IMPROVEMENT OF PEA PROTEINS' PROPERTIES BY MICROWAVE GLYCATION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ÜLKÜ ERTUĞRUL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
FOOD ENGINEERING

SEPTEMBER 2020

Approval of the thesis:

IMPROVEMENT OF PEA PROTEINS' PROPERTIES BY MICROWAVE GLYCATION

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ABSTRACT

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September 2020, 159 pages

The tendency to include plant proteins in the diet has increased significantly as consumers' preference for animal proteins decline. Sustainability goals, low manufacturing cost, and high nutritional value are also triggering this demand. For this reason, pea protein as a form of isolate and concentrate is often used in protein enriched diets due to its nutritive properties. Previously, improvement in its functional properties has been studied from many perspectives. Glycation, which is known as the initial stage of the Maillard reaction can be considered as a modification strategy for improving the functional properties. In this study, microwave was used for glycating pea protein concentrate. The objective of the study was to investigate the extent of microwave glycation between pea protein concentrate (PPC) and reducing sugars of dextrose, fructose, and the rare sugar, Allulose, by determining the soluble proteins, free amino groups, remaining reducing sugars and advanced glycation end products (AGEs). Also, the effect of two different pH values (7 and 10) and two different pea protein to sugar ratios (2:1.25 and 9:1.25) were observed. Solubility and hydration behavior of PPC were investigated

afterwards. Lowry method proved that microwave glycation enhanced the solubility of dextrose samples by 50% compared to control samples. Also, the alkaline environment improved PPC solubility. Besides that, microwave glycation improved the hydration of PPC more when it is compared to water bath glycation. To conclude, the study showed that microwave glycation could be used as an alternative modification method since it improved the water-pea protein concentrate interactions more than water bath glycation.

Keywords: glycation, microwave heating, Allulose, pea protein concentrate, AGEs

ÖZ

BEZELYE PROTEİNİNİN ÖZELLİKLERİNİN MİKRODALGA GLİKASYONU İLE İYİLEŞTİRİLMESİ

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Eylül 2020, 159 sayfa

Tüketicilerin hayvansal proteinleri tercih etme eğilimleri azaldıkça bitki proteinlerine olan eğilim önemli ölçüde artmıştır. Sürdürülebilirlik hedefleri, düşük üretim maliyeti ve yüksek besin değeri de bu talebi tetiklemektedir. Bu nedenle, izolat ve konsantre formu bulunan bezelye proteini, besleyici özelliklerinden dolayı proteinle zenginleştirilmiş diyetlerde sıklıkla kullanılmaktadır. Daha önce, bu proteinlerin fonksiyonel özelliklerindeki iyileştirme birçok açıdan incelenmiştir. Maillard reaksiyonunun ilk aşaması olarak bilinen glikasyon ise, bu fonksiyonel özellikleri iyileştirmek için bir modifikasyon stratejisi olarak değerlendirilebilir. Bu çalışmada, mikrodalga, bezelye proteini konsantresinin glikasyonu için kullanılmıştır. Bu çalışmanın amacı, bezelye protein konsantresi (PPC) ile dekstroz, fruktoz ve nadir şeker olan Allulose arasındaki glikasyon derecesini, çözünebilir proteinlerini, serbest amino gruplarını, kalan indirgenmiş şekerleri ve gelişmiş glikasyon sonu ürünlerini (AGE'ler) belirleyerek araştırmaktır. Ayrıca iki farklı pH değerinin (7 ve 10) ve iki farklı bezelye proteini-şeker oranının (2:1.25 ve 9:1.25) etkisi gözlemlenmiştir. Daha sonra, glike bezelye proteinlerin çözünürlüğü ve hidrasyon davranışı araştırılmıştır. Lowry yöntemi, mikrodalga glikasyonunun, dekstroz numunelerinin çözünürlüğünü, kontrol numunelerine kıyasla %50

artırdığını göstermiştir. Ayrıca, alkali ortamda glikasyon işlemi PPC çözünürlüğünü geliştirmiştir. Bunun yanı sıra, mikrodalga glikasyonu, su banyosu glikasyonuna kıyasla PPC'nin hidrasyon davranışını daha fazla iyileştirmiştir. Sonuç olarak, bu çalışma, mikrodalga ile glikasyonun bir alternatif modifikasyon tekniği olarak kullanılabileceğini göstermektedir çünkü bu metot, su-bezelye proteini konsantresi arasındaki ilişkiyi, su banyosuyla gerçekleştirilen glikasyona kıyasla daha fazla iyileştirmiştir.

Anahtar Kelimeler: glikasyon, mikrodalga ile ısıtma, Allulose, bezelye proteini konsantresi, AGE'ler

Dedicated to all those who encouraged me to fly

toward my dreams, especially my family

ACKNOWLEDGMENTS

Foremost, I would like to express my sincere gratitude to my advisor Assoc. Prof. Dr. Mecit Halil Öztop for the continuous support of my master's study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not imagine having a better advisor and mentor like him. I also would like to thank my co-advisor Prof. Dr. Servet Gülüm Şümnü for her valuable support and guidance throughout my thesis.

This research was financially supported by the Scientific and Technological Research Council of Turkey (TUBITAK) with the proposal number of 118R048. I am deeply grateful to them for giving me this opportunity.

I would like to express my eternal appreciation towards my grandmother, Fatma Ülkü Ertuğrul, and my parents, Abdullah and Ebru Ertuğrul, for their love, carrying and sacrifices for educating and preparing me for my future. I am also very much thankful to my little sister, Fatma Eylül Ertuğrul, who brightens up my every day, for her endless love and support. They have always been there for me and stood beside me in my whole life. A life without them would be meaningless.

I also would like to thank my dear colleagues in "Öztop Lab", especially Serap Namlı, Esmanur İlhan and Selen Güner for enlightening me with their practical and scientific knowledge whenever I faced with a problem during my study. They always made my job easier and never hesitated when I need help.

Some special words of gratitude go to my friends who have always been a significant source of support when things would get a bit discouraging: Eda Ceren Kaya, Muhammad Bin Zia, Şirvan Sultan Uğuz, Kamil Ünsal, Begüm Köysüren, İsmet Baykan, İlayda Nazlı, Berkay Berk, Bilge Baştürk, Gökhan Uğur Atıl and Jón Frederiksen. I am incredibly grateful for having all of you in my life. You always made me laugh louder and brighter. Therefore, I would like to thank every each of you for always being there for me.

Most of all, I would like to thank my closest friend, Ozan Taş, for making me laugh my lungs out even in bad days. Not only working with you but also being a close friend with you cared me a step further in my academic and personal life. I can never pay my debt to him for his guidance, contribution, and incredible friendship for several years.

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ABBREVIATIONS

PPC	: Pea protein concentrate
PPI	: Pea protein isolate
FPI	: Faba bean protein isolate
SPI	: Soybean protein isolate
MR	: Maillard reaction
MW	: Microwave
WB	: Water bath
AGEs	: Advanced glycation end products
OPA	: ortho-Phytalaldehyde
HPLC	: High pressure liquid chromatography
DOG	: Degree of glycation
FAG	: Free amino group
RRS	: Remaining reducing sugar
RID	: Refractive index detector
RF	: Radiofrequency
MS	: Mass spectroscopy
HPLC-MS	: High pressure liquid chromatography-mass spectroscopy
DAD	: Diode array detector
(O/W)	: Oil-in-water

(W/O)	: Water-in-oil
WHC	: Water holding capacity
HMF	: hydroxymethylfurfural
IR	: Infrared
FTIR	: Fourier transform infrared
ATR	: Attenuated total reflectance
SFC	: Solid fat content
NMR	: Nuclear magnetic resonance
TD-NMR	: Time domain nuclear magnetic resonance
CPMG	: Carr–Purcell–Meiboon–Gill
FID	: Free induction decay
FID/IR	: Free induction decay/inversion recovery
ODS	: Octadecylsilane
HILIC	: Hydrophilic interaction liquid chromatography
CML	: N-ε-carboxymethyllysine
CEL	: N-εcarboxyethyllysine
BSA	: Bovine serum albumin
SDS	: Sodium dodecyl sulfate
ESI	: Electrospray ionization
MRM	: Multiple reaction monitoring
ANOVA	: Analysis of variances
AHBL	: Average hydrogen bond length

CHAPTER 1

INTRODUCTION

1.1 Pea Protein

1.1.1 General View

Over the last decade, the tendency for plant protein consumption is increasing due to the unavailability and insufficiency of animal protein sources or preferences due to religious and cultural traditions. Also, there is a high demand for plant proteins due to providing energy and high nutritional value with low cost. Plant proteins such as sunflower, pumpkin seeds, quinoa, sesame, and pea are under the spotlight of nutritionists and consumers looking for a different protein source rather than animals (Du et al., 2018; Feyzi et al., 2018; Nadathur et al., 2017). Among these plant proteins, a significant number of research have been focused on pea proteins and its use in the food industry due to several health benefits (Bajaj et al., 2015; Dickinson, 2013; Lam et al., 2018; Qamar et al., 2019; Tulbek et al., 2016).

To produce pea proteins of 48-90% protein content, dry and wet milling technologies have been used and extraction has been conducted by several methods such as alkaline extraction, salt extraction and micellar precipitation (Tulbek et al., 2016; F. Wang et al., 2020). Total protein content, consisting of 15%-25% albumin and 50%-60% globulin, is mostly affected by both the production and the extraction methods.

Pea protein mainly contains 2S (albumin), 7S (vicilin), 11S (legumin) and 15S protein groups and has a wide range of amino acid profile including glutamic acid,

aspartic acid, tryptophan, threonine, cysteine, methionine, phenylalanine, arginine, leucine, and lysine (Banaszek et al., 2019; J. Boye et al., 2010). The functional properties of pea proteins like solubility, water-binding capacity, foam stability, gelation, emulsion activity, and stability are mostly affected by all these different protein groups and amino acid types (Lu et al., 2019).

1.1.2 Functional Properties of Pea Protein

The use of pea proteins in the food industry is affected mostly by their physical and chemical characteristics. Protein addition to a food formulation can achieve its function only if the physical properties are well-known. They can affect food processing, storage, and consumption (Shevkani et al., 2015). Therefore, a detailed evaluation of pea proteins' functional properties is necessary for its utilization since these properties are affected from the production methods. Multiple intrinsic and extrinsic factors affect physical and chemical properties. Shape, size, structure, hydrophobicity/hydrophilicity, and amino acid composition can be given as examples for intrinsic factors, whereas pH, moisture, temperature, and mechanical processing are the examples for extrinsic factors (Damodaran, 1997).

A detailed table given below shows the functional properties of proteins in several food applications (Söderberg, 2013). Among these properties, the most important ones are solubility, water holding capacity, emulsification, foaming and gelation (Lam et al., 2018). Improving these properties can be achieved using several different modification techniques, which will be explained in the next sections.

Table 1.1 Common functional properties of proteins (Söderberg, 2013)

General property	Functional Properties
Organoleptic	Color, flavor, and odor
Kinesthetic	Texture, mouthfeel, smoothness, grittiness, turbidity
Hydration	Solubility, water absorption, swelling, thickening gelling, syneresis, viscosity
Surface	Emulsification, foaming (aeration, whipping), film formation
Binding	Lipid-binding, flavor-binding
Structural	Elasticity, cohesiveness, chewiness, adhesion, aggregation, dough formation, fiber formation, extrudability
Rheological	Viscosity, gelation
Enzymatic	Coagulation (rennet), tenderization (papain), mellowing (proteinases)
Blendability	Complementarity (wheat-soy, gluten-casein)
Antioxidant	Off-flavor prevention (fluid emulsions)

1.1.2.1 Solubility

Protein solubility can be described as the thermodynamic equilibrium between the protein present in the liquid and solid phases (Hall, 1996). At this equilibrium, the hydrophobic interactions between proteins and hydrophilic interactions between protein and solvent become particularly important. Hydrophobic parts of the proteins orient themselves such that they are buried inside the structure to decrease the free energy, so only the residues at the surface hinder the protein solubility (Lam et al., 2018). Meanwhile, the hydrophilic relationship between protein and solvent increases the solubility through ionic interactions. These two are mostly influenced by environmental factors such as pH, ionic strength, temperature, and the solvent present in the system (Damodaran, 2007).

1.1.2.1.1 Effect of pH

When the effect of pH on the solubility of a protein was considered, the first thing that comes to mind is the isoelectric point because the changes in the electrostatic repulsive forces above, below, and at the isoelectric point (pI) influence the solubility. This implies that the net charge of the protein can be either negative or positive depending on being above or below the isoelectric point and can promote hydrophilic interactions and thus solubility. However, the net charge at the pI becomes zero, which reduces the repulsive forces to the minimum and leads to the aggregation of proteins due to the promoted hydrophobic interactions between proteins. Therefore, the relationship between protein solubility and pH is usually expressed with a U-shaped curve where the lowest value is around pI. For example, the U-shaped curve indicating pea protein solubility at different pH values is given in Figure 1.1 (Fernández-Quintela et al., 1997).

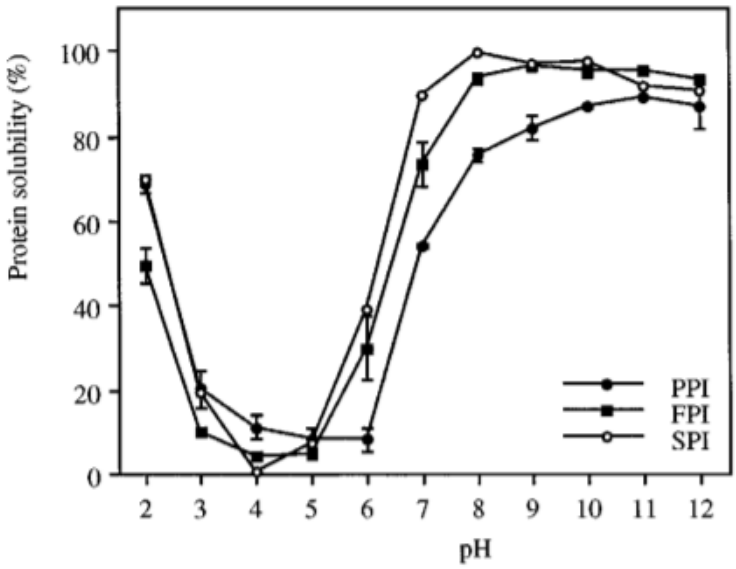

Figure 1.1. Protein solubility (%) of the pea protein isolate (PPI), faba bean protein isolate (FPI) and soybean protein isolate (SPI) at different pH values (Fernández-Quintela et al., 1997)

Pea proteins have a wide range of isoelectric points between pH 4-6 and as seen from the figure, pea protein has the lowest solubility at that pH (Estevinho & Rocha, 2018). Moreover, alkaline pH increases the pea protein solubility due to structural changes in legumin section of the protein. Legumin hexamers in pea protein tend to be broken down to its monomers at alkaline conditions. This partial denaturation results in a rise in the solubility (Estevinho & Rocha, 2018).

1.1.2.1.2 Effect of Ionic Strength

Another critical parameter affecting the protein solubility is the ionic strength of the medium. When the ionic strength is low (< 0.5), the net charge around the proteins is neutralized, and thus aggregation of proteins starts due to the promoted hydrophobic interactions (Damodaran, 2007). On the other hand, at high ionic strength (> 1.0), the type and the amount of the salt present in the solution becomes significantly important due to the changes in the electrostatic repulsive forces (Frazier, 2004). There are two important phenomena: “salting-in,” and “salting-out” that can be described to understand the effect of salts on the solubility. The enhancement of the hydrophilic (protein-solvent) interactions by salts such as calcium, bromide, and iodide is expressed as salting-in, whereas the exposure of hydrophobic (protein-protein) interactions by salts such as sulfate, potassium, and ammonium are defined as salting-out (Lam et al., 2018). According to the previous studies, using different salts at different concentrations during pea protein extraction was found to have an increasing effect on the solubility (Hang et al., 1970).

1.1.2.1.3 Effect of Temperature

It is a well-known fact that temperature has a considerable effect on protein

solubility. Over the decades, heat treatment was applied to the proteins as a modification technique. It was found that heating proteins up to a certain point increased the solubility due to the destabilization of covalent bonds and the loss of tertiary and secondary structures (Damodaran, 2007). On the other hand, when the temperature reaches to denaturation temperature, the unfolding of the protein occurs due to the increase in the kinetic energy. This irreversible change in the structure enhances the hydrophobic interactions leading to a decrease in protein solubility (Lam et al., 2018). However, recent studies have shown that heat treatment can also enhance the functional properties of proteins, including the solubility. A previous research where emulsifying properties of pea proteins were explored showed that heating at 95 ºC for 30 minutes did not decrease the solubility (Peng et al., 2016). In fact, according to the study of Bogahawaththa et. al (2019), heating pea proteins at 121ºC for 2.8 minutes increased protein solubility (Bogahawaththa et al., 2019). Therefore, the critical point here is to find the best temperature-time combination to obtain the high protein solubility.

1.1.2.2 Emulsification

An emulsion is described as a dispersed system created by agitating two or more immiscible liquids (Damodaran, 2007). Oil-in-water (O/W) and water-in-oil (W/O) are examples of emulsions used frequently in the food industry (Sharif et al., 2018). In an emulsion system increase in the system's free energy at the interface creates a thermodynamically unstable system. However, the integration of proteins into the system is an excellent solution for this problem. Because of their amphiphilic nature, proteins can rearrange themselves at the interphase and decrease the interfacial tension (Lam et al., 2018).

The criteria for a protein to create a stable dispersed system is to have a good emulsion capacity and stability, and previous studies showed that pea protein had this ability (Liang & Tang, 2013; Peng et al., 2016; Shao & Tang, 2014; Sijtsma et al., 1998).

Several parameters affect the emulsification properties, but the pH of the medium can be considered as the most important one. It is known that the emulsification capacity is low around isoelectric point, but as pH increases, the capacity increases because of the changes in the pea protein solubility (Karaca et al., 2011). According to the previous studies, it was found that both alkaline and acidic environments increased the emulsion capability and the stability of pea proteins. There are several globulin fractions in pea protein such as legumin and vicilin, and it was found that vicilin, with low molecular weight and a relatively flexible structure, showed more emulsion stability than legumin (Dagorn-Scaviner et al., 1987). Not only the amount of vicilin but also the vicilin to legumin ratio was significant on the emulsifying properties of pea proteins (Barac et al., 2010; Koyoro & Powers, 1987). Moreover, pea proteins was also shown to inhibit the oxidation of emulsions at alkaline conditions (Jiang, Zhu, Liu, & Xiong, 2014).

1.1.2.3 Water-Holding Capacity (WHC)

Water-holding capacity, also known as water-binding capacity, is generally defined as the water absorption and the retention ability of a food product such as protein within its matrix under an external, often gravitational, force (Boye et al., 2010; Shevkani et al., 2015) and it is also considered as a way to understand the hydration behavior of the proteins. The binding of water to a protein occurs in several ways. Water can bind to charged groups via ion-dipole interactions, hydroxyl groups via

dipole-dipole interactions, and nonpolar residues via hydrophobic interactions (Damodaran, 2007).

Like protein solubility, WHC is also strongly affected by the pH of the environment. WHC is minimum when the protein-protein interactions are maximum, which is the case observed at the isoelectric point (Lam et al., 2018). The change in pH can reveal the water binding sites of the proteins, leading to an increase in the water entrapment (Zayas, 1997). Moreover, heating influences the WHC of proteins. According to a study, heat treatment increased the water holding capacity of pea proteins (Swanson, 1990). This increase was explained by the unfolding of the proteins, which dissociates the subunits and exposes the water-binding sites (Abbey & Ibeh, 1988; Owusu-Ansah & McCurdy, 1991). Furthermore, the processing type has an influence on the WHC of proteins. According to a study, it was found that drum-dried pea proteins had the highest WHC, followed by spray-dried and freeze-dried samples (Sumner et al., 1981).

1.1.2.4 Gelation

A protein gel is defined as the transformation of a protein to a three-dimensional and well-established complex, facilitated by heat, enzymes, pH, pressure/shearing, or ions via different mechanisms (Jeantet et al., 2016). However, gelation via heat treatment is the most common method among them (Damodaran, 2007). What happens in heating is that the protein is partially denatured and forms aggregates, and this is followed by the formation of a three-dimensional network containing entrapped water (Gaonkar & McPherson, 2006).

According to the studies about the heat-treated pea protein gels, it has been found that several factors have affected their gelation behavior such as heterogenicity, heating time and temperature, and extraction method (Mession et al., 2013; O'Kane et al., 2004; Shand et al., 2007). Studies showed that the size of the pea aggregates and the network structure is also profoundly affected by the pH of the medium due to the changes in protein-protein interactions (Munialo et al., 2015).

Fractionation can also influence the gelation ability of pea proteins. The chromatographic separation of 7S and 11S can increase the denaturation temperature of pea proteins. A study revealed that the vicilin-enriched samples had advanced gelation abilities than legumin-enriched samples due to the increased elasticity to store energy (Munialo et al., 2015).

1.2 Non-enzymatic Browning Reactions

Non-enzymatic browning reactions, which are responsible for the brown color formation by volatile flavor molecules, can be considered as the most critical phenomena in the food industry because of its many contributions in food stability, technology, as well as in nutrition, and health (Jeantet et al., 2016). These reactions have been used in the production of various foods, such as fermented products including cheeses and beer (Nøddekær & Andersen, 2007; Schwietzke et al., 2009), roasted products such as coffee beans (Liu & Kitts, 2011) and pastry products such as bread and cookies (Bressa et al., 1996; Capuano et al., 2008; Helou et al., 2016). Among non-enzymatic browning reactions such as caramelization, chemical oxidation of phenols, and maderization (Jeantet et al., 2016), Maillard reaction has been the most common reaction which will be discussed in detail in this section.

1.2.1 Maillard Reaction

The Maillard reaction, a condensation reaction between the carbonyl ends of reducing sugars and amino groups on protein, peptides, and amino acids, was named after Louis-Camille Maillard in 1912. Since then, it has been used in many different applications (Feiner, 2006). The primary reason behind this popularity is the enhancement of final product in terms of sensorial properties such as color, flavor, and texture (Starowicz & Zieliński, 2019), as well as the chemical properties such as protein solubility (Zhou et al., 2017), hydration behavior (Ennis & Mulvihill, 1999), emulsification (Zhang, Wu, Yang, He, & Wang, 2012), and antioxidant activity (Vhangani & Van Wyk, 2013). However, the Maillard reaction in foods is not always favored due to the uncontrollable changes (Hoskin & Dimick, 1995). Nutritional value of the food can be easily influenced by the changes in color and flavor during processing and storage, the loss of essential amino acids (most importantly lysine) (Hedegaard & Skibsted, 2013), vitamins (especially vitamin C) (Zhang, Ames, Smith, Baynes, & Metz, 2009) and nutritionally valuable metals (copper, zinc, and iron) (Borrelli et al., 2002), and the formation of toxic compounds like acrylamide and hydroxymethylfurfural (HMF) (Mehta, 2015). Therefore, the priority should be keeping these reactions under control to obtain the desired product with a high nutritional value.

