INVESTIGATION OF STRUCTURAL AND BIOLOGICAL ACTIVITIES OF D-GLUCOSE – L-GLYCINE, D-GLUCOSE – L-ARGININE, AND BREAD MELANOIDINS

A thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by
Ferhat ELCİ

July 2022
İZMİR

ACKNOWLEGEMENTS

I would like to thank my advisor Assoc. Prof. Çağatay Ceylan for his encouragement and assistance during my studies. I was always able to easily reach to him and solve difficulties. Thank you once again for believing in me and generously providing all of the resources he has to accomplish my thesis.

I am grateful to Assistant Prof. Dr. İpek Şahin to be my co-supervisor.

I'd also like to thank the staff at the Biotechnology and Bioengineering Application and Research Center, particularly Yekta Günay and Evrim Paşık, who guided and assisted me during my experiments.

I'd like to express my gratitude to my close friends Yasin Yetişkin, Ömer Taşdelen, Bişenk Paksoy, Mustafa Kaçmaz, and Malik Ölmez, and Ogün Bahçıvanoğlu who had to listen to my constant complains about difficulties and supported me during my thesis.

Finally, I would like to express my endless thanks to my family for understanding my decisions, supporting me, and trusting me.

ABSTRACT

INVESTIGATION OF STRUCTURAL AND BIOLOGICAL ACTIVITIES OF D-GLUCOSE – L-GLYCINE, D-GLUCOSE – L-ARGININE, AND BREAD MELANOIDINS

As a result of Maillard reactions, high molecular weight compounds known melanoidins are formed from the proteins and sugars in food. Amino acids and reducing sugars combine, making them common ingredients in meals, fruit juices, coffee, and baked products that are consumed on a regular basis. Food melanoidins can affect human health, thus it's critical to assess and understand their chemical composition. Understanding the relationship between melanoidins' structural analysis and their activity was the main objective of this work. According to our observations, melanoidins have an antibacterial effect on the microorganisms *E. coli* and *S. aureus.* Melanoidins demonstrated excellent antibacterial action against *E. coli* at low doses, whereas *S. aureus* was more resistant at higher concentrations. These data suggested that melanoidins have a significant level of activity against gram-negative bacteria. The antioxidant capacity of melanoidins was assessed using the ABTS test. The bread melanoid exhibited the lowest antioxidant value, whereas the Gly - Glc melanoid model system had the strongest antioxidant property. Fourier transform Infrared (FTIR) spectroscopy was utilized to identify the functional groups of melanoidins and to analyze these groups. HPLC was used to identify melanoidins. We investigated the structural and functional properties of melanoidins utilizing spectroscopic methods such as FTIR spectroscopy, UV-Visible Spectroscopy, Scanning Electron Microscopy, and biochemical approaches. Studies on their biological activities and structures have enhanced our understanding of them; yet the complexity of the structures and variety of melanoidins provide the greatest obstacle to the knowledge.

ÖZET

D-GLİKOZ – L-GLİSİN, D-GLİKOZ – L-ARJİNİN VE EKMEK MELANOİDİNLERİNİN YAPISAL VE BİYOLOJİK AKTİVİTELERİNİN ARAŞTIRILMASI

Maillard reaksiyonları sonucunda gıdalardaki protein ve şekerlerden melanoidin olarak bilinen yüksek moleküler ağırlıklı bileşikler oluşur. Amino asitler ve indirgeyici şekerler birleşerek yemeklerde, meyve sularında, kahvede ve düzenli bir şekilde tüketilen unlu mamullerde yaygın olarak meydana gelir. Gıda melanoidinleri insan sağlığını etkileyebilir, bu nedenle kimyasal bileşenlerinin yapısını anlamak çok önemlidir. Melanoidinlerin yapısal analizi ile aktiviteleri arasındaki ilişkiyi anlamak bu çalışmanın temel amacıydı. Elde ettiğimiz verilere göre melanoidinler, *E. coli* ve *S. aureus* mikroorganizmaları üzerinde antibakteriyel etki gösterdiler. Melanoidinler, düşük dozlarda *E. coli*'ye karşı etkili antibakteriyel etki gösterirken, *S. aureus* daha yüksek konsantrasyonlarda antibakteriyel özellik gösterdi. Elde edilen bu verilerden yola çıkarak, melanoidinlerin gram-negatif bakterilere karşı önemli düzeyde aktiviteye sahip olduğunu göstermiş oldu. Melanoidinlerin antioksidan kapasitesi ABTS testi kullanılarak değerlendirildi. Ekmek melanoidi en düşük antioksidan değeri sergilerken, Gly - Glc melanoidin model sistemi en güçlü antioksidan özelliği gösterdi. Fourier Dönüşüm Kızılötesi (FTIR) spektroskopisi metodu ile melanoidinlerin fonksiyonel gruplarını belirlemek ve bu grupları analiz etmek için kullanıldı. Melanoidinleri tanımlamak için HPLC kullanıldı. FTIR, UV-Görünür Spektroskopisi, Taramalı Elektron Mikroskobu (SEM) ve biyokimyasal yaklaşımlar gibi spektroskopik yöntemler kullanarak melanoidinlerin yapısal ve fonksiyonel özelliklerini araştırıldı. Biyolojik aktiviteleri ve yapıları üzerine yapılan çalışmalar, melanoidinleri daha iyi anlamamızı sağladı. Ancak, melanoidinlerin yapılarının karmaşıklığı ve çeşitliliği bu bileşiklerin yapıssal analiz çalışmalarınnın önündeki en büyük engel olarak duruyor.

TABLE OF CONTENTS

LIST OF FIGURES v

LIST OF TABLES vii

CHAPTER 1. NTRODUCTION 1

1.1. Maillard Reactions (MR).....2

1.2. Factors involved in the formation of melanoidin.....4

1.2.1. Temperature and heating duration 4

1.2.2. Amino acids and sugar composition.....4

1.2.3. Ratio of sugar and amino acids 5

1.2.4. Effect of pH 5

1.2.5. Water activity 6

1.2.6. Molecular Weight of Melanoidins (MWM).....6

1.2.7. Melanoidins in Foods.....7

1.3. Purification of Melanoidins 8

1.4. Effects of melanoidins on human health 9

1.5. Structural and Chemical Properties.....10

1.6. Isolation of melanoidins 15

1.7. Biological Activity of melanoidins 16

1.7.1. Antimicrobial activity of melanoidins 16

1.7.2. Antioxidant activity of melanoidins 18

1.7.3. Antibacterial and Prebiotic activity 19

CHAPTER 2. MATERIAL AND MEDHODS 22

2.1. Chemicals 22

2.2. Preparation of Melanoidins Model Systems.....22

2.3. Purification of Bread Crust Melanoidins.....23

2.4. Gel Filtration.....23

2.5. UV-vis Spectroscopy 24

2.6. Fouirer Transform Infrared Spektrofotometre (FTIR) Spectroscopy 24

2.7. Antibacterial Assay 24
2.8. Antioxidant Assay.....25
2.9. Scanning Electron Microscopy (SEM).....25
2.10. High Performance Liquid Chromatography (HPLC) Analysis 26
2.11. Statistical Analysis 26

CHAPTER 3. RESULTS AND DISCUSSION.....27
3.1. Antibacterial Effect of Melanoidins.....27
3.2. Antioxidant Activity of Melanoidins 30
3.3. Analysis of Melanoidins Morphological Structure of Melanoidins and Bacteria Membrane by SEM 31
3.4. Fourier Transform Infrared Spectrophotometer (FT-IR) analysis of Melanoidins 37
3.5. Uv-vis Spectrophotometer Analaysis.....42
3.6. High Performance Liquid Chromatography (HPLC) Analysis 43

CHAPTER 4. CONCLUSION 47

REFERENCE 49

LIST OF FIGURES

Figure **Page**

Figure 1.1. Melanoidins produced as a result of Maillard reactions 3

Figure 1.2. Significant factors in formation of melanoidins. 6

Figure 1.3. Foods which contain melanoidins after heat process.....8

Figure 1.4. Consumption of melanoidins in Spanish diet according to amount foods.....9

Figure 1.5. Melanoidin structure that is based on carbohydrates (A) oligodeoxyhexosuloses, (B) oligodeoxyhexosuloses amine.....13

Figure 1.6. The suggested structures of chromophoric LMW and HMW protein cross-linking with melanoidin (A) furyl-imidazolidic dimer-protein, (B) trifuryl-pyrrolinone-protein, and (C) pronyl-protein. 14

Figure 3.1. Changing absorbance at 600 nm. for melanoidins (bread, Arg-Glc and Gly– Glc.) and control group on *S. aureus*……29

Figure 3.2. Changing absorbance at 600 nm. for melanoidins (bread, Arg – Glc and Gly – Glc.) and control group on *E. coli* 29

Figure 3.3. $ABTS^{\bullet+}$ radical scavenging activity (%) of melanoidins. 30

Figure 3.4. Antibacterial action of bread melanoidin on *E. coli* 31

Figure 3.5. Antibacterial action of Arg – Glc melanoidin on *E. coli* 32

Figure 3. 6. Antibacterial action of Gly – Glc melanoidin on *E. coli* 33

Figure 3.7. Antibacterial action of bread melanoidin on *S. aureus*.....33

Figure 3.8. antibacterial action of Arg – Glc melanoidin on *S. aureus* 34

Figure 3.9. Antibacterial action of Gly – Glc melanoidin on *S. aureus.* 35

Figure 3.10. Antibacterial action of Gly – Glc melanoidin on *S. aureus.* 35

Figure 3.11. The morphological structure of Arg – Glc (a) and Gly – Glc (b) melanoidins sample under Scanning Electron Microscopy 36

Figure 3.12. FT-IR spectrums of bread melanoidins. Each number and black arrows which are given in boxes show peak position of melanoidins. 37

Figure 3.13. FT-IR spectra of Glycine – Glucose melanoidins peak position which are indicated by black arrows. 38

Figure 3.14. FT-IR spectrum of Arginine – Glucose melanoidins peak positions which are indicated by black arrows. 38

Figure **Page**

Figure 3.15. Comparison of the FTIR spectra of glucose-glycine (bottom spectrum), arginine-glucose (middle spectrum) and bread melanoidins (top). 42
Figure 3.16. Uv-vis specturum (a) Gly – Glc melanoidin, (b) Bread melanoidin, and (c) Arg – Glc melanoidin detected at 280 nm 43
Figure 3.17. HPLC data spectrum shows bread melanoidins 44
Figure 3.18. HPLC data spectrum shows Arginine – Glucose melanoidins.....45
Figure 3.19. HPLC data spectrum shows Glycine – Glucose melanoidins 45
Figure 3.20. HPLC data spectrum shows PEG-8000 kDa standard.....46

LIST OF TABLES

Table **Page**

Table 3. 1. Minimum inhibitor concentration (MIC) of melanoidins for both *E. coli* and *S. aureus*.....28

Table 3. 2. Peak position of specific groups in bread, Arg-Glc, and Gly-Glc melanoidins. 40

CHAPTER 1

INTRODUCTION

The conversion processes that are now known as the Maillard reaction were initially reported by the French chemist Louis Maillard in 1912. However, it wasn't until 1953 when Hodge proposed the first coherent scheme for the reaction. The reaction is called for after Maillard [1]. The Maillard process is a browning mechanism that does not need enzymes. It is a complicated system of reactions that includes carbonyl and amino substances, such as reducing sugars and amino acids. During the process of food manufacturing, this reaction is the primary one that is important in the formation of precursors into colorants and flavor chemicals [2].

When carbohydrates (usually reducing sugars) come into contact with a free amino group component, the result is the formation of melanoidins, which are polymeric, colored polymers generated by the Maillard process. To further understand how diverse environmental factors such as pH, temperature, water activity, sugar, and amino acid types have an effect on the melanoidins, researchers are examining their chemical characterization and reaction chemistry [3]. Melanoidins' structure and chemical characteristics have been extensively studied, but no confined and described melanoidins have yet been identified. Melanoidins, like other biomolecules, have key functions in taste and color that are profoundly influenced by physical changes and structural transitions [4]. It is possible that the complexity and variability of melanoidins explain why so few studies exist to their physical form.

Melanoidins are significant both because they are found in a broad variety of foods and due to the potential implications that these compounds may have on the quality foods. Melanoidins have the potential to either harm or enhance the overall quality of food products, based on the intensity of the heat-induced response. Melanoidins are the pigments that give cooked and processed foods their color, and they can be found in a variety of common foods and beverages, such as coffee, bread, sausage, and beer. The Maillard reaction is capable of giving artificial syrup its signature golden brown color. This color is achieved by combining corn syrup with

amino acids in a controlled manner before heating the mixture. At the end of the Maillard reaction, there are hundreds of different complex amino/acid sugar combinations. These are used to make artificial flavorings, which are also made through the Maillard reaction [5].

Increasingly, Maillard Reaction Products (MRPs), particularly melanoidins, have attracted considerable interest. Due to their possible health-promoting characteristics, these compounds are deemed beneficial dietary components. In contrast, melanoidins are considered to be the diminished nutritional value of products. In addition, their ability to bind metal ions and their reducing characteristics are extensively investigated. Because melanoidins are found in many foods and the average person eats about 10 g of them every day, it seems important to study their bioactivity and how they affect human health [6].

1.1 Maillard Reactions (MR)

Condensation products N-substituted glycosilamine and the Amadori rearrangement product are generate in the early stages of the reaction when reducing sugars are combined with compounds containing free amino groups [7]. Browning reactions can not take place through the enzymatic process under these situations. The process of nonenzymatic browning may be essentially broken down into three different kinds of reactions. The first reaction, known as the Maillard reaction, needs both a carbonyl molecule, which in this context is frequently a reducing sugar, and an amine, which is generally an amino acid. The second process is called caramelization, and it involves a reaction in which the sugars react with one other on their own. This process often needs more extreme circumstances. The final one is the oxidation of ascorbic acid. The last method, even though it does not necessarily have to involve any enzymes at all, is the one that comes the closest to enzymic browning because that frequently does require ascorbic acid oxidase [8].

Aldehydes and α-aminoketones are formed after reaction between di-carbonyl each with amino acids. This is followed by various reactions such as cyclizations, dehydrations and retroaldolises as well as rearrangements and isomerizations as well as further condensations, all of which culminate in the formation of brown nitrogenous polymers and co-polymers defined as melanoidins in the last step of the reaction

process. In the Maillard reaction, in addition to the usual breakdown that results in the formation of Amadori product, the sugars and amino acids also go through their own independent breakdown [9]. Last, the essential significance of the Amadori product, which was once thought to be the primary intermediate of the reaction, has been considered in the food (Figure 1.2). The mechanism of the Maillard reaction is still a hotspot in despite of all the studies that have been conducted on the subject (Davis A, 1995).

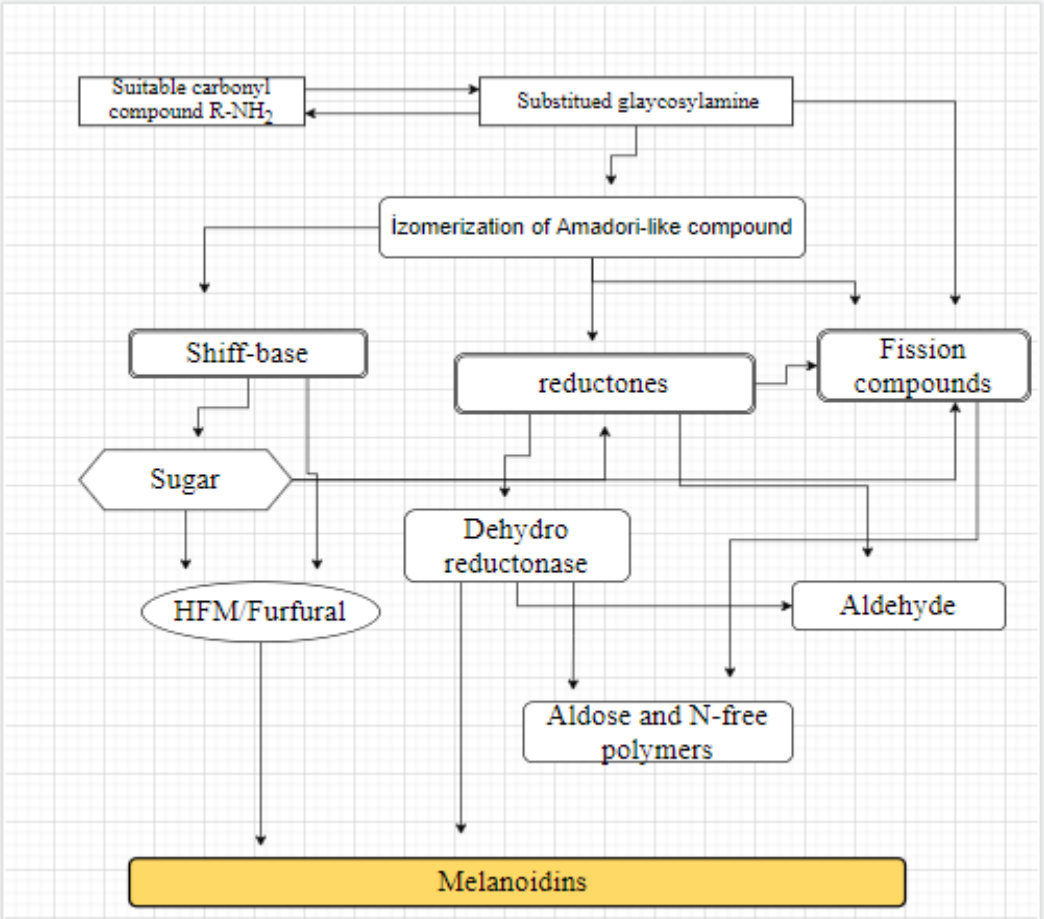

Figure 1. 1. Melanoidins produced as a result of Maillard reactions.

