is also affected significantly by pH due to denaturation; particularly at pH extremes. This could also cause a change on the solubility and consequently on DoG.

3.1.3. Effect of Protein:Sugar Ratio

In this study, protein:sugar ratio was a tested parameter. As explained in Chapter 2, all solutions were prepared at 5% solid concentration initially and then freeze-dried. That 5% solid concentration was adjusted according to the predetermined ratios. So, 1:1 ratio had the highest sugar content and 10:1 ratio had the lowest. While conducting the experiments, glycated samples were added to the solution at 1% ratio and it is obvious that this 1% ratio could also include unglycated sugar. Such an approach was followed to see if the used sugar concentration was enough to glycate the protein. According to the 3 -Way ANOVA results protein:sugar ratio was found significant as expected since substrate concentrations were changing as the ratio changed ($p<0.05$). As protein:sugar ratio increased DoG values decreased significantly ($p<0.05$). This could have been an indication that as protein concentration was increasing sugar concentration started to be insufficient thus sugar concentration started to control the reaction rate. A representative figure has been shown below (Fig. 3.3.).

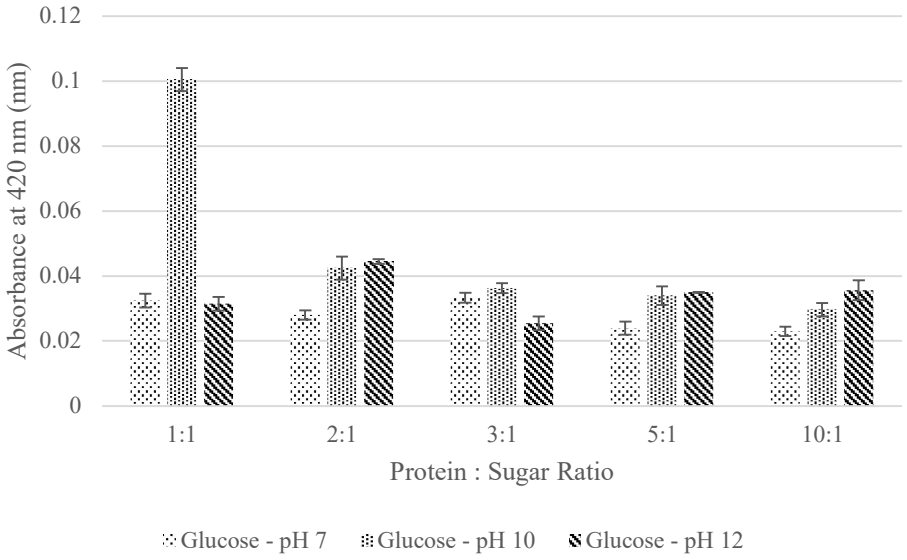

Figure 3.3. Effect of protein:sugar ratio on the Degree of Glycation of the samples glycated with glucose.

According to a study, higher carbonyl group to amino group molar ratios are generally expected to promote better the development of the Maillard reaction Martinez-Alvarenga *et al.*, 2014). On the other hand, another study suggested that the degree of glycation more likely depended on the general crowding of the reactants rather than the molar ratio of them (Zhang *et al.*, 2012).

In the case of this study since the total amount of the reactants by weight was kept constant, the increase in the amount of protein resulted in a decrease in the amount of sugar. In that case although the total reactant concentration of the system was still constant, the molar crowding increased due to large protein molecules with the increase of carbonyl to amino groups ratio.

3.2. Determination of the Free Amino Groups (FAG)

Maillard reaction starts with an initial step called *condensation* where the amino groups from protein source and carbonyl groups from sugar source reacts with each other and bind together to form a glycosyl amine and releases a water group. Thus,

the degradation of primary amino groups of the protein source is widely accepted as an indicator of the extent of early stage Maillard reaction.

In this study, as discussed in Chapter 2, the degradation of free amino groups was determined by OPA method, which is a spectrofluorometric analysis (R. Li *et al.*, 2015).

Similar to DoG data analysis, 3 Way ANOVA and 2 Way ANOVA for each sugar concentration was performed. ANOVA results are given Appendix (Table A.3. and A.4.1. – A.4.5.). The data acquired by OPA method did not follow a normal distribution when 3-Way ANOVA was conducted and did not satisfy the 'equality of variance' assumption of ANOVA. That is why Box-Cox transformation was applied on the data set with a rounded value of 0.5 for the exponent. Transformed form of the data satisfied the assumptions and interpretations were done accordingly. Results of the OPA method are given in Table 3.2. The data in the table is untransformed data. For 2-Way ANOVA analysis, except protein:sugar ratio of 3, all other ratios satisfied the both assumptions of ANOVA and no transformation was applied. For protein:sugar ratio of 3, Box-Cox transformation was applied with an exponent of -0.5.

In both ANOVA (2 and 3-Way), all factors and their corresponding interactions was found to be significant on FAG ($p<0.05$).

3.2.1. Effect of sugar type

As seen in Appendix Table A.3., sugar type was found significant on FAG ($p<0.05$). Since the protein concentrations in each sample was not same, to make a more accurate comparison, FAG values were adjusted with respect to the initial protein amounts in the respective samples as explained in Chapter 2. As glycation occurs it is expected a decrease in the FAG. Results showed that (Table 3.2.), fructose samples had the highest amount of FAG indicating that glycation was not that much. This was consistent with the DoG results as fructose samples were shown the have the lowest amount of DoG values as well. Moreover, glucose samples showed the lowest FAG

values followed by D-Psicose. A representative figure has been shown below (Fig. 3.4.). When the 2 Way ANOVA results were examined (Appendix Table A.4.1. - A.4.5.), the trend was kind of similar. For protein sugar ratios of 2, 5 and 10 glucose and D-Psicose samples were similar in terms of FAG whereas for ratios of 1 and 3, glucose was slightly lower than D-Psicose. Although not perfect there was a significant ($p<0.05$) but positive correlation ($r=\sim0.40$) between DoG and FAG as seen in the correlation plot in Fig 3.5. The reason of the low correlation could also be attributed to solubility of protein at different pHs. As seen Fig. 3.6. for unglycated soy protein FAG changes with pH. This could be due to the unfolding of soy proteins as pH increases. And this would have a direct effect on the overall behavior of the glycation.

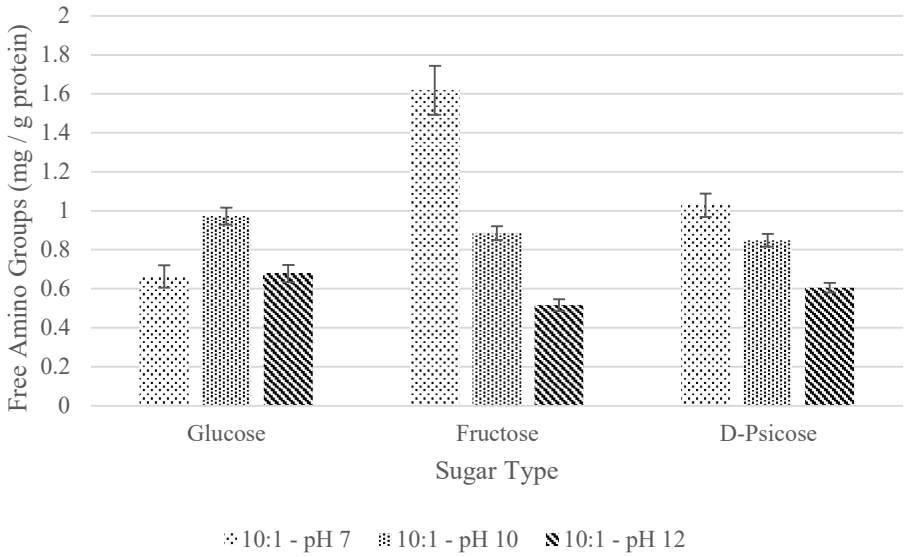

Figure 3.4. Effect of sugar type on the Free Amino Group content of the glycated samples at 10:1 protein:sugar ratio.

Table 3.2. Free Amino Group content of the glycated samples

Protein:Sugar Rate	Free Amino Groups (mg/g protein)								
	Glucose			Fructose			D-Psicose		
	pH 7	pH 10	pH 12	pH 7	pH 10	pH 12	pH 7	pH 10	pH 12
1:1	0.72 ± 0.03^{e}	1.65 ± 0.11^{c}	0.72 ± 0.06^{e}	2.16 ± 0.15^{a}	1.19 ± 0.09^{d}	1.30 ± 0.05^{d}	1.60 ± 0.13^{c}	1.85 ± 0.14^{b}	0.62 ± 0.04^{e}
2:1	0.64 ± 0.05^{efg}	1.20 ± 0.11^{bc}	0.60 ± 0.04^{fg}	1.60 ± 0.04^{a}	1.20 ± 0.05^{bc}	0.60 ± 0.07^{fg}	1.24 ± 0.06^{b}	0.82 ± 0.06^{de}	0.51 ± 0.04^{g}
3:1	0.73 ± 0.03^{c}	0.99 ± 0.05^{b}	0.75 ± 0.02^{c}	1.74 ± 0.21^{a}	0.87 ± 0.01^{b}	0.69 ± 0.02^{c}	0.97 ± 0.02^{b}	0.94 ± 0.09^{b}	0.52 ± 0.02^{d}
5:1	0.59 ± 0.03^{ef}	1.25 ± 0.01^{b}	0.65 ± 0.05^{e}	1.50 ± 0.08^{a}	0.81 ± 0.02^{d}	0.56 ± 0.01^{ef}	0.98 ± 0.05^{c}	0.85 ± 0.09^{cd}	0.48 ± 0.04^{f}
10:1	0.66 ± 0.06^{d}	0.97 ± 0.04^{bc}	0.68 ± 0.05^{d}	1.69 ± 0.13^{a}	0.89 ± 0.03^{c}	0.52 ± 0.03^{e}	1.03 ± 0.06^{b}	0.85 ± 0.03^{c}	0.61 ± 0.02^{de}

*Lettering in the tables indicate the significance differences for the same protein:sugar ratio at 95% significance level (Appendix Table A.4.1.-A.4.5.: Two Way ANOVA)

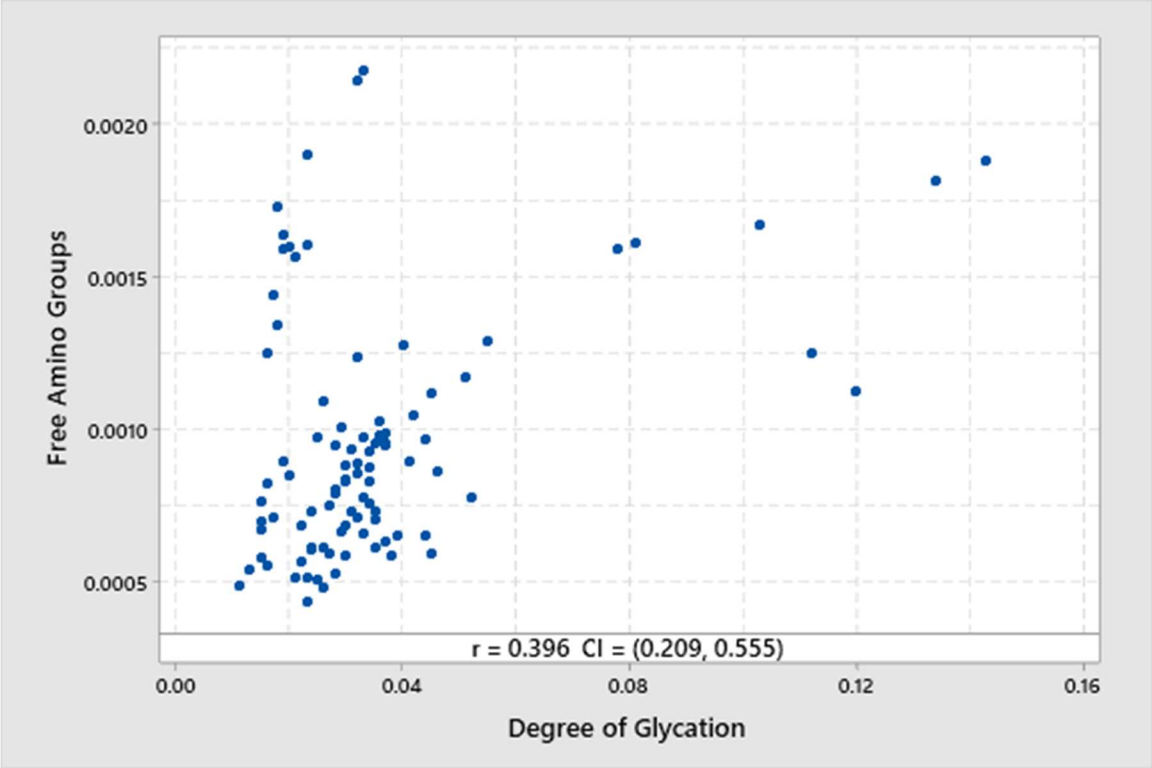

Figure 3.5. Correlation Plot between DoG and FAG.