1.2.1.1 Mechanism

Maillard reaction includes a series of incredibly complex reactions (Jeantet et al., 2016). The mechanism is composed of 4 stages: the condensation reaction, Amadori and Heyns rearrangement, decomposition, and polymerization. The reaction starts with a nucleophilic attack of the free amino group of a specific compound (mainly lysine) to the carbonyl end of the reducing sugar (Figure 1.2) (Hodge, 1953).

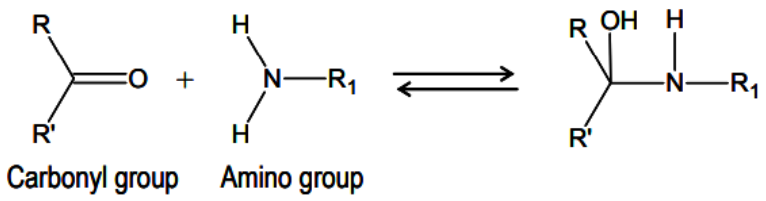

Figure 1.2. The reaction between the free amino group and the carbonyl group (Jeantet et al., 2016)

At the end of this condensation reaction, the formation of a glycosylamine, also known as "Schiff's base" is observed with a release of water (Figure 1.3) (Hellwig & Henle, 2014).

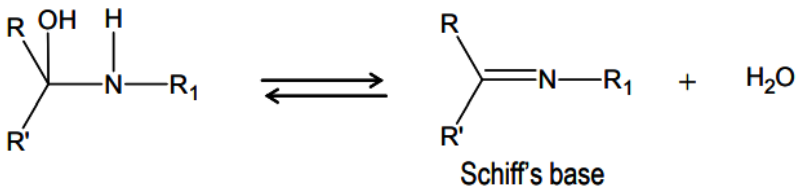

Figure 1.3. The formation reaction of Schiff's base (Jeantet et al., 2016)

At the second stage, due to the relative instability of glycosylamines, the isomerization may lead to a more stable product formation, which are Amadori products (ketosamines) if the substrate is an aldose, and Heyns products (aldosamines) if the substrate is a ketose (Wrodnigg & Eder, 2001). The main changes in this stage could be considered as a slight color and flavor changes, as well as a decrease in the number of available amino groups (Stadler & Studer, 2016).

Later on, no matter how Amadori and Heyns products are stable, a series of parallel reactions such as enolization, dehydration, oxidation, and decarboxylation can still be observed (Friedman, 1996; Ledl & Schleicher, 1975). However, highly reactive carbonyl compound formation, Strecker degradation, and retro-aldolization (carbohydrate unit fragmentation) can be considered as the most important ones (Jeantet et al., 2016). The compounds obtained with these reactions can proceed even further.

Finally, the significant color development in the products can be observed after the successive polymerization reactions, causing high molecular weight, brown color pigments, known as melanoidins (Friedman, 1996; Martins et al., 2001). In addition to melanoidins, several types of compounds such as advanced glycation end products (AGEs), aldehydes, ketones, dicarbonyls, acryl amides, and heterocyclic amines are also formed, which are the main contributors of the several physical and chemical changes in the food product (Andriot et al., 2004; Lindenmeier et al., 2002). The complete mechanism of the Maillard reaction is given in Figure 1.4.

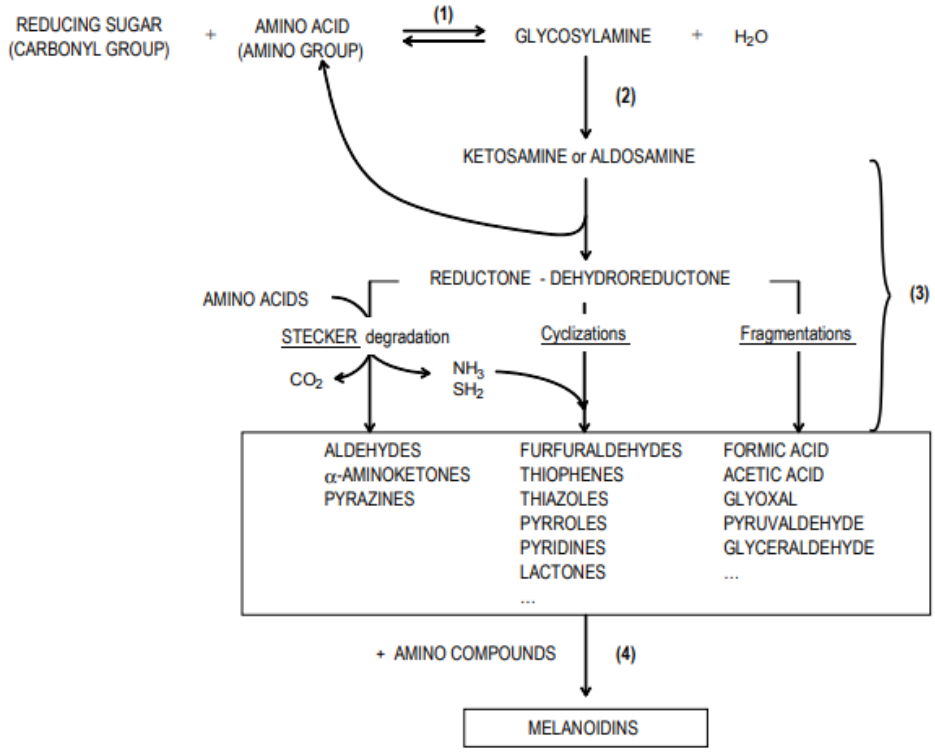

Figure 1.4. Reactions involved in Maillard reaction as 1) the condensation reaction, 2) the Amadori and Heyns rearrangement, 3) the decomposition of ketosamines, 4) polymerization (Jeantet et al., 2016)

1.2.2 Glycation

As mentioned before, besides off-flavor and nutritional loss, the Maillard reaction can cause several toxic compounds to form, which can be dangerous for human health. One of the examples for these compounds could be hydroxymethylfurfural (HMF). It behaves like an indirect mutagen and organotoxic agent and it induces the possibility of carcinogenesis and inhibits significant enzyme activities (Shapla et al., 2018). Secondly, heat treatment can result in acrylamide formation, which is considered as a potential carcinogen (Tamanna & Mahmood, 2015). Due to these

and more other toxic compounds, the Maillard reaction should be kept under control, and one way to achieve this is by stopping the reaction in its early stages.

Stopping the reaction right after the formation of Amadori and Heyns compounds is a way not only to prevent the formation of toxic compounds but also to improve the quality of the products. For instance, the antioxidant activity of protein-sugar mixtures has increased with the formation of the Maillard reaction intermediates (Antony et al., 2000; Chen & Kitts, 2008; Morales & Jimeâ Nez-Peâ Rez, 2001). Based on a study, it has been found that the antioxidant activity of a garlic extract increased after the formation of a fructose-arginine complex, which is an intermediate (Colín-González et al., 2012; Ryu et al., 2001). In addition to anti-oxidative properties, Amadori compounds, which were isolated and synthesized, have been found to have the ability to inhibit the human breast, prostate, and thyroid tumor cells in vitro and in vivo (Glinskii et al., 2012; Glinsky, 1993; Glinsky et al., 1996). In that regard, glycation, which is defined as the early stages of the Maillard reaction between protein and reducing sugars, that leads to the formation of Schiff's bases followed by Amadori and Heyns compounds has gained popularity more than the Maillard reaction (MR) (Taniguchi et al., 2015). Further crosslinking of these Amadori compounds with other proteins results in the development of harmful advanced glycation end products (AGE), so glycation reaction should be performed under control (Poulsen et al., 2013). However, several factors such as the pH of the environment, reducing sugar type and processing time, and temperature can affect the extent and the rate of the glycation reaction (Pan & Melton, 2007; Scaman et al., 2006).

1.2.2.1 Factors Affecting Glycation

1.2.2.1.1 Sugar Type

The rate of the glycation may differ with the size and the structure of the reducing sugars. The first comparison can be made between pentoses and hexoses. Research showed that pentoses had higher reactivity than hexoses (Jeantet et al., 2016). The structural reason why pentoses were more reactive was that pentoses had shorter hydrocarbon chains, which led to a less steric hindrance (Hao Jing & Kitts, 2004; O'Brien & Morrissey, 1989). This less hindrance promoted the reaction with the free amino groups in the system.

Moreover, the availability of pentoses in an open-chain form in an aqueous environment increases as the chain length decreases (Chevalier et al., 2001; Hayward & Angyal, 1977). Therefore, the rise in active pentose amount in the environment can increase the rate of reaction. On the other hand, hexoses have more conformational stability so the rate of chromophore (part that is responsible for the color formation) production might be slower (Burton & McWeeny, 1963).

The second important factor affecting the behaviors of sugars is the structure of the six-carbon sugars. There is a debate going on in the literature about the rate of participation of aldoses and ketoses in the glycation. The first claim is that aldoses (e.g., glucose) are more reactive than ketoses (e.g., fructose) (Jing & Kitts, 2002; Hao Jing & Kitts, 2004; Naranjo, Malec, & Vigo, 1998; Oliver, Melton, & Stanley, 2006; Yen, Tsai, & Lii, 1992). The first explanation of this claim is the more accessibility of the aldehyde group (Jing & Kitts, 2002). This availability favors the crosslinking with amino groups more than ketoses. Also, the nucleophilic attack of free amino groups to the aldoses, which is the very first step in the glycation reaction is facilitated due to the higher electrophilicity of them (Benjakul et al., 2005; Bunn & Higgins, 1981; Naranjo et al., 1998). Furthermore, as mentioned in Section 2.1.1.,

the rate of the glycation may differ with the structure of the sugar since the pathway of the Amadori rearrangement is different for ketoses and aldoses. D-glucose, as an aldose example, crosslinks with the free amino group at position C_1 (Carbon 1), which results in 1-amino-1-deoxyketoses formation (Figure 1.5) (Taniguchi et al., 2015). On the other hand, D-fructose, being a ketose, reacts with free amino groups at position C_2 to form 2-amino-2-deoxyaldoses (Figure 1.6) (Taniguchi et al., 2015). Due to this difference in pathways and products, the Amadori compounds produced from aldoses reacts faster than the Heyns compounds produced from ketoses (Kwak & Lim, 2004). These pathways can also be affected by the differences in the thermal fragmentation abilities of aldoses and ketoses (Cämmerer & Kroh, 1995).

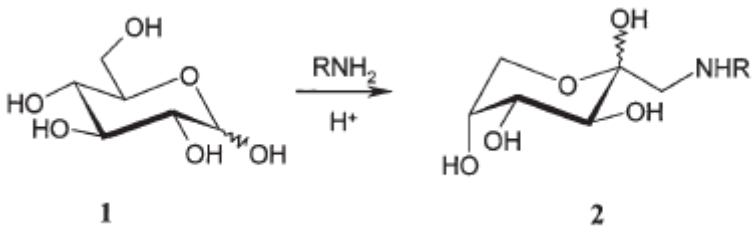

Figure 1.5. The Amadori rearrangement: 1) D-glucose and 2) example of 1-amino-1-deoxyketoses (Jeantet et al., 2016)

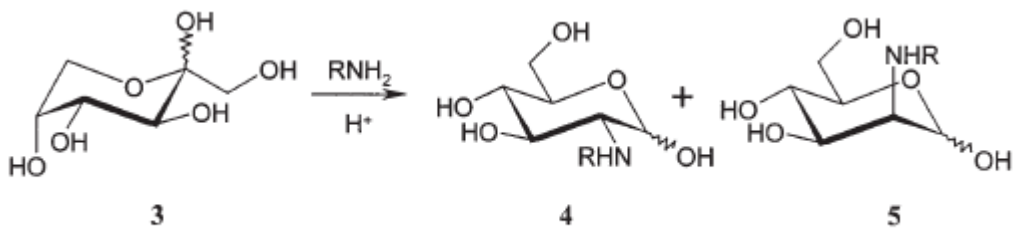

Figure 1.6. The Heyns rearrangement: 3) D-fructose and 4) & 5) examples of 2-amino-2-deoxyaldoses (Jeantet et al., 2016)

On the contrary, several studies have shown that there is a particular case between fructose and glucose use as a reactant in the reaction. It was found that fructose had a higher reactivity than glucose. The first explanation is the higher availability of the fructose in open-chain form than the glucose (Benjakul et al., 2005; Hayward & Angyal, 1977; Naranjo et al., 1998). Therefore, the participation of fructose was found to be more likely. Moreover, the browning degree of the samples glycated with fructose can be overestimated since the caramelization, a contributor to brown color development can also take place at the same time, and it is known that ketoses are more susceptible to caramelization reactions (Ajandouz, Tchiakpe, Dalle Ore, Benajiba, & Puigserver, 2001; Benjakul et al., 2005; Jing & Kitts, 2002). The final products can also be influential on the reactivity of sugars.

In some cases, the products of glycation that formed in the presence of glucose could behave like an inhibitor. Also, with glucose as the reactant, only one Amadori compound is formed (Ajandouz et al., 2001). These both circumstances can decrease the reactivity of glucose. However, most importantly, other factors like pH and the temperature of the environment can affect the reactivity of reducing sugars (Laroque et al., 2008).

1.2.2.1.2 pH

It is particularly important to identify the effect of pH on the glycation since every stage of the reaction has a different optimum pH ranging from 6 to 9 (Jeantet et al., 2016). One of the key factors to find which mechanism is followed during the reaction is the pH of the medium. At the very first step of the reaction, the reactivity of carbonyl ends and free amino groups is found to be higher at alkaline pH values (Perez-Locas & Yaylayan, 2010). After this stage, the possibility of furfural or HMF

formation by the 1,2-enolization reaction in acidic and neutral environments is found to be very high (Liu, Ru, & Ding, 2012). Therefore, hydrolysis and dehydration reactions are more favorable at acidic conditions. On the other hand, the degradation of the compounds by 2,3-enolization is more prone to occur under alkaline conditions, leading to a formation of compounds such as 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one (HMF_{one}), acetol, pyruvaldehyde, and diacetyl (Martins et al., 2001). Thus, the pH of the environment influences the color and flavor intensity developed by the reaction since these parameters are affected by the products obtained by different pathways.

Another point is the effect of pH on the rate of glycation. According to a study performed by Hayashi and Namiki (1986), the amount of free amino group increased as the pH of the environment increased. This increase led to a rise in the reaction rate, so the browning was succeeded in shorter processing times (Hayashi & Namiki, 1986). The idea of increasing rate at alkaline conditions was also supported by another study showing the rate of glucose and lysine loss at different pH values. The study showed that the rate of loss was higher between pH 8-12, indicating the importance of the environmental conditions such as pH not only on the pathway but also on the rate of the reaction (Ames, 1998).

1.2.2.1.3 Temperature and Processing Time

Although the Maillard reaction is a spontaneous reaction that can take place even at mild temperatures, it is known that the rate of reaction can be accelerated by heat treatment (Benzing-Purdie et al., 1985). Therefore, heating has been applied in many food products to obtain the desired outcome in a short period, and the investigation of brown color and flavor formation at high temperatures has been the focus of the majority of researches (Hiller & Lorenzen, 2010; Jiang & Brodkorb, 2012; Lillard,

Clare, & Daubert, 2009; Nie et al., 2013). However, there are several drawbacks of the heat treatment on the final product. The first and the most important problem is the loss of nutritional value and the rise in the toxicity as heating time and temperature increase. The use of extreme temperatures for a long time causes deteriorative reactions whose yields are hydrocarbons, aldehydes, and ketones (Ames, 1992; Fox, 2011). These compounds alter the product's overall quality through the flavor, color, and toxicological perspective (Zhu et al., 2018).

Moreover, high temperatures can cause proteins to denature and form aggregates, which can change the functional properties (Teodorowicz et al., 2017). Heating initiates the disruption of ionic and van der Waals bonds between the proteins, and as heating proceeds over time, the unfolding, aggregation, and precipitation of the proteins occur (Dutson & Orcutt, 1984). Although it depends on the use of the protein in a food system, such a precipitation can decrease the water holding capacity and digestibility of the proteins (Neucere & Cherry, 1982). Thus, the selection of time and temperature should be made by considering the quality, functional properties of the protein, and its nutritional value (Nie et al., 2013).

1.2.2.2 Glycation Methods

Glycation reaction is usually conducted under two different conditions, which are dry heating and wet heating. Both techniques include similar processing steps, but the significant difference is the state of the prepared samples. In the case of dry glycation, the hydrated protein-sugar mixtures are lyophilized and brought into the powder form before glycation. In contrast, in wet glycation, hydrated samples are directly glycated without lyophilization (Sedaghat Doost et al., 2019). Therefore, the preparation of dry glycated samples can take a long time to reach a certain level of dryness. During this time, phase separation or uneven distribution of proteins and

sugars in the mixture could be observed (Zhuo et al., 2013). Also, the extent of the reaction could not be controlled due to the necessity for long reaction time. Thus, it is not wise to apply dry heating glycation to the products that are desired to be industrialized (Zhu, Damodaran, & Lucey, 2008).

On the other hand, wet glycation requires shorter heating times, minutes to hours, to a protein-sugar mixture in a buffer solution. The optimum water activity and temperature range for the wet glycation are found as 0.5-0.8 and 60-95 ºC, respectively (O'Mahony et al., 2017; Van Boekel, 2001). The major advantage of wet glycation over dry heating is the successful conjugation of proteins and sugars due to the improved protein solubility and stability (McGuffey et al., 2005; Seymour & Knapp, 1980). Moreover, wet glycation can be achieved by several cost and time-efficient instruments such as microwave (Wang et al., 2013; Wang, Zhang, Zhang, Ju, & He, 2018).

1.2.2.2.1 Microwave Glycation

Although there are several different claims about the invention of microwave ovens, the first microwave oven in history was created by Percy Spencer, who is an inventor and other designers working in Raytheon Manufacturing Company in 1945-46 (Osepchuk, 2009). However, the idea of "matching some lossy dielectric load to a radiating dipole by an intermediate dielectric body around the dipole" has already existed way before the World War II (Hand et al., 1979). Several pieces of evidence showed that the food manufacturers did not recommend using the high-frequency microwave in food processing even way before its great recognition. Despite the negative views, scientists kept examining and enhancing the microwave ovens to catch their attention. Since then, the microwave oven usage, not only in domestic

and commercial kitchens but also in the food industry, has become universal throughout the world (Osepchuk, 1984).

Microwave ovens work in the radio frequency range of the electromagnetic spectrum, between 300 and 300,000 MHz. Still, the only frequencies allowed are 2,450 MHz for domestic usage and 915 MHz for industrial usage (Graham, 2003). The conversion of electromagnetic energy into heat explains the basic working principle of the microwave oven. There are two different mechanisms of interactions that are based on ionic interactions (Figure 1.7b) and dipolar rotation (Figure 1.7a) between food and microwaves. After the generation of the alternating electric field by a magnetron, the dissociative ions start to oscillate forward and backward and collide with each other, leading to a rise in the kinetic energy and, thus, a rise in the temperature of the food product by ionic conduction. On the other hand, polar molecules start to rotate clockwise and counterclockwise to align with the alternating field. This creates a motion and friction, and the kinetic energy and the temperature of the food products begin to increase with that motion and friction (Decareau, 1992). In both mechanisms, heat transfer occurs from the center of the product to the outside through either conduction or convection (Ibrahim et al., 2012).

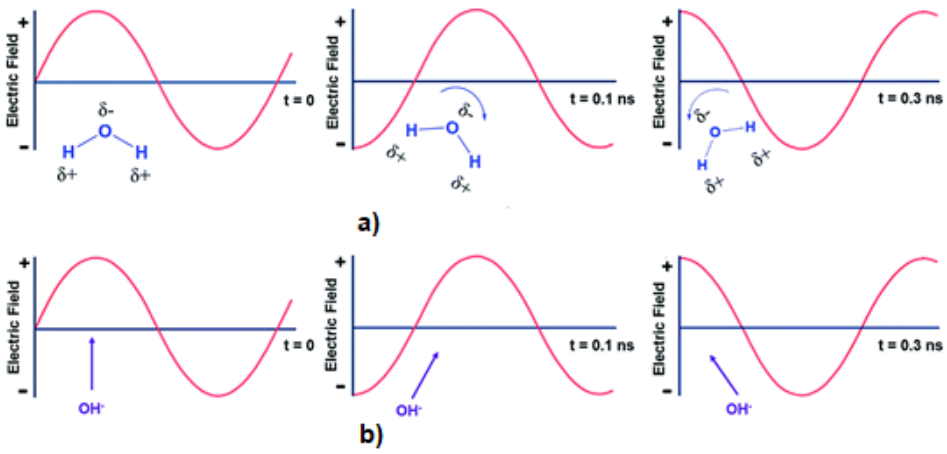

Figure 1.7. Mechanism of microwave heating by a) dipole rotation and b) ionic interaction (Zhang, Rajagopalan, Lei, Ruan, & Sharma, 2017)

Microwave heating has several advantages compared to conventional heating. The most important feature of microwave heating is the higher heating rate due to the polarization, which reduces the heat transfer path (Siddiquey et al., 2011). Thus, processing time becomes significantly lower, leading to a cost-efficient process. Moreover, the heating can be controlled by the selective heating characteristic of microwave heating. Partial heating can be efficiently conducted by the differences in the molecules' dielectric constants (Xing et al., 2014). Furthermore, the problems arising from the overheating of the samples for prolonged times can be overcome by microwave heating. The fresh-like taste of the product can be maintained, and the desired texture can be obtained without any nutrition loss (Mchugh, 2020). Due to all these significant advantages, the microwave has been used in various food-related applications.

The first successful microwave heating was achieved by Frito-Lay to dry the potato-chips (Osepchuk, 1984). After this, microwave has been used in many areas such as baking of the breads (Demirekler et al., 2004; Keskin et al., 2004b, 2004a; Ozkoc et al., 2009), cakes (A. Li & Walker, 1996; Sevimli et al., 2005; Gulum Sumnu et al.,

2005) and several gluten free products (Demirkesen et al., 2013; Turabi et al., 2008, 2010); frying of fruits (Faruq et al., 2019; Jumras et al., 2020) and meat (Barutçu Mazi & Mazi, 2017; Echarte et al., 2003); drying of fruits (Beaudry et al., 2003; Maskan, 2000, 2001) and vegetables (Prabhanjan et al., 1995; Sharma & Prasad, 2006; Soysal et al., 2009); blanching (N. Jiang et al., 2015; Latorre et al., 2013; Moreno et al., 2000; Y. Wang et al., 2012); thawing (Taher & Farid, 2001); tempering (Schaefer, 1999; Seyhun et al., 2009); sterilization and pasteurization (Burfoot et al., 1988; Coronel et al., 2008; Koskiniemi et al., 2011).