(Source: Martins et al., 2002.)

1.2. Factors involved in the formation of melanoidin

1.2.1. Temperature and heating duration

The rate of the non-enzymatic browning is affected by a number of variables, such as the environment and proportion of the reagents, the chemical makeup of the food system, and heating duration. However, temperature is the factor that has the greatest impact on the rate of the Maillard reactions. It is necessary to collect kinetic data on numerous quality-related aspects such as nutrition, color, flavor, and texture throughout the heat processing of foods to optimize the thermal treatment during the heating process. The preservation of natural colors like as chlorophyll, carotenoids, and other pigments, as well as the production of new colors during browning processes, may be desired. The reaction of the sugar and the amino group increases as the temperature rises [11].

1.2.2. Amino acids and sugar composition

The kind of the reducing sugar that is part of the reaction has a substantial impact on the pace at which browning occurs. Type of amino acids and sugars have significant effects on final products which can determine structure and activities. In terms of their level of reactivity, aldopentose come first, followed by aldohexoses, then ketohexoses, and finally disaccharides. The subsequent reactions of protein-disaccharide were slowed down when there was a terminal pyranose group at the C-4 position of the reducing end of the disaccharides. Especially, aldoses have an essentially higher level of reactivity compared to ketoses. Regarding the relative reactivity of glucose and fructose, there have been studies that disagree with one another. Glucose is more reactive than fructose, while at high temperatures and pH 5.5, fructose is more reactive [12].

1.2.3. Ratio of sugar and amino acids

It has been demonstrated that the proportion of reducing sugar to amino acid has a key role in deciding the percentage of Maillard browning. This would have important effects on how foods are produced. The rate of Maillard browning is increased when there is more reducing sugar than amino compounds. The breakdown of sugar and amino acids occurs in various ways. In the aqueous model systems with glucose and aspartic acid or asparagine, the induction time decreased when the amount of glucose increased [13].

1.2.4. Effect of pH

In most situations, the non-enzymatic browning process of a model system can begin in a pH range that is either neutral or very slightly alkaline, and the pH can eventually fall as the reaction progresses. It is required to investigate the effects of pH regulation on the intermediates and melanoidins in a model system in order to match the model melanoidin with dietary melanoidin. This is because a pH reduction of such a significant magnitude is not often seen in the preparation and protection of food. In the same way that temperature plays a significant role, pH has a substantial influence on the level at which the sugar and amino group reactants. When the pH is higher, the open chain form of the sugar and the unprotonated form of the amino group, both of which are regarded to be the reactive forms, are preferred. The quantity of the amino group that is in its protonated form may be connected to the Maillard process. The sugar's nucleophilic interaction with the amino group results in a nucleophilic reaction. A more nucleophilic amino group is more prone to being deprotonated in the most basic of situations. Although the amino group is a weak nucleophile under acidic circumstances, activation of the group is required before the nucleophilic reactions. In the fructose-lysine aqueous model system, a brown polymer and UV-absorbing, colorless substances are generated at higher pH levels during the Maillard reaction. When the pH is lower, the equilibrium has a higher concentration of protonated amino groups, which are less responsive to the sugar molecule [14].

1.2.5. Water activity

Microbial growth, lipid oxidation, and non-enzymatic and enzymatic activity have all been measured using water activity after foods have been produced. Water activity is widely utilized across the food industry as a health and reliability sign since it gives a basic guideline for estimating the longevity of food systems. Because chemical reactions are complicated, it's hard to figure out how water affects enzymatic or non-enzymatic chemical reactions in food [15].

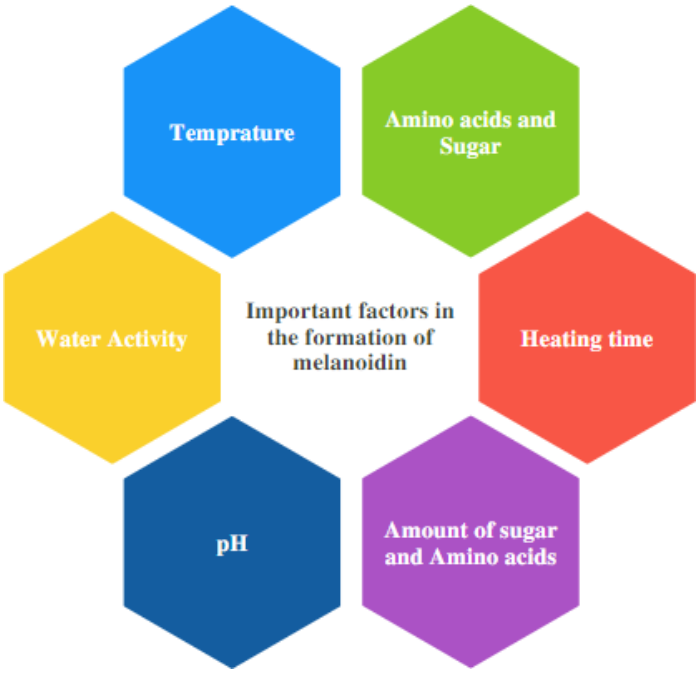

Figure 1.2. Significant factors in formation of melanoidins.

1.2.6. Molecular Weight of Melanoidins (MWM)

High molecular weight compounds predominate among the melanoidins that are formed when proteins and sugars in real foods undergo the Maillard Reaction process. It's possible that during the early stages of the MR, low molecular weight chromophoric melanoidins are formed, which later polymerize or cross-link with other MRPs to produce HMW melanoidins during the MR's final stages. This is attributed to the reason

that the molecular weight of melanoidins synthesized from MRPs is largely dependent on heating intensity, with HMW melanoidins produced at longer reaction durations. The proportion of melanoidins contained in foods have been shown to be HMW molecules[16]. For instance, when heated, ethanol extracts of bread crust become browner due to an increase in molecular weight. Given that the severity of the browning rises with increasing molecular weight, this proves that HMW melanoidins account for a significant amount of the browning. High molecular weight (12–14 kDa) melanoidins make up 59% of coffee's total melanoidin content. It has been discovered that prolonged roasting leads largely to the synthesis of HMW melanoidins, which exhibit a brown color that is more intense than the LMW melanoidins [9]. Similarly, it has been demonstrated that polymers of HMW melanoidins that are separated from sweet wines (12 kDa), roasted malt (60 kDa), and roasted cocoa beans (5 kDa) are responsible for the brown color that is present in these products.

1.2.7. Melanoidins in Foods

Since formation of melanoidins is associated with the heat treatments of foods, it is logical to assume that this kind of compound is present in almost every foodstuff that we consume. However, there is a lack of scientific studies regarding the evaluation of the amounts of melanoidins that may be found in a variety of foodstuffs. Only little information on the key chemicals that contribute to the backbone structure of melanoidins is currently known. It is remarkable that not a specific food melanoidin form has been isolated and properly identified up to that time. Moreover, owing to the exceptional significance of melanoidins in the Asian diet, the first melanoidins to be investigated were those found in soy sauce. To date, research has been conducted on a number of processed foods to determine the amount of melanoidin they contain. Melanoidins have been found in large quantities in roasted coffee beans, bakery products, cocoa, malt, and roasted barley. Due to the obviously high levels of water-soluble melanoidins that are generated in coffee brews and instant coffee, these two types of coffee have been the focus of the most research studies [17].

The absence of a primary source, the absence of a recognized molecular structure, and the lack of a suitable analytical procedure are the three primary obstacles to accurately calculating the amount of melanoidins found in various foods. The content of melanoidins was determined gravimetrically, and the calculation included finding the

difference between the total amounts of known elements in the target foodstuff. Melanoidins were eventually extracted from the low molecular weight material of the food matrix and further defined by their brown color as evaluated for their absorbance at wavelengths between 400 nm. and 450 nm. [18].

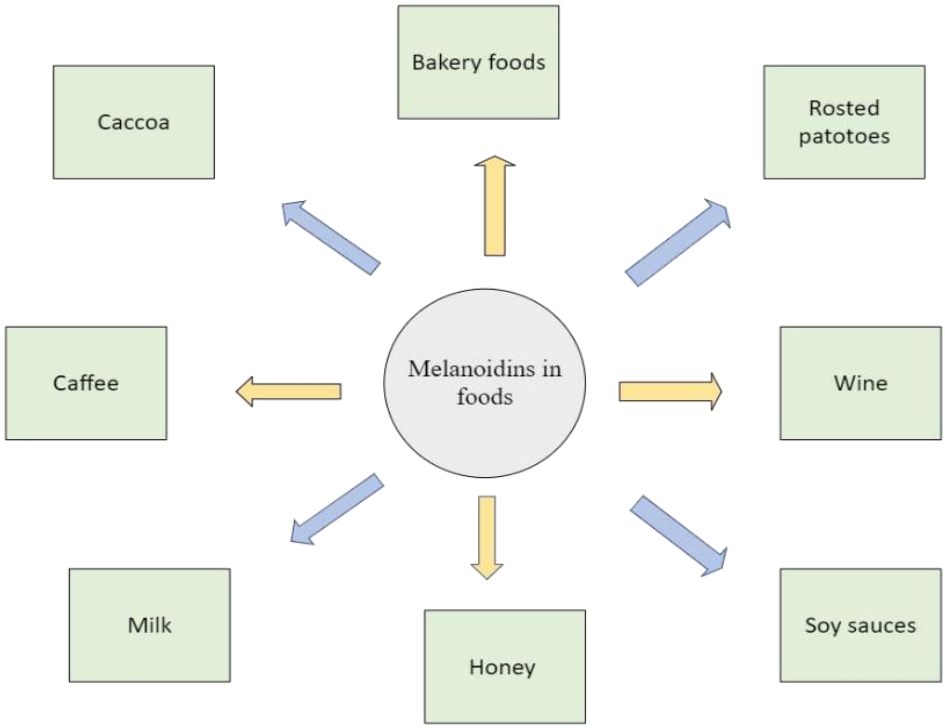

Figure 1.3. Foods which contain melanoidins after heat process.

(Source:Langner & Rzeski, 2014).

1.3. Purification of Melanoidins

The process of isolating melanoidins may be subdivided into three main groups: dialysis, ultrafiltration, and gel filtration methods. Following separation, the melanoidin sections are lyophilized, at which point their composition may be represented as a percentage of the original food's dry mass. For instance, the amount of melanoidin in roasted coffee is thought to be between 30 and 35 percent of its weight. However, this could change based on how much the coffee was roasted and how it was made. An informed estimate that takes into consideration the food intake from behaviors of

drinking regular amounts of coffee predicts an uptake of melanoidins of approximately 0.5 g each day [19].

Combining the average amount consumed with a weighted estimate of the amount of melanoidin found in the product is one way to arrive at an assumption that is comparable to the one described above for bread and cereal products. Melanoidins are mostly found in the crust of bakery foods, but in dry biscuits, they are dispersed throughout the whole product. An enzymatic treatment with proteases is required before isolating and determining the amount of those melanoidins. A consumption of up to 15 g/day may be calculated for bread, while an input of up to 8.5 g/day can be calculated for biscuits. According to these estimations, the average person who consumes a traditional Western diet takes roughly 10 g of melanoidin per day from their diet, which comes mostly from drinking coffee and eating bread [20].

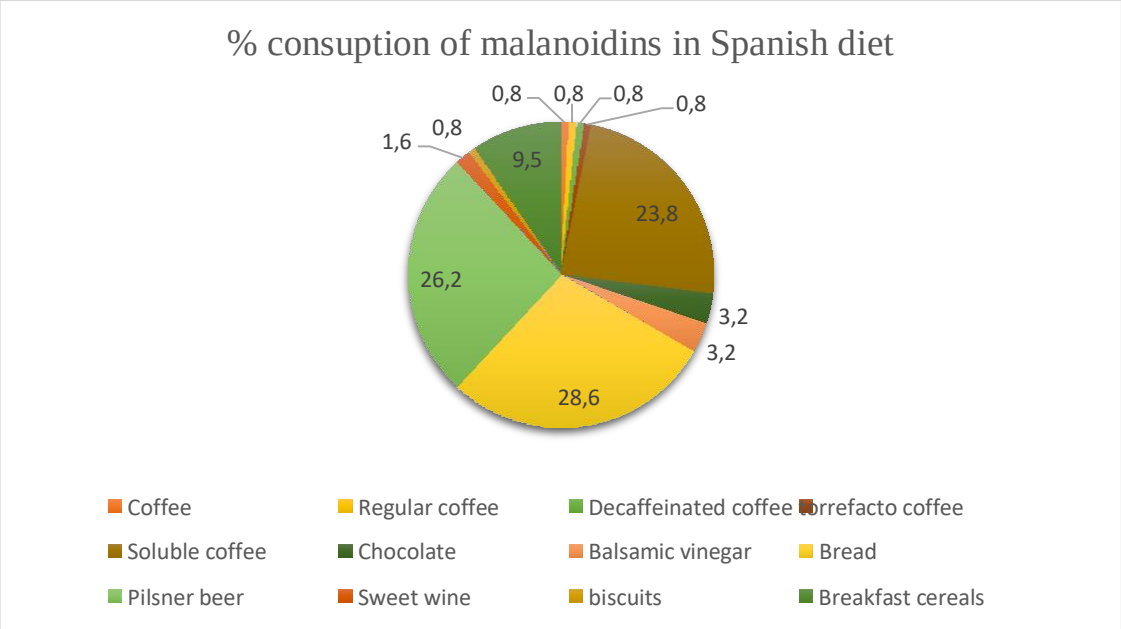

Figure 1.4. Consumption of melanoidins in Spanish diet according to amount foods (Source: Sharma et al. 2021).

1.4. Effects of melanoidins on human health

One way to look at melanoidins' impact on health is to consider the effects they have on the body as either main or secondary, depending on how they interact with

other nutrients. Melanoidins have both primary and secondary effects. The loss of bioavailable amino acids due to the Maillard Reaction is the most significant side effect of heat-treated proteins on human health. A proper amino acid or protein supplement may effectively contribute this impact. COST Action 919's main health impacts, on the other hand, should be studied using chemically defined components, yet most of melanoidins' chemical structures were unknown when this COST Action 919 began [21]. It was essential to characterize food-derived melanoidin structures and MRPs chemically in order to study the physiological consequences. Also required were melanoidins' structure-specific physiological effects *in vivo*. After a certain effect *in vivo* has been proven scientifically, it may be possible to find a biomarker for MRPs and melanoidins that are physiologically active [22].

We intake large amounts of melanoidins, but we don't know much about how they're metabolized in the human gut. As far as we know, few studies have analyzed the role of digestive enzymes or microorganisms in our digestive tracts on melanoidin breakdown. Combination of glucose and glycine that had been roasted had an effect on large intestine bacteria dynamics in research. Using human feces as the precursor material, a batch culture fermenter containing these microorganisms produced a rise in their total numbers within just 24 hours. No breakdown products were found, but it is clear that the melanoidins made when roasted glucose and glycine are mixed together may be used as food by gut bacteria [23]. The stimulation of *Bifidobacteria* growth and the generation of short-chain fatty acids in a fermenter containing human fecal bacteria were further signs of bacterial breakdown of melanoidins obtained from a gluten-glucose combination treated in water for one hour. Since the growth of *Bifidobacteria* was slowed when gluten-glucose mixtures were heated for only two or three hours, it is likely that microbial breakdown is restricted to less complex melanoidin structures, which are more likely to occur in water than in the absence of water and at lower temperatures [24].

1.5. Structural and Chemical Properties

Even though a great number of initiatives have been devoted to isolating and purifying melanoidins from foods like bread crust and coffee, and from sugar–amino acid model systems, very little is known about the general structural features of

melanoidins. However, some preliminary research on the structure of melanoidin has been completed, and it has been revealed that high molecular weight (HMW) melanoidin formations can be derived from water-free model systems as well as from food products. After being subjected to heating process, the uncertain profiles of models such as bread crust, coffee, and tomato sauce were analyzed to determine the composition of the melanoidins. It was discovered that the melanoidins were largely made up of furans, along with carbonyl compounds [24].

In alkaline aqueous xylose–glycine, glucose–glycine, and xylose–β-alanine reactions, which were carried out under the influence of nitrogen (pH 8.1, 60 % ethanol, 26.5 °C, and 48 hours), blue and red pigments with polymerizing activity were identified. It was found that the blue pigments, known as pyrrolopyrrole dimers, have a new chemical structure that is made up of two pyrrolopyrrole rings connected by a methine bridge [25]. Additionally, it was discovered that the red pigments, known as pyrrolopyrrole azepines, have distinct structures that are made up of pyrrolopyrrole, pyrrole, and azepin rings. Both the xylose–glycine and glucose–glycine systems yielded yellow substances with pyridinone or azepinone rings. It also generated a polymer chromophore from neutral pentose and methylamine in an aqueous MR model system (120 °C, 1 hour) with exceptional polycondensation activity. These low molecular weight (LMW) colorants polymerize, indicating they are reaction intermediates in melanoidin production [26].