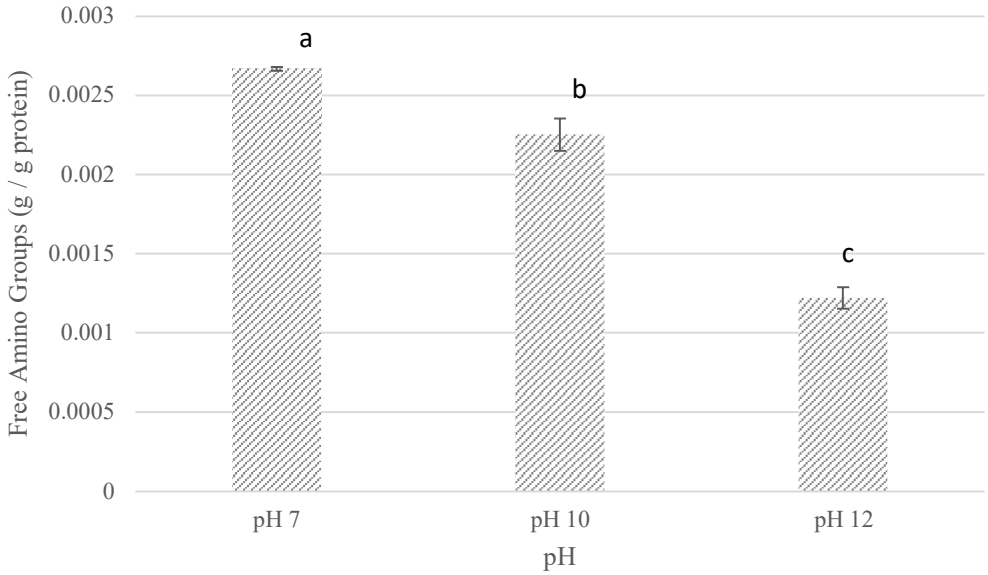

Figure 3.6. FAG of unglycated soy proteins.

In a study, the free amino group percentage of several sugar – egg white protein complexes were measured by OPA method after 48 h of incubation at 50°C and 55% RH. In fact, this was the reference study for determining the glycation parameters in this thesis. According to the study, no significant difference was found between the results of fructose and D-Psicose systems (Charoen *et al*., 2014). Another study examined α-lactalbumin glycation with fructose, glucose and allose and found out that during the condensation period of Maillard reaction, the degradation of free amino groups was highest in the allose systems and lowest in the fructose systems while the free amino group amount of native protein remained same (Sun *et al.*, 2008). This result can be a considered as verification of the hypothesis that the that aldose sugars had higher reactivity than ketoses for Maillard reaction (Nooshkam and Madadlou, 2016).

Li et al (2011) compared the amounts of free amino groups of fructose-lysine and D-Psicose-lysine systems during Maillard reaction (Li *et al*., 2011) and observed that free amino group decrease in the D-Psicose-lysine system was about 10% higher than fructose-lysine system at the end of the 8 h incubation period indicating D-Psicose had higher reactivity than fructose. They also found no correlation between the free amino group loss and browning rate of the glycated samples. This was also a consistent finding with our study.

3.2.2. Effect of pH

pH was found to be significant on the amount of FAG. Three-way ANOVA results showed that pH 12 samples had the lowest amount of FAG followed by 10 and 7 respectively ($p<0.05$). On the other hand, when the results were examined at different *protein:sugar* ratios it was found that for ratios of 2,3 and 10 all pH values were significantly different whereas for ratios of 1 and 5 pH 7 and 10 were same but higher than pH 12.

FAG values being lower at pH 12 was not clearly an indication of the highest glycation since for the native proteins that were not glycated as seen in Fig 3.6., the lowest values

were found for pH 12 as well. As will be discussed later in the *solubility* section this is clearly an indication that in such a complex polymer system, it is not possible to examine glycation just by considering the FAG since denaturation as a result of pH change could significantly affect the results.

Considering the overall results and excluding the pH 12 it is possible to say that glycation have occurred more at pH 10 which was also supported by the DoG results. A representative figure has been shown (Fig. 3.7.)

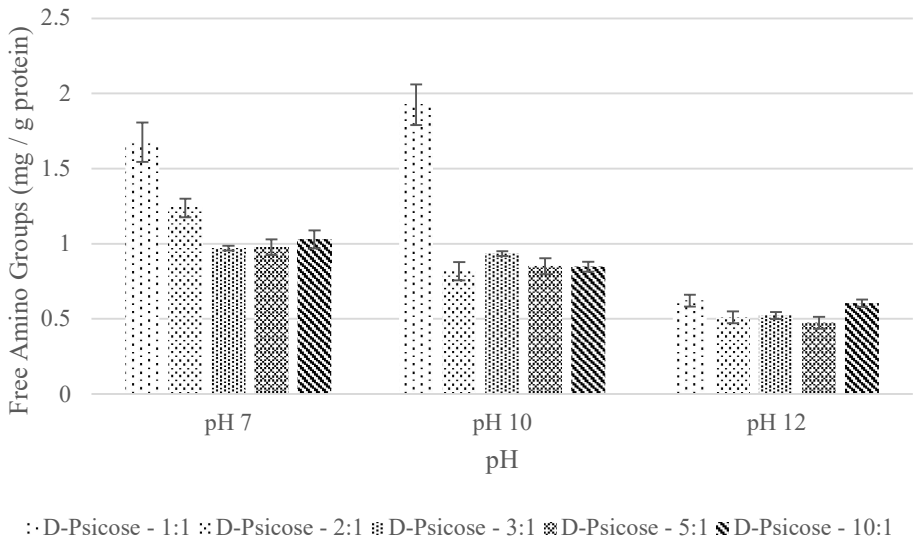

Figure 3.7. Effect of pH on the Free Amino Group Content of the glycated samples with D-Psicose.

3.2.3. Effect of protein sugar ratio

As explained before, in the samples examined in this project, protein concentration was not fixed. So, normally as the ratio increased protein content increased indicating higher FAG values in initial samples. To account for this effect, the final values of the OPA results were corrected with respect to the initial protein concentration. If glycation occurs FAG are expected to decrease but the initial high concentration of the proteins might still have dominated the results. Table 3.3. shows the Tukey

multiple comparison results of the 3-Way ANOVA (also given in Appendix). It was observed from the results that as protein concentration increased, FAG decreased ($p<0.05$) and protein sugar ratio of 5 was enough and results did not change at the ratio of 10. A representative figure has been shown below (Fig. 3.8.). So, increasing the protein concentration to induce higher glycation could be a good strategy as similar results were also observed for DoG. However, it should be pointed that with increasing protein concentration, molecular crowding could also have affected the accessibility of free amino groups thus decreasing their values. This affect should also be considered to make generalized interpretations.

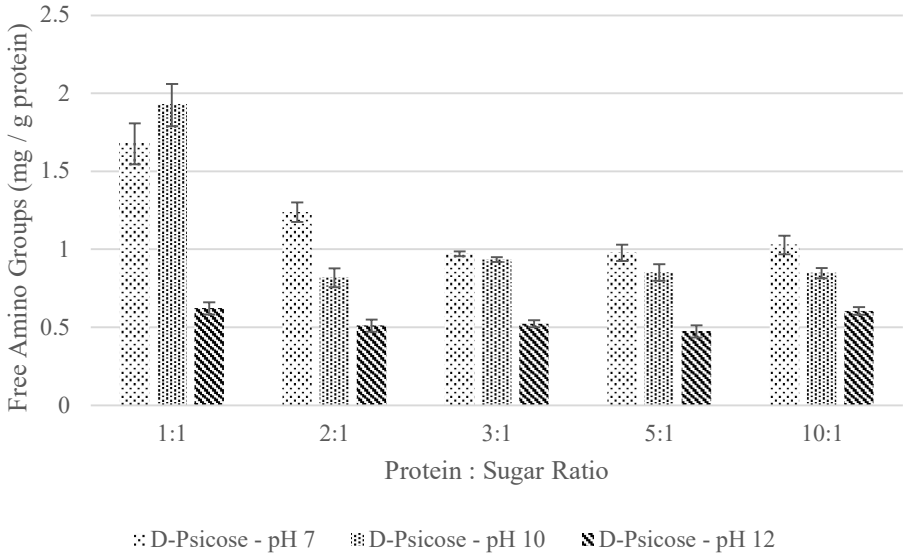

Figure 3.8. Effect of protein:sugar ratio on the Free Amino Group content of the samples glycated with D-Psicose.

Table 3.3. Effect of Protein:Sugar Ratio on FAG (Considering All Results)

Protein:sugar Ratio	N	Mean	Grouping		
1	21	0.0012575	A		
2	24	0.0008990		B	
3	24	0.0008864		B	C
10	25	0.0008494			C D
5	24	0.0008213			D

Means that do not share a letter are significantly different at 95 % CL.

3.3. Determination of the % Reducing Sugar (%RS)

Glycation is a reaction involving both proteins and sugars. Proteins and sugars are depleted, and new products are formed as result of the Maillard Browning. In order to understand the extent of reaction, the amount of reducing sugars attached to the protein could also be a good indicator. In this study, to determine the amount of the sugar that has reacted with the proteins; one of the most commonly used methods of reducing sugar content determination; DNS method was used and then the sugar that has bound to soy protein was calculated. Details of the method were already explained in Chapter 2. Results are given in Table 3.*4*. Similar to DoG and FAG data, 3 Way ANOVA and 2 Way ANOVA for each sugar concentration was conducted on the acquired data. ANOVA results are given in Appendix *(Table* A.5. and A.6.*1. – A.6.5.).* Data satisfied the assumptions of the ANOVA and no transformation was conducted on the data set. All factors and their corresponding interactions were found significant on the results ($p<0.05$).

Table 3.4. Reducing Sugar Content (%) of the glycated samples

Protein:Sugar Rate	Reducing Sugar Content (%)								
	Glucose			Fructose			D-Psicose		
	pH 7	pH 10	pH 12	pH 7	pH 10	pH 12	pH 7	pH 10	pH 12
1:1	35.87±1.65^{d}	50.49±1.71^{b}	59.79±2.08^{a}	39.99±3.62^{cd}	59.24±0.54^{a}	43.39±0.39^{c}	23.29±1.28^{e}	55.38±3.20^{ab}	41.97±1.96^{c}
2:1	40.16±0.58^{d}	58.03±1.74^{b}	64.73±0.66^{a}	49.65±3.30^{c}	63.12±1.19^{a}	62.00±0.91^{ab}	33.72±1.80^{e}	58.18±0.69^{b}	48.00±1.30^{c}
3:1	27.70±3.36^{f}	55.48±3.79^{bc}	50.78±1.67^{cd}	42.29±3.00^{e}	68.19±2.30^{a}	60.77±1.55^{b}	30.28±2.04^{f}	54.82±0.95^{bc}	47.85±0.26^{de}
5:1	59.96±1.60^{a}	61.80±2.59^{a}	61.70±3.19^{a}	48.26±2.91^{b}	61.83±1.17^{a}	58.14±0.96	33.70±1.30^{c}	62.85±0.67^{a}	42.43±2.80^{b}
10:1	78.89±1.10^{a}	71.25±1.09^{b}	67.33±0.96^{bc}	65.30±2.46^{cde}	61.99±1.22^{de}	76.41±0.78^{a}	51.97±1.29^{f}	65.07±0.99^{cd}	60.68±2.88^{e}

*Lettering in the tables indicate the significance differences for the same protein:sugar ratio at 95% significance level (Appendix Table A.6.1.-A.6.5.: Two Way ANOVA)

3.3.1. Effect of sugar type

According to 3 Way ANOVA results, sugar type was found to be significant on the % reducing sugar content ($p<0.05$). The highest amount of bound sugar was found in the fructose samples followed by glucose and D-Psicose. This indicated that the highest amount of glycation occurred on the fructose. Moreover, D-Psicose samples showed the lowest amount of reducing sugar ($p<0.05$). Although RS and DoG results seemed to contradict, a significant correlation was not detected between the DoG and RRS values ($p>0.05$). For the ANOVA results conducted based on individual protein:sugar ratios (Appendix Table A.6.1. – A.6.5.); D-Psicose samples always had the lowest %RS results compared to glucose and fructose samples except at the ratio of 3. For that ratio, D-psicose and glucose were similar which was also a case that was observed in DoG values as explained before. A representative figure has been shown below (Fig. 3.9.). A 2011 study, where the reducing sugar contents of solutions prepared with various monosaccharides and disaccharides were measured by DNS, suggested that different sugars may gave different reaction rates with DNS reagent at the same concentration. Thus, comparability of different sugar types with DNS method may have been deceptive (Saqib and Whitney, 2011). On the other hand, the reducing compounds that have formed as a result of Maillard could have also contributed to the results. Thus, this could be the reason of contradicting results of DNS and OPA analyses on the extent of glycation. Since DNS does not specify the type of the reducing sugar, it is probable that other carbonyl groups have also reacted with the DNS agent.