The usage of the microwave for glycation and Maillard reaction is a rather new method. Therefore, the investigation of microwave glycation has not been performed much in the literature. One of the first studies investigated the effects of microwave treatment on milk (Villamiel et al., 1996). This study showed that microwave heating accelerated the Maillard reaction rate, compared to conventional (oil bath) heating. After this, microwave glycation of other proteins such as soy protein (Guan et al., 2006; Kaye et al., 2001; Žilić et al., 2014), ovalbumin (Hu et al., 2019; J. Sun et al., 2020; H. Wang et al., 2013; Zhong et al., 2020), whey protein (Noma et al., 2009; Nooshkam & Madadlou, 2016) and bovine serum albumin (Nasrollahzadeh et al., 2017) were also studied. These studies revealed that the probability of protein-reducing sugar collision increased after microwave glycation due to the disruption of subunits and protein unfolding (Guan et al., 2006). Another reason for the acceleration in reaction rate was the decrease in the activation energy by the reaction selectivity of microwave (Guan et al., 2011). The overall conclusion of these studies was the higher efficiency and the enhancing ability of microwave glycation over conventional glycation.

In the literature, there are few studies about the glycation of pea proteins, and in these studies, the chosen glycation method was mostly based on the use of climate chamber which is a conventional heating method (Bielikowicz et al., 2012; Kutzli, Beljo, et

al., 2020; Kutzli, Griener, Gibis, Grossmann, et al., 2020; Kutzli, Griener, Gibis, Schmid, et al., 2020; Marciniak-Darmochwal & Kostyra, 2009). On the other hand, microwave heating was applied to pea proteins; however, the interest of those studies was not the glycation or Maillard reaction (Divekar et al., 2017; Xiaohong Sun et al., 2020). Therefore, microwave glycation of pea proteins and the investigation of the functional properties after glycation will be a novel approach.

1.3 Characterization of Wet Glycated Pea Protein

Once proteins are glycated the next question becomes; what has changed on the proteins? To answer these questions; characterization of proteins will be discussed in the following section.

1.3.1 Lowry Method

Lowry method, which can be considered as one of the most accurate methods to determine the protein solubility, was firstly discovered by Oliver H. Lowry in 1951 (Lowry et al., 1951). Since then, it has been used in many studies (Bolat, 2019; Georgieva & Zapryanova, 2009; Kusunoki et al., 2012). Lowry's most distinct advantage over other methods is being moderately sensitive to the amino acid composition (Sapan et al., 1999).

The mechanism of the Lowry method includes two main reaction steps: 1) Biuret reaction between proteins and copper under alkaline conditions and 2) the reduction of Folin reagent by protein-copper complex (Lowry et al., 1951). In the first step, the presence of the copper changes the color of the medium slightly. However, the

intensity of the color mostly depends on the free tyrosine and tryptophan content (Herriott, 1935, 1941).

In the second part, the reaction between Folin and Cu-peptide complex occurs only at about pH 10. Therefore, mixing this solution at the very beginning for 8 seconds is crucial for color development (Folin & Denis, 1912). If all the steps are applied very carefully, the yellow-colored proteins turn to dark blue. The ideal absorbance value to read this color via spectrophotometer is 750 nm (Lowry et al., 1951).

$$Cu^{2+} + \text{Protein (Peptide Bonds)} \rightarrow (Cu^{1+}\text{-Peptide Bond Complex})$$

$$\text{Folin-Phenol Reagent} + (Cu^{1+}\text{-Peptide Bond Complex}) \rightarrow \text{Reduced Folin Phenol Reagent}$$

Although Lowry is a universal method, it has some disadvantages. One should be kept in mind that the results can alter among different proteins (Walker, 2002). Therefore, the comparison between a control sample and modified protein samples should be made to be precise. Also, the intensity of the blue color can be affected by many factors. For example, the overestimation of the protein content is possible due to compounds other than amino acids (Everette et al., 2010). Furthermore, the second step of the reaction is very sensitive to light, so the experiment should be conducted in a light-proof container and in a dark room (Dawson & Heatlie, 1984).

1.3.2 OPA (ortho-Phthalaldehyde) Method

The invention of a new method for the measurement of free amino groups was an urgent matter due to the problems such as being lack of sensitivity and requirements

of a large number of samples while detecting the proteolysis (Hull, 1947). Therefore, a new method was discovered and detailed in 1983 in order to achieve a correct measurement of proteolysis of milk and milk protein isolates (Church et al., 1983). After the discovery, this method has become exceedingly popular to quantitatively determine the free α- and ε-amino groups in amino acids, peptides, and proteins (Benson & Hare, 1975; Cohn & Shore, 1961; García Alvarez-Coque et al., 1989; K. S. Lee & Drescher, 1978; Molnár-Perl, 2001; Roth, 1971).

The name of this method comes from the chemical compound used in the solution, which is ortho-phthalaldehyde (OPA). This compound is known to be highly stable and soluble in water at pH values below 11.5 (Uptima, 2016). The reaction occurs between OPA and free amino groups of the protein in the presence of a thiol component, in this case, β-mercaptoethanol. The final product of this reaction is alkylthio-2-alkylisoindoles, which possess a maximum fluorescence at 340 nm (Nielsen et al., 2001). The reaction is given in Figure 1.8.

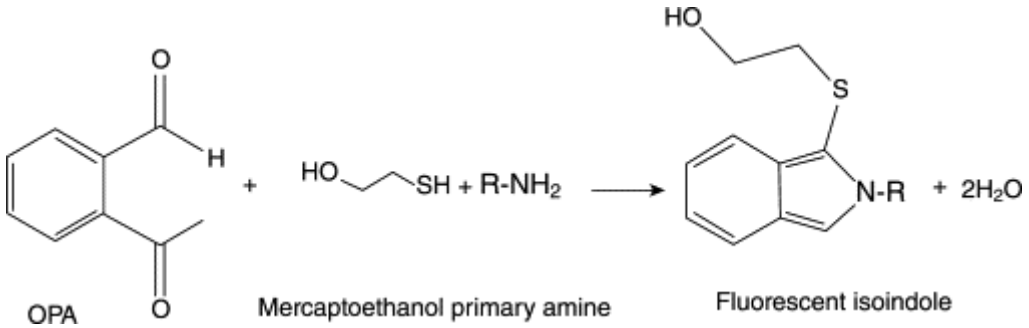

Figure 1.8. The OPA reaction (Perrett & Nayuni, 2013)

The reactivity of OPA depends on several factors, such as pH and the accessibility/availability of lysine residues. Alkaline conditions (pH~9) can enhance

the fluorescence intensity due to the protonation of amino groups, but measurements can also be done at pH values beyond the isoelectric point (Held, 2006). To maintain the desired conditions, several amine-free buffer systems such as sodium borate can be used. Secondly, to obtain accurate results, all the free amino groups should be denatured or solubilized and one way to achieve this is to use sodium dodecyl sulfate (SDS) and β-mercaptoethanol (Goodno et al., 1981).

Besides these factors, some external factors can also manipulate the results. The freshened of the OPA reagent and fluorescence reading are also important. The OPA reagent should be used two hours after preparation, and the OPA-protein mixture should be read within two minutes (Uptima, 2016). Also, OPA reagent is photosensitive, so the experiment should be conducted in a dark place (Held, 2006).

1.3.3 Fourier Transform Infrared (FTIR) Spectroscopy

After discovering infrared (IR) light in the 19th century, scientists tried to utilize it in many studies. Later, the first-generation IR spectrometer was invented in the 1950s, and it was developed further in the 1960s to observe the structural changes of molecules via infrared radiation absorption (Christy et al., 2001). However, obtained spectra were created as a function of time, and its transformation to a frequency domain was accomplished in 1965 (Cooley & Tukey, 1965). This final version of the FTIR system consists of three parts: a radiation source, an interferometer, and a detector (Figure 1.9).

The mechanism of FTIR starts with the production and the reflection of an IR beam. After the beam's emission by a black-body source, it reaches the interferometer, which creates the spectral information. The beam leaving the interferometer, then,

passes through the sample having a unique characteristic to absorb the specific energy. At the same time, background information is collected by the superimposition of another beam. Finally, the signal coming from the detector, which was created by the beam, is digitalized and sent to the computer where Fourier transformation takes place (Smith, 2011).

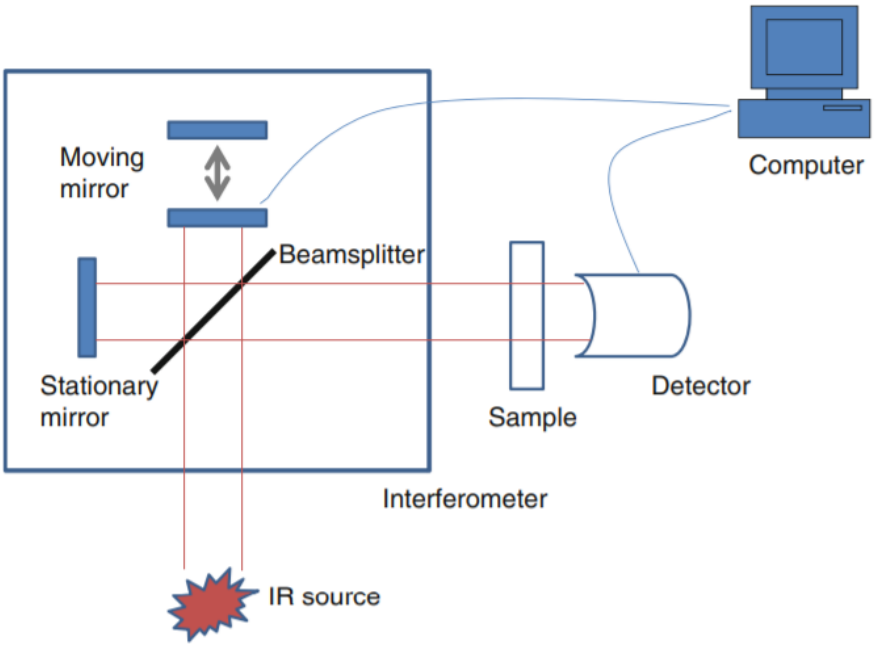

Figure 1.9. Basic components of an FTIR spectrometer (Avelino Alvarez-Ordóñez & Prieto, 2012)

Although IR has a quite wide spectrum range, FTIR usually works in the mid-IR range, which is between 400-4000 cm^{-1} since this range can be used to detect the specific functional groups of compounds via the transition of vibrational energy. There are four main regions on the FTIR spectra: single bond, double bond, triple bond, and fingerprint regions (Mohamed et al., 2017). A demonstrative figure of these regions is given below (Figure 1.10).

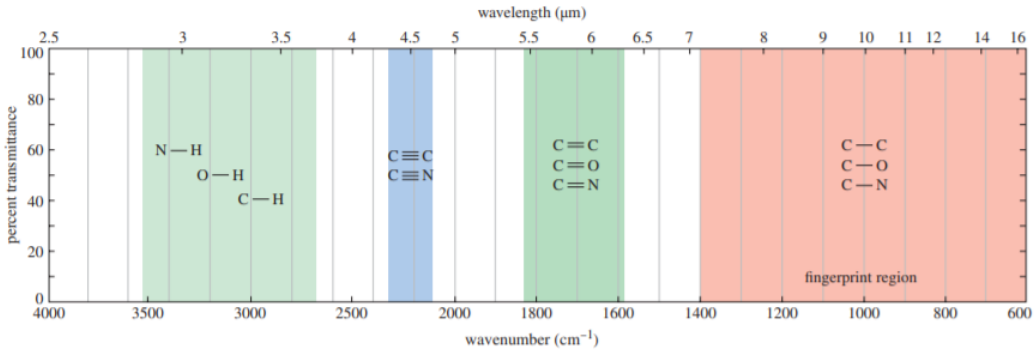

Figure 1.10. Summary of FTIR stretching regions (Wade Jr., 2016)

Due to its several advantages, the FTIR system is used in the food industry to detect several organic compounds such as proteins, carbohydrates, lipids. Some of the benefits are:

- It is a non-destructive, inexpensive, and fast method,
- It is a selective, chemical-sensitive, and information-rich method,
- It can be used on several types of samples such as solids, liquids, gases, powders, polymers, organics, inorganics, and pure substances,
- And it requires relatively less amount of sample (Smith, 2011).

FTIR spectrometer is sometimes preferred to be used with an additional attachment called attenuated total reflectance (ATR) (Figure 1.11). It is usually used if the sample is highly scattering, such as freeze-dried samples (Durak & Depciuch, 2020; Quijano-Ortega et al., 2020). The main difference of this part is the reflection of beam to the sample is achieved through a ZnSe crystal. Besides easy cleaning and minimum sample addition, the significant advantage of ATR is using the samples in their actual state without the requirement to press or heat (Aguilar, 2013).

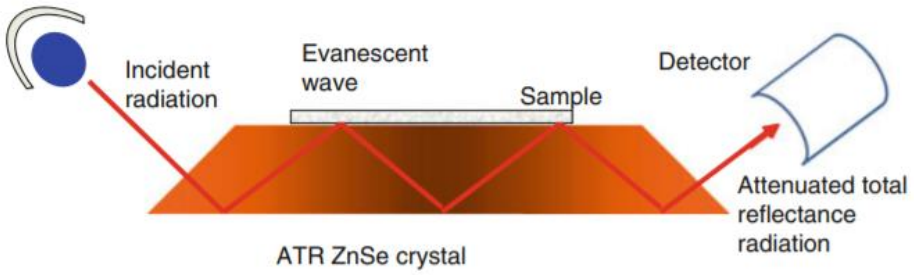

Figure 1.11. FTIR system with attenuated total reflectance (ATR) attachment (A Alvarez-Ordóñez et al., 2011)

1.3.4 Nuclear Magnetic Resonance (NMR) Relaxometry

Over the last decades, Nuclear Magnetic Resonance (NMR) integration has become widespread in several areas since it provides versatile information about the chemical composition and the structure of a material (J. P. M. van Duynhoven, 2010). Besides uncovering the nano and microstructure of the material, NMR can also be used as a quantitative tool to inform about the chemical compounds present in the system (Gil, 2003). For these and many other advantages, such as being a non-destructive, non-invasive, and efficient method with a relatively low-cost, the presence of NMR can also be noticed in various fields of food science and technology (Bayer et al., 2010).

The first benchtop NMR tool was developed to determine the solid fat content (SFC) in fat blends by the collaboration of Bruker and Unilever in 1970 (Van Putte & Van Den Enden, 1974). Then, the measurement of SFC got huge attention among manufacturers, and the use of NMR widened in other food-related areas such as chocolate and seed industry (van Duynhoven et al., 2010). Later, NMR was also recognized by scientists as a suitable tool to conduct research effortlessly. Now, the industry and academia are working on several NMR based qualification projects, and it is still spreading among other areas (Besghini et al., 2019).

The preferred NMR method for foods is Time Domain NMR rather than the high-resolution spectroscopy. The TD-NMR core principle relies on nuclear magnetism, which depends on the atomic charge distribution of a nucleus and the spin of its protons and neutrons. A nucleus with an odd number of nucleons is needed to achieve this magnetism, and hydrogen is mostly used for this purpose due to its high sensitivity and abundance (Konez, 2011). The first important part of the NMR system to obtain a signal is the magnet, where an external magnetic field is generated. When the sample is placed in this magnetic field, the protons randomly distributed in the sample start to line up in the same (in +z-direction) or opposite direction (in -z-direction) of the magnetic field, creating the net magnetization (Figure 1.12a) (Hashemi et al., 2010). The generation of the signal also requires a radio frequency (RF) pulse to flip the protons into the x-y plane. Flipping can cause a decrease in the longitudinal magnetization while transverse magnetization increases. However, since this situation is achieved with an RF pulse, protons relax back to their previous position right after removing the pulse from the system (Figure 1.12b). The signal is recorded when the relaxation of both longitudinal and transverse magnetization occurs (Kirtil & Oztop, 2016). The signals coming from relaxation generates two critical time constants: T_1 relaxation (spin-lattice relaxation or longitudinal relaxation) and T_2 relaxation (spin-spin relaxation or transverse relaxation) time. Use of these time constants for various applications is known as Time Domain (TD) NMR relaxometry.

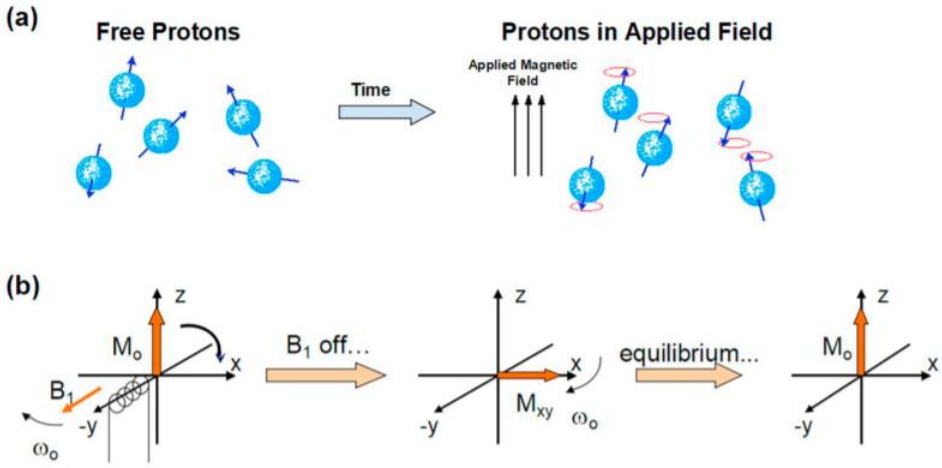

Figure 1.12. The principle of NMR. (a) Positional change in protons with a magnetic field, and (b) magnetization vector changes under the effect of RF pulse (A. Rahman, 1989)

The signals coming from the realignment of the spins along the magnetic field axis create a recovery curve, and the time constant of this curve results is the T_1 relaxation time (Figure 1.13) (Kirtil & Oztop, 2016). Since the total energy of the system variations is associated with T_1, it can be used to observe the interaction between the spin system and the environment and the motion of the segmental dynamics of the side groups (Besghini et al., 2019). Although the information obtained from T_1 may be more useful, T_2 times are preferred because T_1 requires longer measurement times. The reason behind this is the difficulty of T_1 to reach the equilibrium state due to the spin-environment interactions and energy exchange (Kleinberg, 1999).

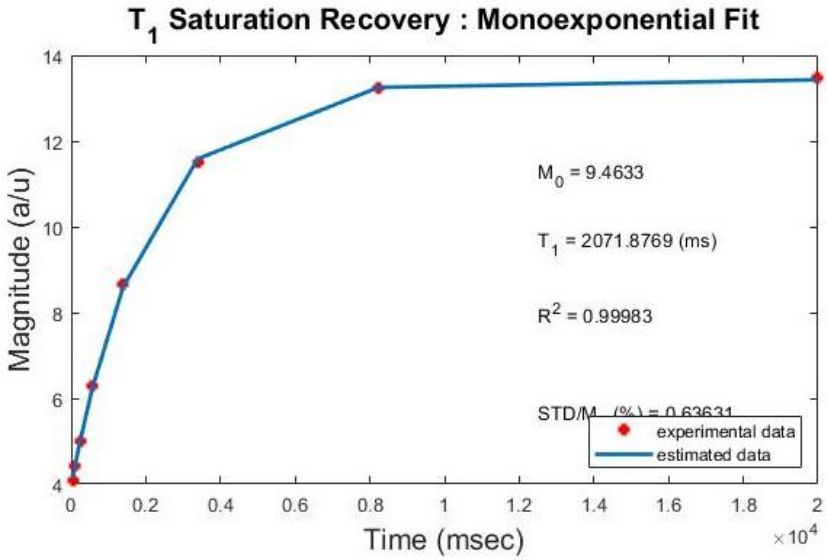

Figure 1.13. An example for T_1 recovery curve

On the other side, T_2 transverse relaxation time is defined as the time required for the magnetization in the x-y axis to decay to the equilibrium value of zero (Figure 1.14) (Kirtil & Oztop, 2016). T_2 is based on the dephasing of magnetization, which is affected by the magnetic field's inhomogeneities. However, the partial elimination of this inhomogeneity problem can be achieved using ideal sequences such as Hahn-echo and Carr–Purcell–Meiboon–Gill (CPMG) sequences based on the formation of spin echoes (Kleinberg, 1999).

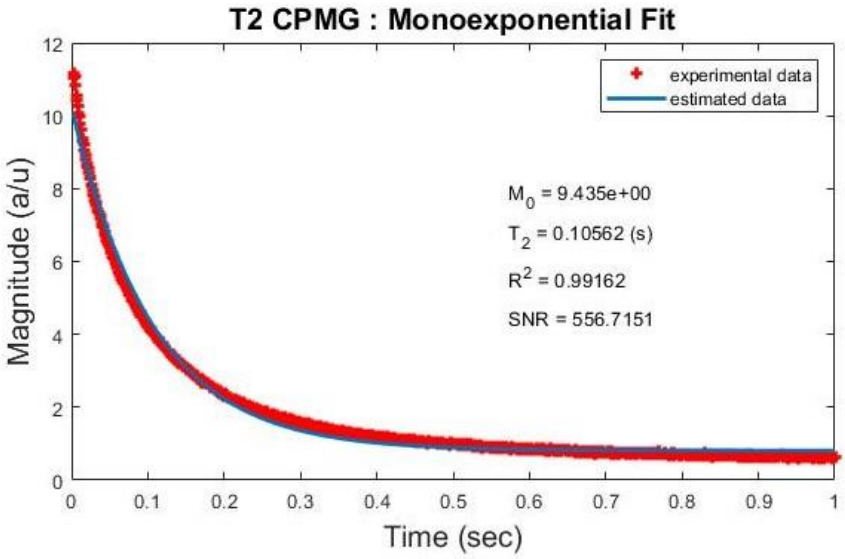

Figure 1.14. An example T_2 decay curve

Hydration behavior and the composition of the food materials, including lipids, carbohydrates, and proteins, have been studied by benchtop TD-NMR besides other well-known techniques (Table 1.2) (van Duynhoven et al., 2010). Most of the studies are aimed to measure the water population via T_2 relaxation time since the amount of free water in the system is directly associated with T_2. One of the examples is the hydration behavior of the proteins. Hydration of a protein is based on water absorption by protein via hydrophilic interactions (Damodaran, 2007). Previous studies proved that the qualification of the maximum water absorption in modified proteins can be measured simply by TD-NMR relaxometry (Belton et al., 1994, 1995; Le Botlan & Helie-Fourel, 1994; Namli, 2019; Tas, 2019). Therefore, NMR relaxometry can be used in many studies to investigate the protein-water interactions, as well as with the interactions of water with other compounds.