The pH value seems to be a major component that determines the structure of the chromophoric melanoidins. Moreover, the temperature and time of the reaction limit the melanoidins' molecular weight. The formation of HMW melanoidins has been explained in three different ways. One theory indicates that higher molecular weight-colored structures are formed in the later stages of the Maillard process by polymerization of lower molecular weight Maillard reaction intermediates. The polymerizing actions of low molecular weight colorants may function as reaction intermediates during polycondensation (Figure 1.7). As a result, the molecular weight of the final melanoidins would be dependent on the amount of time that the mixture was heated [27].

In the early phases of the MR, reducing sugars combine with amino compounds to generate a schiff base adduct, which is later stabilized by an Amadori rearrangement. -dicarbonyls, aldehydes, furaldehydes, and furanone are produced, which rapidly react with each other in an aldol-type condensation. Finally, these intermediate products react

with amino acids to generate LMWs, which then polymerize to form HMWs. HMW MRPs can also be created by modifying proteins using intermediate products. Because of the more varied pool of reactants in real foods, melanoidin composition is more complicated than in model systems. The synthesis of LMW and HMW melanoidins is dependent on the degree of response time, which is around 2 hours. In foods with a higher water content, such as beer, sweet wine, and grape syrup [28].

No matter how different their chemical makeup, investigations show that the of melanoidins present in both foodstuffs and model systems are generally negatively charged. The anionic nature of the melanoidins in coffee was discovered to be distinct and to contain a negative charge. High molecular weight (HMW) melanoidins in coffee were shown to be more negatively charged than low molecular weight (LMW) melanoidins. The source of the negative charges in coffee melanoidins has been proposed to be chlorogenic acids. But even without chlorogenic acids, melanoidins made from sugar-amino acid model systems demonstrated anionic characteristics. Melanoidins are produced by reacting glucose with lysine, for instance, might be separated into 14 bands spanning a pI range of 3.5–4.85, showing that they were negatively charged at neutral pH. Under these circumstances, the anionic characteristics of the melanoidins were defined by the type of amino acid present during the heating process [29].

In the water-free conditions, transglycosylation reactions of saccharides and aldol condensations of highly reactive α-dicarbonyl compounds, which are the essential precursors during the early stages of MRPs formation, are thought to have produced the postulated carbohydrate-based skeleton visible in Figure 1.5. This structure has carbonyl groups that could combine with amino acids to make melanoidins with branches (Figure 1.5).

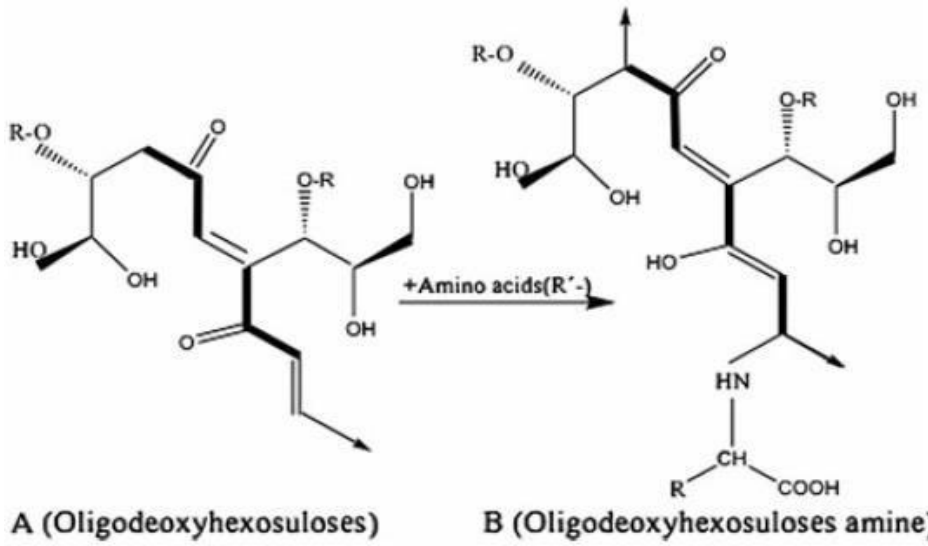

Figure 1.5. Melanoidin structure that is based on carbohydrates (A) oligodeoxyhexosuloses, (B) oligodeoxyhexosuloses amine (Source: H. Wang et al., 2011.)

It has been commonly agreed that melanoidins are colored molecules with a high molecular weight (HMW), as well as having nitrogen. On the other hand, the latest study revealed that melanoidins also contain an LMW proportion. High molecular weight (HMW) melanoidins make up over 59% of coffee's total melanoidin content (>12–14 kDa) [30]. It has been discovered that extended heating promotes largely the development of HMW melanoidins, which exhibit a brown color that is much more intense than that of LMW melanoidins. Likewise, it has been demonstrated that polymers of HMW melanoidins that are extracted from sweet wines (> 12 kDa), roasted malt (> 60 kDa), and roasted cocoa beans (> 5 kDa) are responsible for the brown color that is exhibited by these foods [31].

Additionally, other reaction mixtures and conditions, such as the heating of glucose and fructose-amino acids to 100 °C for two hours, the heating of lactose and glycine to vaporizing conditions for 5 hours, and the heating of glucose and an alanine/glycine mixture to 95 °C for four hours, all produced low molecular weight colored substances (3.5 kDa). Moreover, when casein and glucose are mixed, a large number of colored melanoidins with high molecular weight are generated. Melanoidins are high-molecular-weight molecules that mostly result from the Maillard reactions that take place between proteins and carbohydrates in real foods. Low molecular weight (LMW) chromophoric melanoidins may develop in the first stages of the MR. During

the later phases of the MR, these LMW melanoidins may subsequently polymerise or cross-link with other MRPs to produce high molecular weight (HMW) melanoidins (Figure 1.6). Given that the heating process has a significant impact on the molecular weight of melanoidins made from MRs [32].

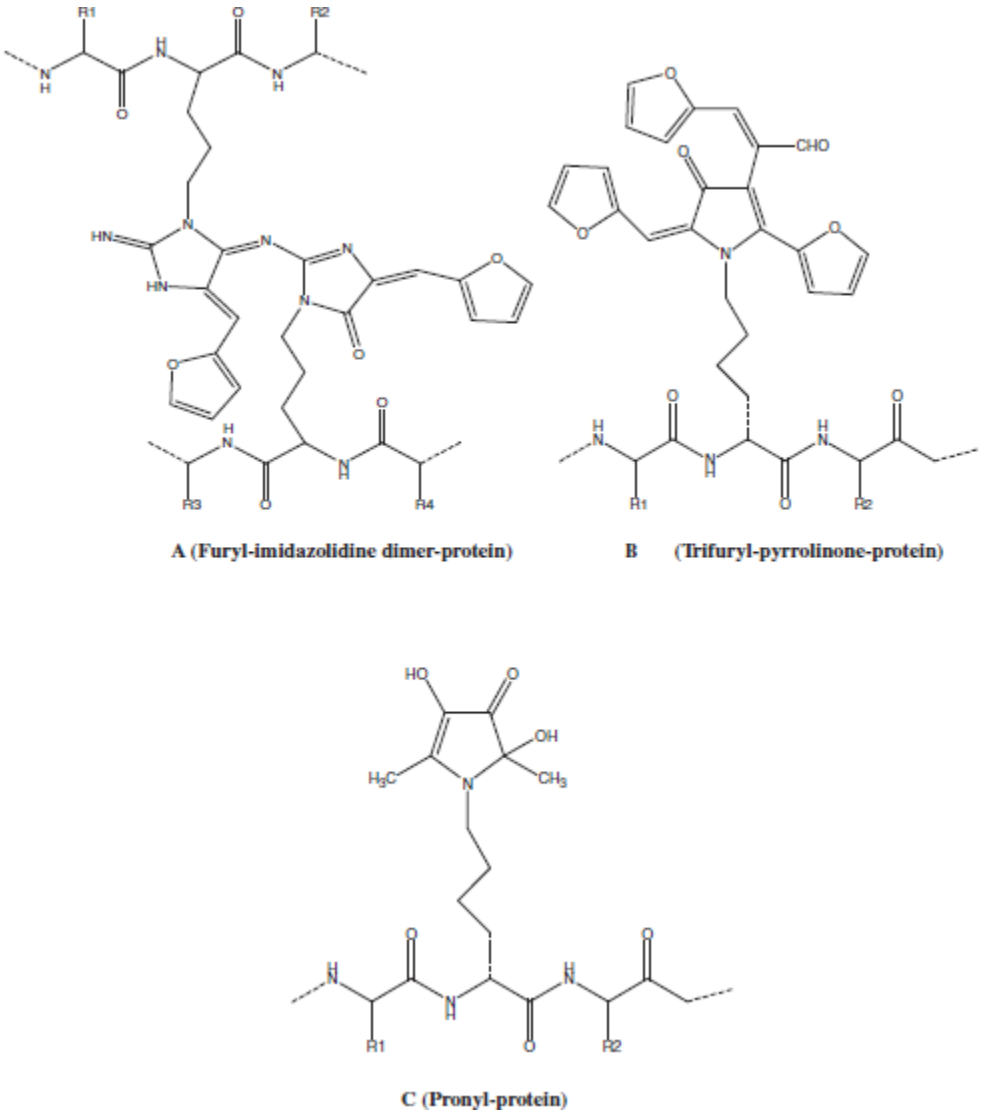

Figure 1.6. The suggested structures of chromophoric LMW and HMW protein cross-linking with melanoidin (A) furyl-imidazolidic dimer-protein, (B) trifuryl-pyrrolinone-protein, and (C) pronyl-protein (Source: H. Wang et al., 2011).

1.6. Isolation of melanoidins

The chromatographic and electrophoretic procedures have been used to separate and purify melanoidins from various food items, such as coffee [33], vinegar [34], bread [35], and dark beer [36]. In a model system, ultrafiltration using membranes with a molecular weight cut-off limit ranging from 1 to over 300 kDa is a typical technique for isolating melanoidins' high and low molecular weights (MW cut-off 12–14 kDa). Gel filtration, anion-exchange chromatography, copper-chelating chromatography, Sephadex LH-20, Sephadex G-25 on octyl Sepharose and chromatography were some of the other forms of column chromatography that were used in the process of further purifying melanoidins [37]. Studies have shown that there is a polysaccharide backbone that is present, along with proteins and amino acid-derived substances. Phenolic chemicals are covalently bonded to the polysaccharide backbone. Because of the various origins these compounds have, it is still rather difficult to establish the origin of volatile chemicals that are essential for flavor. Recently, the food industry has focused on this topic, so it would want to be able to manufacture and manage the unique odors and colors that are produced via baking, roasting, and cooking. Capillary zone Electrophoresis is used to separate water-soluble melanoidins from carbohydrate/amino acid model systems and medium-roasted coffee. [38].

After being separated, the melanoidins were analyzed to establish whether or not they had a saturated or aromatic character. Melanoidins produced from various solutions and then subjected to the same heating process have the same apparent molecular weight, but their charge-to-mass ratios are distinct from one another, indicating that they have varying levels of saturation. Melanoidins isolated from model systems including lysine had a lower saturation than melanoidins isolated from coffee or other model systems containing glycine, alanine, or tryptophan, which exhibited a higher saturation. Melanoidins might be primarily composed of a stable structure or a common core that changes according to the temperature conditions that are applied via a more saturated structure. This depends on the kind of amino acid that is used as the reactant [39].

1.7. Biological Activity of melanoidins

MRPs are found in a wide range of foods, so it's vital to examine if they are secure and how they affect the food's nutrient benefit. Melanoidins can change the odor, taste, and appearance of foods, although they can also have an effect on health. Melanoidins are hard to give clear features to because they come in many different forms. They are arduous to purify and identify. They don't dissolve well in water or organic solvents. Melanoidins are often associated with low-molecular-weight molecules, which may affect their properties. Also, most organisms tend to break down and use the substance [40].

Potential advantages and disadvantages of melanoidins' presence in foods are currently being considered. In the past, melanoidins were mostly considered to be the primary factor responsible for the decline in the nutritional content of food. This is primarily attributable to the deactivation or degradation of amino acids or proteins, a decreased digestibility of nitrogen, and poor absorption of brown-colored substances in the gut as a consequence of the inactivation of enzymes [41]. Melanoidins have been found to reduce the function of many proteolytic enzymes in the gastrointestinal system, including trypsin. This was demonstrated both *in vitro* and *in vivo*. Melanoidins exhibit a wide array of advantageous benefits, in contrast to the Maillard reaction's end-products, which have a negative impact on the quality of the foodstuff. They promote health by functioning as antioxidants, antibacterial agents, antihypertensive agents, antiallergenic agents, and prebiotic agents. Melanoidins not only have the capacity to bind metal ions, but they are also thought to be substances that limit the formation of tumors and prevent mutations from occurring. Products with melanoidins in them may have a longer shelf life [42].

1.7.1. Antimicrobial activity of melanoidins

The fact that melanoidins normally occur throughout the digestion process makes their antioxidant action more noteworthy. Melanoidins are typically generated. The majority of research that has been done on the impact that MRPs have on microorganisms has been conducted in specialized microbial growth medium. These studies have shown that MRPs may either promote or impede the development of

microorganisms. According to the findings of the most comprehensive study to date on the effect of MRPs on the growth of microbes, Bacteriostatic action of MRPs is influenced by their kind and concentration, as well as by the pH and temperature of the culture medium and their molecular weight (MW). In this way, compared to products with an LMW, those with a MW larger than 1,000 Da show a higher level of activity. In addition, the research that was referred to showed that the antimicrobial effect of the MRPs was regulated by a reduction in the solubility of iron, which led to a reduction in the amount of glucose and oxygen that was taken in by the microorganisms. Also, a study of the antibacterial properties of different melanoidins made from glucose-amino acid model systems [43].

According to research published in the literature, melanoidins' antibacterial effect may be linked to their anionic charge and their capacity to chelate certain cations, such as Fe, Zn, and Cu, that are necessary for the development and viability of harmful bacteria. Because they are negatively charged, melanoidins can also break the bilayer plasma membrane of *Escherichia coli* by chelating the stabilizing cation Mg^{+2}. At smaller doses of melanoidins, the antimicrobial activity was shown to be bacteriostatic, but at greater concentrations, it was found to be bactericidal. The fact that the microbicidal process is dependent on the concentration of melanoidin may have something to do with the metal-chelating characteristics of melanoidin. There have been three main proposals put forward on the methods by which melanoidins exert their antimicrobial property. Melanoidins, when present in low quantities, are capable of exerting a bacteriostatic action that is primarily mediated by the removal of iron from the culture medium. Chelation of the siderophore-Fe^{3+} complex by melanoidins has been reported in bacterial species capable of producing siderophores for iron absorption. This phenomenon, which might lead to a reduction in the pathogenicity of harmful bacteria, has been seen in bacterial species. When present in high quantities, melanoidins have the ability to damage both the outer and inner membranes. This is done by removing Mg^{2+} ions from the outer membrane, which makes the inner membrane unstable in the end [44].

The study found that gram-positive bacteria lack an outer barrier, making them more susceptible to antimicrobial agents. The antimicrobial effect of Maillard reaction products is achieved through reducing microbial growth. While food-derived melanoidins have a strong antibacterial effect, the antibacterial effect of model system

melanoidins depends on the low molecular weight (LMW) Maillard-derived chemicals related to their melanoidin center. This is an important distinction. Melanoidins found in foods may have such potent antibacterial properties because they are synthesized from a complex reaction medium that contains an array of amino acids, sugars, and proteins. This results in polymers with a significant level of complexity. Simultaneously, melanoidins had more powerful antibacterial action than LMW compounds in gluten-glucose models [45].

1.7.2. Antioxidant activity of melanoidins

Harm to proteins and DNA may be caused by reactive oxygen species, which can ultimately result in the death of the affected cells. In a healthy body, cells might have a set of enzymes that are responsible for regulating the amount of oxidants present. However, it is also of the utmost need to offer exogenous antioxidants together with food. In addition to their positive effects on health, the antioxidants that are produced during processing and storage are thought to improve the product's overall quality and make it tastier. Melanoidins have the potential to act as additional antioxidants that are developed during the heating processing of food [46]. This capacity has been extensively studied via the use of synthetic model melanoidins, in addition to melanoidins that are acquired from foods such as coffee [47] and bread [48]. The antioxidant activity of melanoidins *in vitro* has been studied using a variety of different methods. These techniques include the assessment of antioxidant activity ($ABTS^+$ test), free radical scavenging activity (DPPH assay), and ferric reducing capacity (FRAP). MRPs have been demonstrated to have the ability to chelate metal cations, notably iron, and scavenge free radicals, such as hydroxyl, superoxide, and peroxyl radicals. This capacity enables MRPs to have an antioxidant capacity. Because the form of MRPs is still mostly unclear, it is hard to ascertain whether proportion, high or low molecular weight, is responsible for this activity. This is due to the fact that MRPs are still totally unprecedented [49].