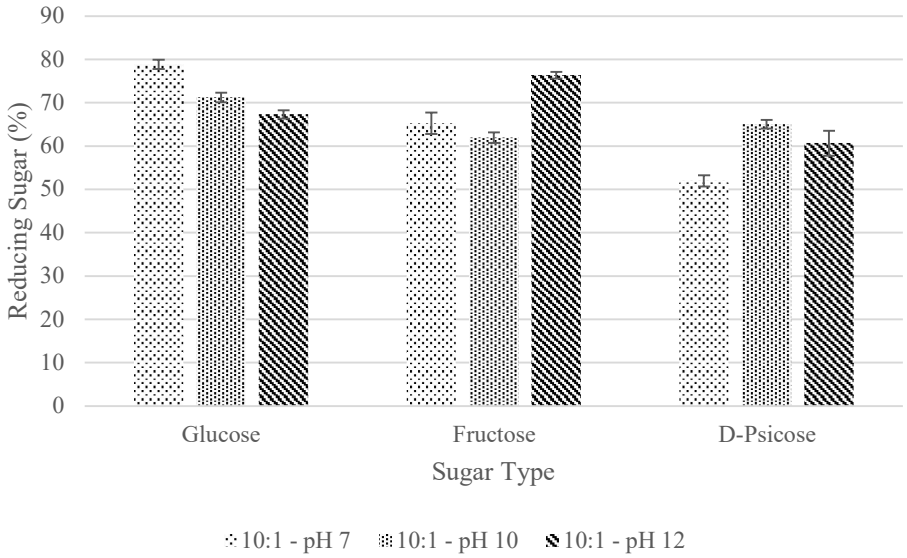

Figure 3.9. Effect of sugar type on the % Reducing Sugar content of the glycated samples at 10:1 protein:sugar ratio.

3.3.2. Effect of pH

pH was found to be a significant factor on the % reducing sugar content. As expected, pH 10 samples had the highest amount of % reducing sugar which was the pH where the highest amount of glycation was observed by other analyses. pH 10 was followed by 12 and 7 respectively ($p<0.05$). A representative figure has been shown below (Fig. 3.10.). To isolate the effect of pH, Pearson correlation analysis was conducted between the DoG values and %RS values at different pHs. A significant negative correlation of around 60 % was detected for pH 7 and pH 10 samples (Fig. 3.12.a-b) whereas correlation faded away at pH 12 ($p<0.05$). It was interesting to see a negative correlation and it was not expected since both responses were indicative of the extent of glycation. Negative correlation between these responses was a clear evidence that it was not only Maillard, but other factors were controlling the reaction.

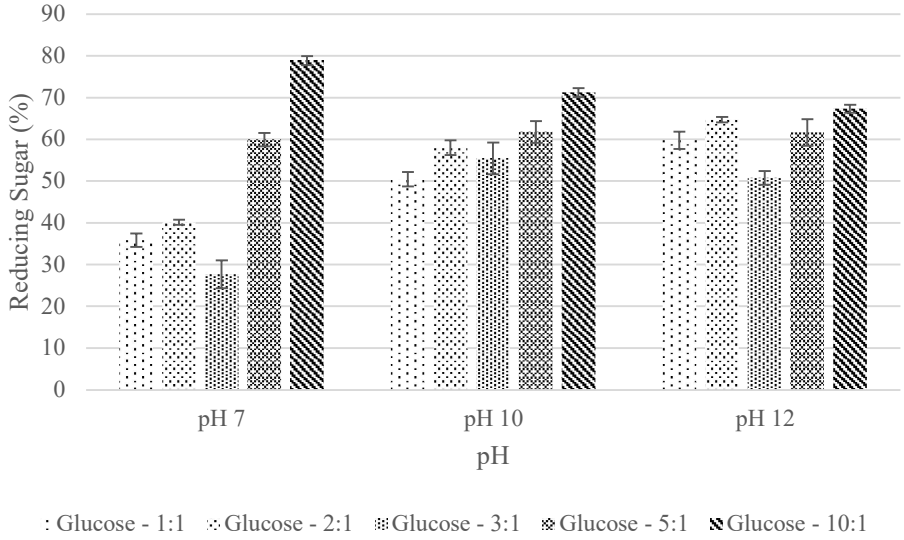

Figure 3.10. Effect of pH on the % Reducing Sugar content of the samples glycated with glucose.

3.3.3. Effect of protein sugar ratio

Protein:sugar ratio was found to be a significant factor on the % reducing sugar content ($p<0.05$). As protein concentration increased RS values increased indicating that higher protein concentrations caused an increase on the glycation rate. A representative figure has been shown below (Fig. 3.11.). Although the DoG values at high protein concentrations were the lowest, FAG results confirmed the results of RS. The contradiction between the results of DoG and other analyses may actually have been a delusion since DoG indicated the degree of browning originally and gave information about the rate of final stages of Maillard reaction while FAG and RS results represented mostly the initial stage (condensation) of the Maillard reaction.

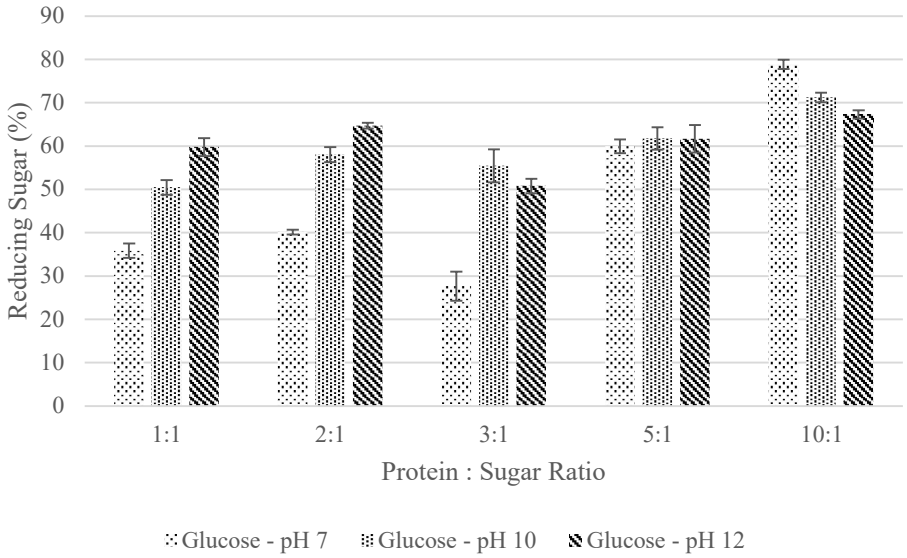

Figure 3.11. Effect of protein:sugar ratio on the % Reducing Sugar content of the samples glycated with glucose.

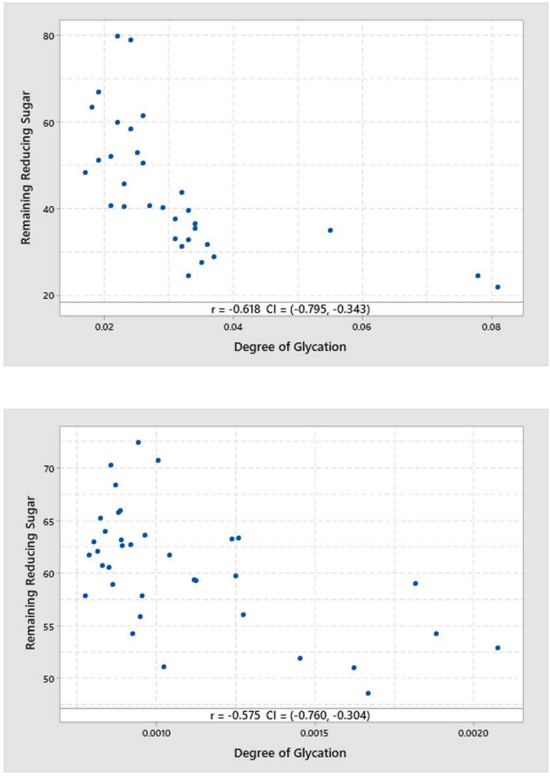

Figure 3.12. Correlation plot between DoG and RRS a) pH 7 b) pH 10

3.4. Determination of the Solubility of the Glycated Proteins

Solubility of the proteins is important for their utilization in food formulations. It is known that controlled Maillard reactions could change some of the functional properties of proteins including solubility (de Oliveira et al., 2016a). Moreover, solubility has a direct effect on other functional properties such as emulsifying activity, foaming capacity, and gelling property (de Oliveira et al., 2016a). One of the biggest disadvantages of soy protein is that its solubility is not that high in the solutions. That is why, modifying soy protein with glycation is quite important to change its solubility.

Similar to the other analysis, 3-Way and 2-Way NAOVA was conducted for the solubility of the glycated proteins. No transformation was needed on the data since the obtained data satisfied the assumptions of ANOVA. Results are given in Table 3.*5.* and ANOVA results are provided in Appendix *(Table* A.7. and A.8.*1. – A.8.5.).*

All factors and their corresponding interactions were found significant on the solubility of the glycated soy proteins ($p<0.05$).

Table 3.5. Solubility of the glycated samples

Protein:Sugar Rate	Solubility (%)								
	Glucose			Fructose			D-Psicose		
	pH 7	pH 10	pH 12	pH 7	pH 10	pH 12	pH 7	pH 10	pH 12
1:1	39.64±2.68^{e}	63.05±2.21^{c}	39.53±2.96^{e}	92.88±7.20^{a}	96.27±1.47^{a}	87.79±5.14^{ab}	50.68±0.00^{d}	79.42±2.57^{b}	26.43±1.29^{f}
2:1	41.33±2.21^{e}	47.76±1.68^{de}	31.78±2.36^{f}	62.28±4.71^{bc}	77.62±4.55^{a}	65.77±4.21^{b}	47.86±0.84^{de}	52.86±2.53^{cd}	31.78±1.28^{f}
3:1	34.76±2.34^{ef}	48.18±0.56^{cd}	56.06±0.69^{bc}	61.52±1.48^{b}	72.67±5.24^{a}	51.81±0.85^{c}	41.63±2.31^{de}	43.81±2.19^{d}	30.35±2.52^{f}
5:1	43.57±1.39^{cd}	49.60±2.22^{c}	39.54±0.39^{cd}	79.95±5.73^{a}	67.49±4.83^{b}	28.70±1.83^{e}	38.56±2.78^{d}	45.32±2.01^{cd}	27.10±1.39^{e}
10:1	44.50±2.34^{c}	48.77±2.99^{c}	35.81±2.40^{e}	84.68±1.30^{a}	64.56±3.35^{b}	34.21±1.35^{e}	38.51±0.99^{de}	44.10±1.63^{cd}	34.08±0.90^{e}

*Lettering in the tables indicate the significance differences for the same protein:sugar ratio at 95% significance level (Appendix Table A.8.1-A.8.5.: Two Way ANOVA)

3.4.1. Effect of sugar type

According to 3 Way ANOVA results sugar type was found significant and fructose samples had the highest solubilities ($p<0.05$) while D-Psicose ones had the lowest. Two-Way ANOVA results were also similar and fructose samples had the highest solubility for all protein:sugar ratios. On the other hand, for protein:sugar ratios of 1 and 2 glucose samples were higher, whereas for 3, 5 and 10 D-Psicose samples had the lowest solubility. A representative figure has been shown below (Fig. 3.13.).

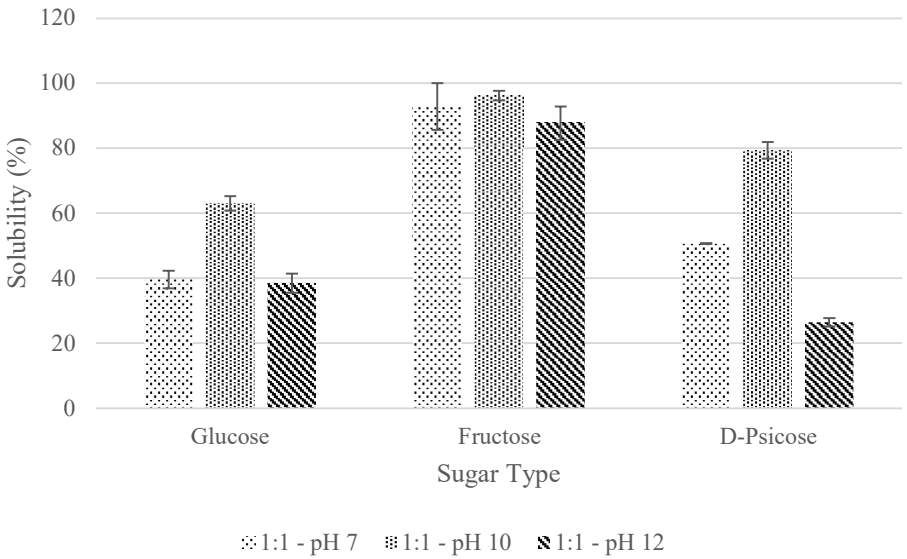

Figure 3.13. Effect of sugar type on the Solubility of the samples glycated at 1:1 protein:sugar ratio.