Table 1.2 NMR studies on several food products (van Duynhoven et al., 2010)

Material	Application	Acquisition	Reference
Proteins	Interaction with polysaccharides	CPMG	(Goetz & Koehler, 2005)
	Denaturation	CPMG	(Lambelet et al., 1992) (Coelho et al., 2007)
	Aggregation	CPMG	(Coles, 1980) (Namli, 2019; Tas, 2019)
	Protein content	CPMG	
	Hydration	CPMG	
Carbohydrates	Mobility in low moisture sugar	FID	(Derbyshire et al., 2004)
	Sugar-protein interactions	CPMG	(Ducel et al., 2008)
Lipids	Milk fat solid content	FID/IR	(Le Botlan & Helie-Fourel, 1995)
	Level of unsaturation	CPMG	(Le Botlan & Helie-Fourel, 1994)

1.3.5 High Pressure Liquid Chromatography (HPLC)

In the early years of chemistry and food science, the separation of the desired compounds was achieved by using several chemical-based techniques such as extraction and distillation. The qualification of these compounds was then carried out to ensure a correct separation by colorimetric and volumetric methods. However, the quantification of these compounds was also challenging due to the massive amount of chemical waste production (Townshen, 1983). The errors arising from under- or overestimating the titrant volume and the time consumed to achieve a correct result directed people to seek for other novel methods (Swadesh, 1998). This gave rise to the discovery of the chromatography by Russian botanist Mikhail S. Tswett, and it was first used to separate plant pigments (Ali et al., 2010). Then, this method was evolved with the following years, and several chromatographic methods

were created. High-performance liquid chromatography, a method based on the identification, qualification, and quantification of each compound in material, can be given as an example to those chromatographic methods (Townshen, 1983).

There are several chromatographic modes, depending on the type of compound that is desired to be separated. Adsorption, reversed-phase, ion-exchange, ion pair, size exclusion, affinity, and hydrophilic interaction liquid chromatography are some examples of these modes (Meyer, 2010).

Reversed-phase chromatography contains two phases: a relatively less polar stationary phase and a mobile phase. The basic principle of this mode is the separation and the detection of compounds via the difference in the polarity (Meyer, 2010). The mobile phase mostly consists of water or water-immiscible solvent mixtures. Methanol, ethanol, acetonitrile, and isopropanol can be the examples of mobile phase solvents (Buszewski & Noga, 2012). The stationary phase is usually preferred as chemically bonded octadecylsilane (ODS), containing 18 carbon atoms, to obtain stronger retention. Several compounds, such as caffeine amount in drinks and sugar amount in solutions, phenols, halophenols, and polyphenolic flavonoids, vitamins and amino acids are examined by this method (Emara, 2004; Muramoto et al., 1987; Odriozola-Serrano et al., 2007; Swadesh, 1998). When the desired compound has weak absorbance in UV, such as alcohols, ethers, and sugars the preferred detector is the refractive index detector (RID). Detection of these compounds can be achieved by measuring the refractive index (Silveira et al., 2015). The illustration of the RID optical system is given in Figure 1.15.

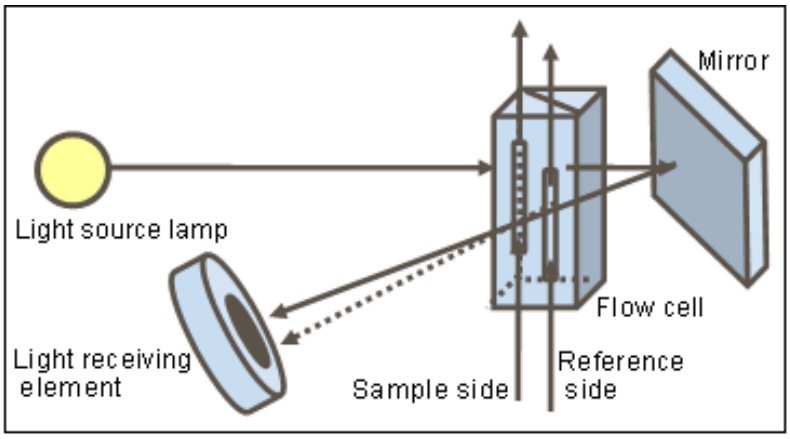

Figure 1.15. Diagrammatic illustration of RID optical system (Hitachi High-Tech corporation, 2001)

Hydrophilic interaction liquid chromatography (HILIC) is another mode that is suitable for the efficient separation of small polar compounds (Buszewski & Noga, 2012). The integration of this mode to the reversed-phase chromatography is usually considered as a solution to the solubility problems; therefore, the combination is preferred in many studies (Hemström & Irgum, 2006). Also, mass spectroscopy (MS) and diode array detector (DAD) can be easily coupled with HILIC.

The HPLC-MS combination consists of three parts: the interface, mass analyzer, and the detector (Figure 1.16). This combination is usually preferred to preserve the chemical identity while obtaining the desired separation (Willoughby et al., 1998). The main working principle of this mode is remarkably similar to the other adsorption methods except for a hydrophilic stationary phase. The hydrophilicity degree of the stationary phase shifts the equilibrium towards the stationary phase, leading to an increase in retention (Alpert, 1990; Hemström & Irgum, 2006). The interaction of the analyte, stationary phase, and mobile phase can be achieved through physical interactions (e.g., ion-dipole, dipole-dipole, dipole-induced dipole) and chemical interactions (e.g., hydrogen bonding). Several compounds such as

amino acids, peptides and proteins, carbohydrates, and advanced glycation end products such as furosine, N-ε-carboxymethyllysine (CML) and N-ε-carboxyethyllysine (CEL) can be easily determined by this mode (Buszewski & Noga, 2012).

The stationary phase of HILIC systems can be created by any polar chromatic surface such as acrylamide, polyethylene, and zwitterionic sulfobetaine bonded phases. On the other hand, the mobile phase can be water or water-immiscible solvent (e.g. acetonitrile and formic acid) mixture (Meyer, 2010).

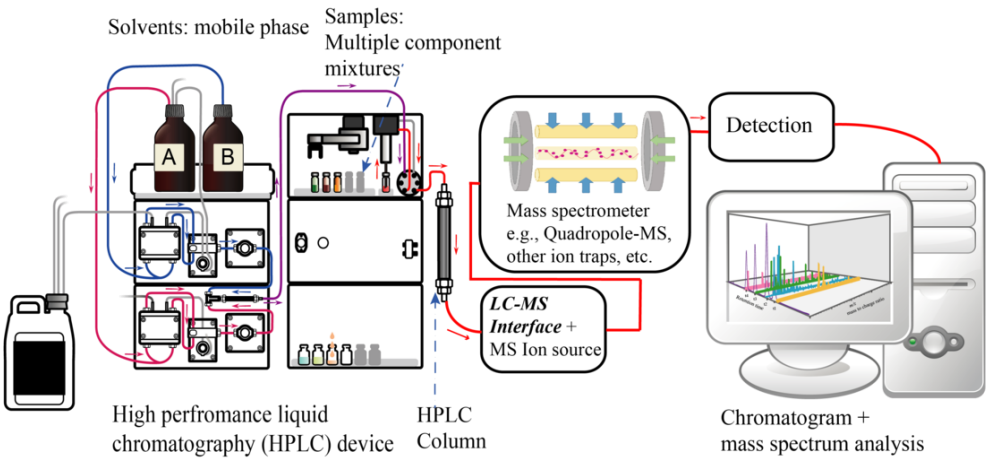

Figure 1.16. Diagrammatic illustration of HPLC-MS system (Niessen, 2006)

Moreover, DAD is also preferred due to the ease of chemical quantification by several wavelengths simultaneously (Mattila et al., 2000). The principle of this detector is based on the estimation of the wavelengths that are coming from the dispersed light by the polychromator. The wavelengths are read by a large number (100–1,000) of light-sensitive diodes. The signal that is obtained by diodes is, then used to identify the specific compounds in the system (Meyer, 2010).

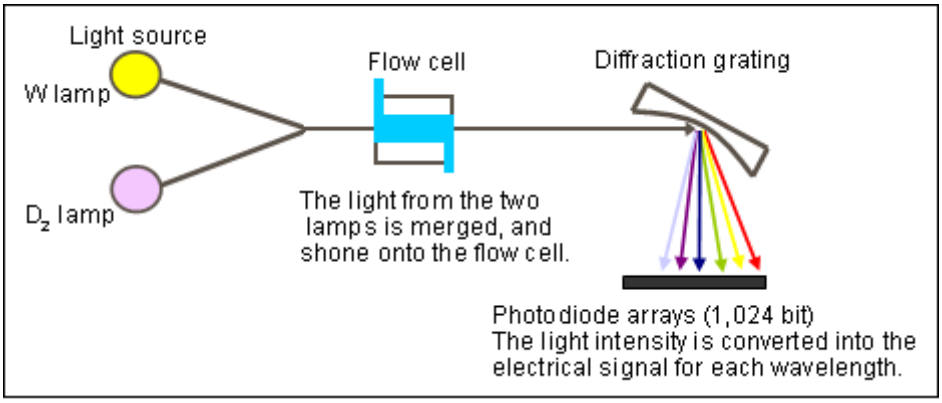

Figure 1.17. Diagrammatic illustration of DAD optical system (Hitachi High-Tech corporation, 2001)

1.4 Objectives

Over the last decade, there has been an increasing trend by the millennials and the young population, who favor healthy, ethically, and naturally sourced, and minimally processed foods. And this changing consumer tendency created a significant demand for increased plant-based protein consumption. Among these proteins, pea proteins have been under the spotlight for a long time due to several health benefits. However, adding these proteins to food formulations is still a challenge due to their low functional properties. The enhancement of these proteins, however, can be simply done by several modification techniques. Glycation, which is defined as the reaction between proteins and reducing sugars, can be considered as a proper modification technique to improve the functional properties of pea proteins. Conventional glycation is not the most appropriate method to modify the pea proteins since it requires long processing times and high energy consumption.

In this study, the main objective is to improve the functional properties of pea proteins such as solubility and hydration via microwave glycation, which has not been studied before on pea proteins and to compare the results with the conventional water bath glycation method. Furthermore, effects of different conditions such as pH, reducing sugar type, protein-sugar ratio, and processing time were also examined to see their effect both on the extent of glycation and also on the functional properties.

The hypothesis of the study can be described as;

If pea protein is modified via microwave glycation, physicochemical properties like solubility and hydration behavior will improve more compared to water bath glycation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Pea protein concentrate (PPC) with a 65% protein content was purchased from NorCal Organic (Crescent City, California), sugars (fructose and dextrose) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and Allulose was purchased from Lang's Chocolates (Pennsylvania, USA).

Copper (II) sulfate pentahydrate, sodium potassium tartrate tetrahydrate, sodium hydroxide (NaOH), Folin-Ciocalteau's phenol reagent, Trolox, ortho-phthalaldehyde (OPA), Bovine Serum Albumin (BSA), Boric acid, hydrochloric acid, sodium dodecyl sulfate (SDS), sodium carbonate, ethanol (C_2H_5OH), β-mercaptoethanol, glycine, potassium hexacyanoferrate (II) trihydrate, zinc sulfate heptahydrate, sodium borohydride, sodium borate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2 Methods

2.2.1 Sample Preparation

The first step was to prepare the sample solutions at different protein to sugar ratios and pHs. To do so, pea protein concentrate (PPC) was mixed with different sugars (dextrose, fructose and Allulose) at two different ratios as 2:1.25 and 9:1.25 in dry basis. These ratios were determined by preliminary studies to achieve the most ideal

comparison (Namli, 2019). These dry mixtures were dissolved in 100 mL buffers prepared at pH 7 (0.1 M *potassium phosphate buffer*) and pH 10 (0.1 M *carbonate-bicarbonate buffer*). The total solid content of the solutions was kept at 10% (w/v). Then, to ensure ideal mixing and hydration, high shear homogenizer was used (IKA®-Werke GmbH & Co. KG, Germany) at 7,200 rpm for 2 minutes.

2.2.2 Glycation

Glycation was conducted to hydrated solutions in a microwave oven (Arcelik Co., Istanbul, Turkey). Before glycation, the maximum power of the microwave was measured as 600 W by IMPI-2L test (Sakiyan et al., 2007). Microwave was applied to the solutions for 4 minutes at 180W to reach 90ºC, which is the preferred glycation temperature, in the light of other studies (Guan et al., 2006, 2011; Nasrollahzadeh et al., 2017). These power and time combinations were determined by preliminary trials to ensure that no overboiling or excess heating occurred in the samples. Following heating, solutions were placed in an ice bath to decrease the temperature and stop the reaction. Afterwards, samples were freeze dried (Beijing Songyuan Huaxing Technology Development Co., Ltd., China) for 48 hours. Finally, microwave (MW) glycated samples were grinded and stored at 4 ºC for further analysis.

As the control**,** glycation was conducted in a water bath (WB) at 90 ºC. These experiments were conducted at pH 7. Glycation time was selected as 4 and 30 minutes. Short time was equivalent to MW heating time and longer time was the usual time used for conventional glycation (Ayrancı & Dalgıç, 1990). After water bath, same cooling, freezing, drying, and grinding steps were followed as in MW samples.

2.2.3 Glycated Pea Protein Characterization

2.2.3.1 Determination of AGEs

2.2.3.1.1 Quantification of Furosine

Firstly, 50 mg sample was mixed with 5 mL 8 M HCl solution in a glass tube. For hydrolysis, the tubes were closed with screw caps (with PTFE lined silicone septum) after purging headspace with nitrogen, and then incubated at 110 ˚C for 24 hours. Then, 50 µL hydrolysate was evaporated under a gentle stream of nitrogen and the residue was dissolved with 1 mL deionized water. The solution was passed through a preconditioned Oasis HLB cartridge (Waters Corporation, USA). The first 8 drops of sample were discarded and the rest was collected to an autosampler vial. HLB cartridges were conditioned with 1 mL methanol followed by 1 mL water before usage.

An Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump, a diode array detector, an autosampler and a temperature-controlled column oven was used to quantitate furosine. Chromatographic separation was performed on an Atlantis HILIC column (150 mm × 4.6 mm id., 5 µm, Waters Corporation, USA) using isocratic elution of 1% formic acid in water at a flow rate of 1 mL/min at 40 °C. Furosine was monitored in diode array detector at 280 nm. An external calibration curve built in the range of 0.5-5 mg/L was used to quantitate furosine.

2.2.3.1.2 Quantification of CML (N-ε-carboxymethyllysine) and CEL (N-ε-carboxyethyllysine)

Samples (20 mg) were mixed with 100 µL deionized water, 450 µL 0.2 M sodium borate buffer at pH 9.2 and 500 µL of sodium borohydride (1 M solution in 0.1 M aqueous NaOH). After incubating at room temperature for 4 hours, 2 mL 8 M HCl was added and the headspace was purged with nitrogen. Then, the tubes were incubated at 110 ˚C for 24 hours. A part of the hydrolysate (20 µL) was dried with a gentle stream of nitrogen and dissolved with 1 mL deionized water. The solution was passed through a preconditioned Oasis HLB cartridge (Waters Corporation, USA). The first 8 drops of sample were discarded and the rest was collected to an autosampler vial. HLB cartridges were conditioned with 1 mL methanol followed by 1 mL water before usage.

CML and CEL content were determined by using an Agilent Ultivo LC-MS/MS system with (Electrospray Ionization) ESI source. Chromatographic separation was performed on a SeQuant ZIC-HILIC column (150 mm × 4.6 mm id., 3.5 µm, Merck KGaA, Darmstadt, Germany) using a gradient mixture of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as mobile phases at a flow rate of 1 mL/min at 60 °C. Initially mobile phase A was at 30% for 10 min and then increased to 50% in 1 min and kept at 50% for 2 min, then decreased to 30% in 1 min and kept at 30% for 1 min. The run time was 15 min. The injection volume was 3 µL. ESI source had the following settings in positive ionization mode: gas flow 6 L/min at 300 °C, nebulizer 60 psi, nozzle voltage 500 V, capillary voltage 2000 V, fragmentor voltage 90 V, sheath gas flow 11 L/min at 300 °C. The (Multiple Reaction Monitoring) MRM transitions of m/z 205→84 (N_2 collision energy (CE), 12 V) and 205→130 (CE, 2 V) were measured for CML, 219→84 (CE, 12 V) and 219→130 (CE, 6 V) were measured for CEL. Matrix match calibration curves were built to quantitate CML and CEL between 1-50 µg/L.

2.2.3.2 Soluble Protein Content by Lowry Method

Since dialysis was not performed on the glycated samples, to make a correct comparison between the samples, total protein content of each glycated samples were measured first by using Kjeldahl Method (ASTM Standard E258, 2007).

To measure the soluble protein content, samples were dissolved in pH 7 and pH 10 buffer solutions. Each sample was prepared at different concentrations so that the total protein amount was kept constant in each solution.

Lowry method was used to determine the soluble protein content in the samples (Lowry et al., 1951). The first step in this method was to prepare the necessary reagents. The list of the reagents and its details are given in the table below.

Table 2.1 Lowry Reagents

Reagent Name	Details
A	2% (w/v) Na_2CO_3 dissolved in 0.1 N NaOH
1	2% $CuSO_4.5H_2O$
2	2% Na-K Tartrate
B	Mixture of Reagent A:1:2 with a 100:1:1 ratio
C	Diluted Folin-ciocalteu's Phenol Reagent with a 1:1 ratio

Following the preparation all the reagents, 0.5 ml of diluted supernatant was mixed with 2.5 ml Lowry reagent and the mixtures were kept at room temperature for 10 minutes. Then, 0.25 ml Folin reagent was added to the mixtures, and the mixture was

stirred with a vortex-mixer (VM-10, Witeg Labortechnik GmbH, Germany) for 8 seconds and kept in dark for 30 minutes. Finally, the absorbance values at 750 nm was recorded by spectrophotometer (Optizen Pop Nano Bio, Mecasys Co., Ltd., Korea). The calibration curve was prepared with 1 g/L BSA (Bovine Serum Albumin) stock solution. Serial dilution was applied five times to the BSA solution until the final diluted stock had a concentration of 0.03125 g/L. The calibration equation was found by absorbance vs BSA concentration (g/L) graph. The calibration graph and the equation were given in the Appendix.

2.2.3.3 Degree of Glycation by OPA Method

Like in Lowry, the concentration of each sample was arranged and mixed in an orbital shaker (Daihan Scientific Co., Ltd., Korea) at 200 rpm for 24 hours. The well-mixed sample solutions then centrifugated (MF-80, Hanil Science Industrial Co., Ltd., South Korea) at 1789 xg for 5 minutes. Finally, supernatant of the solutions was used for further analysis.

To measure the free amino groups, OPA method was used with small modifications (Nielsen, 2001). The first step was to dissolve 80 mg of o-phthalaldehyde (OPA) in 2 ml 95% (v/v) ethanol solution. After dissolving OPA carefully, 50 ml 100 mM borax buffer at pH 9.75, 5 ml 20% (w/v) sodium dodecyl sulfate (SDS) solution, 200 μL β-mercaptoethanol and OPA solution were mixed in a 100 mL volumetric flask. The volume of the mixture was brought to 100 mL with distilled water and the final solution was mixed. The final mixture that is the OPA reagent (1.5 ml) and 0.5 ml supernatant was mixed kept at room temperature for 2 minutes. The absorbance values were measured at 340 nm (Optizen Pop Nano Bio, Mecasys Co., Ltd., Korea).

1% (w/v) glycine solution was prepared and diluted to different concentrations as 0.8%, 0.4%, 0.2%, 0.1%. Procedure explained above was also applied to glycine solutions and absorbance values was used to draw absorbance vs glycine concentration (g/100 mL) graph. The calibration graph and the equation were given in the Appendix.

2.2.3.4 Protein and Sugar Structure by Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra of glycated powders were also obtained. To do so, IR Affinity-1 Spectrometer with Attenuated Total Reflectance (ATR) attachment (Shimadzu Corporation, Kyoto, Japan) was used. The measurements were collected within a 600-4000 cm^{-1} spectral range and 10 scans were applied at a resolution of 4 cm^{-1}. Absorbance vs wavelength plots were further analyzed and compared with native pea protein and sugars.

2.2.3.5 Hydration Behavior by Nuclear Magnetic Resonance (NMR) Relaxometry

NMR Relaxometry experiments were conducted on the samples prepared by mixing 0.15 g sample powder with 0.45 mL distilled water. A 0.5 T (20.34 MHz) bench top NMR system (Spin Track, Resonance Systems GmbH, Kirchheim/Teck, Germany) was used to obtain T_2 (spin-spin relaxation) times. CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence was used for the measurements. The table for acquisition parameters is given below. Mono-exponential analysis was conducted in MATLAB (R2019b, The MathWorks Inc., USA) to calculate relaxation times.

Table 2.2 NMR Relaxometry System Parameters

Parameters	Values
Echo Time	1000 ms
Number of Echo	800-1000 ms
Number of Scans	16
Repetition Time	1000 ms

2.2.3.6 Reducing Sugar Content by High Pressure Liquid Chromatography (HPLC)

The first step of this experiment was to mix 0.25 g sample powder with 9 mL double distilled water. These solutions were mixed in an orbital shaker (Daihan Scientific Co., Ltd., Korea) at 200 rpm for 24 hours. After complete hydration, two solutions, Carrez I and Carrez II, were prepared and added to precipitate the proteins in the sample solutions. 15 g potassium hexacyanoferrate (II) trihydrate was dissolved in 100 mL double distilled water to obtain Carrez I while 30 g zinc sulfate heptahydrate was dissolved in 100 mL double distilled water to obtain Carrez II. The final solutions with a volume of 10 mL were vortexed and centrifugated at 1,789 xg for 5 minutes. Supernatant of the solutions were filtered with 0.45 μm nylon filters and collected to HPLC vials before HPLC-RID (Shimadzu Scientific Instruments, Japan) analysis.

The HPLC system consisted of an inertsil NH_2 column (dimensions of 250 x 4.6, 5μm) was (Shimadzu Sci. Ins., Japan), degasser, pump, auto-sampler, column oven, and refractive index detector. Acetonitrile and water mixture at a ratio of 80:20 (v/v) was used as a mobile phase. Flow rate, injection volume, and oven temperature were 1 mL/min, 20 μl, and 40 ºC, respectively.

To prepare the calibration curve, sugar solutions at different concentrations as 5, 7.5, 10, 15, 20 g/L were prepared. The obtained calibration curves and the equations were given in the Appendix.

2.2.3.7 Statistical Analysis

Each experiment was conducted in three replicates and statistical analysis were conducted by MINITAB (Version 19, Minitab Inc., Coventry, UK). Analysis of variances (ANOVA) was performed to observe the effect of variables on results by using a general liner model regression approach. Before comparison was made, assumptions of ANOVA such as normality of the residuals, constant variance, independence, influential points, multicollinearity were tested, and transformations were applied when necessary. Tukey's comparison test at 95% confidence interval was applied to observe the significance when necessary.