It has been shown that the antioxidant capacity is the product of intermediate and low molecular weight (LMW) MRPs, and the impacts of these molecules have been the focus of a significant amount of study with regard to the conservation of foods. In any

event, the mechanism behind how melanoidins exert their antioxidant action is not completely understood since the molecular structure of melanoidins has not been determined. It is generally accepted that the process relies on the capacity of melanoidins to either scavenge oxygen radicals, trap positively charged electrophilic species, or perform outright metal chelation in order to produce non-active compounds [50]. Browning that does not involve the action of enzymes may lead to the synthesis of D-amino acids in many plant- and animal-based meals. This happens when amino acids are present in close proximity to one another. Concerning the racemization process, it is hypothesized that the creation of reversible Schiff bases is the first step in the interaction of amino acids with glucose or fructose. This is where the reaction begins. The steric and electrical features of the amino acid side chains are particularly important factors that determine the level to which racemization occurs. It is important to note that the first phases of the MR happen under modest circumstances and do not need an alkaline or acidic environment in order to progress. This novel racemization process, which is based on the comparatively stable Amadori compounds, has been conducted to analyze the formation of free D-amino acid in foodstuffs such as fruits, fruit juices, and fortified wines. Current findings involving the heating of synthetic Amadori compounds indicate that these products are providers of the enantiomers of amino acids [51].

Furthermore, melanoidins have a protective role for the cells they are present in. It was determined that the capacity of HMW compounds to scavenge free radicals was responsible for their preventive activity. In addition to this, it has been shown that melanoidins extracted from meals may protect cells *in vitro* against oxidative stress. It was shown that the soluble portion of melanoidins derived from biscuits and coffee, when ingested by replicating the circumstances of stomach and pancreatic digestion, protected cultured human hepatoma HepG2 cells from oxidative stress. There has been a lot of research done on the coffee brew melanoidins and their antioxidant activities [52].

1.7.3. Antibacterial and Prebiotic activity

The Salmonella mutagenic test system was used in the first investigation to demonstrate the inhibitory mechanism of antibacterial MRPs. According to the findings, the MRPs that were examined did not have a mutagenic impact, and the antimicrobial action of the MRPs was primarily caused by the MRPs' interaction with iron, which

reduced oxygen absorption. Coffee melanoidins' capacity to inhibit the growth of multiple harmful microorganisms has recently been put to the test. The findings demonstrated that melanoidins, which were produced by iron chelation from the culture media, served as bacteriostatic agents at low levels. Melanoidins, however, showed a bactericidal action at high concentrations by removing Mg^{2+} cations from the outer membrane, which encouraged the breaking of the cell membrane. Additionally, the bactericidal capabilities of cocoa fractions of varying molecular weights extracted from roasted cocoa have been investigated [53]. The results reveal that the growth of several species *Bifidobacteria* was also suppressed, despite the fact that almost all of the components were able to prevent the development of harmful bacteria [54]. Infection with *H. pylori* is the major cause of peptic ulcers as well as gastric cancer, and the colonization of *H. pylori* in the stomach may be inhibited by the presence of anionic polymers such as dietary melanoidins. Extracellular urease proteins are gastric mucin-targeted adhesins that may be found on the surface of *Helicobacter pylori*. These adhesins serve a significant role in the process of infection and colonization of the host. Furthermore, it has been shown that melanoidins, which are produced by heating casein and lactose, have an anti-colonization impact in both an animal model and in human individuals who have been infected with these bacteria. They concluded that melanoidins might be used as an alternative to antibiotic-based treatment to limit the development of *H. pylori*. This therapy would have to be harmless, simple to administer, and effective. The fact that the antibacterial activity increased in proportion to the severity of the heat treatment lends support to the idea that melanoidins have a critical role in this process [55].

Melanoidins from coffee, which are fermented by the microbiota in the digestive tract, act as a kind of soluble dietary fiber. The microbial breakdown of HMW molecules in coffee generated large levels of acetate and propionate. A possible explanation for this is that coffee's polysaccharide-rich melanoidins, such as those found in coffee, are more likely to be digested by *Bifidobacterium* than protein-rich melanoidins, like those found in protein-glucose mixes or milk-like systems. The LMWs, colored molecules connected to the gluten polymer, make up the bread melanoidins [56]. In a static batch culture experiment, melanoidins from various bread kinds were tested for their possible prebiotic action. The results suggest that anaerobic bacteria, notably *Bifidobacteria*, may utilize bread melanoidins as a carbon source. Depending on the kind of melanoidins sample, the growth of bacteria is varied, which

indicates that the starting ingredients and processing circumstances have a significant effect on the bread melanoidins' prebiotic capability. Bacterial growth was shown to be reduced when bread was used instead of other sources, such as the silver skin of coffee beans. Melanoidins, on the other hand, have been shown to raise the percentage of anaerobic bacteria found in the human colon, namely clostridia and lactobacilli. This is contrary to previous studies that have shown that melanoidins have a particular impact on certain anaerobic bacteria. Melanoidins, on the other hand, may not have prebiotic effects [57].

CHAPTER 2

MATERIAL AND MEDHODS

2.1. Chemicals

D-glucose (Sigma, G8270), Glycine (AppliChem, A1076), Arginine (Sigma – Aldrich, A5006), Potassium persulfate (K_2S2O_8), Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), ABTS (2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid), Trichloroacetic acid (TCA) (Rieder-de Haen, 24529), Trypsin (Sigma, 93610), Calcium chloride (AppliChem, A3652), NAOH (Merck, 106498), Nutrient Broth (NB) (Merck, 105443), Agar (Fluka, 05039), Tryptic Soy Broth (TSB) (Merck, 105459), Gelatin powder (Merck, 104078).

2.2. Preparation of Melanoidins Model Systems

Model melanoidin systems were prepared according to [58] Two model systems are developed using a sugar (D-glucose) and amino acids in combination (arginine and glycine). Glucose (0.05 mol, 9.00 g), arginine (0.05 mol, 8.76 g), and glycine (0.05 mol, 3.75 g) were dissolved in distilled water (20 mL) before freeze-dried at temperature of 20 ℃ for 24 h. The freeze-dried samples were taken in a beaker and baked for 2 hours in an oven at 125 ℃. Following the heating process, the baker was taken from the oven and left in a desiccator to cool at room temperature. The solid was placed in a mortar and thoroughly ground to a powder form. 5 g of the powder was dissolved in 20 mL of distilled water, and the solution was mixed to dissolve as much material as needed. The filtrate was obtained after the combination was filtered with Whatman 595 filter paper. The solid particles were washed thoroughly (2x10 mL) over filter paper, and all filtrates were mixed to yield soluble melanoidins. Finally, melanoidins were stored at -20 ℃.

2.3. Purification of Bread Crust Melanoidins

The bread crust preparation procedure was carried out way described by [59]. The baking and preparation of the bread was done in the laboratory. 150 mL of water, 6 g of sugar, 3 g of salt (NaCl), 6 g of oil, 150 g of white wheat flour, and 2 g of yeast (Saccharomyces cerevisiae) were used in the preparation of the bread. The material was mixed with the aid of a mixer for 5 minutes in order to distribute it homogeneously. The bread was kept at room temperature for 90 min to leaven. Bread was heated at 200 °C for 15 minutes. 85 g of bread crust powder was digested for 72 hours at 37 °C in 490 mL of 0.2 M Tris-HCl buffer (pH 8.0) containing 0.367 mg (0.7 U/mL) of trypsin and 0.77 g $CaCl_2$. To stop the reaction, 15 ml of TCA at a 40% concentration was added to the solution at the end of the procedure. The filtrate was collected through a Whatman 595 filter paper and ultrafiltration (10 kDa ultrafiltration membrane) was used after centrifugation (10.000 x g, 4 °C, 15 min) was carried out. The solution was then lyophilized, and kept at -20 °C, and ready for use.

2.4. Gel Filtration

Gel filtration method was used to separate melanoidins according to their molecular weight. 4 gr sephadex-50 was dissolved in 50 ml of distilled water in the beaker. The solution was stirred gently and left overnight at room temperature to allow complete swelling of the gel. then slowly transferred to a column of x dimension. The column was washed with 2 times the column volume with distilled water. 1 g of melanoidin was dissolved in 3 ml of water, the samples were placed on the column and separated. The absorbance of the eluent was measured using a UV-Vis photometer (SHIMADZU UV-2450) at 280 nm and 305 nm to provide an accurate reading. The column was washed with distilled water and placed in the tris-buffer in the beaker and stored.[47].

2.5. UV-vis Spectroscopy

The UV-vis (SHIMADZU UV-2450) spectra of the melanoidins were measured by absorbance spectrum detection at wavelengths ranging from 200 nm to 700 nm. The compounds were dissolved in distilled water at 0.1 mg/mL concentration [60].

2.6. Fouirer Transform Infrared Spektrofotometre (FTIR) Spectroscopy

Lyophilization, which took place in a freeze dried overnight, was used in order to remove all water from melanoidins samples (Labconco, FreeZone 18-liter freeze dry equipment). The spectra of the samples powder were analyzed with the use of a Perkin-Elmer spectrometer that had a MIR TGS detector (Spectrum 100 Instrument, Perkin Elmer Inc., Norwalk, CT, USA). The FTIR spectra of the materials were obtained by collecting them at different frequencies ranging from 4000 to 450 cm^{-1}. It was established that the resolution of the interferograms was 2 cm^{-1}, and a total of 20 scans were carried out. Before any additional processing could be done to any of the spectra, the spectrum of the background was extracted from each sample. The program Spectrum 100 was used to make all of the necessary adjustments to the data (produced by Perkin Elmer). On each sample, we performed at least three different scans, each of which resulted in an equal spectrum, and then we averaged the results. The spectra of these duplicates were first mean, and then the resulting averaged spectra were used for further data analysis and data analysis. A total of three copies of each sample were carried out as a replication. After that, the spectra were given an interactive baseline by starting from two different locations that had been picked at random.

2.7. Antibacterial Assay

E. coli (ATCC 25922) and *S. aureus* (RSKK 1009 strain) were used to investigate the melanoidins' antibacterial activity. These bacterial were taken from IZTECH's Environmental Research and Biotechnology Center stocks, and the Bioengineering Research and Application Center in Izmir, Turkey. The bacteria were cultured and grown overnight. The optical density of the bacteria was calibrated to the

0.5 McFarland standard bacterial concentration: $2.5x10^7$ and $3.4x10^7$ CFU/mL for both strains. Subsequently, bacterial concentrations were adjusted in NB and TSB to 10^5 CFU/mL bacterial by dilution. The minimum doses for these tests were identified to be between 15 and 25 mg mL and 15 to 45 mg mL for *E. coli* and *S. aureus*, respectively. In addition, no melanoidins were added to the control samples. Three replicate tests were performed for each sample and bacterial culture, and the bacterial cultures were grown in an incubation shaker at 37 C and 100 rpm for 24 hours. The turbidity of the bacteria was measured using by Varioskan (Thermo) [61]. Finally, *E. coli* and *S. aureus* cells were cultured on NB and TSB medium with different concentrations of melanoidins to determine their antibacterial activity.

2.8. Antioxidant Assay

The procedure that was developed by [62] was used to test the scavenging activity of ABTS radical cation, which is abbreviated as $ABTS^{\bullet+}$. $ABTS^{\bullet+}$ was produced by exposing ABTS at a concentration of 7 mM, potassium persulfate at a concentration of 2.55 mM, and 20 mL of distilled water to a reaction at a temperature of 26.3 degrees Celsius for a period of 12-16 hours. During this time, the mixture was kept in the dark. ABTS cation solution was diluted with ethanol – water (50:50) solution to an absorbance 0.70 at 743 nm. Next, Trolox (2 mM) was dissolved into 10 ml ethanol and the calibration curves were created using Trolox at 5 serial different concentrations (between 2 mM and 0.25mM). Readings of absorbance were obtained every 60 seconds for 5 minutes using a UV- Visible Spectrophotometer (SHIMADZU UV-2450) with temperature control (30 °C). At least three times the test was carried out.

2.9. Scanning Electron Microscopy (SEM)

SEM imaging was used for two main purposes; first was to analysis the bacterial membrane of E. coli and S. aureus. Second was compared to morphological structure of melanoidins. Initially, melanoidin-treated bacterium strains were incubated for 24 hours. Pellet 1 milliliter of a bacterial culture with an optical density of 0.5 600 nm was centrifuged at 4,500 rcf. The same amount of filtered and deionized water was used by Peller for the washing process (2x). Immediately after being removed from the

centrifuge, the pellet was resuspended in 500 µL of nano-pure deionized water or buffer. A pipette tip was used to spread the cell suspension throughout the surface of the aluminum foil after it had been coated with the cell suspension (10-20 µL). After letting its surface sit undisturbed for ten minutes, a flow of water was then used to wash it. For SEM imaging, samples were coated with gold and studied using a scanning electron microscope (Phillips XL–30S FEG) at a vacuum of 0.09 mbar for 90 seconds at 15 kV in argon gas at a power of 15 mA.

2.10. High Performance Liquid Chromatography (HPLC) Analysis

Bread, Arg – Glc, and Gly – Glc melanoidins have their MWs measured using high-performance liquid chromatography (HPLC). The obtained samples were filtered through a membrane with a pore size of 0.22 µm. Chromatographic separations were performed using a GL Science Inertsil ODS3 (5 µm, 250 mm 4.6 mm) maintained at a constant temperature of 30 °C. The LC mobile system consisted of a gradient of water (eluent A), acetonitrile (eluent B), and eluent B, kept at 1% during the whole run), with a constant flow rate of 1.0 mL/min. Prior to injection, the column was equilibrated with 95% A and 5% B. After 20 min injection of the sample, this proportion was decreased to 75% A, 25% B, and in 40 min, 50% A, and 50% B. For the last 10 min, final portion flow rates were decreased by 95% A and 5% B. To re-equilibrate, 10-minute intervals were employed between injections. Melanoidins were detected by comparing their retention periods and The HPLC was calibrated with polyethylene glycol (PEG-8000 kDa) standards with known MW. The findings were represented in mg of chemical per 100 g [63].

2.11. Statistical Analysis

Using the Mann-Whitney U Test, the differences between the groups were examined. The findings of the statistical analysis are presented as means standard deviation. Statistical significance was set at $p < 0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

It is important to know their structural and functional properties of melanoidins in order to understand to antimicrobial effects of melanoidins Therefore, we investigated the structural and functional properties by using several spectroscopic methods, Fourier Transform Infrared Spectroscopy and UV-Visible spectroscopy and Scanning Electron Microscopy and biochemical techniques to determine potential activities of melanoidins.

3.1. Antibacterial Effect of Melanoidins

In this study, the bactericidal characteristics of bread melanoid were investigated and compared to model systems including Arginine – Glucose and Glycine – Glucose. The relationship between the complexity and simplicity of melanoidins' structure was analyzed. The antibacterial impact of melanoidins on gram negative (*E. coli*) and gram positive (*S. aureus*) bacteria was examined in this study. According to the findings obtained from experiments, there is a significant antibacterial activity for bread melanoidins, Arg – Glc, and Gly – Glc melanoidins. Melanoidins distrupted the bacteria's membrane structure (Table 3.1). Melanoidin concentrations were adjusted and treated to bacteria at dosages ranging from 5 to 50 mg/ml. Melanoidins had a stronger antibacterial activity on E. coli (gram negative) bacteria than on *S.aureus* gram positive bacteria. Unexpectedly, the minimum inhibitory concentration concentration of bread melanoidins was 5 mg/ml, whereas the model systems of Arg – Glc and Gly – Glc melanoidins were 10 mg/ml. Gly – Glc, bread melanoidins, and Arg – Glc melanoidins on the other hand, inhibited *S. aureus* bacteria at minimum inhibitory doses of 15, 25, and 30 mg/ml, respectively. Melanoidins were found to have higher activity against *E. coli* (gram negative) bacteria as a results. Furthermore, despite having a more complicated structure than simple model systems, bread melanoidins had a high antibacterial effect. Interestingly, melanoidins from bread *E. coli* bacteria showed

higher antibacterial effect at lower concentration. On the other hand, the Gly – Glc model system showed higher antibacterial activity at low dose against *S. aureus* bacteria. However, the Arg – Glc model system was expected to show highest activity at beginig design of the antibacterial assay for melanoidins. Because the positively charged guanidine group in the arginine structure was thought to play an important role in the destruction of the bacterial membrane structure [64]. In general, melanoidin groups showed antibacterial effect, but there were no big differences minimum ınhibitor concentration (MIC) among melanoidins. In addition, a significant finding of this study is that bread melanoidins have an effective antibacterial property.