3.4.2. Effect of pH

Isoelectric point of soy protein is around 4.5 and at that pH values, soy protein coagulates (Hefnawy and Ramadan, 2011). Around this pH, soy protein also does not unfold so solubility of the protein will be lowest (Hefnawy and Ramadan, 2011). However, unfolding does not necessarily increase the solubility. It is a balance between the hydrophobic and hydrophilic residues present in the protein. In a study

where soy protein isolate films were examined at pH values of 2, 8 and 11, it was observed out that extreme pH values (below 2 and over 11) disrupted the protein structure and even at buffer solutions, the final pH of the solution was different due to the buffer effect of the soy protein. The results of the study showed that the highest solubility was observed at the pH value of 8. Around pH 8, the protein kept its native form and protein denaturation occurred at pH 11 with the exposure of insoluble aggregates. Consequently, these insoluble compounds caused a decrease in the solubility of the protein at the end (Veliyulin *et al.*, 2008). Fig. 3.15. shows the solubility of unglycated native soy protein at different pHs. Similar to the previous studies, soy protein had the lowest solubility at pH 12 due to denaturation whereas pH 7 and 10 were not statistically different form each other ($p<0.05$). According to 3 Way ANOVA results pH was also significant and pH 10 samples had the highest solubility followed pH 7 and 12. Two-way ANOVA results showed that for all protein:sugar ratios, pH 12 samples had the lowest and except the ratio of 10, pH 10 samples all had the highest solubility ($p<0.05$). Other experiments have shown that at pH 10 the glycation was the highest amount. A representative figure has been shown below (Fig. 3.14.).

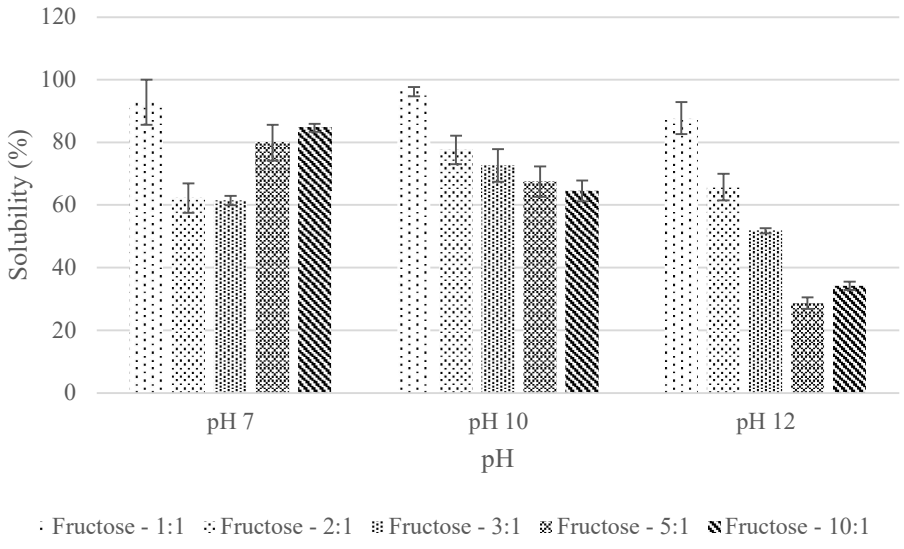

Figure 3.14. Effect of pH on the solubility of the samples glycated with fructose.

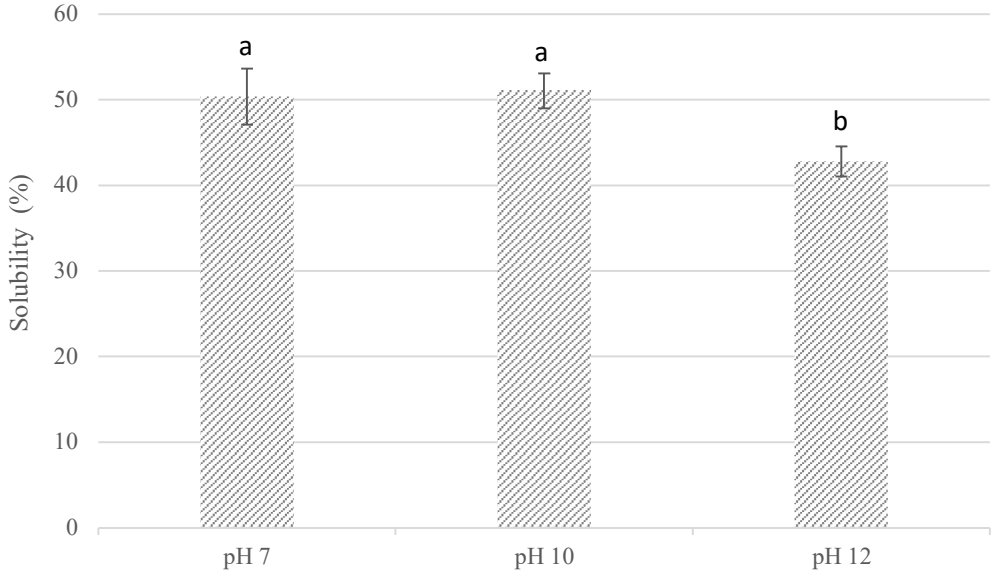

Figure 3.15. pH dependence of unglycated soy proteins.

3.4.3. Effect of protein:sugar ratio

Protein:sugar ratio was found significant on the solubility of glycated proteins and as the protein ratio, and thus concentration in our case, increased solubility decreased (Table 3.*6.*). *A representative figure also has been shown below (Fig 3.16.).* As protein concentration increases it becomes much more difficult to hydrate the proteins and they tend to be less soluble. Even though protein concentration increased the glycation rate, it decreased the solubility possibly due low hydration rates. By prolonged glycation time, Maillard reaction proceeds to advanced stage where advanced glycation end products (AGEs) is produced in. The solubility of AGEs is low due to their high molecular weight. The decrease in the solubility of glycated proteins might be due to the prolonged glycation time as well as the increased protein concentration.

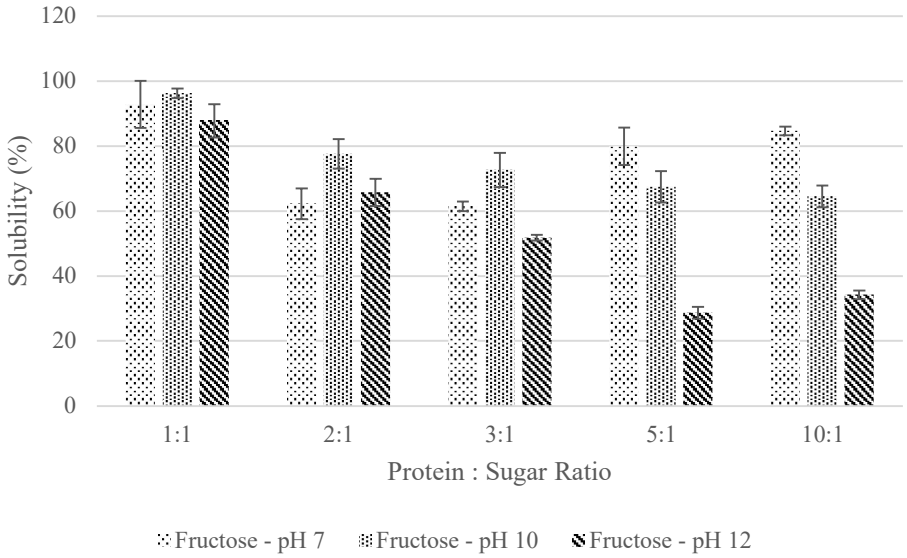

Figure 3.16. Effect of protein:sugar ratio on the solubility of the samples glycated with fructose.

Table 3.6. Effect of Protein:sugar Ratio on Solubility (Considering All Results)

Protein:sugar Ratio	N	Mean	Grouping
1	22	63.8533	A
2	24	51.5559	B
3	25	49.2498	C
10	27	47.6907	C D
5	21	46.3514	D

Means that do not share a letter are significantly different at 95% CL.

CHAPTER 4

CONCLUSION AND RECOMMENDATION

In the present study, the effects of different parameters on the glycation of soy protein were investigated. The parameters were sugar type (Glucose, Fructose, D-Psicose), pH of the glycation environment (7, 10, 12) and protein:sugar ratio (1:1, 2:1, 3:1, 5:1, 10:1). To comprehend the physico-chemical characteristics of the glycated soy protein, investigations including the degree of glycation, free amino group content (OPA method), reducing sugar content (DNS method) and solubility has been conducted.

The degree of glycation analyses showed contradicting results with other analyses that aim to investigating the extent of glycation. The reason for that is considered to be the lack of reliability of the degree of glycation analysis.

The determination of % reducing sugar content in the glycated product has also shown contradicting results with free amino group content analysis. Furthermore, some results were indicating completely opposite conclusions on the extent of glycations. This problem may also have been caused by the poor selectivity of the analysis.

By the free amino group determination, it was aimed to get information on the extent of the glycation. OPA method was used in this regard. The consistency of the OPA results of the present study with the corresponding literature, verified the reliability of the analysis.

Since the DNS and DoG results were not reliable enough to make strong arguments on the extent of glycation, the regarding interpretation has been done over the FAG results. It is known that FAG analysis provides information about the condensation step of the Maillard reaction. In this regard, the extent of glycation, in fact, should be perceived as the extent of conjugation rate or early stage Maillard reaction rate.

Primarily considering the information obtained from FAG analysis and under the conditions of present study, it can be said that the increase of protein:sugar ratio increases the extent of glycation. Similarly, as the pH of the environment shifts from neutral to extreme alkaline conditions, glycation extent also increases. The results emphasizing the effect of sugar type on the glycation were as expected and consistent with the literature. While glucose led to the highest rate of glycation as being an aldose, ketoses left behind it in terms of glycation extent. On the other hand, the rare sugar D-Psicose met the expectations by presenting better glycation results than fructose and did not contradict with the literature. Although even slight changes in the experimental conditions may change the statistical results and this situation depresses the meaning of selecting the best combination of parameters for the best condition for glycation, under present limitations, the best condition for the glycation of soy protein seems to be with glucose at pH 12 and with the protein:sugar ratio of 10:1.

The evaluation of the effect of glycation on the functionality of the soy protein has been done by investigating the solubility of glycated and native proteins. The results showed that there was a positive correlation between the amounts of FAG and solubility. Since the increase of FAG indicates lower glycation extent, it can be suggested that glycation has decreased the solubility of soy protein under these conditions. In the advanced stage of Maillard reaction the advanced glycation end products (AGEs) are produced. AEGs are known to be responsible for the decreased rates of solubility mostly because of their higher molecular weights. In the present study, the time of glycation was set to 24 hours while in most of the other studies it was lower than that. Thus, production of AGEs in the present study may be the reason of the contradiction with the literature.

In order to increase the consistency and reliability of the study alternative methods of measurement might be added. Also, glycation time might be added as a parameter and instead of keeping the total amount of sample constant, the amount of protein might be kept constant to make comparison better. Thus, further research studies are needed to certainly specify the physico-chemical characteristics of the glycated soy protein.

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APPENDICES

A. ANOVA TABLES

Table A.1. *ANOVA Results for Degree of Glycation (3-Way ANOVA)*

Method

Factor coding	(-1, 0, +1)
Rows unused	29

Factor Information:

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12
Protein:sugar Ratio	Fixed	5	1, 2, 3, 5, 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	0.004085	0.002042	440.76	0.000
pH	2	0.012722	0.006361	1372.73	0.000
Protein:sugar Ratio	4	0.020988	0.005247	1132.32	0.000
Sugar Type*pH	4	0.002085	0.000521	112.49	0.000
Sugar Type*Protein:sugar Ratio	8	0.002296	0.000287	61.94	0.000
pH*Protein:sugar Ratio	8	0.018285	0.002286	493.26	0.000
Sugar Type*pH*Protein:sugar Ratio	16	0.001203	0.000075	16.22	0.000
Error	61	0.000283	0.000005		
Total	105	0.058521			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0021526	99.52%	99.17%	98.36%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
Psicose	34	0.0438667	A		
Glucose	35	0.0370667		B	
Fructose	37	0.0284667			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
10	38	0.0516889	A
7	36	0.0319556	B
12	32	0.0257556	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Protein:sugar Ratio	N	Mean	Grouping
1	19	0.0651111	A
2	20	0.0356481	B
3	25	0.0292963	C
5	20	0.0274630	C
10	22	0.0248148	D

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping
Psicose 10	13	0.0585667	A
Glucose 10	12	0.0486000	B
Fructose 10	13	0.0479000	B C
Psicose 7	11	0.0453333	C
Glucose 12	11	0.0344333	D
Glucose 7	12	0.0281667	E
Psicose 12	10	0.0277000	E
Fructose 7	13	0.0223667	F
Fructose 12	11	0.0151333	G

Tukey Pairwise Comparisons: Sugar Type*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Protein:sugar Ratio	N	Mean	Grouping
Psicose 1	6	0.0851667	A
Fructose 1	7	0.0553333	B
Glucose 1	6	0.0548333	B
Psicose 2	7	0.0421667	C
Glucose 2	6	0.0383333	C
Psicose 3	8	0.0328333	D
Glucose 3	8	0.0317222	D E
Glucose 5	7	0.0310000	D E
Psicose 5	6	0.0306667	D E F
Glucose 10	8	0.0294444	D E F
Psicose 10	7	0.0285000	E F

Fructose 2	7	0.0264444	F G
Fructose 3	9	0.0233333	G H
Fructose 5	7	0.0207222	H
Fructose 10	7	0.0165000	I