2.3 Experimental Design

Table 2.3 Parameters of the experimental design

Experiments	***Factors***	***Levels***	
1. *Determination of AGEs by LC* • *Quantification of Furosine* • *Quantification of CML and CEL* 2. *Total and Soluble Protein Content by Lowry Method* 3. *Degree of Glycation by OPA Method* 4. *Protein and Sugar Structure by FTIR Spectroscopy* 5. *Hydration Behavior by NMR Relaxometry* 6. *Reducing Sugar Content by HPLC*	Sugar Type	Dextrose Fructose Allulose	
	Protein-Sugar Ratio	2:1.25 9:1.25	
	Processing Type	Microwave Glycation (for 4min)	pH 7 pH10
		Water Bath Glycation (at pH 7)	4min 30min

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Advanced Glycation End Products (AGEs): Furosine, CML & CEL Contents

Furosine is obtained by acid hydrolysis of the Amadori product of protein bound lysine with dextrose (or maltose and lactose if present) and used as a marker of the early stage of Maillard reaction (MR), and may also indicate nutritional quality loss in proteins (Çelik & Gökmen, 2020; Erbersdobler & Hupe, 1991; Li et al., 2019). On the other hand, CML and CEL are indicative of the advance stage of MR. CML and CEL are formed by the reaction of lysine residues with glyoxal and methylglyoxal, respectively, or by the oxidation of the lysine Amadori products (Ahmed et al., 1988).

Table 3.1 Furosine, CML and CEL amounts of MW glycated samples at pH7 and pH10

	Sugar Type	PPC:Sugar	Furosine (ppm)	CML (ppb)	CEL (ppb)
pH7	Dextrose	2:1.25	10713.3±292.9[a]	81.6±5.5[c]	6.5±0.1[d]
		9:1.25	8974.6±420.2[b]	102.7±1.9[ab]	6.4±0.03[d]
	Fructose	2:1.25	331±15.2[c]	58.1±1.9[d]	8.04±0.4[cd]
		9:1.25	186.01±1.7[c]	101.4±4.9[abc]	11.4±0.7[c]
	Allulose	2:1.25	1141.3±79.7[c]	90.3±0.5[bc]	40.2±0.3[a]
		9:1.25	269.5±9.1[c]	120.6±3.7[a]	21.1±1.4[b]
	Sugar Type	**PPC:Sugar**	**Furosine (ppm)**	**CML (ppb)**	**CEL (ppb)**
pH10	Dextrose	2:1.25	9040.2±306.8[b]	71.3±1.9[c]	0.7±0.05[f]
		9:1.25	15911.04±559.3[a]	478.8±7.6[b]	88.1±0.1[e]
	Fructose	2:1.25	4827.3±21.3[d]	430.1±26.9[b]	272.1±8.5[b]
		9:1.25	7003.1±19.8[c]	343.2±24.5[b]	235.5±10.4[c]
	Allulose	2:1.25	5785.8±150.5[cd]	646.4±47.9[a]	381.4±1.7[a]
		9:1.25	5433.2±242.7[cd]	348.5±0.3[b]	182.9±3.6[d]

Letters indicate significant difference in each pH value (a-e)

When the table above investigated, sugar type was found to have a significant effect on the MR products ($p<0.05$) but protein-sugar ratio did not have a significant effect on the MR products ($p>0.05$). Furosine was found to be the highest in dextrose samples and it was considerably low in fructose and Allulose samples while Allulose samples showed the highest CML and CEL. As mentioned before, different sugars have different reaction pathways. Furosine can usually be obtained by acid hydrolysis of the Amadori compounds with dextrose while CML and CEL can be obtained more by fructose and Allulose due to the higher possibility of these sugars to form methylglyoxal than dextrose (Guerra-Hernandez et al., 1999). Furosine amounts observed in fructose and Allulose might be due to the isomerization of those sugars to dextrose which further converted to Furosine (Carraher et al., 2015). This hypothesis was confirmed by the results obtained in this study as will be explained afterwards.

There has been an ongoing debate about the effect of different sugars on the rate of Maillard reaction. One of the theories is that the reactivity of aldoses is higher than the ketoses due to different reaction pathways (Brands & Van Boekel, 2001; O'Brien & Morrissey, 1989). On the other hand, some researchers support the opposite theory stating that ketoses are more reactive than aldoses (Jing & Kitts, 2002; Jing & Kitts, 2004; Yen et al., 1992). Ketoses, being a higher contributor to Maillard reaction due to the involvement of caramelization and isomerization reactions, are also considered as a better reactant (Laroque et al., 2008). Therefore, there is not a universal conclusion to identify which sugars are reacting more and fast. Thus, it is not possible to make a comparison for the rate of MR for different sugars.

Table 3.2 Furosine, CML and CEL amounts of WB glycated samples at 4min and 30min

	Sugar Type	PPC:Sugar	Furosine (ppm)	CML (ppb)	CEL (ppb)
4min	Dextrose	2:1.25	5415.2±428.8[a]	58.8±1.4[c]	3.9±0.2[d]
		9:1.25	1296.9±29.9[b]	116.3±4.9[a]	11.7±0.8[b]
	Fructose	2:1.25	19.2±0.1[c]	66.7±0.7[c]	4.8±0.2[cd]
		9:1.25	22.1±0.2[c]	90.7±1.8[b]	7.3±0.5[c]
	Allulose	2:1.25	514.8±25.8[bc]	62.4±1.2[c]	11.2±0.2[b]
		9:1.25	39.5±0.9[c]	107.9±0.4[a]	15.5±0.5[a]
	Sugar Type	**PPC:Sugar**	**Furosine (ppm)**	**CML (ppb)**	**CEL (ppb)**
30min	Dextrose	2:1.25	3756.7±134.6[a]	74.1±3.3[c]	1.5±0.1[c]
		9:1.25	566.8±2.1[c]	132.8±2.1[a]	13.5±0.9[b]
	Fructose	2:1.25	58.9±2.04[d]	115.5±6.9[ab]	31.3±0.3[a]
		9:1.25	55.6±1.7[d]	101.6±7.4[b]	12.2±0.8[b]
	Allulose	2:1.25	1286.1±36.2[b]	124.9±4.7[ab]	31.9±0.1[a]
		9:1.25	123.8±2.9[d]	136.9±0.2[a]	28.5±1.9[a]

Letters indicate significant difference in each processing time (a-d)

As stated before, in addition to MW, samples were also glycated in a water bath. This was basically the comparison method to MW. The highest furosine amounts in WB glycated samples were found in dextrose samples. Moreover, the lowest furosine and the highest CML and CEL amounts were observed in the samples glycated for 30 minutes. Therefore, as the processing time increased, MR proceeded more towards advanced stages. When two processing types were compared, it was seen that MW samples had higher CML and CEL amounts. Therefore, it can be concluded that the rate of MR was higher during MW heating. In literature, it was found that the probability of protein-reducing sugar collision increased after microwave glycation due to the disruption of subunits and protein unfolding (Guan et al., 2006). Also, the decrease in the activation energy by the reaction selectivity of microwave accelerated the reaction (Guan et al., 2011). Therefore, the higher efficiency and the enhancing ability of microwave glycation over conventional glycation resulted in participation to MR more.

Baked products (Ait-ameur, 2007; He et al., 2014), meat (Hull, Woodside, Ames, & Cuskelly, 2012; Lima, Magee, & Ames, 2009; Xiaohua Sun et al., 2015) and dairy products (Delatour et al., 2009; Visani et al., 2006) have been reported to include a wide range of CML. The CML results obtained in this study were also in this range (0-300 ppb) and in some cases, it was even higher. The results obtained confirmed the occurrence of glycation and also the last stages of Maillard for certain conditions.

3.2 Soluble Protein Content

It is known that solubility is a common problem among the plant proteins due to the high content of globulins and is affected by several parameters such as pH of the medium, modification methods and the presence of salts (Lam et al., 2018). In this study, Lowry method was used to observe the influence of pH (7 and 10), sugar type

(dextrose, fructose and Allulose), process type (MW and WB), processing time (4 min and 30 min) and protein to sugar ratio (2:1.25 and 9:1.25) on the solubility of microwave glycated pea proteins.

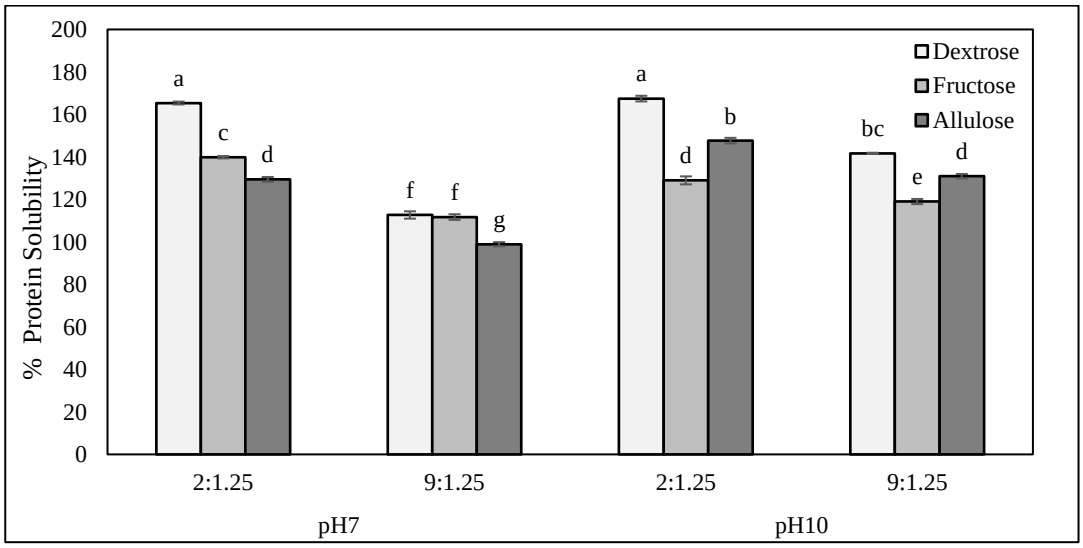

Figure 3.1. % Protein Solubility of MW samples

The solubility of MW glycated samples was significantly influenced by all the factors ($p<0.05$). The highest solubility was observed for dextrose samples followed by fructose and Allulose which were equally lower. Also, samples with a 2:1.25 protein-sugar ratio had a higher solubility than 9:1.25 samples and alkaline pH had an increasing effect on the solubility more compared to neutral conditions. For a protein, the lowest protein solubility is usually obtained around isoelectric point (pI) due to the zero net-charge around the proteins and it increases as we get further from this point (Fernández-Quintela et al., 1997). According to a study where functional properties of pea, chickpea and lentil protein concentrates were examined; the highest solubilities were observed at pH values between 1 to 3 and 7 to 10 (Boye et al., 2010). Therefore, the increase in the solubility of PPC samples with increase in pH was not unusual.

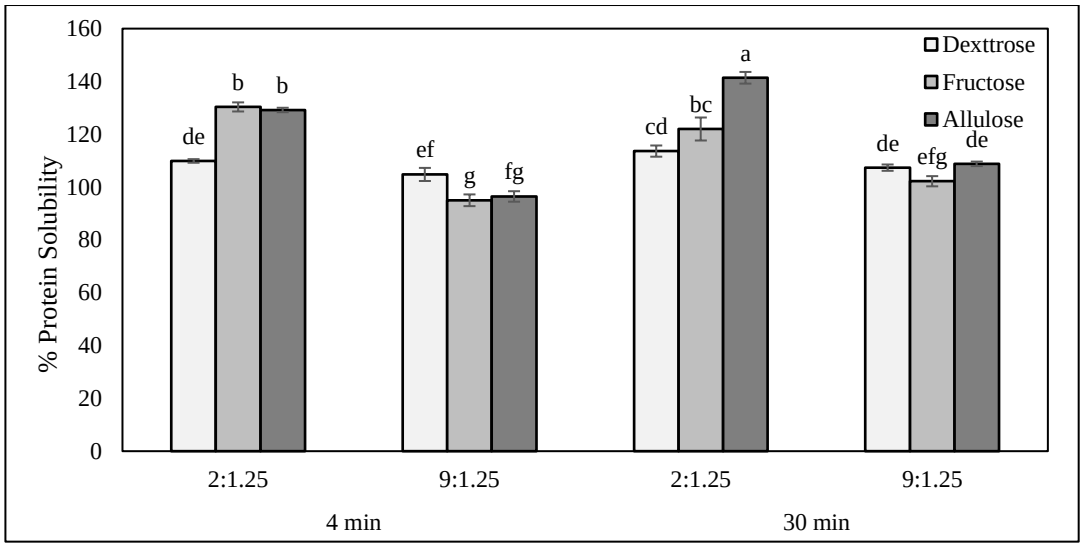

Figure 3.2. % Protein Solubility of WB samples

In WB samples, all the variables had a significant effect on the solubility ($p<0.05$). Allulose samples had the highest solubility and it was followed by fructose and dextrose, respectively. Similar to MW samples, WB samples with a 2:1.25 ratio had higher protein solubility compared to 9:1.25 samples. Moreover, as process time increased, the solubility of the WB samples improved. When the effect of MW was evaluated, MW samples resulted in a higher solubility than WB samples. Based on previous studies, it has been found that the extent of agglomeration can be less with microwave heating, which results in improved protein-water interactions (Nasrollahzadeh et al., 2017). Also, the higher amount of protein- hydrophilic sugar complex generated during MR can increase the solubility, and it was known from AGE results that MW samples participated in MR more. Therefore, the increase in solubility by microwave glycation was expected. Overall, Allulose samples with a sugar to protein ratio of 2:1.25 had the highest solubility.

Also, based on the previous studies, it can be said that the relation between sugar type and the protein solubility is extremely complicated. The rate of the glycation may differ due to the structure of the sugar since the pathway of the Amadori rearrangement is different for ketoses and aldoses. D-glucose, for example,

crosslinks with the free amino group at position C_1 (Carbon 1), which results in 1-amino-1-deoxyketoses formation. On the other hand, D-fructose, being a ketose, reacts with free amino groups at position C_2 to form 2-amino-2-deoxyaldoses (Taniguchi et al., 2015). Due to this difference in pathways and products, the Amadori compounds produced from aldoses reacts differently than the Heyns compounds produced from ketoses (Kwak & Lim, 2004). The products formed with these different methods could affect the solubility in different ways.

3.3 Degree of Glycation (DOG)

OPA is a quite common method to observe the degree of glycation (Nielsen et al., 2001) and to measure the remaining free amino group (FAG) in a protein mixture following necessary modifications (Sajib et al., 2020; Xiaohong Sun et al., 2020). The decrease in the FAG amount during glycation can be observed by comparing the FAG amounts of glycated samples with the control samples.

Table 3.3 Free amino group (FAG) amount of MW and WB glycated samples

Sugar Type	PPC:Sugar Ratio	MW		WB	
		FAG (g/L) [pH7]	FAG (g/L) [pH10]	FAG (g/L) [4 min]	FAG (g/L) [30 min]
Control	2:1.25	2.97± 0.04[a]	3.55 ± 0.070[a]	2.70± 0.020[a]	2.70± 0.020[a]
	9:1.25	2.94± 0.05[a]	3.52 ± 0.060[a]	2.71± 0.018[a]	2.71± 0.018[a]
Dextrose	2:1.25	1.92± 0.033[d]	2.30 ± 0.025[c]	1.36± 0.007[d]	1.77± 0.035[b]
	9:1.25	2.44± 0.038[b]	3.62 ± 0.035[a]	1.93± 0.043[b]	0.52 ± 0.012[d]
Fructose	2:1.25	2.33 ± 0.054[b]	1.77 ± 0.013[d]	0.86 ± 0.006[e]	1.50 ± 0.030[c]
	9:1.25	2.51 ± 0.022[b]	3.00 ± 0.069[b]	0.94 ± 0.041[e]	1.73 ± 0.021[b]
Allulose	2:1.25	2.11± 0.027[c]	2.51 ± 0.040[c]	1.46± 0.008[d]	1.36± 0.045[c]
	9:1.25	2.14 ± 0.017[c]	3.49 ± 0.026[a]	1.67 ± 0.023[c]	1.49 ± 0.038[c]

Letters indicate significant difference in each pH value (a-e)

All factors (sugar type, protein-sugar ratio, and pH) were found to have an effect on the FAG ($p<0.05$). The highest FAG amount at pH7 and pH10 was observed on native protein samples as expected. These results might be an evidence that glycation was successful because the decrease in FAG amount was directly related to the amino acids that have participated to the Maillard reaction during glycation (Tas, 2019).

When samples with two different protein to sugar ratio were compared, it was observed that the 9:1.25 samples had higher FAG amounts, which confirmed the occurrence of less glycation on these samples. Moreover, there was a significant difference in FAG amounts of samples prepared at different pH values. The FAG amount was higher at pH 10 samples because of the increase on the solubility at alkaline pHs. This finding was also in accordance with the Lowry results, showing an increasing solubility trend with pH.

The WB samples showed a similar trend as MW samples. When all the variables were considered, there was no significant effect of concentration and processing time on FAG results ($p>0.05$). According to the comparison among different sugars and control, it was observed that fructose samples had the lowest FAG amount while control samples had the highest FAG amounts.

Furthermore, it can be clearly seen that MW samples had higher amount of FAG then the WB glycated samples. The higher solubility of MW samples might lead to an increase in the free amino group amount. Moreover, protein unfolding can be facilitated by microwave heating and the unfolding might result in higher amount of FAG in MW samples. Furthermore, the internal heat generation which is the basic heating mechanism of microwave might be another reason for higher release of FAG (Sumnu, 2001; Uygun, Yildiz, Sumnu, & Sahin, 2020).

3.4 Remaining Reducing Sugar (RRS) Contents

Reducing sugar concentrations were measured in the glycated samples. Isomerization was also observed, and it was only in pH10 samples. It was found that dextrose and Allulose were converted to fructose whereas fructose was converted to both dextrose and Allulose at alkaline conditions. From the previous researches, it is known that the soluble bases like triethylamine did not induce an efficient isomerization but the addition of some catalysts like borates or NaOH to the system may increase the yield of conversion (Barker et al., 1973; Mendicino, 1960). Therefore, the presence of isomerization at alkaline pH which was provided by buffers (prepared with NaOH) was likely. Moreover, the higher amount of available sugar in mixtures increase the possibility and the efficiency of isomerization (Delidovich & Palkovits, 2016; Oshima et al., 2014). Thus, 2:1.25 samples resulted in higher isomerization rates.

On the other hand, as can be seen from the Table 3.4, isomerization was not observed in WB glycated samples. As stated before WB experiments were only performed at pH 7 and neutral conditions were not promoting isomerization (Carraher et al., 2015).

Table 3.4 Percentage remained reducing sugar amounts after MW glycation

pH	Sugar Type	P:S Ratio	MW		
			Dextrose (%)	Fructose (%)	Allulose (%)
7	Dextrose	2:1.25	100.0 ± 0.0	ND	ND
7		9:1.25	100.0 ± 0.0	ND	ND
7	Fructose	2:1.25	ND	100.0 ± 0.0	ND
7		9:1.25	ND	100.0 ± 0.0	ND
7	Allulose	2:1.25	ND	ND	100.0 ± 0.0
7		9:1.25	ND	ND	100.0 ± 0.0
10	Dextrose	2:1.25	73.7 ± 1.0	26.3 ± 1.0	ND
10		9:1.25	100.0 ± 0.0	ND	ND
10	Fructose	2:1.25	23.7 ± 1.8	67.1 ± 2.0	9.2 ± 0.7
10		9:1.25	ND	100.0 ± 0.0	ND
10	Allulose	2:1.25	ND	47.4 ± 1.5	52.6 ± 1.5
10		9:1.25	ND	19.9 ± 0.1	80.1 ± 0.1

ND: not detected, D: Dextrose, F: Fructose, A: Allulose.

The results were expressed as the mean of three replicates ± standard error

Table 3.5 Percentage remained reducing sugar amounts after WB glycation

Time (min)	Sugar Type	P:S Ratio	WB		
			Dextrose (%)	Fructose (%)	Allulose (%)
4	Dextrose	2:1.25	100.0 ± 0.0	ND	ND
4		9:1.25	100.0 ± 0.0	ND	ND
4	Fructose	2:1.25	ND	100.0 ± 0.0	ND
4		9:1.25	ND	100.0 ± 0.0	ND
4	Allulose	2:1.25	ND	ND	100.0 ± 0.0
4		9:1.25	ND	ND	100.0 ± 0.0
30	Dextrose	2:1.25	100.0 ± 0.0	ND	ND
30		9:1.25	100.0 ± 0.0	ND	ND
30	Fructose	2:1.25	ND	100.0 ± 0.0	ND
30		9:1.25	ND	100.0 ± 0.0	ND
30	Allulose	2:1.25	ND	ND	100.0 ± 0.0
30		9:1.25	ND	ND	100.0 ± 0.0

ND: not detected, D: Dextrose, F: Fructose, A: Allulose.

The results were expressed as the mean of three replicates ± standard error

The sugars in the MW glycated samples, no matter what type they are, were considered and divided into the initial amount to find the percentage remaining sugar amount. All factors had a significant effect on the RRS content ($p<0.05$). Allulose samples had the lowest remaining sugar amount while fructose and dextrose were equally higher. The first reason might be the higher amount of isomerization in Allulose samples at pH10 while the other reason might be the glycation of the samples.

Moreover, samples prepared at pH10 had lower remaining sugar amounts which was also associated with isomerization. Since the isomerization of monosaccharides at alkaline conditions can result in several types of byproducts, the amount of remaining sugar can be less than the non-isomerized ones (Knill & Kennedy, 2002; Novotný et al., 2008; B. Y. Yang & Montgomery, 2005).

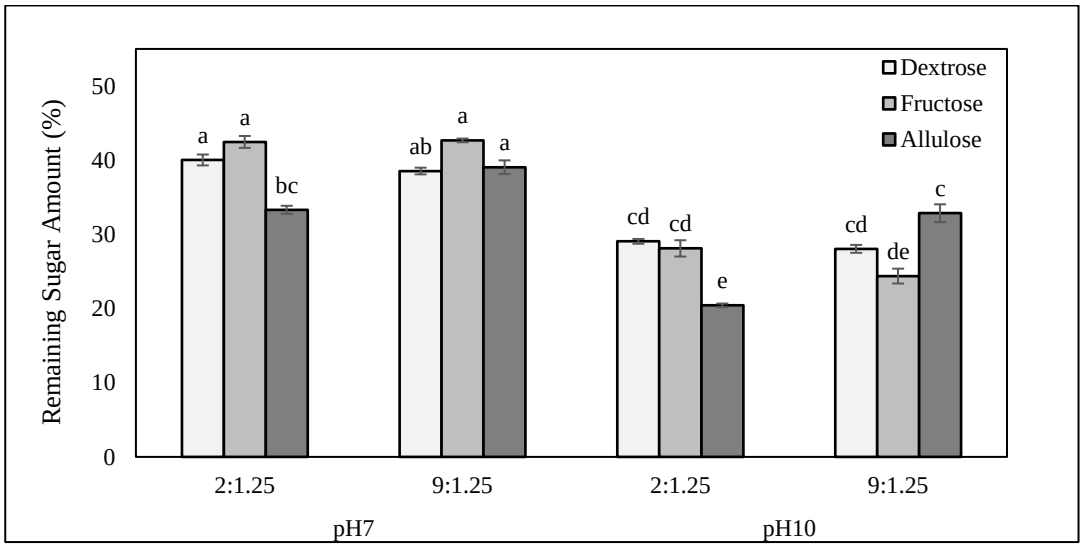

Figure 3.3. Remaining Reducing Sugar Amount in MW samples

When the remaining sugar amounts of WB glycated samples were investigated, it was observed that processing time and the sugar type had an unignorable effect on the results ($p<0.05$) whereas protein to sugar ratio was insignificant ($p>0.05$). Allulose samples had the lowest amount of remaining reducing sugar as in WB samples.