Table 3.1. Minimum inhibitor concentration (MIC) of melanoidins for both *E. coli* and *S. aureus.*

Type of melanoidin	**Bread (mg/ml)**	**Glucose – Glycine (mg/ml)**	**Arginin – glucose (mg/ml)**
E. coli	5	10	10
S. aureus	25	15	30

To determine the change in absorbance of melanoidin-exposed bacterial cultures, absorbance was measured at 600 nm. every hour with a varioscan instrument overnight. Results were calculated to determine the difference between the first and last measurements. When melanoidins were used to treat *S. aureus* and *E. coli* ba, Arg – Glc melanoidins model systems showed highest absorbance changing and Gly – Glc and bread, respectively. However, the change in melanoidin absrobance appears to be rather low when compared to the control group (Figure 3.1). Since melanoidins prepared at minimum inhibition doses were added to bacteria, low absorbance change was an expected result. However, a high change occurred in the model system of Arg – Glc melanoid, where the change in Gly – Glc and bread melanoidins was low. This result may be due to the intense color of Arg – Glc melanoidins (Figure 3.2).

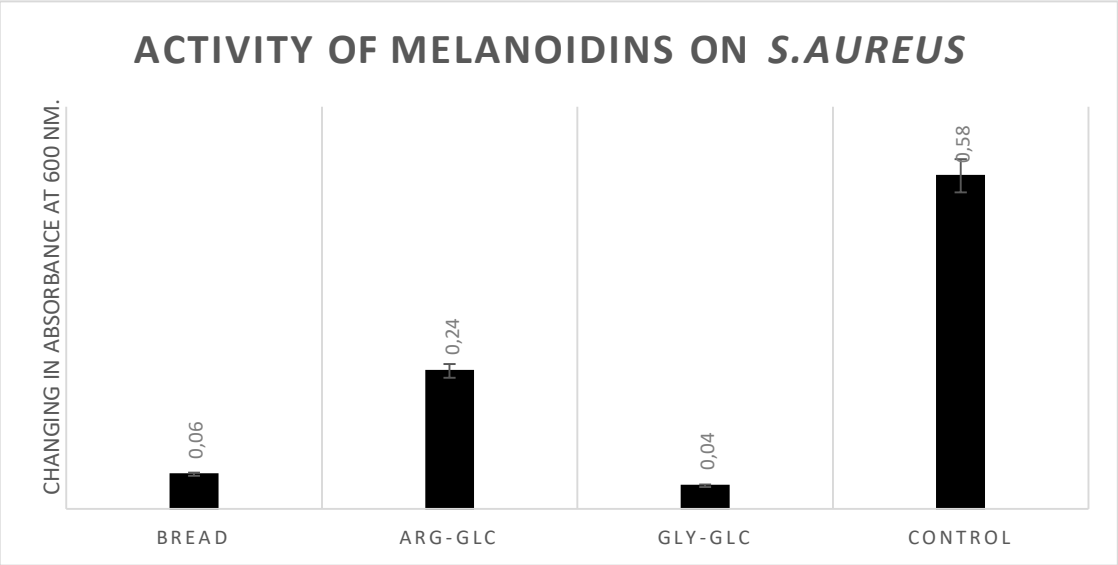

Figure 3.1. Changing absorbance at 600 nm. for melanoidins (bread, Arg – Glc and Gly – Glc.) and control group on *S. aureus*.

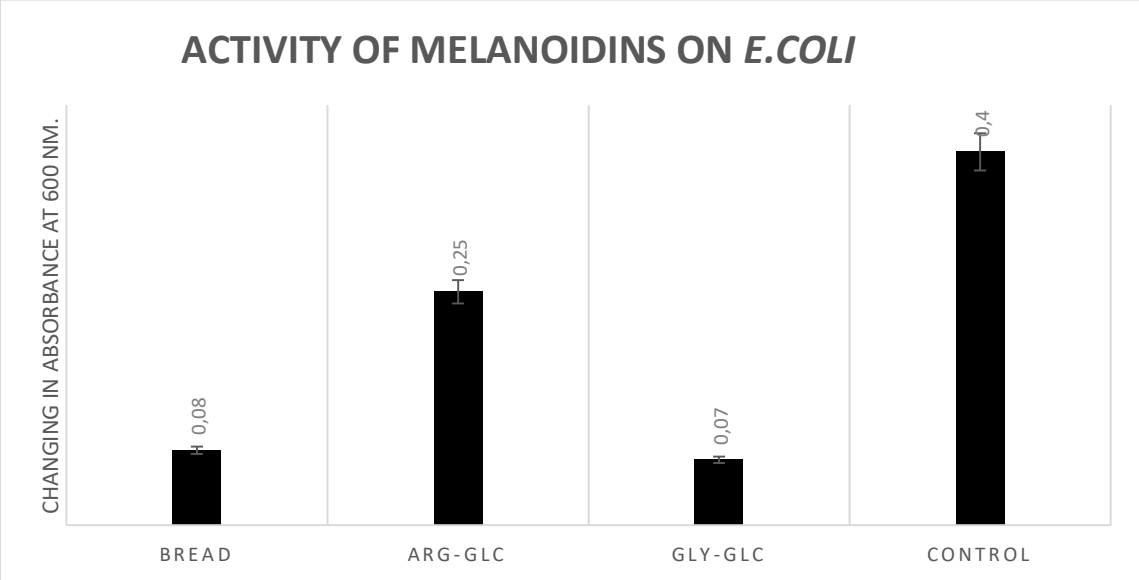

Figure 3.2. Changing absorbance at 600 nm. for melanoidins (bread, Arg – Glc and Gly – Glc.) and control group on *E. coli*.

3.2. Antioxidant Activity of Melanoidins

Antioxidant activity of melanoidins were measured by ABTS radical scavenging. Concentration of melanoidins were adjused 20 mg/ml. statistical data were calculated as ABTS radical scavenging percentage and the equation formulation is as follows: $(A_0 - A_1) /A_0 \times 100$. A_0 is control absorbace, A_1 is absorbance of sample. While the model system of Gly-glc melanoid showed the highest antioxidant activity with a rate of 73.66%, the model system of Arg-Glc melanoid showed antioxidant activity with a rate of 46.35% and bread melanoid with a rate of 13.65%, respectively (Figure 3.3). The ABTS method is a technique that uses decolorization to identify the samples' antioxidant capacities. The antioxidant qualities of the samples are assessed using the color change in ABTS. When compared to bread melanoidins, the color intensity of the Gly – Glc and Arg – Glc melanoid model systems was much greater. Therefore, the large differences in the findings may be due to the color intensity melanoidin model systems.

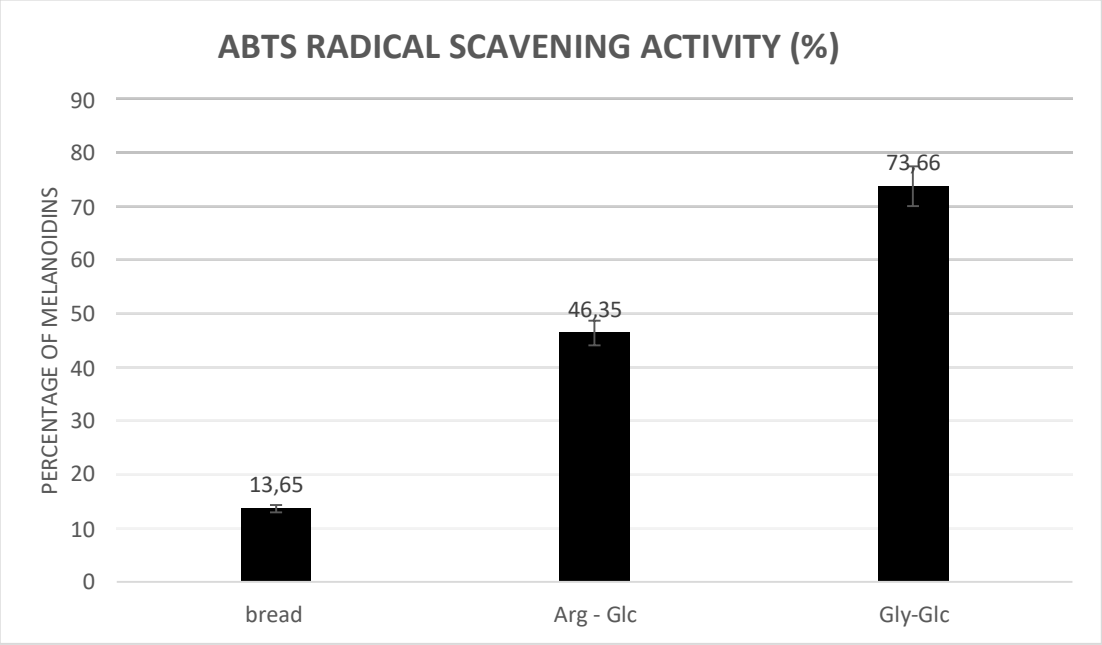

Figure 3.3. $ABTS^{\bullet+}$ radical scavenging activity (%) of melanoidins.

3.3. Analysis of Melanoidins Morphological Structure of Melanoidins and Bacteria Membrane by SEM

Several approaches have been taken to understand the antibacterial properties of melanoidins on bacteria. In the first place, the antibacterial action of melanoidins, even at low concentrations, was due to the chelation of iron from the growth medium. Bacterial strains with the capacity to produce siderophores to acquire iron were affected by this. The siderophore-Fe^{3+} complex will be chelated by melanoidins in the following stage, which might reduce the effects of the bacteria. In the end, it was discovered that large dosages of coffee melanoidins had powerful antibacterial effects. Removed Mg^{2+} ions from the cell's outer membrane were used to accomplish this. This resulted in a rupture in the cell membrane, causing cytosolic materials to leak [65]. Based on these findings, SEM imaging technique was used to better understand the interaction between melanoidins and bacterial membrane.

Data demonstrated that bread melanoidins performed antibacterial effect against *E. coli.* It caused severely disruption bacterial membrane and spread intracellular materials out of the cells which is seen below (Figure 3.4).

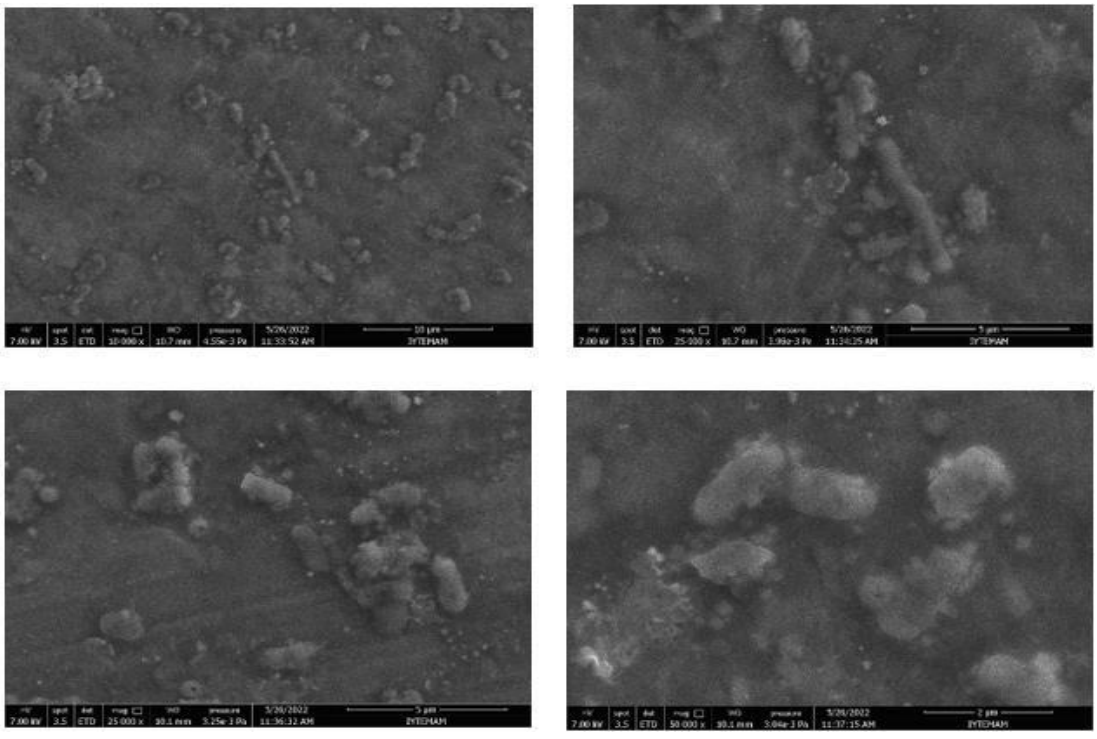

Figure 3.4. Antibacterial action of bread melanoidin on *E. coli*.

On the other hand, Arginine – Glucose melanoidins system shown significant bacterial effects. they caused the formation of various cavities on the *E. coli* membrane and adversely affected the integrity of bacteria. In addition, it is seen that there are significant deteriorations in the bacterial cell structure (Figure 3.5.).

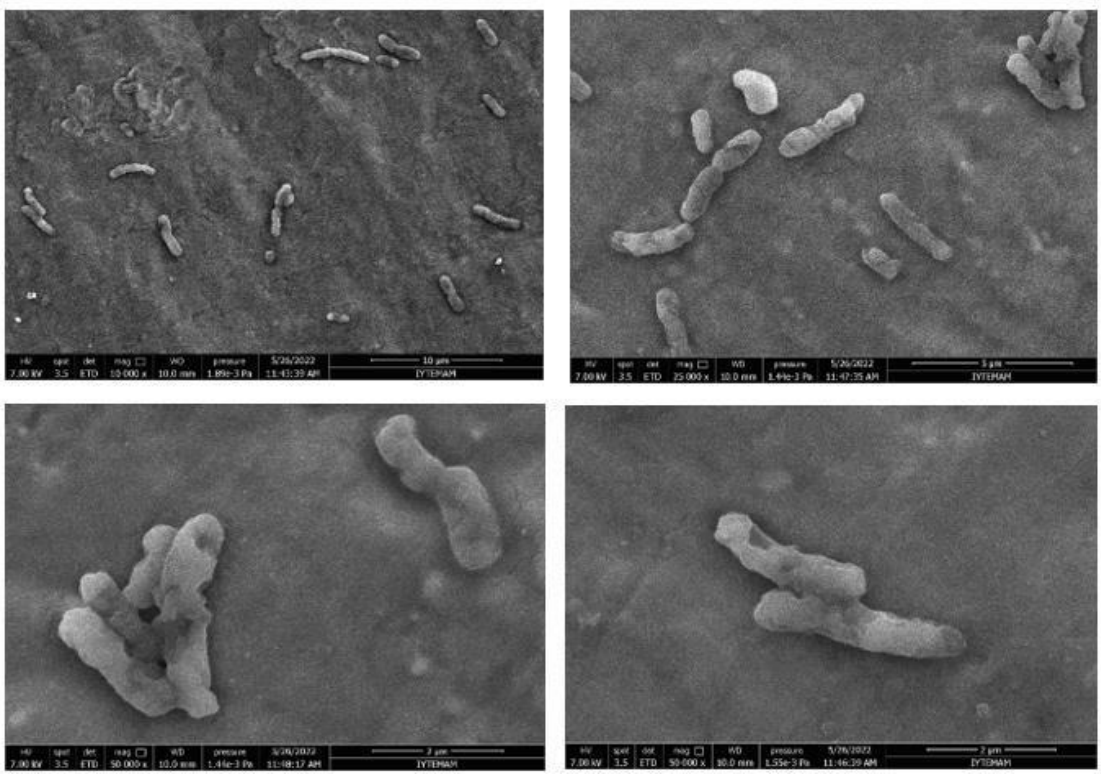

Figure 3.5. Antibacterial action of Arg – Glc melanoidin on *E. coli.*

Lastly, the Glycine – Glucose melanoidins model system exhibited antibacterial characteristics by disturbing the membrane structure of *E. coli* bacteria. Although the bacterial membrane can malfunction by Glycine – Glucose melanoidins, bacterial integrity appears to be preserved when compared to bread and Arginine – Glucose melanoidins (Figure 3.6).

Bread melanoidins were shown to totally disrupt the bacterial membrane while inhibiting the growth of *S. aureus* bacteria. It had a similar impact to that seen in *E. coli* bacteria (Figure 3.7).

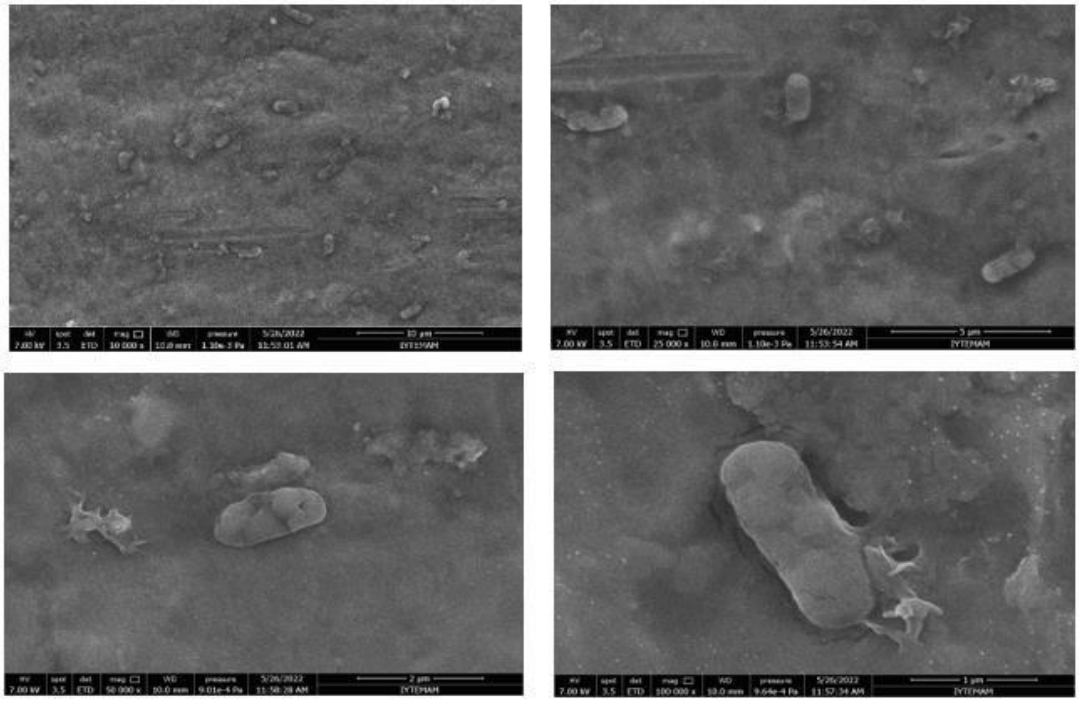

Figure 3.6. Antibacterial action of Gly – Glc melanoidin on *E. coli*.