Tukey Pairwise Comparisons: pH*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

pH*Protein:sugar Ratio	N	Mean	Grouping
10 1	6	0.118333	A
7 1	7	0.048333	B
10 2	8	0.044944	B
10 3	9	0.034556	C
7 2	6	0.033833	C D
10 5	7	0.033556	C D
7 3	9	0.030778	D E
12 1	6	0.028667	E F
12 2	6	0.028167	E F G
10 10	8	0.027056	E F G
12 10	7	0.025222	F G H
7 5	7	0.024667	F G H
12 5	6	0.024167	G H
12 3	7	0.022556	H
7 10	7	0.022167	H

Tukey Pairwise Comparisons: Sugar Type*pH*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH*Protein:sugar Ratio	N	Mean	Grouping
Psicose 10 1	2	0.138500	A
Fructose 10 1	2	0.116000	B
Glucose 10 1	2	0.100500	C
Psicose 7 1	2	0.079500	D
Psicose 7 2	2	0.053000	E
Psicose 10 2	3	0.049000	E F
Glucose 12 2	2	0.044500	E F G
Fructose 10 2	3	0.043333	F G H
Glucose 10 2	2	0.042500	F G H I
Psicose 10 5	2	0.038000	G H I J
Psicose 12 1	2	0.037500	G H I J K
Psicose 7 3	3	0.036667	G H I J K
Glucose 10 3	3	0.036333	H I J K
Glucose 12 10	3	0.035667	I J K L
Psicose 10 3	3	0.035333	I J K L
Glucose 12 5	2	0.035000	I J K L M
Glucose 10 5	2	0.034000	I J K L M N
Glucose 7 3	3	0.033333	J K L M N

Fructose 7 1	3	0.033000	J K L M N
Glucose 7 1	2	0.032500	J K L M N O
Psicose 10 10	3	0.032000	J K L M N O
Psicose 7 5	2	0.032000	J K L M N O P
Fructose 10 3	3	0.032000	J K L M N O
Glucose 12 1	2	0.031500	J K L M N O P Q
Glucose 10 10	3	0.029667	K L M N O P Q R
Fructose 10 5	3	0.028667	L M N O P Q R S
Glucose 7 2	2	0.028000	L M N O P Q R S
Psicose 12 10	2	0.028000	L M N O P Q R S
Psicose 12 3	2	0.026500	M N O P Q R S
Psicose 7 10	2	0.025500	N O P Q R S
Glucose 12 3	2	0.025500	N O P Q R S
Psicose 12 2	2	0.024500	O P Q R S
Glucose 7 5	3	0.024000	P Q R S
Glucose 7 10	2	0.023000	Q R S
Fructose 7 3	3	0.022333	S
Psicose 12 5	2	0.022000	R S
Fructose 7 2	2	0.020500	
Fructose 10 10	2	0.019500	
Fructose 7 10	3	0.018000	
Fructose 7 5	2	0.018000	
Fructose 12 1	2	0.017000	
Fructose 12 3	3	0.015667	
Fructose 12 5	2	0.015500	
Fructose 12 2	2	0.015500	
Fructose 12 10	2	0.012000	

Sugar Type*pH*Protein:sugar Ratio	**Grouping**
Psicose 10 1	
Fructose 10 1	
Glucose 10 1	
Psicose 7 1	
Psicose 7 2	
Psicose 10 2	
Glucose 12 2	
Fructose 10 2	
Glucose 10 2	
Psicose 10 5	
Psicose 12 1	
Psicose 7 3	
Glucose 10 3	
Glucose 12 10	
Psicose 10 3	
Glucose 12 5	
Glucose 10 5	
Glucose 7 3	
Fructose 7 1	
Glucose 7 1	
Psicose 10 10	
Psicose 7 5	
Fructose 10 3	
Glucose 12 1	

Glucose 10 10						
Fructose 10 5						
Glucose 7 2	T					
Psicose 12 10	T					
Psicose 12 3	T	U				
Psicose 7 10	T	U	V			
Glucose 12 3	T	U	V			
Psicose 12 2	T	U	V			
Glucose 7 5	T	U	V			
Glucose 7 10	T	U	V	W		
Fructose 7 3	T	U	V	W		
Psicose 12 5	T	U	V	W		
Fructose 7 2	T	U	V	W	X	
Fructose 10 10	T	U	V	W	X	
Fructose 7 10			V	W	X	
Fructose 7 5		U	V	W	X	
Fructose 12 1			V	W	X	
Fructose 12 3				W	X	
Fructose 12 5				W	X	
Fructose 12 2				W	X	
Fructose 12 10					X	

Table A.2.1. *ANOVA Results for Degree of Glycation (2-Way ANOVA) @ Protein:sugar Ratio of 1:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	8

Factor Information

Factor	Type	Levels	Values
pH	Fixed	3	7, 10, 12
Sugar Type	Fixed	3	Fructose, Glucose, Psicose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	0.003690	0.001845	179.11	0.000
pH	2	0.026856	0.013428	1303.71	0.000
pH*Sugar Type	4	0.001222	0.000306	29.67	0.000
Error	10	0.000103	0.000010		
Total	18	0.032553			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0032094	99.68%	99.43%	98.75%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Psicose	6	0.0851667	A
Fructose	7	0.0553333	B
Glucose	6	0.0548333	B

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
10	6	0.118333	A
7	7	0.048333	B
12	6	0.028667	C

Tukey Pairwise Comparisons: pH*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

pH*Sugar Type	N	Mean	Grouping
10 Psicose	2	0.1385	A
10 Fructose	2	0.1160	B
10 Glucose	2	0.1005	C
7 Psicose	2	0.0795	D
12 Psicose	2	0.0375	E
7 Fructose	3	0.0330	E
7 Glucose	2	0.0325	E
12 Glucose	2	0.0315	E
12 Fructose	2	0.0170	F

Table A.2.2. *ANOVA Results for Degree of Glycation (2-Way ANOVA) @ Protein:sugar Ratio of 2:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	3

Factor Information

Factor	Type	Levels	Values
pH	Fixed	3	7, 10, 12
Sugar Type	Fixed	3	Fructose, Glucose, Psicose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	0.000907	0.000453	28.30	0.000

pH	2	0.000919	0.000460	28.68	0.000
pH*Sugar Type	4	0.001301	0.000325	20.30	0.000
Error	15	0.000240	0.000016		
Total	23	0.003542			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0040028	93.22%	89.60%	84.09%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping	
Psicose	7	0.0421667	A	
Glucose	8	0.0388333	A	
Fructose	9	0.0278889		B

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping		
10	9	0.0441111	A		
7	8	0.0360000		B	
12	7	0.0287778			C

Tukey Pairwise Comparisons: pH*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

pH*Sugar Type	N	Mean	Grouping			
7 Psicose	2	0.0530000	A			
10 Psicose	3	0.0490000	A			
12 Glucose	2	0.0445000	A	B		
10 Fructose	3	0.0433333	A	B		
10 Glucose	3	0.0400000	A	B		
7 Glucose	3	0.0320000		B	C	
12 Psicose	2	0.0245000			C	D
7 Fructose	3	0.0230000			C	D
12 Fructose	3	0.0173333				D

Table A.2.3. *ANOVA Results for Degree of Glycation (2-Way ANOVA) @ Protein:sugar Ratio of 3:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	2

Factor Information

Factor	Type	Levels	Values
pH	Fixed	3	7, 10, 12
Sugar Type	Fixed	3	Fructose, Glucose, Psicose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	0.000460	0.000230	99.35	0.000
pH	2	0.000566	0.000283	122.40	0.000
pH*Sugar Type	4	0.000097	0.000024	10.52	0.000
Error	16	0.000037	0.000002		
Total	24	0.001274			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0015207	97.10%	95.64%	92.23%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Psicose	8	0.0328333	A
Glucose	8	0.0317222	A
Fructose	9	0.0233333	B

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
10	9	0.0345556	A
7	9	0.0307778	B
12	7	0.0225556	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

pH*Sugar Type	N	Mean	Grouping		
7 Psicose	3	0.0366667	A		
10 Glucose	3	0.0363333	A	B	
10 Psicose	3	0.0353333	A	B	
7 Glucose	3	0.0333333	A	B	
10 Fructose	3	0.0320000		B	
12 Psicose	2	0.0265000			C
12 Glucose	2	0.0255000			C
7 Fructose	3	0.0223333			C

12	Fructose	3	0.0156667	D

Table A.2.4. *ANOVA Results for Degree of Glycation (2-Way ANOVA) @ Protein:sugar Ratio of 5:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	7

Factor Information

Factor	Type	Levels	Values
pH	Fixed	3	7, 10, 12
Sugar Type	Fixed	3	Fructose, Glucose, Psicose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	0.000452	0.000226	57.65	0.000
pH	2	0.000368	0.000184	46.92	0.000
pH*Sugar Type	4	0.000286	0.000072	18.23	0.000
Error	11	0.000043	0.000004		
Total	19	0.001071			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0019810	95.97%	93.04%	85.61%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping	
Glucose	7	0.0310000	A	
Psicose	6	0.0306667	A	
Fructose	7	0.0207222		B

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping	
10	7	0.0335556	A	
7	7	0.0246667		B
12	6	0.0241667		B

Tukey Pairwise Comparisons: pH*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

pH*Sugar Type	N	Mean	Grouping			
10 Psicose	2	0.0380000	A			
12 Glucose	2	0.0350000	A	B		
10 Glucose	2	0.0340000	A	B		
7 Psicose	2	0.0320000	A	B		
10 Fructose	3	0.0286667		B	C	
7 Glucose	3	0.0240000			C	D
12 Psicose	2	0.0220000			C	D E
7 Fructose	2	0.0180000				D E
12 Fructose	2	0.0155000				E

Table A.2.5. *ANOVA Results for Degree of Glycation (2-Way ANOVA) @ Protein:sugar Ratio of 10:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	4

Factor Information

Factor	Type	Levels	Values
pH	Fixed	3	7, 10, 12
Sugar Type	Fixed	3	Fructose, Glucose, Psicose

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	0.000734	0.000367	102.06	0.000
pH	2	0.000087	0.000044	12.11	0.001
pH*Sugar Type	4	0.000244	0.000061	16.98	0.000
Error	14	0.000050	0.000004		
Total	22	0.001175			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0018961	95.72%	93.27%	89.62%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Glucose	8	0.0294444	A
Psicose	8	0.0285000	A

Fructose	7	0.0165000	B

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
10	8	0.0270556	A
12	8	0.0252222	A
7	7	0.0221667	B

Tukey Pairwise Comparisons: pH*Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

pH*Sugar Type	N	Mean	Grouping				
12 Glucose	3	0.0356667	A				
10 Psicose	3	0.0320000	A	B			
10 Glucose	3	0.0296667		B	C		
12 Psicose	3	0.0280000		B	C	D	
7 Psicose	2	0.0255000			C	D	E
7 Glucose	2	0.0230000				D	E	F
10 Fructose	2	0.0195000					E	F
7 Fructose	3	0.0180000						F	G
12 Fructose	2	0.0120000							G

Table A.2.6. *ANOVA Results for Degree of Glycation (Control-Non-Glycated Samples)*

General Linear Model: Degree of Glycation versus pH

Method

Factor coding	(-1, 0, +1)
Rows unused	2

Factor Information

Factor	Type	Levels	Values
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.000601	0.000301	58.66	0.001
Error	4	0.000020	0.000005		
Total	6	0.000622			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0022638	96.70%	95.05%	90.75%

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping		
7	2	0.0455	A		
10	3	0.0340		B	
12	2	0.0210			C

Table A.3. *ANOVA Results for Free Amino Groups (3-Way ANOVA)*

Method

Factor coding	(-1, 0, +1)
Rows unused	17
Box-Cox transformation	
Rounded λ	0.5
Estimated λ	0.333108
95% CI for λ	(0.00760842, 0.646608)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12
Protein:sugar Ratio	Fixed	5	1, 2, 3, 5, 10

Analysis of Variance for Transformed Response

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	0.000399	0.000199	306.94	0.000
pH	2	0.001618	0.000809	1245.65	0.000
Protein:sugar Ratio	4	0.000640	0.000160	246.30	0.000
Sugar Type*pH	4	0.001342	0.000335	516.59	0.000
Sugar Type*Protein:sugar Ratio	8	0.000090	0.000011	17.25	0.000
pH*Protein:sugar Ratio	8	0.000070	0.000009	13.51	0.000
Sugar Type*pH*Protein:sugar Ratio	16	0.000272	0.000017	26.16	0.000
Error	73	0.000047	0.000001		
Total	117	0.004292			

Model Summary for Transformed Response

S	R-sq	R-sq(adj)	R-sq(pred)
0.0008058	98.90%	98.23%	96.82%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Fructose	40	0.0011016	A
Psicose	40	0.0008879	B
Glucose	38	0.0008308	C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
7	40	0.0011422	A
10	34	0.0010551	B
12	44	0.0006520	C

Tukey Pairwise Comparisons: Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Protein:sugar Ratio	N	Mean	Grouping
1	21	0.0012575	A
2	24	0.0008990	B
3	24	0.0008864	B C
10	25	0.0008494	C D
5	24	0.0008213	D