When two processing types were compared, it was found that MW samples have less RRS. Since the rate of MR was higher in MW samples, this result was expected, and it can be further confirmed by the AGEs.

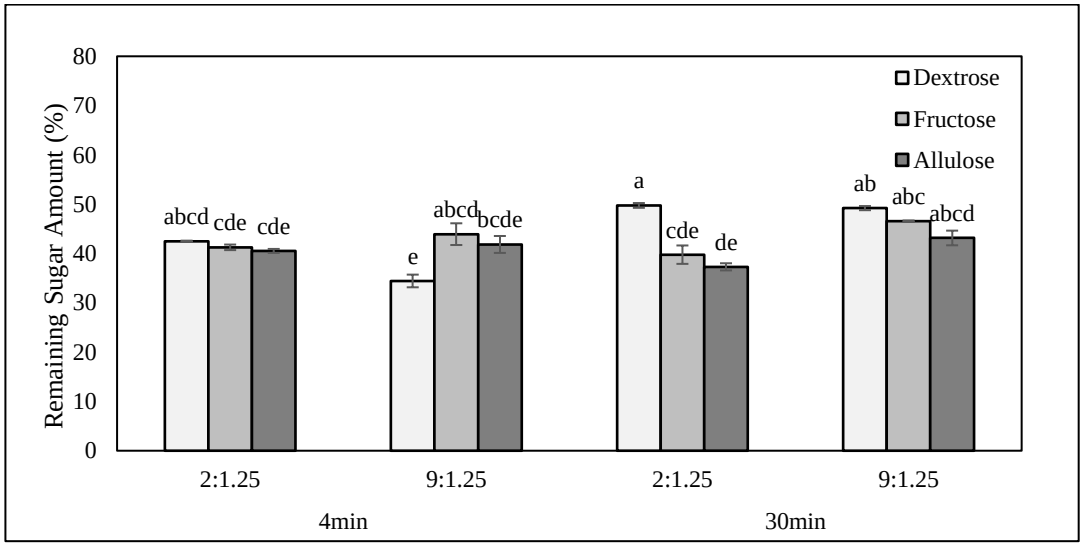

Figure 3.4. Remaining Reducing Sugar Amount in WB samples

3.5 Hydration Behavior

Hydration behavior of proteins is explained as the continuous movement of a "water cloud" around the protein molecules at thermodynamic equilibrium (Timasheff,

2002). In this study, hydration behavior was explored using Nuclear Magnetic Resonance (NMR) relaxometry (Kuntz, 1971; Mallamace et al., 2015; Mora-Gutierrez et al., 1995; Namli, 2019; Tas, 2019). T_2 relaxation times can be used to estimate the water state (bound/free, mobile/immobile) and dynamics in food systems. Since glycation influences the physical properties of water (i.e. hydrophilicity/water binding), the change in relaxation times is also expected. The presence of more free water results in longer relaxation times.

All factors had a significant effect on the T_2 results ($p<0.05$). T_2 times of samples prepared at protein to sugar ratio of 2:1.25 were higher than 9:1.25 samples. Also, pH7 samples were found to have longer T_2 times than pH10 samples. Among the sugar types, fructose samples resulted in longer T_2 times while dextrose and Allulose samples were equally lower. It is known that the higher the free water content the longer the relaxation times are (Kirtil & Oztop, 2016). Thus, less hydrated samples will result in longer T_2 times. Based on this, it might be said that fructose samples were found to be less hydrated than control samples.

The presence of another solute such as carbohydrates as a third component in protein-water systems can affect the protein stability and the hydration behavior (Gekko & Timasheff, 1981; Lee & Timasheff, 1981; Timasheff, 1993). Several studies have reported the effect of sugars in these systems. One of the mechanisms was explained by the presence of a glassy environment, created by the sugar, around the flexible protein structure. This new environment might reduce the possibility of unfolding, so it might induce more hydration (Ding et al., 1996; Lopez-Diez & Bone, 2000). The other mechanism, however, explained as the interruption of protein-water interactions by the formation of the hydrogen bonds between protein and sugar or between sugar and water in an aqueous environment (Carpenter & Crowe, 1989). This mechanism might be the reason why less hydration could have occurred in samples containing fructose since it might interrupt the water and pea protein

interaction and decrease the hydration of pea proteins. Moreover, the average hydrogen bond length (AHBL) of the protein-sugar complex was another reason of this behavior of the fructose samples. It was discovered that AHBL is positively correlated with the hydration behavior of the sugars and the AHBL of fructose samples was less (Imberti et al., 2019), which resulted in decreased hydration and longer T_2 times (Bruni et al., 2018; Imberti et al., 2019; Maugeri et al., 2017). T_2 times of WB glycated samples were also significantly affected by all the parameters ($p<0.05$). Like MW samples, a similar trend was observed in WB samples.

Table 3.6 T_2 (spin-spin relaxation) times of MW and WB glycated samples

	Sugar Type	PPC:Sugar	T_2 Results (ms) [pH7]	T_2 Results (ms) [pH10]	T_2 Results (ms) [Control]
MW	Dextrose	2:1.25	73.14 ± 1.68^{b}	67.53 ± 1.73^{a}	83.05 ± 1.32^{a}
		9:1.25	59.39 ± 1.66^{c}	37.94 ± 1.15^{b}	50.22 ± 0.98^{b}
	Fructose	2:1.25	107.38 ± 3.13^{a}	77.87 ± 2.48^{a}	80.97 ± 0.44^{a}
		9:1.25	66.59 ± 0.61^{c}	47.63 ± 0.67^{b}	49.40 ± 0.63^{b}
	Allulose	2:1.25	80.20 ± 2.07^{b}	67.72 ± 1.96^{a}	80.81 ± 1.60^{a}
		9:1.25	56.23 ± 0.67^{c}	39.92 ± 0.45^{b}	49.87 ± 0.29^{b}
	Sugar Type	**PPC:Sugar**	**T_2 Results (ms) [4 min]**	**T_2 Results (ms) [30 min]**	**T_2 Results (ms) [Control]**
WB	Dextrose	2:1.25	71.5 ± 1.012^{c}	73.25 ± 0.32^{c}	83.05 ± 1.32^{a}
		9:1.25	96.24 ± 0.97^{b}	59.1 ± 1.65^{d}	50.22 ± 0.98^{b}
	Fructose	2:1.25	108.19 ± 1.4^{a}	107.46 ± 1.66^{a}	80.97 ± 0.44^{a}
		9:1.25	76.54 ± 1.86^{c}	78.85 ± 0.5^{c}	49.40 ± 0.63^{b}
	Allulose	2:1.25	106.2 ± 4.36^{a}	97.56 ± 1.27^{b}	80.81 ± 1.60^{a}
		9:1.25	74.83 ± 0.56^{c}	99.16 ± 1.68^{b}	49.87 ± 0.29^{b}

Letters indicate significant difference in each processing time and control (a-d)

All sugars except fructose samples had lower T_2 times than control samples. It was already discussed that solubility of glycated samples were generally higher than control samples, which support the hypothesis of "Glycation enhances the protein solubility". Therefore, the decrease in T_2 times in glycated samples was unavoidable because the amount of free water will be less as the soluble protein amount increases. On the other hand, this effect might also be explained with a different mechanism that involves the entrapment of the water in protein-sugar complex (Belton & Gil, 1994; Lopez-Diez & Bone, 2000). The water trapped in the complex might decrease the free water amount and this might lead to a reduction in T_2 times. When all the possible mechanisms were considered, it can be clearly stated that glycation induced more hydration in pea protein.

3.6 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Investigating the structural changes on protein after Maillard reaction by FTIR has been quite popular (Calabrò & Magazù, 2020; Ioannou, 2016; Z. Yang et al., 2010). In this study, the changes in protein and sugar structures after MW and WB glycation were examined with a wavenumber range of 600-4000 cm^{-1}.

The table given below demonstrates the wavelengths of the important peaks observed in the samples. A detailed examination of these peaks for each FTIR spectrum was conducted. Each spectrum was prepared for each sugar type and control pea protein was also supplied in the spectrum to compare the absorbance values of the peaks easily. FTIR spectrum of MW glycated dextrose samples were given in Figure 3.5 while the others were given in Appendix.

Table 3.7 FTIR peaks of control and glycated samples, and corresponding functional groups

Wavenumber (cm^{-1})	**Assignment**	**Functional groups**
~1600	C= O Stretching	Proteins (Amide I)
~1500	N−H Bending	Proteins (Amide II)
~1200	C-N Stretching	Proteins (Amide III)
~ 3300	N-H stretching	Proteins (Amide A)
~ 3100		Proteins (Amide B)
~1150	C-O/C-C Stretching	Carbohydrates

One of the most important peaks observed in proteins are Amide I (~1600 cm^{-1}) and Amide II (~1500 cm^{-1}) bands. Secondary structures of the proteins can be observed by the changes in the C=O stretching vibration of the Amide I band and by comparing the peak heights of the samples (Arrondo et al., 1993; Fabian et al., 1992; Jackson & Mantsch, 1995). Amide II band is also used to observe the conformational sensitivity and unfolding of the proteins by monitoring the C-N stretching vibrations together with N-H bending (Kong & Yu, 2007; Krimm & Bandekar, 1986). The decrease in the intensities of these bands was expected due to the consumption of the functional groups containing NH_2, especially lysine (Gu et al., 2009; Su et al., 2010). Based on the comparison made between glycated and control samples, the highest peak was observed in control samples, which indicated the presence of more functional groups. Thus, the occurrence of glycation in samples was also confirmed by FTIR results. Moreover, the changes in the Amide III (~1200 cm^{-1}) band that indicates the C-N stretching and N-H deformation also shows the presence of the structural changes in the samples after glycation (Chang & Tanaka, 2002; Gu et al., 2009).

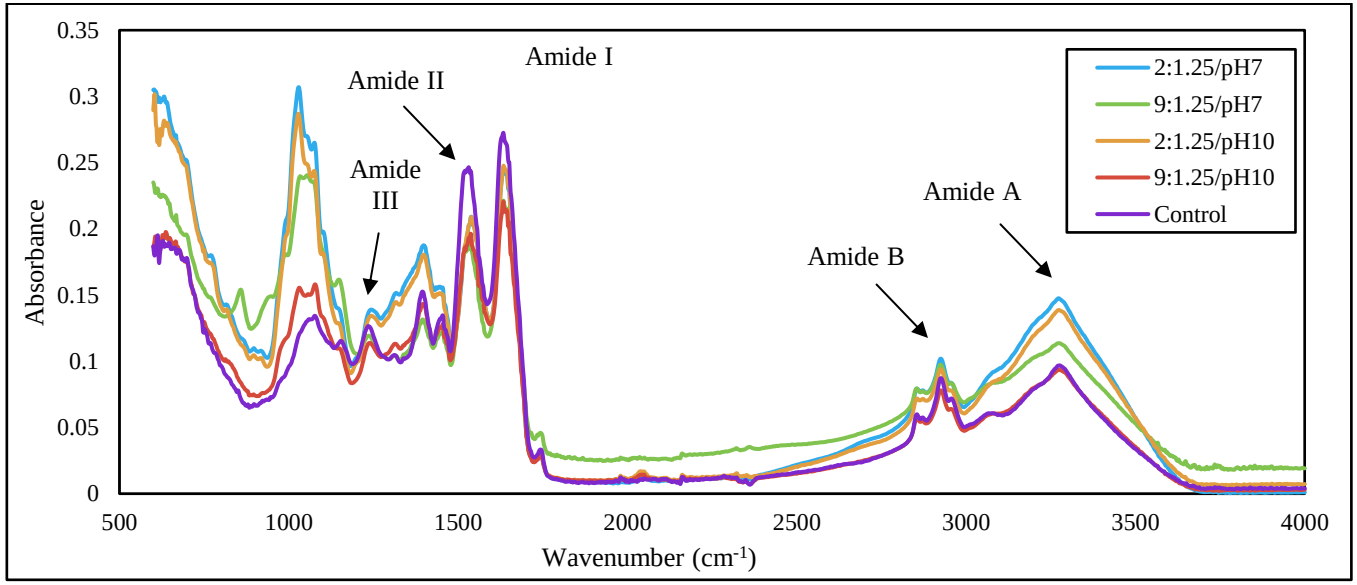

Figure 3.5. FTIR spectrum of MW glycated dextrose samples and control

The other important bands that should be considered are Amide A (~3300 cm^{-1}) and Amide B (~3100 cm^{-1}) bands. Water absorption by the samples can be discussed by the changes in these bands because the vibration mode in these bands highly depended on the strength of a hydrogen bonding in the samples (Bachmann et al., 2005; H. Zhang et al., 2015). As can be seen from the Figure 3.5., control samples had the lowest absorbance intensity, and glycated samples had higher intensities which means stronger hydrogen bond formation in glycated samples and thus, higher rate of water absorption in the system. This result was also in accordance with the enhanced solubility of the proteins with glycation.

Carbohydrates can be detected in a FTIR spectrum at the range of 1000-1100 cm^{-1} (Gu et al., 2009). The intensity of this peak can also be used to detect the presence of the cross-linkages between sugars and proteins by Maillard reaction (Han et al., 2017). As can be seen from the figure, the lowest intensity belonged to control samples, and as the linkages between monosaccharides and proteins were formed, the intensities of these peaks increased.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In this study, microwave glycation of pea protein with three different sugars (dextrose, fructose and Allulose) was conducted by using microwave heating and conventional water bath heating methods. Effect of pH, protein-sugar ratio was also examined. To monitor the Maillard reaction, soluble protein amount, free amino group content, remaining reducing sugar content, and advanced glycation end products (AGEs) concentrations (Furosine, CML and CEL) were measured. Solubility and hydration behavior were assessed, and structural changes were evaluated by FTIR.

It should be noted that the complex mechanism of the Maillard reaction is significantly affected by the intrinsic and extrinsic factors (*especially sugar type is a particularly important factor*). These factors also affect one another, which creates an even more complex system in countless ways. Therefore, effect of different sugar types on the glycation reaction cannot be compared with each other easily. However, Furosine, CML and CEL which are classified as AGEs can be used to observe the occurrence of MR and in this study, and AGEs results confirmed the occurrence of MR in the samples.

Since solubility is the biggest weakness of pea proteins, achieving improvement on the solubility was the priority of this study, and the Lowry method proved that microwave glycation enhanced the solubility of dextrose samples by 50% compared to control samples. Water bath glycation also increased the solubility, but microwave glycation was more useful. Due to the higher possibility of protein-sugar interaction,

samples with less protein participated in the reaction and dissolved more. Moreover, the alkaline environment improved the pea protein solubility.

Another critical measurement was the OPA method because the comparison of FAG amounts in control and glycated samples might indicate that the glycation took place. In this study, to ensure that the comparison can be made successfully, the total protein amount of the samples was kept constant for all samples. Based on the results, control samples had the highest free amino group amount. This result supported the idea that the glycation reaction occurred.

HPLC was preferred to detect the presence of isomerization, and to measure the remaining reducing sugar amounts in the samples due to its high accuracy (up to 90%). It has been proved that the alkaline environment increased the chance of isomerization in the samples. The presence of more reducing sugars in the environment increased the rate of conversion. Moreover, the high consumption of reducing sugars indicated that glycation occurred.

The examination of the hydration behavior by TD-NMR relaxometry and the structural changes of proteins by FTIR was another important part of this study since both methods were non-destructive techniques that can be used in many food products. The comparison of T_2 times of control and glycated samples indicated that glycation enhanced the hydration behavior of pea proteins. Moreover, the intensities of Amide I, II, A, and B bands proved that there was an immense structural change, a decrease in protein amounts, and an enhancement on the water absorption abilities of proteins.

Overall, this study showed that use of microwave as a wet glycation method could be an alternative method since it improves the solubility and hydration behavior of the pea protein relatively more than water bath glycation. Although the reaction rate differed in all parameters, glycation was achieved in all samples, no matter what they were.

As a further step, the other critical functional properties of MW glycated samples, such as emulsification activity and stability, gelling, and foaming ability, might be investigated. Also, the degree of denaturation in the samples might be detected by DSC or native page methods to be sure on the influence of denaturation.

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APPENDICES

A. Calibration Curves

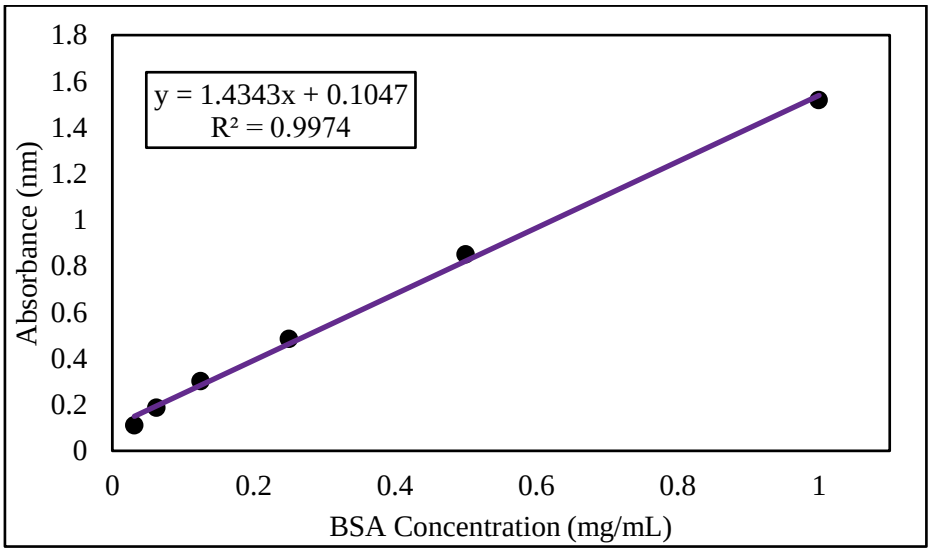

Figure A.1. Calibration curve of Lowry method prepared by Bovine Serum Albumin (BSA)