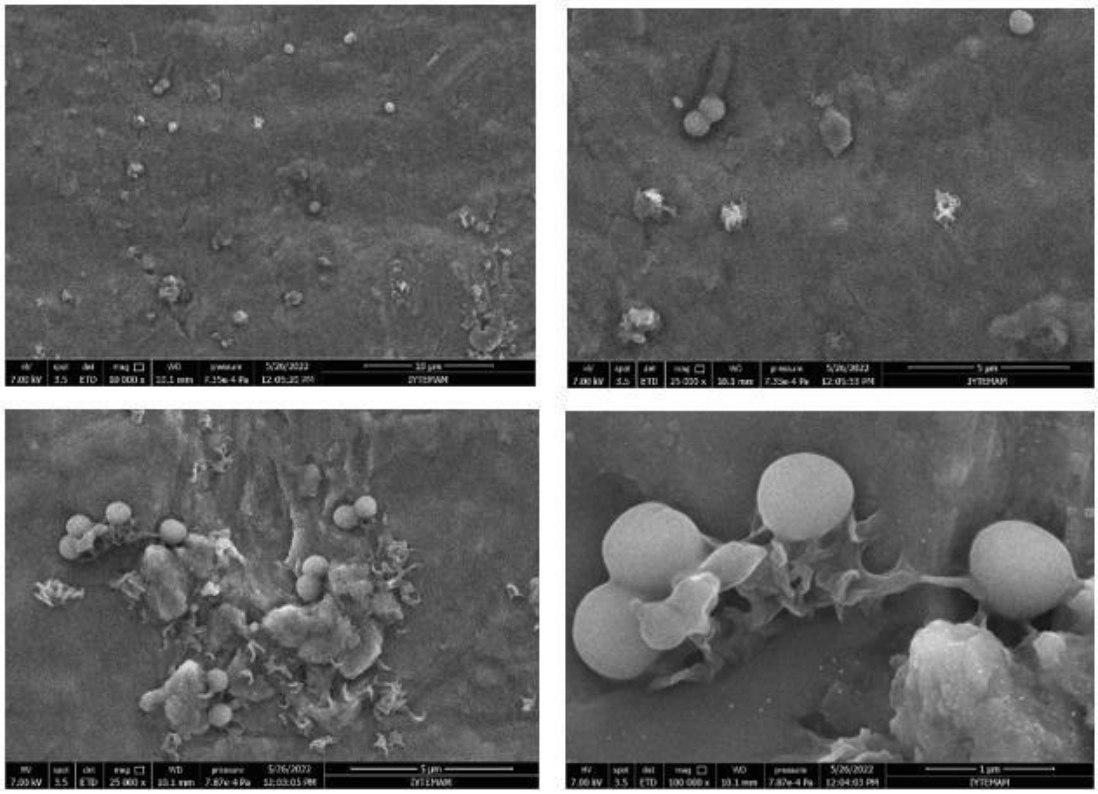

Figure 3.7. Antibacterial action of bread melanoidin on *S. aureus.*

The Arginine – Glucose Melanoidins System has demonstrated strong bacterial impacts. They were responsible for the development defection on the membrane of E. coli, which had a negative impact on the bacterial cell wall. In addition to this, it has been shown that the structure of the bacterial cells has significantly deteriorated (Figure 3.8).

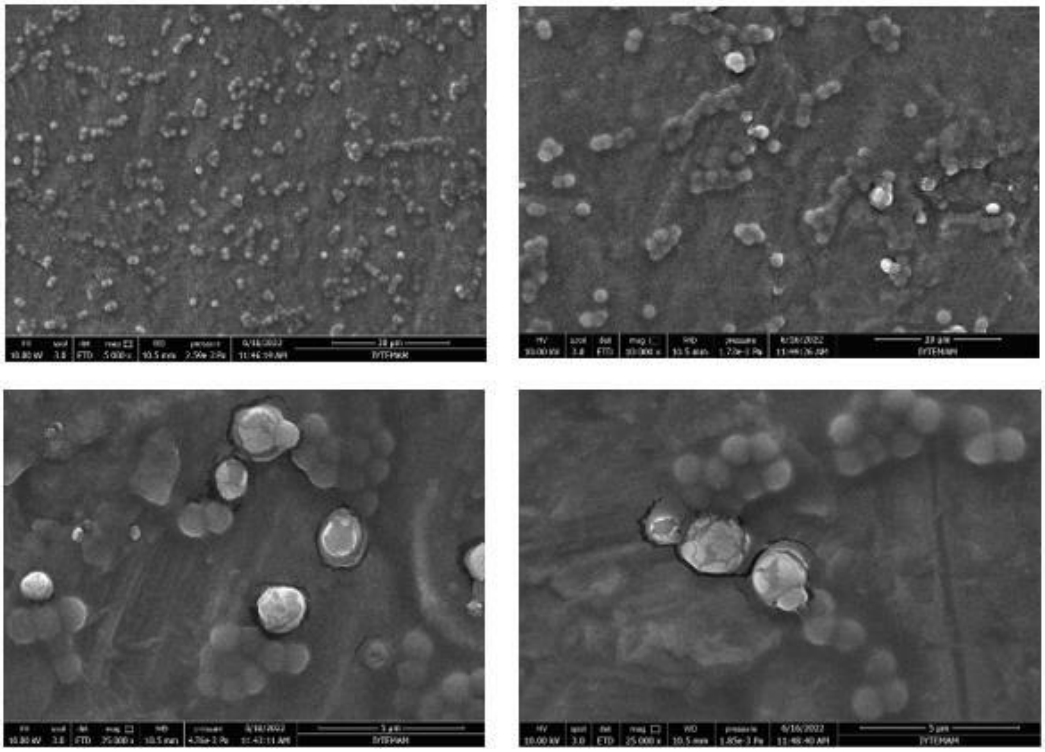

Figure 3.8. antibacterial action of Arg – Glc melanoidin on *S. aureus.*

Glycine-glucose melanoidins have been shown to kill bacteria by destroying their cell membranes and breaking up their cells (Figure 3.9).

Overall, the bread melanoidins were determined to be the most harmful melanoidin to *E. coli* and bacteria, whereas the Glycine – Glucose melanoidin model systems caused highest damage on *S. aureus* membrane structure at lower concentration of melanoidins. Bacterial membrane structures show no degeneration in the absence of melanoidin (Figure 3.9).

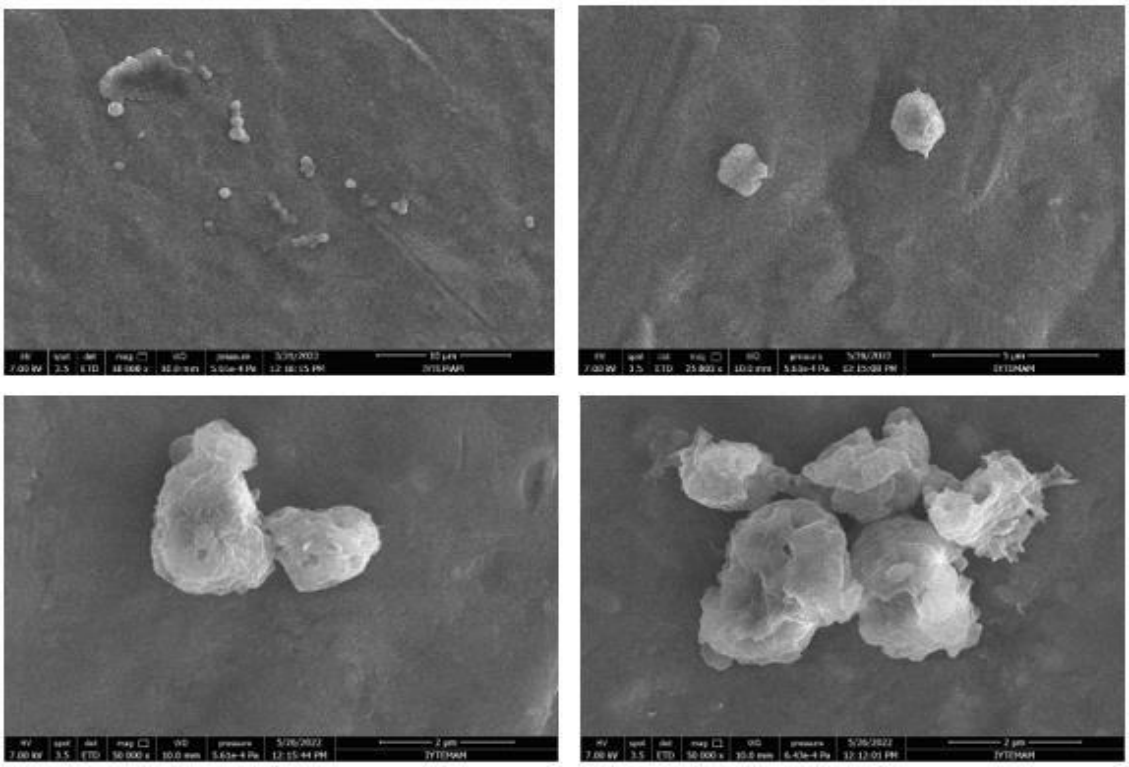

Figure 3.9. Antibacterial action of Gly – Glc melanoidin on *S. aureus*.

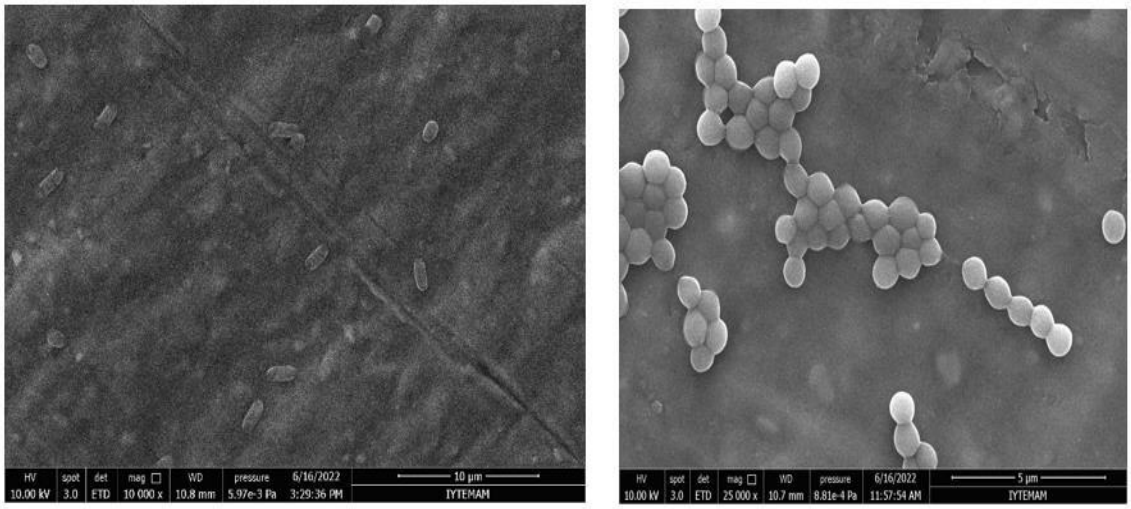

Figure 3.10. Antibacterial action of Gly – Glc melanoidin on *E. coli* (left) and *S. aureus* (right).

The morphological structure of melanoidins was claimed to be effective on their antibacterial activities and it was examined by SEM based on the hypothesis. However, no substantial morphological differences were found between the Arg – Glc (a) and Gly

– Glc (b) melanoidin model systems (Figure 3.11) respectively. The structure of bread melanoidins, on the other hand, could not be analyzed since it was not appropriate for SEM analysis. As a result of the findings, it's suggested that there isn’t significant a relation between melanoidins' morphological structure and their antibacterial characteristics.

These studies indicated the crystal structure of the model melanoidins (Glucose – Glycine and Glucose – Arginine) studied. The SEM images of bread melanoidins could not be obtained. This could be because of the compositional heterogeneity of the bread crust material.

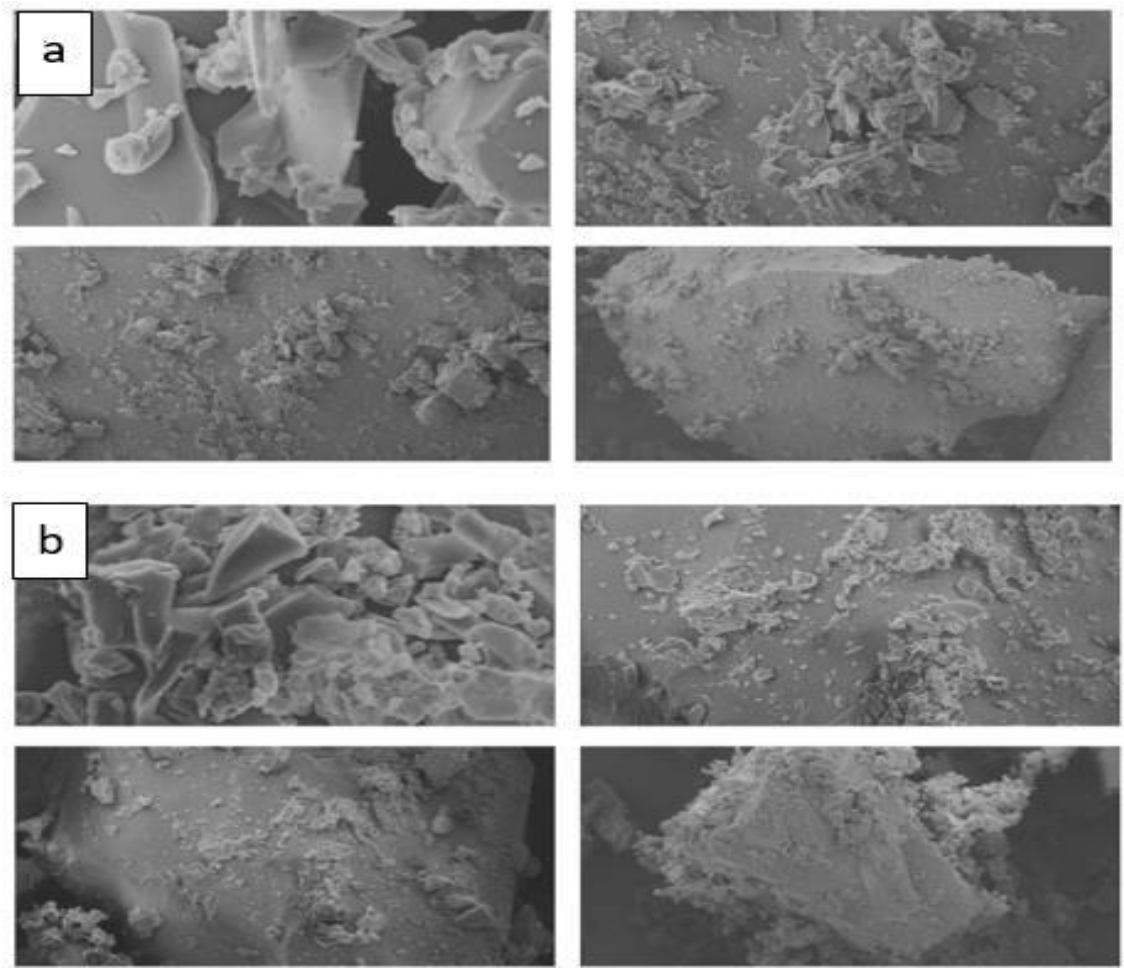

Figure 3.11. The morphological structure of Arg – Glc (a) and Gly – Glc (b) melanoidins sample under Scanning Electron Microscopy.

3.4. Fourier Transform Infrared Spectrophotometer (FT-IR) analysis of Melanoidins

To understand the relation between antimicrobial effects of melanoidins and their structural properties, FTIR spectroscopy were used as seen in the figures below. The definitions of the corresponding bands are given in Table 3.2.