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping
Fructose 7	12	0.0017319	A
Glucose 10	10	0.0011982	B
Psicose 7	14	0.0011520	B
Psicose 10	11	0.0010301	C
Fructose 10	13	0.0009449	D
Fructose 12	15	0.0007408	E
Glucose 12	14	0.0006774	F
Glucose 7	14	0.0006673	F
Psicose 12	15	0.0005456	G

Tukey Pairwise Comparisons: Sugar Type*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Protein:sugar Ratio	N	Mean	Grouping

Fructose 1	7	0.0015189	A						
Psicose 1	7	0.0012950		B					
Fructose 2	8	0.0010908			C				
Fructose 3	8	0.0010569			C	D			
Glucose 1	7	0.0009874				D	E		
Fructose 10	8	0.0009721				D	E		
Fructose 5	9	0.0009160					E		
Psicose 2	8	0.0008289						F	
Glucose 3	8	0.0008183						F	
Psicose 10	9	0.0008180						F	
Glucose 5	7	0.0008011						F	
Psicose 3	8	0.0007955						F	
Glucose 2	8	0.0007914						F	
Glucose 10	8	0.0007648						F	
Psicose 5	8	0.0007512						F	

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

pH*Protein:sugar Ratio	**N**	**Mean**	**Grouping**						
10 1	6	0.0015479	A						
7 1	7	0.0014274	A						
7 2	9	0.0011223		B					
7 3	8	0.0011129		B					
7 10	8	0.0010863		B	C				
10 2	6	0.0009986			C	D			
7 5	8	0.0009847				D	E		
10 5	7	0.0009584				D	E		
10 3	7	0.0009296				D	E	F	
10 10	8	0.0009015					E	F	
12 1	8	0.0008561						F	
12 3	9	0.0006487							G
12 2	9	0.0006175							G H
12 10	9	0.0005978							G H
12 5	9	0.0005589							H

Tukey Pairwise Comparisons: Sugar Type*pH*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH*Protein:sugar Ratio	**N**	**Mean**	**Grouping**			
Fructose 7 1	2	0.0021621	A			
Psicose 10 1	2	0.0018502		B		
Fructose 7 3	2	0.0017482		B		
Fructose 7 10	2	0.0016860		B	C	
Glucose 10 1	2	0.0016462		B	C	
Fructose 7 2	3	0.0016020		B	C	
Psicose 7 1	2	0.0016009		B	C	
Fructose 7 5	3	0.0014964			C	D

Fructose 12 1	3	0.0012945	D	E																
Glucose 10 5	2	0.0012478		E																
Psicose 7 2	3	0.0012381		E																
Glucose 10 2	2	0.0011944		E	F															
Fructose 10 1	2	0.0011863		E	F	G														
Psicose 7 10	3	0.0010286			F	G	H													
Fructose 10 2	2	0.0010020			F	G	H	I												
Glucose 10 3	2	0.0009876			F	G	H	I												
Psicose 7 5	3	0.0009776					H	I												
Glucose 10 10	2	0.0009728				G	H	I	J											
Psicose 7 3	3	0.0009715					H	I												
Psicose 10 3	2	0.0009350					H	I	J											
Fructose 10 10	3	0.0008856					H	I	J	K										
Fructose 10 3	3	0.0008682					H	I	J	K	L									
Psicose 10 5	2	0.0008513					H	I	J	K	L	M								
Psicose 10 10	3	0.0008483						I	J	K	L									
Psicose 10 2	2	0.0008171						I	J	K	L	M	N							
Fructose 10 5	3	0.0008049							J	K	L	M	N							
Fructose 12 2	3	0.0007508								K	L	M	N	O						
Glucose 12 3	3	0.0007447								K	L	M	N	O	P					
Glucose 7 3	3	0.0007344								K	L	M	N	O	P	Q				
Glucose 12 1	2	0.0007217								K	L	M	N	O	P	Q				
Glucose 7 1	3	0.0007201									L	M	N	O	P	Q				
Fructose 12 3	3	0.0006892										M	N	O	P	Q	R			
Glucose 12 10	3	0.0006777											N	O	P	Q	R			
Glucose 7 10	3	0.0006628											N	O	P	Q	R			
Glucose 12 5	3	0.0006451												O	P	Q	R	S		
Glucose 7 2	3	0.0006396												O	P	Q	R	S		
Psicose 12 1	3	0.0006216												O	P	Q	R	S		
Psicose 12 10	3	0.0006053													P	Q	R	S		
Glucose 12 2	3	0.0006026														Q	R	S		
Glucose 7 5	2	0.0005851														Q	R	S		
Fructose 12 5	3	0.0005636															R	S		
Psicose 12 3	3	0.0005229																S		
Fructose 12 10	3	0.0005159																S		
Psicose 12 2	3	0.0005109																		
Psicose 12 5	3	0.0004744																		

Sugar Type*pH*Protein:sugar Ratio	**Grouping**
Fructose 7 1	
Psicose 10 1	
Fructose 7 3	
Fructose 7 10	
Glucose 10 1	
Fructose 7 2	
Psicose 7 1	
Fructose 7 5	
Fructose 12 1	
Glucose 10 5	
Psicose 7 2	
Glucose 10 2	
Fructose 10 1	
Psicose 7 10	

Fructose 10 2		
Glucose 10 3		
Psicose 7 5		
Glucose 10 10		
Psicose 7 3		
Psicose 10 3		
Fructose 10 10		
Fructose 10 3		
Psicose 10 5		
Psicose 10 10		
Psicose 10 2		
Fructose 10 5		
Fructose 12 2		
Glucose 12 3		
Glucose 7 3		
Glucose 12 1		
Glucose 7 1		
Fructose 12 3		
Glucose 12 10		
Glucose 7 10		
Glucose 12 5		
Glucose 7 2	T	
Psicose 12 1	T	
Psicose 12 10	T	
Glucose 12 2	T	
Glucose 7 5	T	U
Fructose 12 5	T	U
Psicose 12 3	T	U
Fructose 12 10	T	U
Psicose 12 2	T	U
Psicose 12 5		U

Table A.4.1. *ANOVA Results for the Free Amino Groups (2-Way ANOVA) @ Protein:sugar Ratio of 1:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	6

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	0.000001	0.000000	224.71	0.000
pH	2	0.000002	0.000001	489.20	0.000

Sugar Type*pH	4	0.000003	0.000001	311.69	0.000
Error	12	0.000000	0.000000		
Total	20	0.000006			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0000454	99.55%	99.26%	98.52%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
Fructose	7	0.0015480	A		
Psicose	7	0.0013578		B	
Glucose	7	0.0010296			C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping	
10	6	0.0015612	A	
7	7	0.0014945	A	
12	8	0.0008797		B

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping			
Fructose 7	2	0.0021621	A			
Psicose 10	2	0.0018503		B		
Glucose 10	2	0.0016463			C	
Psicose 7	2	0.0016009			C	
Fructose 12	3	0.0012948				D
Fructose 10	2	0.0011871				D
Glucose 12	2	0.0007222				E
Glucose 7	3	0.0007203				E
Psicose 12	3	0.0006221				E

Table A.4.2. *ANOVA Results for the Free Amino Groups (2-Way ANOVA) @ Protein:sugar Ratio of 2:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	3

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	0.000000	0.000000	64.66	0.000
pH	2	0.000001	0.000001	208.20	0.000
Sugar Type*pH	4	0.000001	0.000000	81.20	0.000
Error	15	0.000000	0.000000		
Total	23	0.000003			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0000572	98.40%	97.54%	95.32%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Fructose	8	0.0011188	A
Psicose	8	0.0008559	B
Glucose	8	0.0008129	B

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
7	9	0.0011603	A
10	6	0.0010052	B
12	9	0.0006221	C

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping
Fructose 7	3	0.0016022	A
Psicose 7	3	0.0012386	B
Glucose 10	2	0.0011956	B C
Fructose 10	2	0.0010024	C D
Psicose 10	2	0.0008177	D E
Fructose 12	3	0.0007518	E F

Glucose 7	3	0.0006402	E	F	G
Glucose 12	3	0.0006030		F	G
Psicose 12	3	0.0005113			G

Table A.4.3. *ANOVA Results for the Free Amino Groups (2-Way ANOVA) @ Protein:sugar Ratio of 3:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	3
Box-Cox transformation	
Rounded λ	-0.5
Estimated λ	-0.66071
95% CI for λ	(-1.35221, -0.0532103)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance for Transformed Response

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	72.990	36.495	76.33	0.000
pH	2	337.010	168.505	352.41	0.000
Sugar Type*pH	4	230.613	57.653	120.58	0.000
Error	15	7.172	0.478		
Total	23	587.706			

Model Summary for Transformed Response

S	R-sq	R-sq(adj)	R-sq(pred)
0.691484	98.78%	98.13%	96.44%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
Fructose	8	0.0009767	A		
Glucose	8	0.0008103		B	
Psicose	8	0.0007640			C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping

7	8	0.0010415	A		
10	7	0.0009281		B	
12	9	0.0006411			C

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping		
Fructose 7	2	0.0017420	A		
Glucose 10	2	0.0009870		B	
Psicose 7	3	0.0009714		B	
Psicose 10	2	0.0009349		B	
Fructose 10	3	0.0008682		B	
Glucose 12	3	0.0007446			C
Glucose 7	3	0.0007339			C
Fructose 12	3	0.0006890			C
Psicose 12	3	0.0005226			D

Table A.4.4. *ANOVA Results for the Free Amino Groups (2-Way ANOVA) @ Protein:sugar Ratio of 5:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	3

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	0.000000	0.000000	36.22	0.000
pH	2	0.000001	0.000001	248.41	0.000
Sugar Type*pH	4	0.000001	0.000000	140.28	0.000
Error	15	0.000000	0.000000		
Total	23	0.000003			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0000461	98.77%	98.11%	96.95%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Fructose	9	0.0009553	A
Glucose	7	0.0008263	B
Psicose	8	0.0007682	B

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
7	8	0.0010202	A
10	7	0.0009682	A
12	9	0.0005614	B

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping				
Fructose 7	3	0.0014971	A				
Glucose 10	2	0.0012478		B			
Psicose 7	3	0.0009781			C		
Psicose 10	2	0.0008517			C	D	
Fructose 10	3	0.0008050				D	
Glucose 12	3	0.0006457					E
Glucose 7	2	0.0005853					E F
Fructose 12	3	0.0005637					E F
Psicose 12	3	0.0004749					F

Table A.4.5. *ANOVA Results for the Free Amino Groups (2-Way ANOVA) @ Protein:sugar Ratio of 10:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	2

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	0.000000	0.000000	72.00	0.000
pH	2	0.000001	0.000001	293.88	0.000
Sugar Type*pH	4	0.000001	0.000000	139.58	0.000
Error	16	0.000000	0.000000		

Total	24	0.000002

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0000446	98.59%	97.89%	96.35%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Fructose	8	0.0010295	A
Psicose	9	0.0008277	B
Glucose	8	0.0007716	B

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
7	8	0.0011264	A
10	8	0.0009025	B
12	9	0.0006000	C

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping			
Fructose 7	2	0.0016864	A			
Psicose 7	3	0.0010291	B			
Glucose 10	2	0.0009731	B	C		
Fructose 10	3	0.0008858		C		
Psicose 10	3	0.0008485		C		
Glucose 12	3	0.0006782			D	
Glucose 7	3	0.0006637			D	
Psicose 12	3	0.0006055			D	E
Fructose 12	3	0.0005162				E

Table A.4.6. *ANOVA Results for the Free Amino Groups (Control-Non-Glycated Samples)*

Method

Factor coding	(-1, 0, +1)
Rows unused	1

Factor Information

Factor	Type	Levels	Values
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.000003	0.000001	239.61	0.000
Error	5	0.000000	0.000000		
Total	7	0.000003			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0000777	98.97%	98.55%	97.67%

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping		
7	2	0.0026684	A		
10	3	0.0022517		B	
12	3	0.0012218			C

Table A.5. *ANOVA Results for the % Reducing Sugar (3-Way ANOVA)*

Method

Factor coding	(-1, 0, +1)
Rows unused	4

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12
Protein:sugar Ratio	Fixed	5	1, 2, 3, 5, 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	2567.6	1283.79	340.23	0.000
pH	2	6083.9	3041.97	806.19	0.000
Protein:sugar Ratio	4	6708.3	1677.07	444.46	0.000
Sugar Type*pH	4	919.6	229.90	60.93	0.000
Sugar Type*Protein:sugar Ratio	8	1065.5	133.18	35.30	0.000
pH*Protein:sugar Ratio	8	1821.3	227.66	60.34	0.000
Sugar Type*pH*Protein:sugar Ratio	16	1524.6	95.29	25.25	0.000
Error	86	324.5	3.77		
Total	130	21087.1			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.94249	98.46%	97.67%	96.34%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Fructose	43	57.3702	A
Glucose	45	56.2636	B
Psicose	43	47.3454	C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
10	45	60.5132	A
12	45	56.3977	B
7	41	44.0682	C

Tukey Pairwise Comparisons: Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Protein:sugar Ratio	N	Mean	Grouping
10	26	66.5417	A
5	27	54.5185	B
2	25	53.0662	B
3	26	48.6822	C
1	27	45.4901	D