Absorbance (at 750 nm) = 1.4343 * (mg BSA/mL) + 0.1047 where R^2 = 0.9974

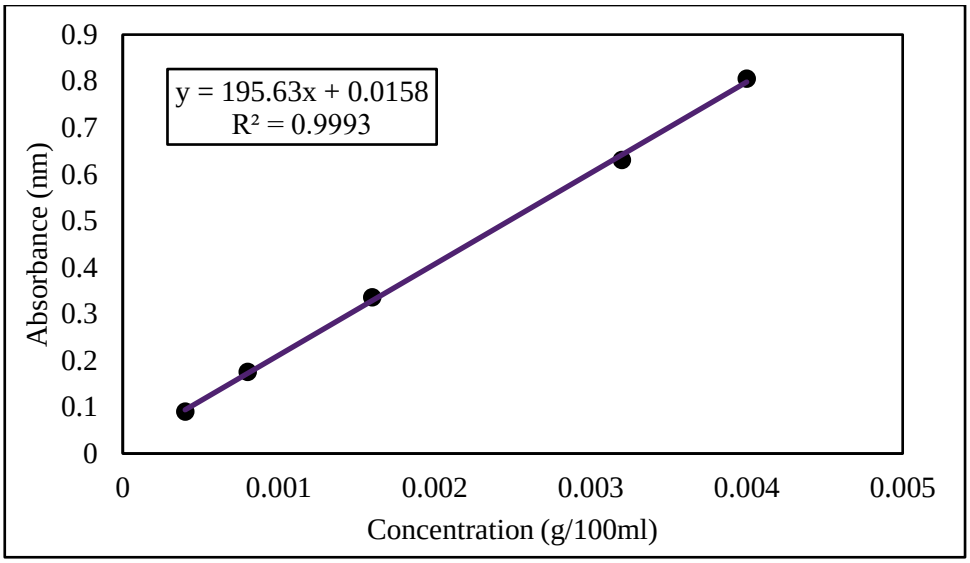

Figure A.2. Calibration curve of OPA method prepared by glycine

Absorbance (at 340 nm) = 195.63 * (g glycine/100 mL) + 0.0158 where R^2 = 0.9993

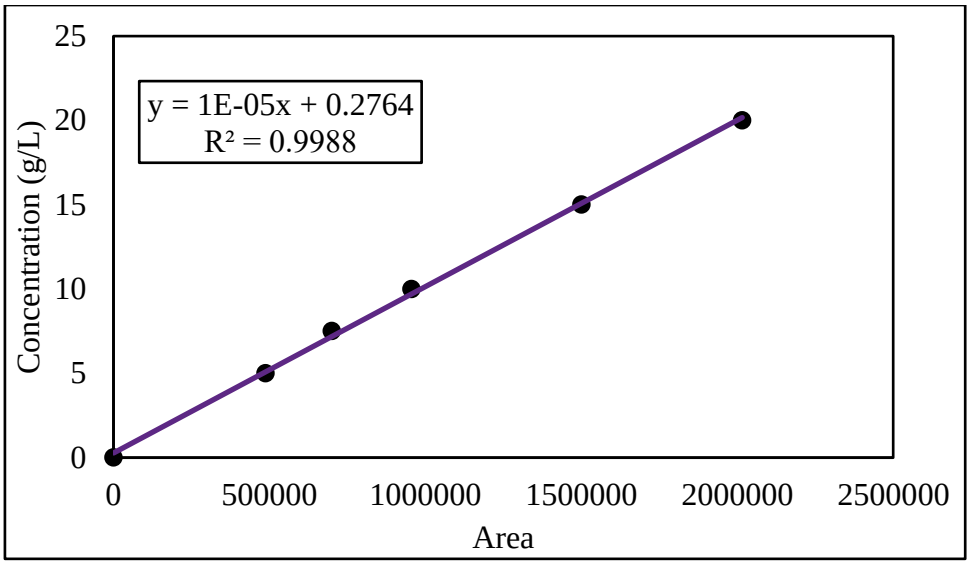

Figure A.3. Calibration curve of glucose prepared from HPLC

Concentration (g/L) = 10^{-5} * (Area) + 0.2764 where R^2 = 0.9988

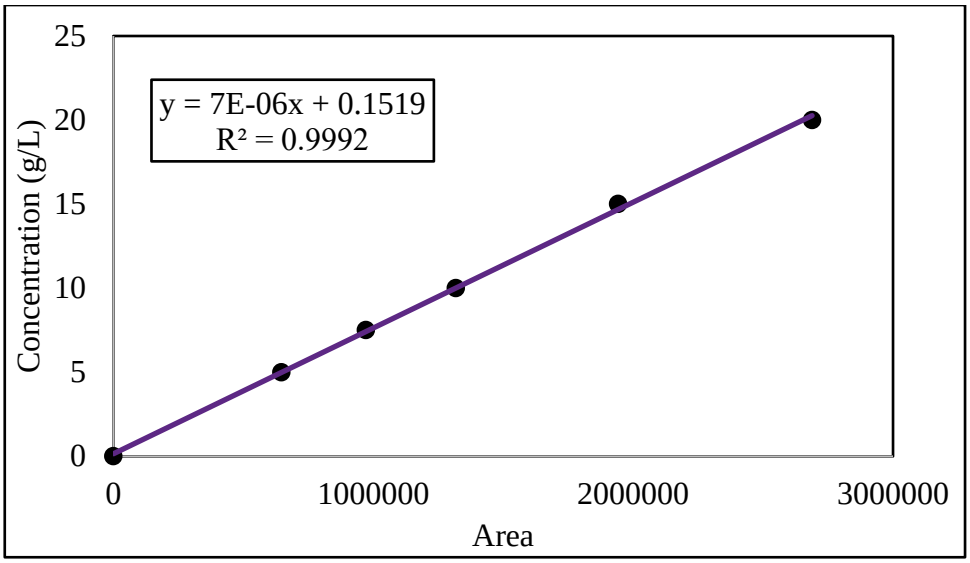

Figure A.4. Calibration curve of fructose prepared from HPLC

Concentration (g/L) = $7*10^{-6}$ * (Area) + 0.1519 where R^2 = 0.9992

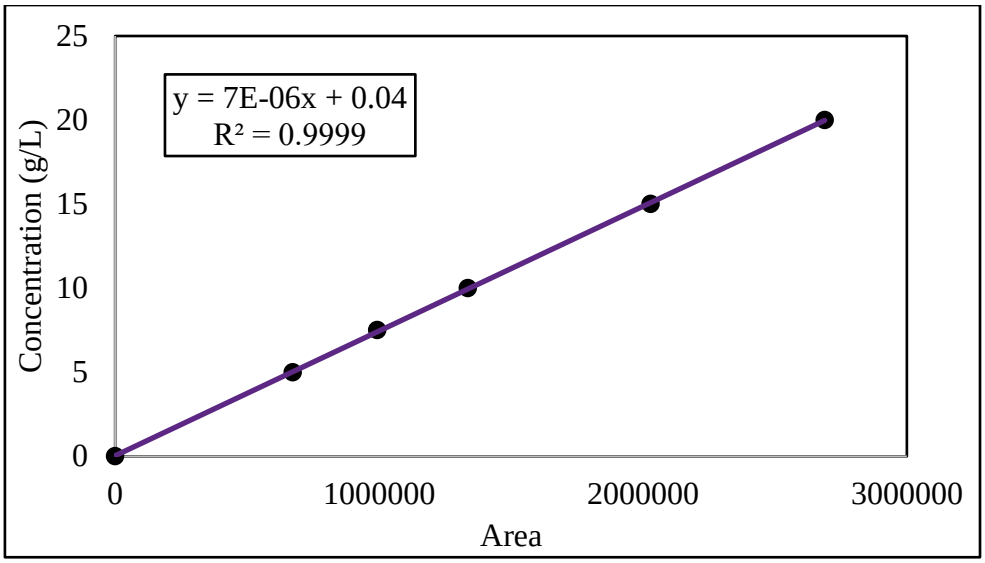

Figure A.5. Calibration curve of Allulose prepared from HPLC

Concentration (g/L) = $7*10^{-6}$ * (Area) + 0.04 where R^2 = 0.9999

B. Comparative Figures

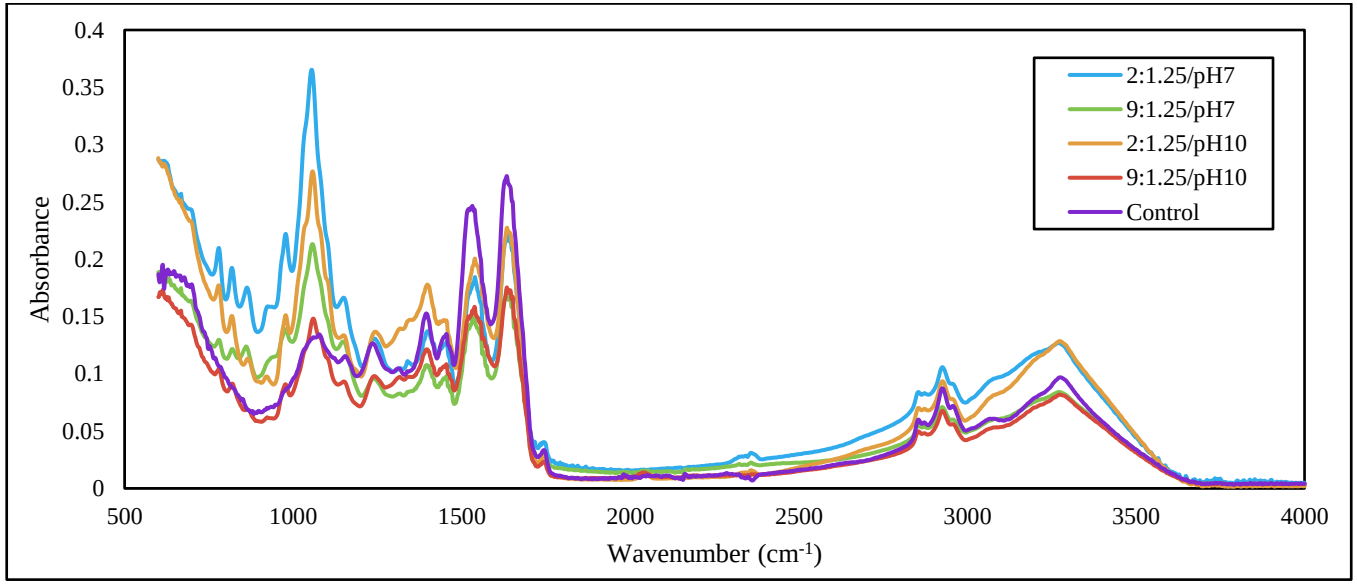

Figure B.1. FTIR spectrum of MW glycated fructose samples and control

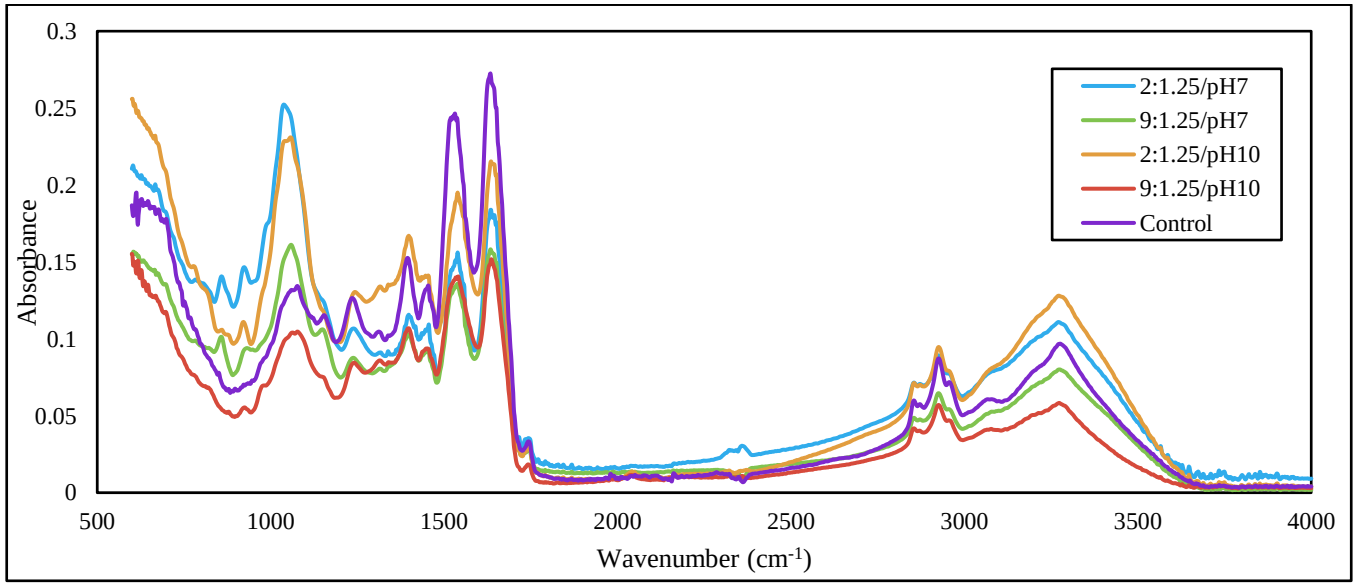

Figure B.2. FTIR spectrum of MW glycated Allulose samples and control

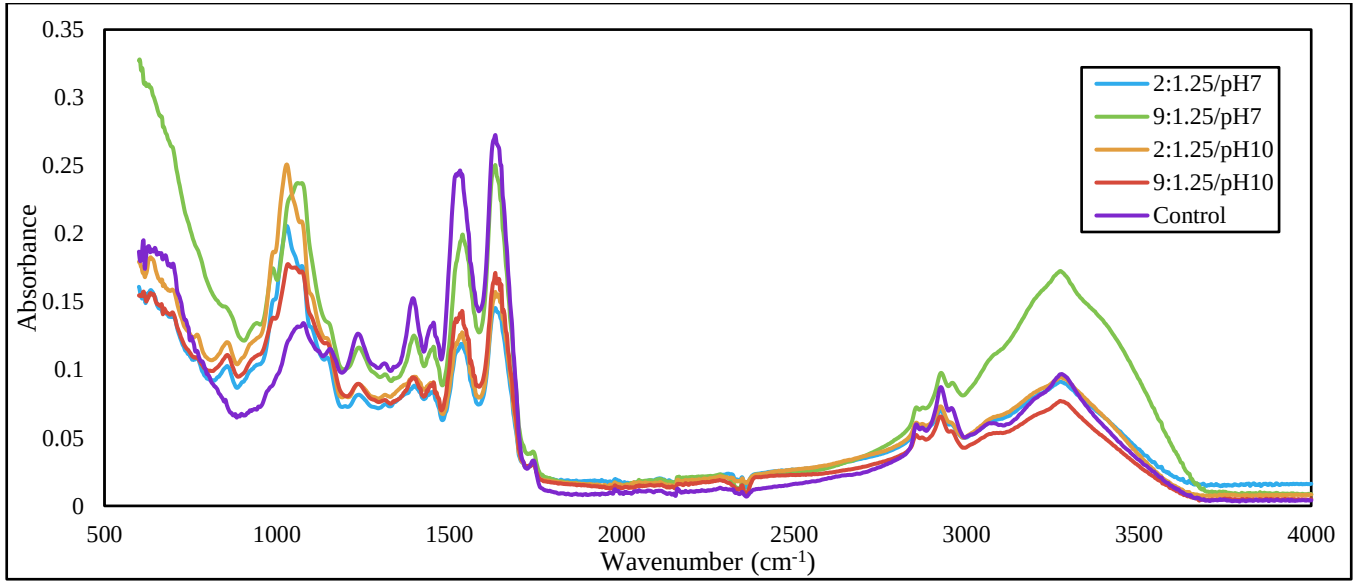

Figure B.3. FTIR spectrum of WB glycated dextrose samples and control

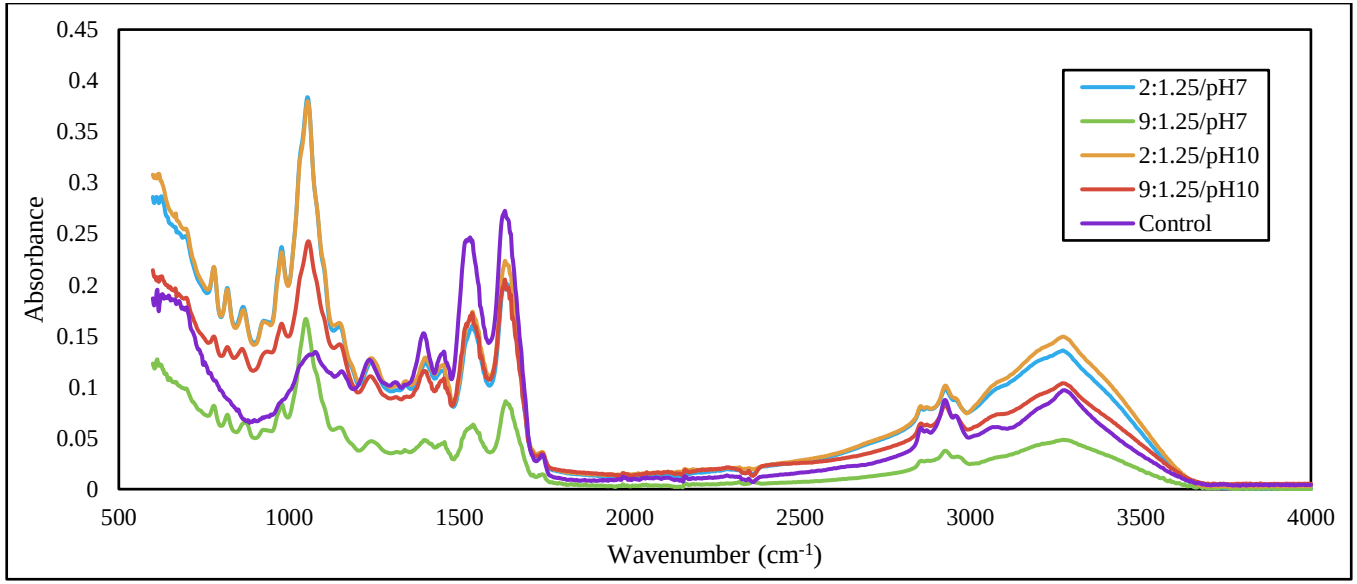

Figure B.4. FTIR spectrum of WB glycated fructose samples and control

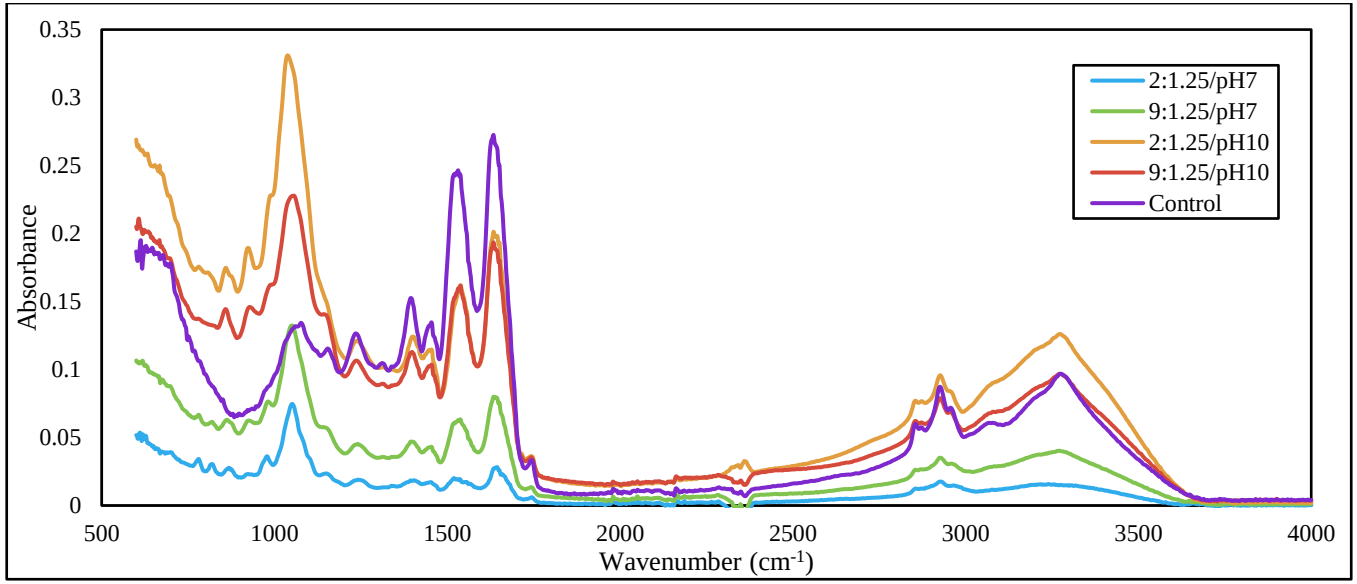

Figure B.5. FTIR spectrum of WB glycated Allulose samples and control

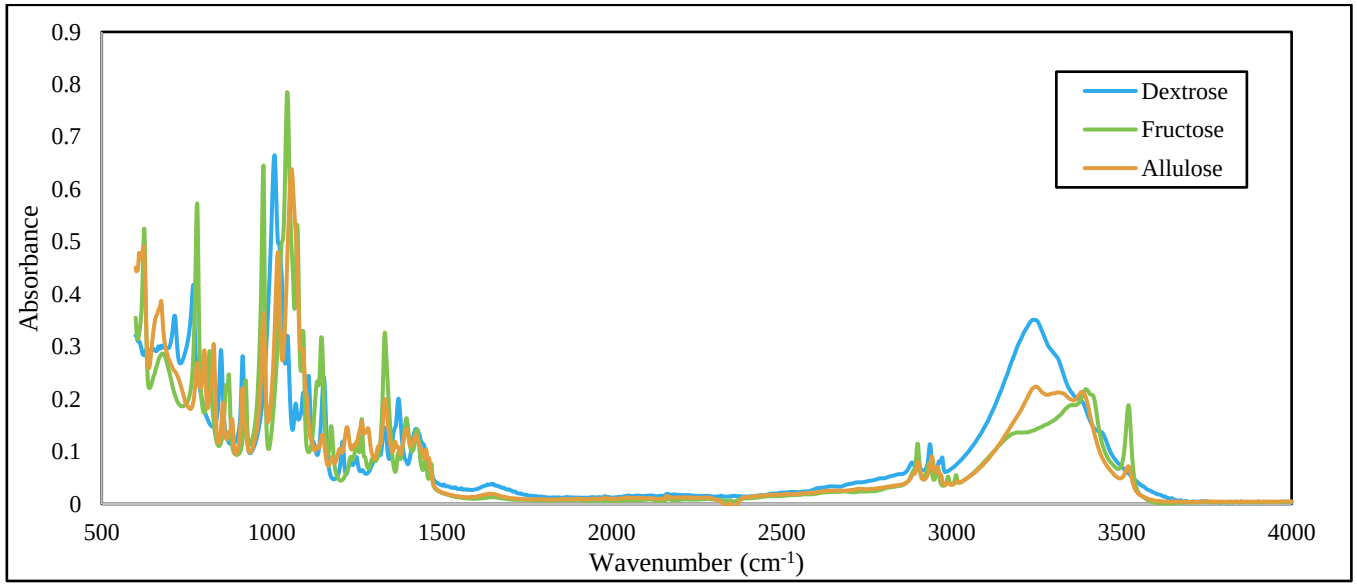

Figure B.6. FTIR spectrum of reducing sugars

C. Statistical Analyses

Table C.1. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing furosine amounts in MW glycated pH7 samples

General Linear Model: Furosine Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	234122057	117061028	1305.31	0.000
Ratio	1	2530840	2530840	28.22	0.002
Sugar Type*Ratio	2	1273245	636623	7.10	0.026
Error	6	538082	89680		
Total	11	238464224			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
299.467	99.77%	99.59%	99.10%

Comparisons for Furosine Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping	
D	4	9843.90	A	
A	4	705.42		B
F	4	258.50		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping	
2:1.25	6	4061.85	A	
9:1.25	6	3143.36		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping		
D 2:1.25	2	10713.3	A		
D 9:1.25	2	8974.5		B	
A 2:1.25	2	1141.3			C
F 2:1.25	2	331.0			C
A 9:1.25	2	269.5			C
F 9:1.25	2	186.0			C

Means that do not share a letter are significantly different.

Table C.2. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing furosine amounts in MW glycated pH10 samples

General Linear Model: Furosine Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	120367233	60183616	368.96	0.000
Ratio	1	25195326	25195326	154.46	0.000
Sugar Type*Ratio	2	26872010	13436005	82.37	0.000
Error	6	978709	163118		
Total	11	173413278			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
403.879	99.44%	98.97%	97.74%

Comparisons for Furosine Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping	
D	4	12475.6	A	
F	4	5915.2		B
A	4	5609.5		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping	
9:1.25	6	9449.10	A	
2:1.25	6	6551.10		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping		
D 9:1.25	2	15911.0	A		
D 2:1.25	2	9040.2		B	
F 9:1.25	2	7003.1			C
A 2:1.25	2	5785.8			C D
A 9:1.25	2	5433.2			C D
F 2:1.25	2	4827.3			D

Means that do not share a letter are significantly different.

Table C.3. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing furosine amounts in WB glycated 4min samples

General Linear Model: Furosine Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	27559790	13779895	222.93	0.000
Ratio	1	7024710	7024710	113.65	0.000
Sugar Type*Ratio	2	10161579	5080790	82.20	0.000
Error	6	370871	61812		
Total	11	45116950			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
248.620	99.18%	98.49%	96.71%

Comparisons for Furosine Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping

D	4	3356.01	A	
A	4	277.14		B
F	4	20.65		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping	
2:1.25	6	1983.05	A	
9:1.25	6	452.83		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping		
D 2:1.25	2	5415.16	A		
D 9:1.25	2	1296.87		B	
A 2:1.25	2	514.81		B	C
A 9:1.25	2	39.48			C
F 9:1.25	2	22.14			C
F 2:1.25	2	19.17			C

Means that do not share a letter are significantly different.

Table C.4. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing furosine amounts in WB glycated 30min samples

General Linear Model: Furosine Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	9294618	4647309	716.86	0.000
Ratio	1	6323491	6323491	975.42	0.000
Sugar Type*Ratio	2	5202802	2601401	401.28	0.000
Error	6	38897	6483		
Total	11	20859808			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
80.5160	99.81%	99.66%	99.25%

Comparisons for Furosine Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
D	4	2161.78	A		
A	4	704.94		B	
F	4	57.24			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping	
2:1.25	6	1700.57	A	
9:1.25	6	248.74		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping			
D 2:1.25	2	3756.73	A			
A 2:1.25	2	1286.07		B		
D 9:1.25	2	566.83			C	
A 9:1.25	2	123.81				D
F 2:1.25	2	58.92				D
F 9:1.25	2	55.56				D

Means that do not share a letter are significantly different.