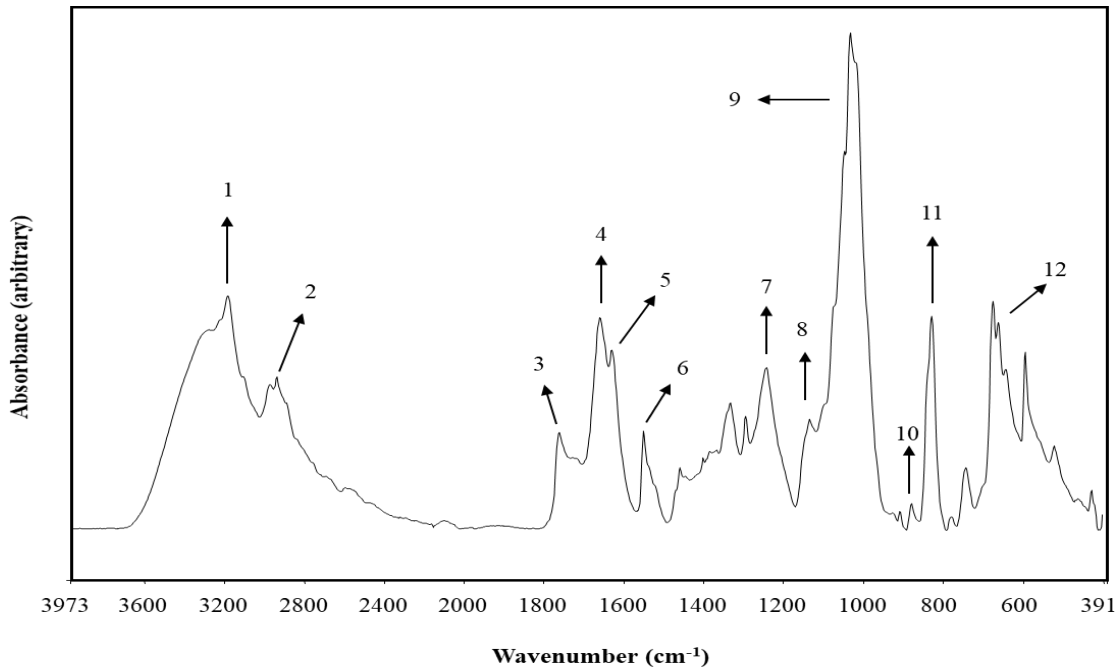

Figure 3.12. FT-IR spectrums of bread melanoidins. Each number and black arrows which are given in boxes show peak position of melanoidins.

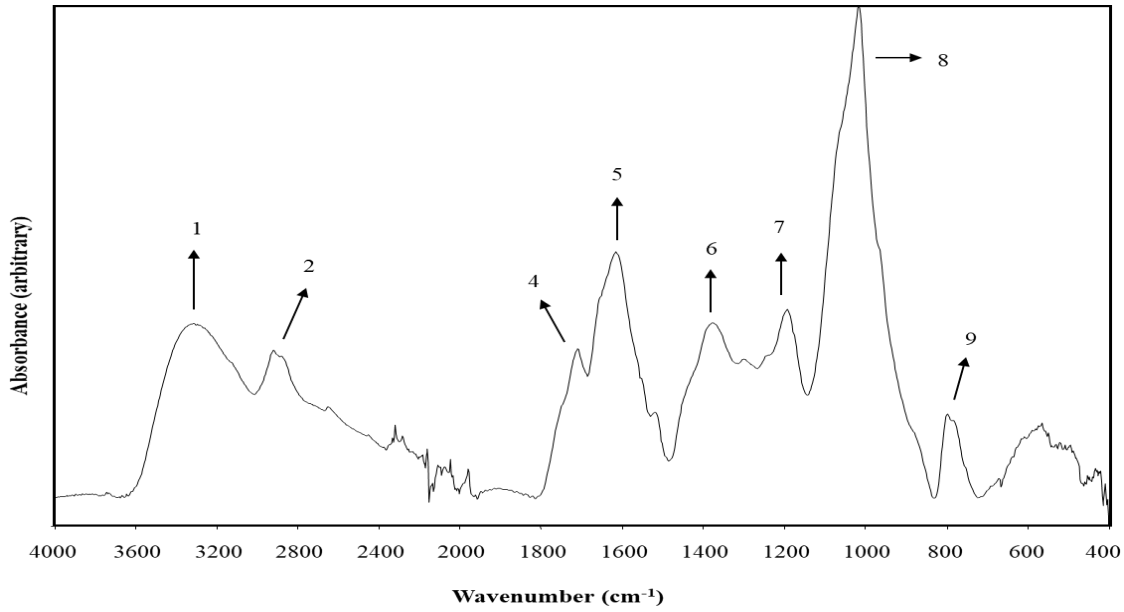

Figure 3.13. FT-IR spectra of Glycine – Glucose melanoidins peak position which are indicated by black arrows.

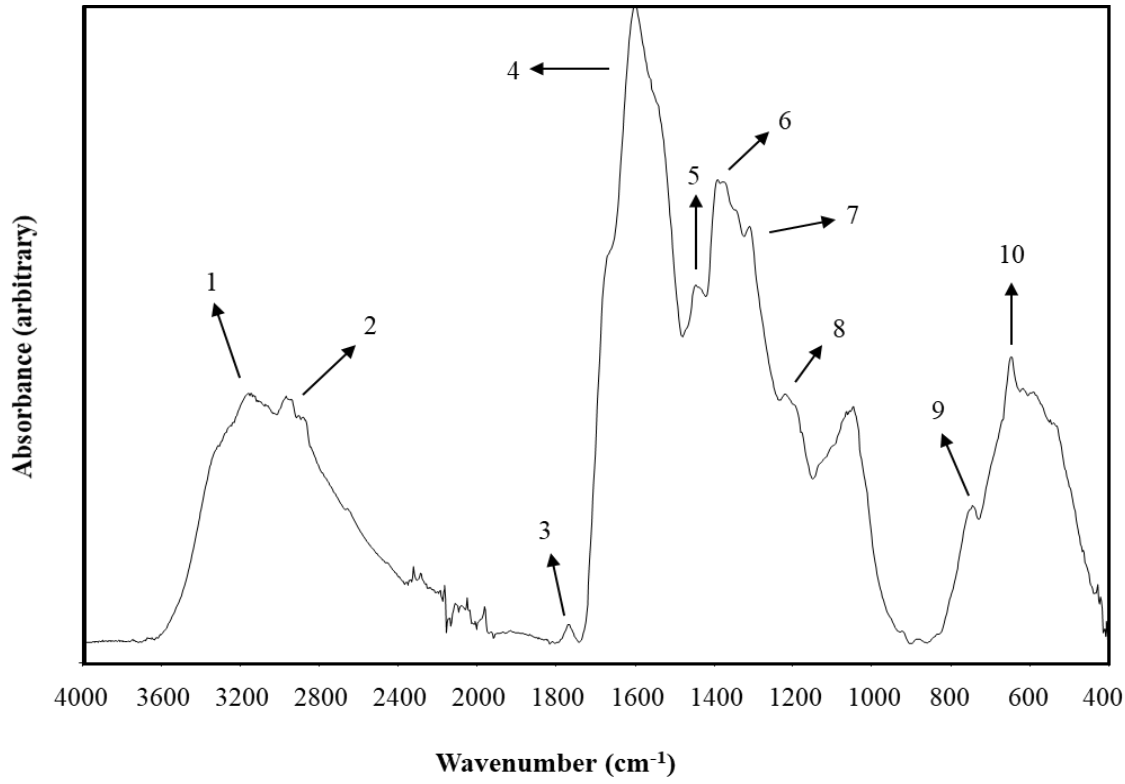

Figure 3.14. FT-IR spectrum of Arginine – Glucose melanoidins peak positions which are indicated by black arrows.

The general properties of melanoidins are determined by the kind of sugar and amino acid, the time of heating, and the pH. Heat-treated compounds have a variety of actions and characteristics. Because of their fingerprint form, Fourier transform infrared (FTIR) spectroscopy can be utilized to explain and recognize the changes that occur in these molecules. The FTIR spectrum of a sample correlates to the sample's distinctive chemical groups, and the method can offer accurate data on structural changes and contents of biological molecules, as well as significant information about melanoidins.

FTIR technique was used to detect functional groups in the structure of bread, Gly - Glc, and Arg - Glc melanoidins. In the bread melanoidins characteristic bands occur at 3186 cm^{-1} N-H stretching vibration, 2946 cm^{-1} $–CH_3$ symmetric, 1769 cm^{-1} C=O and the OH bond stretching, 1661 cm^{-1} vibration of the amide carbonyl group, 1630 cm^{-1} Amide I region, 1551 cm^{-1} α-helical structure, 1244 cm^{-1} asymmetric phosphate stretching (vasPO2), 1136 cm^{-1} oligosaccharide C–OH stretching band, 1034 cm^{-1} C-O stretching vibration, 881 cm^{-1} C–C, and 663 cm^{-1} CH_2 and ring puckering. Functional group peaks occurred in the Arginine – Glucose Melanoidins model system at 3159 cm^{-1} hydroxyl, 2970 cm^{-1} asymmetric stretching of methoxy, 1769 cm^{-1} aromatic ring stretch, 1447 cm^{-1} aromatic C=C stretching, 1408 cm^{-1} aromatic skeletal stretching, 1392 cm^{-1} vibration of C-O (Amide I, C--O stretching), 1310 cm^{-1} Amide III, 1219 cm^{-1} stretching vibration of C-O (C-O-C), 746 cm^{-1} C–O4’ band locations.

In the model system Glycine – Glucose melanoidins functional groups are formed at peak positions 3320 cm^{-1} N-H band, 2924 cm^{-1} stretching C–H, 1711 cm^{-1} C= O (carbonyl), 1617 cm^{-1} stretching vibration of C=O bond, 1379 cm^{-1} δCH_3, 1195 cm^{-1} C-H in-plane bending, and 1019 cm^{-1} C-H in-plane bending (Figure 3.12). Although the exact structure of melanoidins is uncertain, the FTIR data show that there are substantial similarities between the functional groups of melanoidins. It is also possible that these identical peak positions are attributable to melanoidin antimicrobial activities. More peaks were found in the structure of bread melanoidins based on the FTIR data. Interestingly, in the structure of Gly - Glc and Arg - Glc melanoidins, a close number of peaks occurred, and the peak positions are shown to be close.

Table 3.2. Peak position of specific groups in bread, Arg-Glc, and Gly-Glc melanoidins.

Number	Peak Position (cm^{-1})	Functional Group	Reference
Bread Melanoidins			
1	3186	N-H stretching vibration	[66]
2	2946	$-CH_3$ symmetric	[67]
3	1769	C=O and the OH bond stretching	[68]
4	1661	Vibration of the amide carbonyl group	[69]
5	1630	Amide I region	[70]
6	1551	α-helical structure	[71]
7	1244	Asymmetric phosphate stretching (ν as PO2)	[72]
8	1136	Oligosaccharide C–OH stretching band	[73]
9	1034	Phenylalanine	[74]
10	881	Tryptophan δ ring	[75]
11	830	Tyrosine	
12	663	Cysteine	[76]
Arginine – Glucose Melanoidins			
1	3159	hydroxyl	[77]
2	2970	Asymmetric stretching of methoxy	[78]
3	1769	Carbonyl (C=O)	[79]
4	1447	Aromatic C=C stretching	[80]
5	1408	Aromatic skeletal stretching	[81]
6	1392	Vibration of C--O (Amide I, C--O stretching)	[82]
7	1310	Amide III	[82]

(cont. on next page)

Table 3.2. (cont.)

8	1219	Stretching vibration of C-O	[83]
9	746	C–O4' bant /	[84]
10	648	Unknown peak	
Glycine – Glucose melanoidins			
1	3320	NH band	[85]
2	2924	Stretching C–H	[86]
3	1711	C= O (Carbonyl)	[87]
4	1617	Stretching vibration of C=O bond	[88]
5	1379	δCH_3	[89]
6	1195	C-H in-plane bending	[90]
7	1019	Ring stretching vibrations mixed strongly with CH in-plane bending	[91]
8	800	Unknown peak	

Additionally, glycine, arginine, and Glucose samples that were not exposure heat were evaluated and compared to the model systems of melanoid in order to determine structural alterations in the model systems of melanoidins. It is found that there are structural changes, and the formation of new functional groups occur after heat treatment of Gly, Glc, and Arg.

The changes in the FTIR spectrum occurring before and after the heat treatment are shown below (Figure 3.13). From the structure of the Arg - Glc melanoidins, three new distinct bands emerged, 1769 cm^{-1} (carbonyl), 1392 cm^{-1} (Amide I) and 1310 cm^{-1} (amide III), respectively. On the other hand, in the model system of Gly - Glc melanoidins, 1712 cm^{-1} (carbonyl), 1511 cm^{-1} (shoulder), and 800 cm^{-1} (unknown) bands were detected (Figure 3.14). There might be critical links between the function of the melanoidins and the biological activity of the newly formed bands. The FTIR data

results demonstrate that major changes occur in the structures of the melanoidins. However, FTIR data are insufficient for gaining a better knowledge of melanoidins structural alterations. It requires more clarification using analytical analysis procedures.

In the Figure 3.15 a comparison of the three melanoidin systems are presented. The bread specific melanoidins had some specific bands namely, a band around 1034 cm^{-1} representing phenylalanine, a band around 881 cm^{-1} representing tryptophan δ ring, a band around 830 cm^{-1} representing tyrosine, a band around 663 cm^{-1} representing cysteine and a band around 524 cm^{-1} S-S disulfide stretching in proteins. These band correspond to hydrophobic amino acid bands allowing to penetrate into the cell membrane and also taking part in the oxidation-reduction reactions.

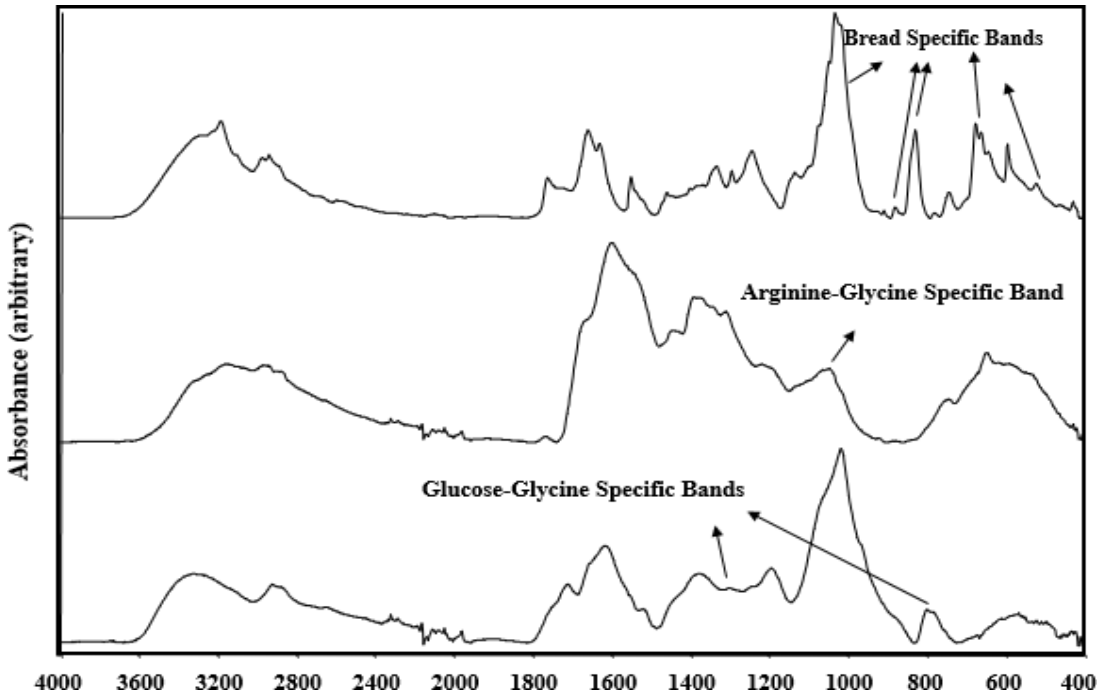

Figure 3.15. Comparison of the FTIR spectra of glucose-glycine (bottom spectrum), arginine-glucose (middle spectrum) and bread melanoidins (top).

3.5. Uv-vis Spectrophotometer Analysis

Figure 3.15 illustrates the browning progression of Arg-Glc (c), Gly-Glc (a), and bread (b) melanoidins. By observing the rise in absorbance at 280 nm, the final step of the browning process was seen. Each peak exhibits a fixed absorbance 280 nm, which is typical of melanoidins. After passing through the UV region, the absorption curve

gradually entered the visible spectrum's blue-absorbing region, where yellow-brown colors developed. The UV is absorbed by the chemicals generated early in the Maillard process.