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping
Fructose 10	15	62.8721	A
Glucose 12	15	60.8665	A B
Fructose 12	15	60.1411	B
Glucose 10	15	59.4086	B
Psicose 10	15	59.2590	B
Fructose 7	13	49.0974	C
Glucose 7	15	48.5157	C
Psicose 12	15	48.1855	C
Psicose 7	13	34.5917	D

Tukey Pairwise Comparisons: Sugar Type*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Protein:sugar Ratio	N	Mean	Grouping
Glucose 10	9	72.4875	A
Fructose 10	8	67.8977	B
Glucose 5	9	61.1536	C
Psicose 10	9	59.2400	C D
Fructose 2	8	58.2594	C D
Fructose 3	9	57.0801	D E
Fructose 5	9	56.0745	D E
Glucose 2	9	54.3056	E
Glucose 1	9	48.7170	F
Fructose 1	9	47.5393	F G
Psicose 2	8	46.6337	F G
Psicose 5	9	46.3274	F G
Glucose 3	9	44.6543	G
Psicose 3	8	44.3120	G
Psicose 1	9	40.2139	H

Tukey Pairwise Comparisons: pH*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

pH*Protein:sugar Ratio	N	Mean	Grouping
12 10	9	68.1390	A
10 10	9	66.1013	A
7 10	8	65.3849	A B
10 5	9	62.1585	B C
10 2	9	59.7757	C D
10 3	9	59.4944	C D
12 2	9	58.2427	D
10 1	9	55.0363	E
12 5	9	54.0913	E
12 3	9	53.1305	E
12 1	9	48.3850	F
7 5	9	47.3056	F
7 2	7	41.1803	G
7 3	8	33.4216	H
7 1	9	33.0489	H

Tukey Pairwise Comparisons: Sugar Type*pH*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH*Protein:sugar Ratio	N	Mean	Grouping
Glucose 7 10	3	78.8874	A
Fructose 12 10	3	76.4117	A B
Glucose 10 10	3	71.2467	B C
Fructose 10 3	3	68.1877	C D

Glucose 12 10	3	67.3284	C D
Fructose 7 10	2	65.2962	C D E F
Psicose 10 10	3	65.0720	C D E
Glucose 12 2	3	64.7278	D E
Fructose 10 2	3	63.1217	D E F G
Psicose 10 5	3	62.8502	D E F G
Fructose 12 2	3	62.0022	D E F G
Fructose 10 10	3	61.9852	D E F G
Fructose 10 5	3	61.8248	D E F G H
Glucose 10 5	3	61.8006	D E F G H
Glucose 12 5	3	61.7035	D E F G H
Fructose 12 3	3	60.7647	E F G H I
Psicose 12 10	3	60.6770	E F G H I
Glucose 7 5	3	59.9566	E F G H I
Glucose 12 1	3	59.7922	E F G H I
Fructose 10 1	3	59.2412	E F G H I
Psicose 10 2	3	58.1797	F G H I J
Fructose 12 5	3	58.1369	F G H I J
Glucose 10 2	3	58.0258	G H I J
Glucose 10 3	3	55.4810	H I J K
Psicose 10 1	3	55.3786	H I J K
Psicose 10 3	3	54.8145	I J K
Psicose 7 10	3	51.9710	J K L
Glucose 12 3	3	50.7808	K L
Glucose 10 1	3	50.4891	K L
Fructose 7 2	2	49.6543	K L M
Fructose 7 5	3	48.2619	L M N
Psicose 12 2	3	47.9981	L M N
Psicose 12 3	3	47.8460	L M N
Fructose 12 1	3	43.3901	M N O
Psicose 12 5	3	42.4336	M N O
Fructose 7 3	3	42.2880	N O P
Psicose 12 1	3	41.9726	N O P
Glucose 7 2	3	40.1633	O P Q
Fructose 7 1	3	39.9865	O P Q
Glucose 7 1	3	35.8698	P Q R
Psicose 7 2	2	33.7233	Q R S
Psicose 7 5	3	33.6982	Q R S
Psicose 7 3	2	30.2755	R S T
Glucose 7 3	3	27.7012	S T
Psicose 7 1	3	23.2904	T

Table A.6.1. *ANOVA Results for the % Reducing Sugar (2-Way ANOVA) @ Protein:sugar Ratio of 1:1*

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose

pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	382.06	191.03	43.84	0.000
pH	2	2288.63	1144.31	262.63	0.000
Sugar Type*pH	4	775.89	193.97	44.52	0.000
Error	18	78.43	4.36		
Total	26	3525.01			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.08737	97.78%	96.79%	94.99%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Glucose	9	48.7170	A
Fructose	9	47.5393	A
Psicose	9	40.2139	B

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
10	9	55.0363	A
12	9	48.3850	B
7	9	33.0489	C

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping			
Glucose 12	3	59.7922	A			
Fructose 10	3	59.2412	A			
Psicose 10	3	55.3786	A	B		
Glucose 10	3	50.4891		B		
Fructose 12	3	43.3901			C	
Psicose 12	3	41.9726			C	
Fructose 7	3	39.9865			C	D
Glucose 7	3	35.8698				D
Psicose 7	3	23.2904				E

Table A.6.2. *ANOVA Results for the % Reducing Sugar (2-Way ANOVA) @ Protein:sugar Ratio of 2:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	2

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	540.96	270.481	141.50	0.000
pH	2	1571.03	785.513	410.94	0.000
Sugar Type*pH	4	276.39	69.097	36.15	0.000
Error	16	30.58	1.912		
Total	24	2461.07			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.38258	98.76%	98.14%	96.20%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
Fructose	8	58.2594	A		
Glucose	9	54.3056		B	
Psicose	8	46.6337			C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping	
10	9	59.7757	A	
12	9	58.2427	A	
7	7	41.1803		B

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping		
Glucose 12	3	64.7278	A		
Fructose 10	3	63.1217	A		
Fructose 12	3	62.0022	A	B	
Psicose 10	3	58.1797		B	
Glucose 10	3	58.0258		B	

Fructose 7	2	49.6543	C		
Psicose 12	3	47.9981	C		
Glucose 7	3	40.1633		D	
Psicose 7	2	33.7233			E

Table A.6.3. *ANOVA Results for the % Reducing Sugar (2-Way ANOVA) @ Protein:sugar Ratio of 3:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	1

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	926.85	463.42	81.71	0.000
pH	2	3011.80	1505.90	265.52	0.000
Sugar Type*pH	4	25.17	6.29	1.11	0.384
Error	17	96.41	5.67		
Total	25	4050.42			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.38148	97.62%	96.50%	94.46%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Fructose	9	57.0801	A
Glucose	9	44.6543	B
Psicose	8	44.3120	B

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping		
10	9	59.4944	A		
12	9	53.1305		B	
7	8	33.4216			C

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping				
Fructose 10	3	68.1877	A				
Fructose 12	3	60.7647		B			
Glucose 10	3	55.4810		B	C		
Psicose 10	3	54.8145		B	C		
Glucose 12	3	50.7808			C	D	
Psicose 12	3	47.8460				D	E
Fructose 7	3	42.2880					E
Psicose 7	2	30.2755						F
Glucose 7	3	27.7012						F

Table A.6.4. *ANOVA Results for the % Reducing Sugar (2-Way ANOVA) @ Protein:sugar Ratio of 5:1*

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	1021.86	510.929	114.53	0.000
pH	2	995.21	497.606	111.54	0.000
Sugar Type*pH	4	649.30	162.326	36.39	0.000
Error	18	80.30	4.461		
Total	26	2746.68			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.11217	97.08%	95.78%	93.42%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
Glucose	9	61.1536	A		
Fructose	9	56.0745		B	
Psicose	9	46.3274			C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping		
10	9	62.1585	A		
12	9	54.0913		B	
7	9	47.3056			C

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping		
Psicose 10	3	62.8502	A		
Fructose 10	3	61.8248	A		
Glucose 10	3	61.8006	A		
Glucose 12	3	61.7035	A		
Glucose 7	3	59.9566	A		
Fructose 12	3	58.1369	A		
Fructose 7	3	48.2619		B	
Psicose 12	3	42.4336		B	
Psicose 7	3	33.6982			C

Table A.6.5. *ANOVA Results for the % Reducing Sugar (2-Way ANOVA) @ Protein:sugar Ratio of 10:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	1

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	812.07	406.035	178.03	0.000
pH	2	34.95	17.473	7.66	0.004
Sugar Type*pH	4	777.42	194.355	85.22	0.000
Error	17	38.77	2.281		
Total	25	1668.45			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.51020	97.68%	96.58%	94.14%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Glucose	9	72.4875	A
Fructose	8	67.8977	B
Psicose	9	59.2400	C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
12	9	68.1390	A
10	9	66.1013	B
7	8	65.3849	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping				
Glucose 7	3	78.8874	A				
Fructose 12	3	76.4117	A				
Glucose 10	3	71.2467		B			
Glucose 12	3	67.3284		B	C		
Fructose 7	2	65.2962			C	D	E
Psicose 10	3	65.0720			C	D	
Fructose 10	3	61.9852				D	E
Psicose 12	3	60.6770					E
Psicose 7	3	51.9710					F

Table A.7. *ANOVA Results for the Solubility of the Glycated Proteins (3-Way ANOVA)*

Method

Factor coding	(-1, 0, +1)
Rows unused	16

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12
Protein:sugar Ratio	Fixed	5	1, 2, 3, 5, 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value

Sugar Type	2	16480.4	8240.20	1407.41	0.000
pH	2	6928.6	3464.32	591.70	0.000
Protein:sugar Ratio	4	4276.3	1069.07	182.59	0.000
Sugar Type*pH	4	1763.9	440.97	75.32	0.000
Sugar Type*Protein:sugar Ratio	8	2612.9	326.61	55.79	0.000
pH*Protein:sugar Ratio	8	2253.1	281.64	48.10	0.000
Sugar Type*pH*Protein:sugar Ratio	16	4082.6	255.16	43.58	0.000
Error	74	433.3	5.85		
Total	118	38785.1			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.41968	98.88%	98.22%	96.69%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
Fructose	38	68.8640	A		
Glucose	41	44.1910		B	
Psicose	40	42.1657			C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping		
10	39	60.2352	A		
7	41	53.6694		B	
12	39	41.3161			C

Tukey Pairwise Comparisons: Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Protein:sugar Ratio	N	Mean	Grouping			
1	22	63.8533	A			
2	24	51.5559		B		
3	25	49.2498			C	
10	27	47.6907			C	D
5	21	46.3514				D

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping		
Fructose 7	12	76.8022	A		
Fructose 10	12	76.1335	A		
Fructose 12	14	53.6563		B	
Psicose 10	12	53.1027		B	

Glucose 10	15	51.4694	B					
Psicose 7	15	43.4459		C				
Glucose 7	14	40.7601		C	D			
Glucose 12	12	40.3434			D			
Psicose 12	13	29.9485				E		

Tukey Pairwise Comparisons: Sugar Type*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Protein:sugar Ratio	**N**	**Mean**	**Grouping**							
Fructose 1	7	92.3127	A							
Fructose 2	7	70.2094		B						
Fructose 3	8	62.8218			C					
Fructose 10	9	61.1500			C	D				
Fructose 5	7	57.8260				D				
Psicose 1	7	52.1752					E			
Glucose 1	8	47.0719						F		
Glucose 3	8	46.3308						F		
Glucose 5	7	44.2333						F	G	
Psicose 2	8	44.1661						F	G	
Glucose 10	9	43.0266						F	G	
Glucose 2	9	40.2923							G	H
Psicose 10	9	38.8955								H
Psicose 3	9	38.5967								H
Psicose 5	7	36.9950								H

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

pH*Protein:sugar Ratio	**N**	**Mean**	**Grouping**							
10 1	8	79.5796	A							
7 1	8	61.0661		B						
10 2	7	60.1651		B	C					
7 10	9	55.8962			C	D				
10 3	8	55.7075			C	D	E			
7 5	7	54.0272				D	E	F		
10 5	7	53.2480				D	E	F		
10 10	9	52.4758				D	E	F		
7 2	8	51.3916					E	F		
12 1	6	50.9141						F		
12 3	8	46.0762							G	
7 3	9	45.9657							G	
12 2	9	43.1111							G	
12 10	9	34.7000								H
12 5	7	31.7791								H