Table C.5. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing CML amounts in MW glycated pH7 samples

General Linear Model: CML Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F

Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	1318.4	659.21	26.22	0.001
Ratio	1	2987.1	2987.09	118.81	0.000
Sugar Type*Ratio	2	248.5	124.26	4.94	0.054
Error	6	150.9	25.14		
Total	11	4704.9			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
5.01416	96.79%	94.12%	87.17%

Comparisons for CML Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
A	4	105.442	A		
D	4	92.112		B	
F	4	79.774			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping	
9:1.25	6	108.220	A	
2:1.25	6	76.665		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping			
A 9:1.25	2	120.586	A			
D 9:1.25	2	102.660	A	B		
F 9:1.25	2	101.414	A	B	C	
A 2:1.25	2	90.299		B	C	
D 2:1.25	2	81.564			C	
F 2:1.25	2	58.133				D

Means that do not share a letter are significantly different.

Table C.6. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing CML amounts in MW glycated pH10 samples

General Linear Model: CML Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	98885	49443	40.22	0.000
Ratio	1	171	171	0.14	0.722
Sugar Type*Ratio	2	262209	131105	106.64	0.000
Error	6	7377	1229		
Total	11	368642			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
35.0633	98.00%	96.33%	92.00%

Comparisons for CML Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
A	4	497.432	A		
F	4	386.618		B	
D	4	275.076			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping
9:1.25	6	390.152	A
2:1.25	6	382.599	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping		
A 2:1.25	2	646.401	A		
D 9:1.25	2	478.828		B	
F 2:1.25	2	430.073		B	
A 9:1.25	2	348.464		B	
F 9:1.25	2	343.162		B	
D 2:1.25	2	71.323			C

Means that do not share a letter are significantly different.

Table C.7. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing CML amounts in WB glycated 4min samples

General Linear Model: CML Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	167.42	83.71	7.86	0.021
Ratio	1	5378.09	5378.09	504.95	0.000
Sugar Type*Ratio	2	574.68	287.34	26.98	0.001
Error	6	63.90	10.65		
Total	11	6184.09			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3.26356	98.97%	98.11%	95.87%

Comparisons for CML Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
D	4	87.5396	A		
A	4	85.1516	A	B	
F	4	78.6968		B	

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping	
9:1.25	6	104.966	A	
2:1.25	6	62.626		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping		
D 9:1.25	2	116.278	A		
A 9:1.25	2	107.912	A		
F 9:1.25	2	90.709		B	
F 2:1.25	2	66.685			C
A 2:1.25	2	62.391			C
D 2:1.25	2	58.802			C

Means that do not share a letter are significantly different.

Table C.8. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing CML amounts in WB glycated 30min samples

General Linear Model: CML Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	1714.0	857.00	18.18	0.003
Ratio	1	1077.9	1077.95	22.87	0.003
Sugar Type*Ratio	2	2705.2	1352.58	28.69	0.001
Error	6	282.8	47.14		
Total	11	5779.9			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
6.86562	95.11%	91.03%	80.43%

Comparisons for CML Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping

A	4	130.943	A	
F	4	108.541		B
D	4	103.421		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping	
9:1.25	6	123.779	A	
2:1.25	6	104.824		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping		
A 9:1.25	2	136.915	A		
D 9:1.25	2	132.788	A		
A 2:1.25	2	124.970	A	B	
F 2:1.25	2	115.447	A	B	
F 9:1.25	2	101.635		B	
D 2:1.25	2	74.054			C

Means that do not share a letter are significantly different.

Table C.9. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing CEL amounts in MW glycated pH7 samples

General Linear Model: CEL Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	1378.35	689.176	727.35	0.000
Ratio	1	82.60	82.596	87.17	0.000
Sugar Type*Ratio	2	291.99	145.993	154.08	0.000
Error	6	5.69	0.948		

Total	11	1758.62

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.973407	99.68%	99.41%	98.71%

Comparisons for CEL Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
A	4	30.6460	A		
F	4	9.7256		B	
D	4	6.4514			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping	
2:1.25	6	18.2312	A	
9:1.25	6	12.9841		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping			
A 2:1.25	2	40.1755	A			
A 9:1.25	2	21.1164		B		
F 9:1.25	2	11.4085			C	
F 2:1.25	2	8.0426			C	D
D 2:1.25	2	6.4754				D
D 9:1.25	2	6.4273				D

Means that do not share a letter are significantly different.

Table C.10. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing CEL amounts in MW glycated pH10 samples

General Linear Model: CEL Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F

Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	134816	67408.2	1023.67	0.000
Ratio	1	7268	7268.4	110.38	0.000
Sugar Type*Ratio	2	41085	20542.7	311.96	0.000
Error	6	395	65.8		
Total	11	183565			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
8.11479	99.78%	99.61%	99.14%

Comparisons for CEL Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
A	4	282.119	A		
F	4	253.791		B	
D	4	44.450			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping	
2:1.25	6	218.064	A	
9:1.25	6	168.842		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping				
A 2:1.25	2	381.353	A				
F 2:1.25	2	272.060		B			
F 9:1.25	2	235.521			C		
A 9:1.25	2	182.884				D	
D 9:1.25	2	88.121					E
D 2:1.25	2	0.779					F

Means that do not share a letter are significantly different.

Table C.11. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing CEL amounts in WB glycated 4min samples

General Linear Model: CEL Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	115.867	57.9333	145.23	0.000
Ratio	1	70.421	70.4215	176.54	0.000
Sugar Type*Ratio	2	14.254	7.1271	17.87	0.003
Error	6	2.393	0.3989		
Total	11	202.936			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.631583	98.82%	97.84%	95.28%

Comparisons for CEL Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
A	4	13.3261	A		
D	4	7.8270		B	
F	4	6.0191			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping	
9:1.25	6	11.4799	A	
2:1.25	6	6.6349		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping

A 9:1.25	2	15.4657	A			
D 9:1.25	2	11.7031		B		
A 2:1.25	2	11.1865		B		
F 9:1.25	2	7.2709			C	
F 2:1.25	2	4.7674			C	D
D 2:1.25	2	3.9509				D

Means that do not share a letter are significantly different.

Table C.12. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing CEL amounts in WB glycated 30min samples

General Linear Model: CEL Results versus Sugar Type, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Ratio	Fixed	2	2:1.25, 9:1.25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	1061.17	530.584	289.21	0.000
Ratio	1	37.27	37.272	20.32	0.004
Sugar Type*Ratio	2	485.93	242.964	132.43	0.000
Error	6	11.01	1.835		
Total	11	1595.37			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.35447	99.31%	98.74%	97.24%

Comparisons for CEL Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
A	4	30.2721	A		
F	4	21.7535		B	
D	4	7.4787			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping
2:1.25	6	21.5971	A

9:1.25	6	18.0724	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Ratio	N	Mean	Grouping		
A 2:1.25	2	31.9828	A		
F 2:1.25	2	31.3352	A		
A 9:1.25	2	28.5614	A		
D 9:1.25	2	13.4840		B	
F 9:1.25	2	12.1717		B	
D 2:1.25	2	1.4734			C

Means that do not share a letter are significantly different.

Table C.13. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing soluble protein contents in MW glycated samples

General Linear Model: Lowry Results versus Sugar Type, Concentration, pH

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Concentration	Fixed	2	1.6, 7.2
pH	Fixed	2	7, 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	3546.6	1773.32	405.02	0.000
Concentration	1	6713.0	6713.02	1533.22	0.000
pH	1	1510.0	1509.99	344.87	0.000
Sugar Type*Concentration	2	671.0	335.51	76.63	0.000
Sugar Type*pH	2	1115.1	557.55	127.34	0.000
Concentration*pH	1	867.6	867.63	198.16	0.000
Sugar Type*Concentration*pH	2	67.2	33.60	7.67	0.003
Error	24	105.1	4.38		
Total	35	14595.7			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.09246	99.28%	98.95%	98.38%

Comparisons for Lowry Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
D	12	146.795	A		
A	12	126.723		B	
F	12	124.878		B	

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
1.6	18	146.455	A	
7.2	18	119.144		B

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	18	139.275	A	
7	18	126.323		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration	N	Mean	Grouping				
D 1.6	6	166.413	A				
A 1.6	6	138.540		B			
F 1.6	6	134.411			C		
D 7.2	6	127.178				D	
F 7.2	6	115.345					E
A 7.2	6	114.907					E

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

D 10	6	154.567	A				
A 10	6	139.275		B			
D 7	6	139.023		B			
F 7	6	125.773			C		
F 10	6	123.983			C		
A 7	6	114.171				D	

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration*pH

Grouping Information Using the Tukey Method and 95% Confidence

Concentration*pH	N	Mean	Grouping			
1.6 10	9	148.022	A			
1.6 7	9	144.887		B		
7.2 10	9	130.529			C	
7.2 7	9	107.758				D

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration*pH

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration*pH	N	Mean	Grouping					
D 1.6 10	3	167.452	A					
D 1.6 7	3	165.373	A					
A 1.6 10	3	147.648		B				
D 7.2 10	3	141.683		B	C			
F 1.6 7	3	139.858			C			
A 7.2 10	3	130.903				D		
A 1.6 7	3	129.431				D		
F 1.6 10	3	128.965				D		
F 7.2 10	3	119.002					E	
D 7.2 7	3	112.674						F
F 7.2 7	3	111.688						F
A 7.2 7	3	98.911							G

Means that do not share a letter are significantly different.

Table C.14. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing soluble protein contents in WB glycated samples

General Linear Model: Lowry Results versus Sugar Type, Concentration, Time

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values

Sugar Type	Fixed	3	A, D, F
Concentration	Fixed	2	1.6, 7.2
Time	Fixed	2	4, 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	624.24	312.12	34.83	0.000
Concentration	1	4344.58	4344.58	484.82	0.000
Time	1	220.29	220.29	24.58	0.000
Sugar Type*Concentration	2	1231.46	615.73	68.71	0.000
Sugar Type*Time	2	262.12	131.06	14.63	0.000
Concentration*Time	1	53.06	53.06	5.92	0.023
Sugar Type*Concentration*Time	2	129.68	64.84	7.24	0.003
Error	24	215.07	8.96		
Total	35	7080.51			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.99354	96.96%	95.57%	93.17%

Comparisons for Lowry Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
A	12	118.951	A		
F	12	112.383		B	
D	12	108.909			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
1.6	18	124.400	A	
7.2	18	102.429		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Time

Grouping Information Using the Tukey Method and 95% Confidence

Time	N	Mean	Grouping	
30	18	115.888	A	
4	18	110.941		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration	N	Mean	Grouping				
A 1.6	6	135.281	A				
F 1.6	6	126.163		B			
D 1.6	6	111.755			C		
D 7.2	6	106.063				D	
A 7.2	6	102.622				D	E
F 7.2	6	98.602					E

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Time

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Time	N	Mean	Grouping		
A 30	6	125.086	A		
A 4	6	112.817		B	
F 4	6	112.672		B	C
F 30	6	112.093		B	C
D 30	6	110.485		B	C
D 4	6	107.333			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration*Time

Grouping Information Using the Tukey Method and 95% Confidence

Concentration*Time	N	Mean	Grouping		
1.6 30	9	125.660	A		
1.6 4	9	123.140	A		
7.2 30	9	106.117		B	
7.2 4	9	98.741			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration*Time

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration*Time	N	Mean	Grouping					
A 1.6 30	3	141.375	A					
F 1.6 4	3	130.344		B				
A 1.6 4	3	129.187		B				
F 1.6 30	3	121.983		B	C			
D 1.6 30	3	113.621			C	D		
D 1.6 4	3	109.890				D	E	
A 7.2 30	3	108.797				D	E	
D 7.2 30	3	107.349				D	E	
D 7.2 4	3	104.777					E	F
F 7.2 30	3	102.204					E	F G
A 7.2 4	3	96.447						F G

F 7.2 4	3	95.000	G

Means that do not share a letter are significantly different.

Table C.15. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing free amino group amounts in MW glycated pH7 samples

General Linear Model: OPA Results versus Sugar Type, Concentration

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	4	A, C, D, F
Concentration	Fixed	2	1.6, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	3	2.73160	0.910534	216.96	0.000
Concentration	1	0.19711	0.197109	46.97	0.000
Sugar Type*Concentration	3	0.25480	0.084934	20.24	0.000
Error	16	0.06715	0.004197		
Total	23	3.25067			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0647833	97.93%	97.03%	95.35%

Comparisons for OPA Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
C	6	2.97446	A		
F	6	2.42196		B	
D	6	2.17696			C
A	6	2.12196			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
7.2	12	2.51446	A	
1.6	12	2.33321		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration	N	Mean	Grouping			
C 1.6	3	2.97446	A			
C 7.2	3	2.97446	A			
F 7.2	3	2.51112		B		
D 7.2	3	2.43612		B		
F 1.6	3	2.33279		B		
A 7.2	3	2.13612			C	
A 1.6	3	2.10779			C	
D 1.6	3	1.91779				D

Means that do not share a letter are significantly different.

Table C.16. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing free amino group amounts in MW glycated pH10 samples

General Linear Model: OPA Results versus Sugar Type, Concentration

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	4	A, C, D, F
Concentration	Fixed	2	1.6, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	3	4.0778	1.35927	183.71	0.000
Concentration	1	4.6200	4.62004	624.42	0.000
Sugar Type*Concentration	3	1.6348	0.54494	73.65	0.000
Error	16	0.1184	0.00740		
Total	23	10.4511			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0860172	98.87%	98.37%	97.45%

Comparisons for OPA Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping

C	6	3.54946	A	
A	6	3.00029		B
D	6	2.95862		B
F	6	2.38446			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
7.2	12	3.41196	A	
1.6	12	2.53446		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration	N	Mean	Grouping			
D 7.2	3	3.61612	A			
C 1.6	3	3.54946	A			
C 7.2	3	3.54946	A			
A 7.2	3	3.48612	A			
F 7.2	3	2.99612		B		
A 1.6	3	2.51446			C	
D 1.6	3	2.30112			C	
F 1.6	3	1.77279				D

Means that do not share a letter are significantly different.

Table C.17. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing free amino group amounts in WB glycated 4min samples

General Linear Model: OPA Results versus Sugar Type, Concentration

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	4	A, C, D, F
Concentration	Fixed	2	1.6, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	3	10.0560	3.35200	1833.57	0.000
Concentration	1	0.2773	0.27735	151.71	0.000

Sugar Type*Concentration	3	0.2821	0.09402	51.43	0.000
Error	16	0.0292	0.00183		
Total	23	10.6446			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0427565	99.73%	99.60%	99.38%

Comparisons for OPA Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping			
C	6	2.70779	A			
D	6	1.64529		B		
A	6	1.56612			C	
F	6	0.90029				D

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
7.2	12	1.81237	A	
1.6	12	1.59737		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration	N	Mean	Grouping				
C 7.2	3	2.70779	A				
C 1.6	3	2.70779	A				
D 7.2	3	1.92946		B			
A 7.2	3	1.66946			C		
A 1.6	3	1.46279				D	
D 1.6	3	1.36112				D	
F 7.2	3	0.94279					E
F 1.6	3	0.85779					E

Means that do not share a letter are significantly different.

Table C.18. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing free amino group amounts in WB glycated 30min samples

General Linear Model: OPA Results versus Sugar Type, Concentration

Method

Factor coding	(-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	4	A, C, D, F
Concentration	Fixed	2	1.6, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	3	8.4418	2.81395	1095.90	0.000
Concentration	1	0.2937	0.29371	114.39	0.000
Sugar Type*Concentration	3	2.1563	0.71875	279.92	0.000
Error	16	0.0411	0.00257		
Total	23	10.9329			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0506726	99.62%	99.46%	99.15%

Comparisons for OPA Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping			
C	6	2.70779	A			
F	6	1.61196		B		
A	6	1.42279			C	
D	6	1.14446				D

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
1.6	12	1.83237	A	
7.2	12	1.61112		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration	N	Mean	Grouping			
C 1.6	3	2.70779	A			
C 7.2	3	2.70779	A			
D 1.6	3	1.76946		B		
F 7.2	3	1.72612		B		
F 1.6	3	1.49779			C	
A 7.2	3	1.49112			C	
A 1.6	3	1.35446			C	
D 7.2	3	0.51946				D

Means that do not share a letter are significantly different.

Table C.19. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing reducing sugar amounts in MW glycated samples

General Linear Model: Sugar amount versus Sugar Type, pH, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
pH	Fixed	2	7, 10
Ratio	Fixed	2	1.6, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	41.26	20.629	10.25	0.003
pH	1	892.52	892.516	443.60	0.000
Ratio	1	24.52	24.521	12.19	0.004
Sugar Type*pH	2	52.50	26.252	13.05	0.001
Sugar Type*Ratio	2	149.75	74.877	37.22	0.000
pH*Ratio	1	1.73	1.725	0.86	0.373
Sugar Type*pH*Ratio	2	28.47	14.236	7.08	0.009
Error	12	24.14	2.012		
Total	23	1214.89			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.41844	98.01%	96.19%	92.05%

Comparisons for Sugar amount

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping

F	8	34.4048	A
D	8	33.9207	A
A	8	31.4132	B

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	
7	12	39.3445	A	
10	12	27.1480		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping	
7.2	12	34.2570	A	
1.6	12	32.2355		B

Means that do not share a letter are significantly different.

Table C.20. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing reducing sugar amounts in WB glycated samples

General Linear Model: Sugar amount versus Sugar Type, Time, Ratio

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Time	Fixed	2	4, 30
Ratio	Fixed	2	1.6, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	44.178	22.089	6.08	0.015
Time	1	75.373	75.373	20.76	0.001
Ratio	1	10.737	10.737	2.96	0.111
Sugar Type*Time	2	169.974	84.987	23.41	0.000
Sugar Type*Ratio	2	96.711	48.355	13.32	0.001
Time*Ratio	1	43.553	43.553	12.00	0.005
Sugar Type*Time*Ratio	2	3.408	1.704	0.47	0.636

Error	12	43.565	3.630
Total	23	487.497	

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.90536	91.06%	82.87%	64.25%

Comparisons for Sugar amount

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping	
D	8	43.9673	A	
F	8	42.8723	A	B
A	8	40.7024		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Time

Grouping Information Using the Tukey Method and 95% Confidence

Time	N	Mean	Grouping	
30	12	44.2862	A	
4	12	40.7419		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Ratio

Grouping Information Using the Tukey Method and 95% Confidence

Ratio	N	Mean	Grouping
7.2	12	43.1829	A
1.6	12	41.8452	A

Table C.21. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing T_2 results in MW glycated pH7 samples

General Linear Model: T2 Results versus Sugar Type, Concentration

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Concentration	Fixed	2	1.6, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	4004.6	2002.32	92.34	0.000
Concentration	1	8181.0	8180.96	377.27	0.000
Sugar Type*Concentration	2	1354.7	677.36	31.24	0.000
Error	46	997.5	21.68		
Total	51	14167.7			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4.65668	92.96%	92.19%	90.97%

Comparisons for T2 Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping	
F	17	85.7385	A	
A	18	68.2163		B
D	17	65.9349		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
1.6	25	85.8589	A	
7.2	27	60.7343		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration	N	Mean	Grouping			
F 1.6	8	104.892	A			
A 1.6	9	80.202		B		
D 1.6	8	72.483			C	
F 7.2	9	66.585			C	
D 7.2	9	59.387				D
A 7.2	9	56.231				D

Means that do not share a letter are significantly different.

Table C.22. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing T_2 results in MW glycated pH10 samples

General Linear Model: T2 Results versus Sugar Type, Concentration

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Concentration	Fixed	2	1.6, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	1087.4	543.7	24.21	0.000
Concentration	1	11517.8	11517.8	512.93	0.000
Sugar Type*Concentration	2	14.4	7.2	0.32	0.728
Error	48	1077.8	22.5		
Total	53	13697.4			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4.73867	92.13%	91.31%	90.04%

Comparisons for T2 Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping	
F	18	62.7489	A	
A	18	53.8188		B
D	18	52.7337		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
1.6	27	71.0384	A	
7.2	27	41.8293		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration	N	Mean	Grouping			
F 1.6	9	77.8669	A			
A 1.6	9	67.7179		B		
D 1.6	9	67.5303		B		
F 7.2	9	47.6309			C	
A 7.2	9	39.9198				D
D 7.2	9	37.9371				D

Means that do not share a letter are significantly different.

Table C.23. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing T_2 results in WB glycated 4min samples

General Linear Model: T2 Results versus Sugar Type, Concentration

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Concentration	Fixed	2	1.6, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	319.2	159.60	18.28	0.000
Concentration	1	976.8	976.82	111.85	0.000
Sugar Type*Concentration	2	4217.5	2108.73	241.46	0.000
Error	18	157.2	8.73		
Total	23	5670.7			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.95518	97.23%	96.46%	95.07%

Comparisons for T2 Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping	
F	8	92.3646	A	
A	8	90.5163	A	
D	8	83.8715		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
1.6	12	95.2972	A	
7.2	12	82.5378		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration	N	Mean	Grouping		
F 1.6	4	108.187	A		
A 1.6	4	106.200	A		
D 7.2	4	96.239		B	
F 7.2	4	76.542			C
A 7.2	4	74.833			C
D 1.6	4	71.504			C

Means that do not share a letter are significantly different.

Table C.24. ANOVA and Tukey's Comparison Test with 95% confidence level for comparing T_2 results in WB glycated 30min samples

General Linear Model: T2 Results versus Sugar Type, Concentration

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Sugar Type	Fixed	3	A, D, F
Concentration	Fixed	2	1.6, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar Type	2	4774.9	2387.44	348.63	0.000
Concentration	1	1129.6	1129.60	164.95	0.000
Sugar Type*Concentration	2	913.2	456.59	66.67	0.000
Error	18	123.3	6.85		
Total	23	6940.9			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.61689	98.22%	97.73%	96.84%

Comparisons for T2 Results

Tukey Pairwise Comparisons: Sugar Type

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type	N	Mean	Grouping		
A	8	98.3560	A		
F	8	93.1508		B	
D	8	66.1735			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
1.6	12	92.7539	A	
7.2	12	79.0329		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Sugar Type*Concentration

Grouping Information Using the Tukey Method and 95% Confidence

Sugar Type*Concentration	N	Mean	Grouping			
F 1.6	4	107.455	A			
A 7.2	4	99.157		B		
A 1.6	4	97.556		B		
F 7.2	4	78.847			C	
D 1.6	4	73.251			C	
D 7.2	4	59.096				D

Means that do not share a letter are significantly different.