Tukey Pairwise Comparisons: Sugar Type*pH*Protein:sugar Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH*Protein:sugar Ratio	N	Mean	Grouping
Fructose 10 1	3	96.2741	A
Fructose 7 1	2	92.8786	A B
Fructose 12 1	2	87.7854	A B C
Fructose 7 10	3	84.6805	B C
Fructose 7 5	2	79.9487	C D
Fructose 10 2	2	79.8710	C D
Psicose 10 1	2	79.4179	C D
Fructose 10 3	2	75.1372	D
Fructose 12 2	3	65.7691	E
Fructose 7 2	2	64.9882	E F
Fructose 10 5	2	64.8268	E F
Fructose 10 10	3	64.5581	E F
Glucose 10 1	3	63.0468	E F
Fructose 7 3	3	61.5148	E F G
Glucose 12 3	2	56.0618	F G H
Psicose 10 2	2	52.8615	G H I
Fructose 12 3	3	51.8135	H I
Psicose 7 1	3	50.6776	H I
Glucose 10 5	3	49.5954	H I J
Glucose 10 10	3	48.7668	H I J K
Glucose 10 3	3	48.1754	H I J K L
Psicose 7 2	3	47.8546	H I J K L
Glucose 10 2	3	47.7628	H I J K L M
Psicose 10 5	2	45.3218	I J K L M N
Glucose 7 10	3	44.5024	I J K L M N
Psicose 10 10	3	44.1026	I J K L M N
Psicose 10 3	3	43.8098	I J K L M N O
Glucose 7 5	2	43.5685	I J K L M N O P
Psicose 7 3	3	41.6270	J K L M N O P Q
Glucose 7 2	3	41.3319	K L M N O P Q
Glucose 7 1	3	39.6423	M N O P Q R
Glucose 12 5	2	39.5360	L M N O P Q R
Psicose 7 5	3	38.5644	N O P Q R
Glucose 12 1	2	38.5266	N O P Q R S
Psicose 7 10	3	38.5057	N O P Q R
Glucose 12 10	3	35.8105	O P Q R S T
Glucose 7 3	3	34.7552	P Q R S T
Fructose 12 10	3	34.2114	Q R S T
Psicose 12 10	3	34.0781	Q R S T
Psicose 12 2	3	31.7821	R S T
Glucose 12 2	3	31.7821	R S T
Psicose 12 3	3	30.3532	S T
Fructose 12 5	3	28.7023	T
Psicose 12 5	2	27.0988	T
Psicose 12 1	2	26.4302	

Sugar Type*pH*Protein:sugar Ratio	Grouping

Fructose 10 1	
Fructose 7 1	
Fructose 12 1	
Fructose 7 10	
Fructose 7 5	
Fructose 10 2	
Psicose 10 1	
Fructose 10 3	
Fructose 12 2	
Fructose 7 2	
Fructose 10 5	
Fructose 10 10	
Glucose 10 1	
Fructose 7 3	
Glucose 12 3	
Psicose 10 2	
Fructose 12 3	
Psicose 7 1	
Glucose 10 5	
Glucose 10 10	
Glucose 10 3	
Psicose 7 2	
Glucose 10 2	
Psicose 10 5	
Glucose 7 10	
Psicose 10 10	
Psicose 10 3	
Glucose 7 5	
Psicose 7 3	
Glucose 7 2	
Glucose 7 1	
Glucose 12 5	
Psicose 7 5	
Glucose 12 1	
Psicose 7 10	
Glucose 12 10	
Glucose 7 3	U
Fructose 12 10	U
Psicose 12 10	U
Psicose 12 2	U
Glucose 12 2	U
Psicose 12 3	U
Fructose 12 5	U
Psicose 12 5	U
Psicose 12 1	U

Table A.8.1. *ANOVA Results for the Solubility of the Glycated Proteins (2-Way ANOVA) @ Protein:sugar Ratio of 1:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	5

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	8547.7	4273.86	448.52	0.000
pH	2	2949.4	1474.69	154.76	0.000
Sugar Type*pH	4	1252.1	313.03	32.85	0.000
Error	13	123.9	9.53		
Total	21	12955.7			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3.08689	99.04%	98.46%	96.56%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Fructose	7	92.3127	A
Psicose	7	52.1752	B
Glucose	8	47.0719	C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
10	8	79.5796	A
7	8	61.0661	B
12	6	50.9141	C

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping
Fructose 10	3	96.2741	A
Fructose 7	2	92.8786	A
Fructose 12	2	87.7854	A B
Psicose 10	2	79.4179	B
Glucose 10	3	63.0468	C
Psicose 7	3	50.6776	D
Glucose 7	3	39.6423	E
Glucose 12	2	38.5266	E
Psicose 12	2	26.4302	F

Table A.8.2. *ANOVA Results for the Solubility of the Glycated Proteins (2-Way ANOVA) @ Protein:sugar Ratio of 2:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	1

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	4162.9	2081.47	222.77	0.000
pH	2	1104.3	552.13	59.09	0.000
Sugar Type*pH	4	303.8	75.96	8.13	0.001
Error	17	158.8	9.34		
Total	25	5885.0			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3.05676	97.30%	96.03%	93.74%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
Fructose	9	68.5558	A		
Psicose	8	44.1661		B	
Glucose	9	40.2923			C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping		
10	8	59.4148	A		
7	9	50.4882		B	
12	9	43.1111			C

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping				
Fructose 10	3	77.6202	A				
Fructose 12	3	65.7691		B			
Fructose 7	3	62.2781		B	C		
Psicose 10	2	52.8615			C	D	
Psicose 7	3	47.8546				D	E
Glucose 10	3	47.7628				D	E
Glucose 7	3	41.3319					E
Psicose 12	3	31.7821						F
Glucose 12	3	31.7821						F

Table A.8.3. *ANOVA Results for the Solubility of the Glycated Proteins (2-Way ANOVA) @ Protein:sugar Ratio of 3:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	1

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	2549.7	1274.85	205.08	0.000
pH	2	460.2	230.10	37.02	0.000
Sugar Type*pH	4	995.5	248.88	40.04	0.000
Error	17	105.7	6.22		
Total	25	4275.5			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.49323	97.53%	96.37%	94.42%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
Fructose	9	61.9999	A		
Glucose	8	46.3308		B	
Psicose	9	38.5967			C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
10	9	54.8856	A
12	8	46.0762	B
7	9	45.9657	B

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping				
Fructose 10	3	72.6714	A				
Fructose 7	3	61.5148		B			
Glucose 12	2	56.0618		B	C		
Fructose 12	3	51.8135			C		
Glucose 10	3	48.1754			C	D	
Psicose 10	3	43.8098				D	
Psicose 7	3	41.6270				D	E
Glucose 7	3	34.7552					E F
Psicose 12	3	30.3532					F

Table A.8.4. *ANOVA Results for the Solubility of the Glycated Proteins (2-Way ANOVA) @ Protein:sugar Ratio of 5:1*

Method

Factor coding	(-1, 0, +1)
Rows unused	5

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	1785.2	892.60	96.98	0.000
pH	2	2290.1	1145.03	124.41	0.000
Sugar Type*pH	4	1534.9	383.71	41.69	0.000
Error	13	119.6	9.20		
Total	21	5717.1			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3.03377	97.91%	96.62%	94.04%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
Fructose	8	58.7148	A		
Glucose	7	44.2333		B	
Psicose	7	36.9950			C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping		
10	8	54.1368	A		
7	7	54.0272	A		
12	7	31.7791		B	

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping			
Fructose 7	2	79.9487	A			
Fructose 10	3	67.4933		B		
Glucose 10	3	49.5954			C	
Psicose 10	2	45.3218			C	D
Glucose 7	2	43.5685			C	D
Glucose 12	2	39.5360			C	D
Psicose 7	3	38.5644				D
Fructose 12	3	28.7023				E
Psicose 12	2	27.0988				E

Table A.8.5. *ANOVA Results for the Solubility of the Glycated Proteins (2-Way ANOVA) @ Protein:sugar Ratio of 10:1*

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	Fructose, Glucose, Psicose
pH	Fixed	3	7, 10, 12

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	2522.37	1261.18	288.01	0.000
pH	2	2330.88	1165.44	266.15	0.000
Sugar Type*pH	4	1955.11	488.78	111.62	0.000
Error	18	78.82	4.38		

Total	26	6887.18

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.09259	98.86%	98.35%	97.42%

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping
Fructose	9	61.1500	A
Glucose	9	43.0266	B
Psicose	9	38.8955	C

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping
7	9	55.8962	A
10	9	52.4758	B
12	9	34.7000	C

Tukey Pairwise Comparisons: Sugar Type*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*pH	N	Mean	Grouping			
Fructose 7	3	84.6805	A			
Fructose 10	3	64.5581	B			
Glucose 10	3	48.7668		C		
Glucose 7	3	44.5024		C		
Psicose 10	3	44.1026		C	D	
Psicose 7	3	38.5057			D	E
Glucose 12	3	35.8105				E
Fructose 12	3	34.2114				E
Psicose 12	3	34.0781				E

B. CALIBRATION CURVES

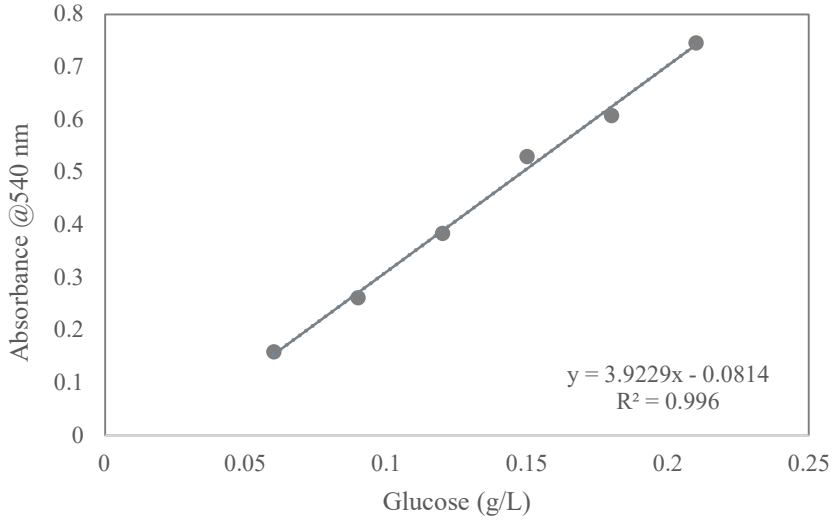

Figure B.1. *Calibration curve for DNS assay prepared by Glucose to determine the attached reducing sugar percentage in the glycated soy protein.*

$$\text{Absorbance (@540 nm)} = 3.9229 \times (g\ Glucose/L) - 0.0814\ where\ R\text{^}2 = 0.996$$

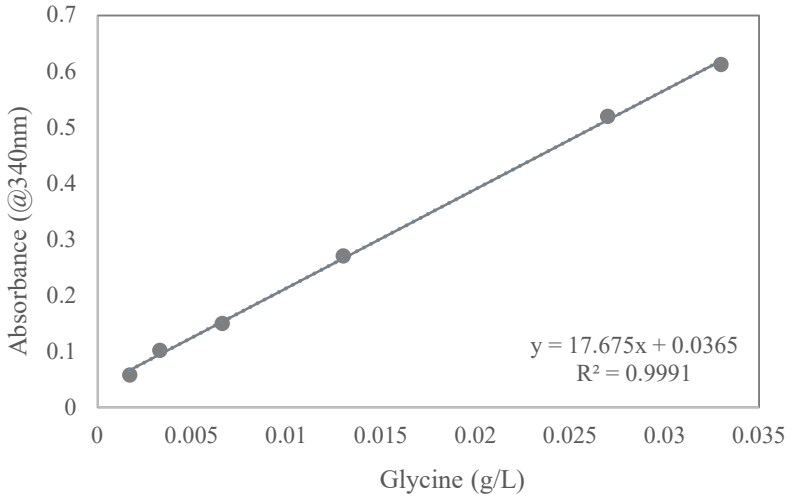

Figure B.2. *Calibration Curve for OPA Method prepared by glycine to determine the free amino group (FAG) content in glycated soy protein.*

$$\text{Absorbance (@340 nm)} = 17.675 \times (g\ Glycine/L) + 0.0365\ where\ R\text{^}2 = 0.9991$$

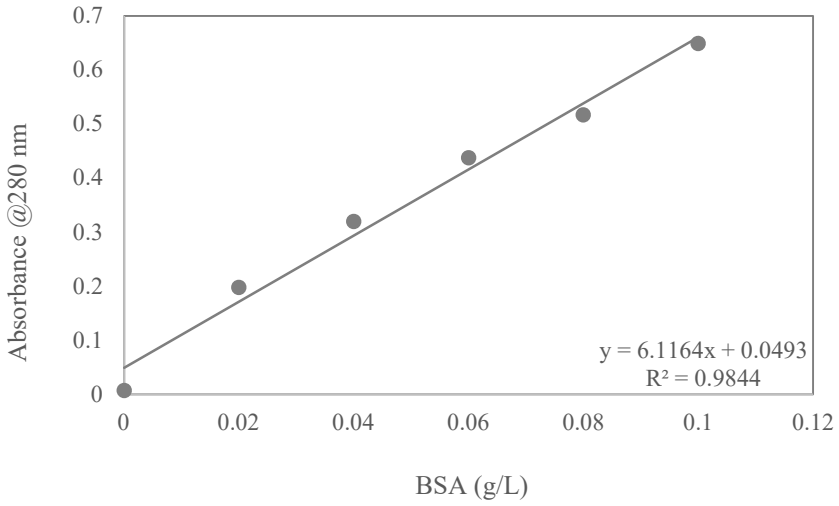

Figure B.3. *Calibration Curve for solubility assay prepared by Bovine Serum Albumin (BSA) to determine the total soluble protein content in the glycated soy protein*

$$\text{Absorbance (@280 nm)} = 6.1164 \times (g\ BSA/L) + 0.0493\ where\ R\text{^}2 = 0.9844$$