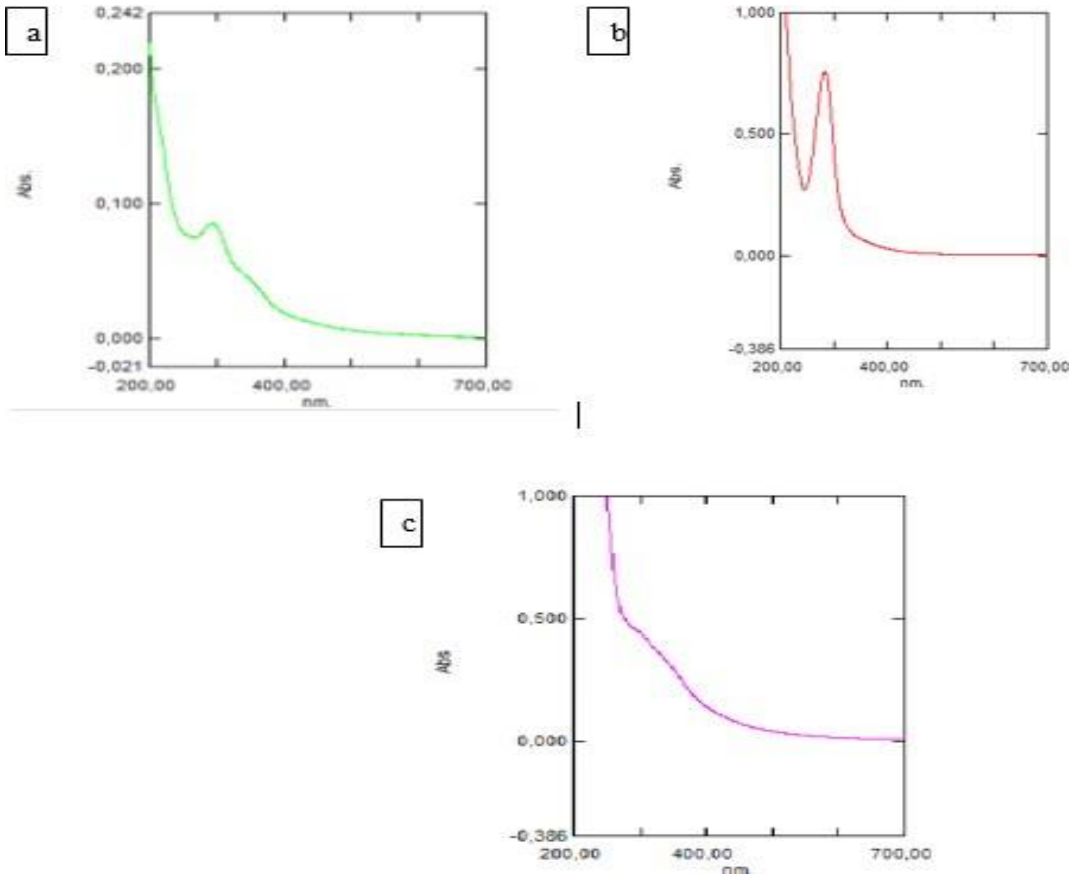

Figure 3.16. Uv-vis specturum (a) Gly – Glc melanoidin, (b) Bread melanoidin, and (c) Arg – Glc melanoidin detected at 280 nm.

3.6. High Performance Liquid Chromatography (HPLC) Analysis

By analyzing their retention times, HPLC analysis can be utilized to identify substances using reference compounds. Changing the standard reference to the extract to accurately show the peak's retention period. HPLC analysis is an effective tool for identifying unknown compounds. However, for identification, a recognized reference compound should be utilized. Melanoidins are difficult to identify since they are complicated and lack a well-defined structure. Melanoidins do not consist of a single

molecule. They can have various numbers and structures. As a result, the primary purpose of our HPLC approach was to detect a common molecule found in melanoidins. Reference molecule polyethylene glycol (PEG-8000) was used and the retetion time was compared with melanoidins (Figure 3.19). The PEG retention time was determined to be 47 minutes. Surprisingly, the identical peak was achieved in all melanoidins. However, the peak absorbance value in the model system of Gly - Glc melanoidin (Figure 3.18) was found to be lower than that of other melanoidins. Despite this interesting findings, multiple undetected peaks occurred in all melanoidin groups during the first 20 minutes. Also, a complex HPLC result was expected due to the presence of many different substances from the bread content (Figure 3.16). However, according to the data obtained from melanoidins, many of peak formation was observed in the model system of Gly - Glc melanoidin, while less peak formation occurred in Arg – Glc (Figure 3.17) and bread melanoidins. A comparable size peak of Dulce de Leche melanoidins was observed at around 40 min [92]. This finding is significant since it is possible that melanoidins have a comparable peak.

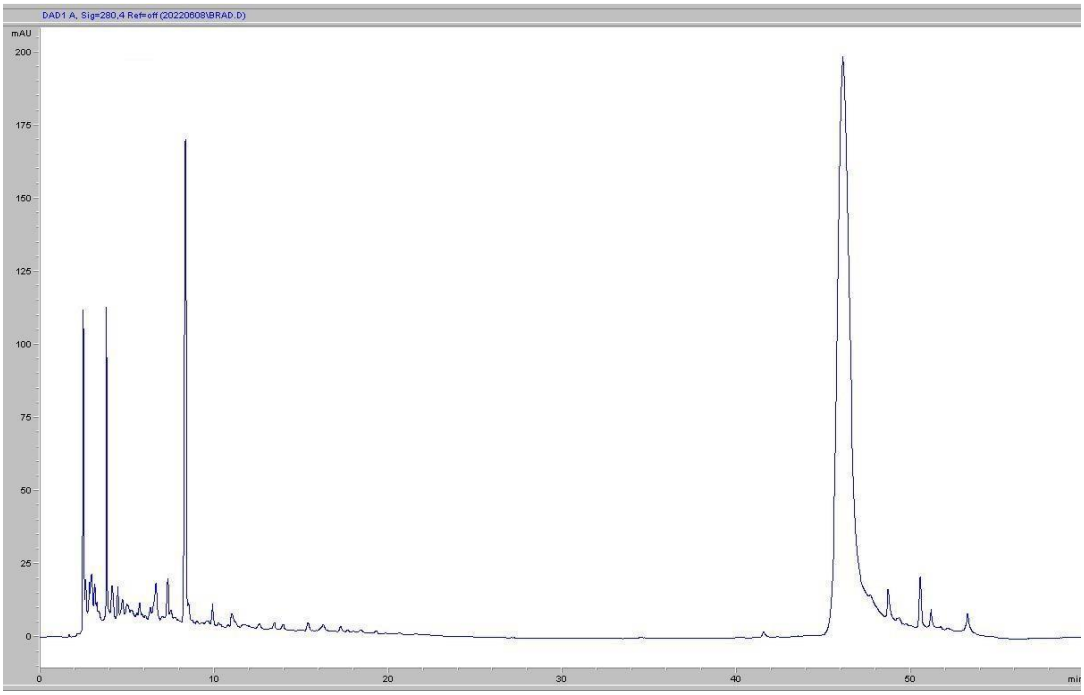

Figure 3.17 HPLC data spectrum shows bread melanoidins.

.

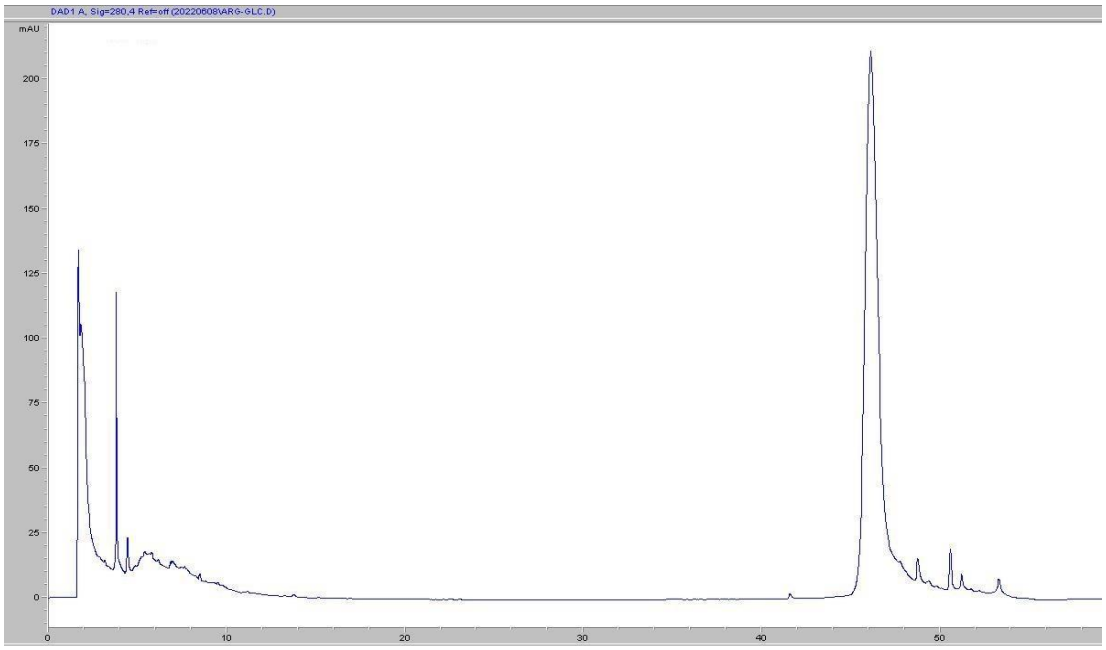

Figure 3.18. HPLC data spectrum shows Arginine – Glucose melanoidins.

.

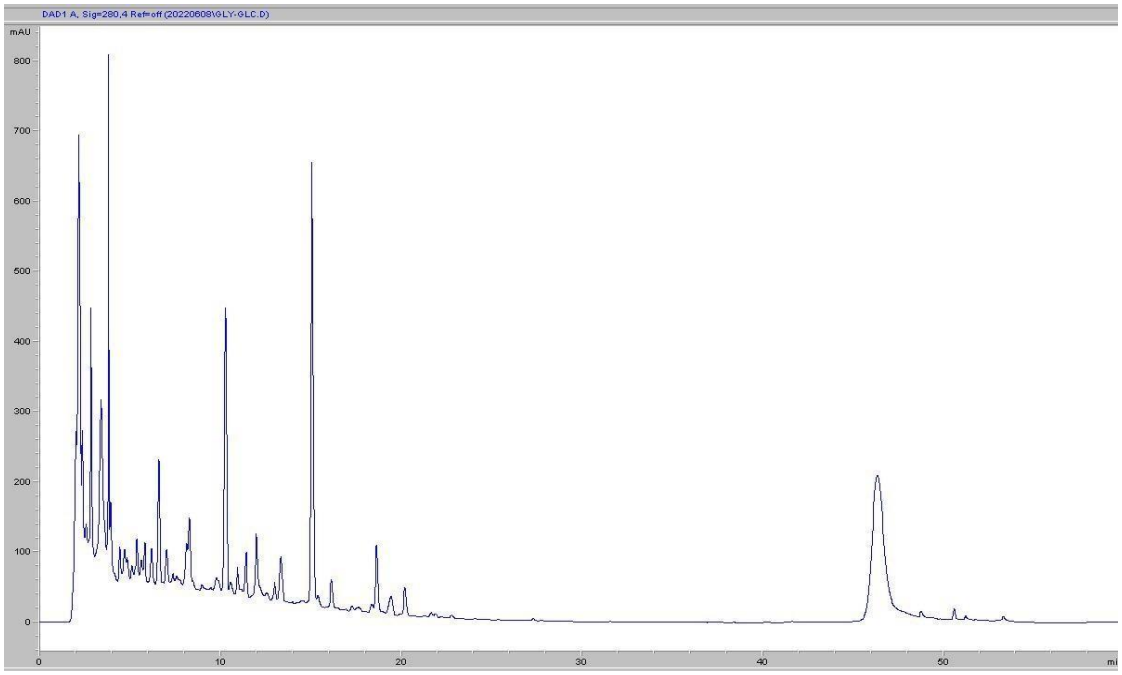

Figure 3.19. HPLC data spectrum shows Glycine – Glucose melanoidins.

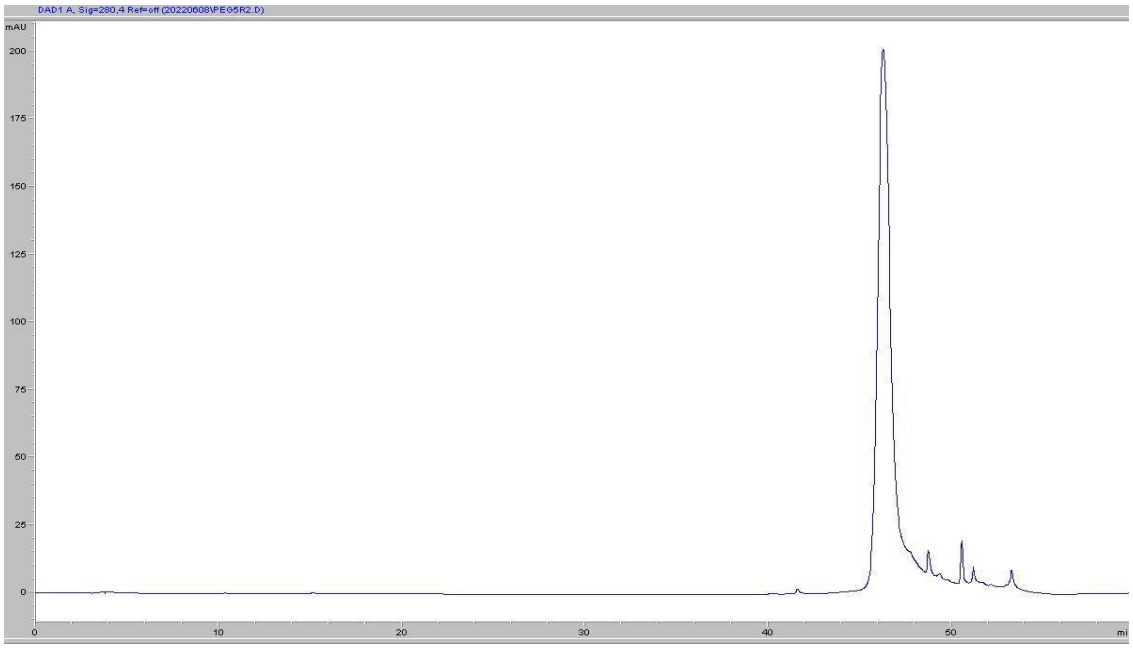

Figure 3.20. HPLC data spectrum shows PEG-8000 kDa standard.

CHAPTER 4

CONCLUSION

High molecular weight molecules known melanoidins are formed during Maillard reactions from the proteins and sugars in food. Since melanoidins are formed as a result of the reaction between amino acids and reducing sugars at high temperature, they are found in high amounts in foods, fruit juices, coffee and bakery products, which are key components of daily consumption. Melanoidins produced from MRs have a strong relationship between heat duration and density and their molecular weight. Longer response periods result in the formation of HMW melanoidins. Melanoidins in food have effects on human health, hence it is essential to evaluate and identify their chemical makeup. Melanoidins' influence on health should also be classified in accordance with their structural composition. This study's major goal was to understand how the structural analysis of melanoidins, and their activity related to one another. Due to the fact that the precise chemical structure of melanoidins has not yet been established. Temperature, amino acid type, reducing sugar type, water activity, pH, and heat length of exposure are significant factors in the final Maillard reaction product. Three different melanoidins were obtained; model systems of bread melanoid, Arg – Glc and Gly – Glc melanoid, respectively. These common points can be evaluated as the general properties (especially structural) of melanoidins, on the other hand more amino acids and reducing sugar combinations can provide more detailed and accurate information in the characterization of melanoidins by analytical methods.

Based on the findings of this study findings, melanoidins had an antibacterial action on the bacteria *E. coli* and *S. aureus*. Melanoidins had strong antibacterial activity against *E. coli* at lower concentrations, whereas *S. aureus* was more resistant to these effects at higher concentrations. According to these results it was found that melanoidins had a high level of action against gram-negative bacteria. The Gly – Glc melanoidin model system (15 mg/ml) for *S. aureus* demonstrated the maximum efficacy, whereas the bread 5 (mg/ml) melanoid demonstrated the highest inhibition for *E. coli*. On the other hand, the model system of Arg – Glc melanoidins was prepared at higher doses than other melanodin concentrations to show antibacterial effect. As can be

seen from the images obtained from the SEM analysis, while melanoidins exerted an antibacterial effect, they damaged the membrane structures in different ways, but it is not fully understood why this works in a different way.

Melanoidins' antioxidant ability was evaluated using the ABTS assay. The bread melanoid had the lowest antioxidant property, whereas the model system of Gly – Glc melanoid demonstrated the highest antioxidant property. FTIR was used to detect the functional groups of melanoidins and to find similarities and differences between these groups. No significant similarity was found between the functional groups of melanoidins, but it was determined that new functional groups were formed after heat treatment in the melanoid model systems of Gly – Glc and Arg – Glc.

Finally, melanoidins were identified using HPLC. Identification was performed using PEG-8000 Da. Unexpectedly, all melanoidins showed a considerable peak at around 47 minutes. In addition, during the first 20 minutes, there were several smaller and unidentified peaks. However, Arg – Glc melanoidins were shown to contain the highest number of melanoidins peaks within the first 20 minutes.

Overall, melanoidins are the topic of an increasing number of investigations. Studies on their biological functions and structures have improved our understanding of it, however the main challenge to the knowledge is the complexity of the structures and diversity of melanoidins.

REFERENCE

(1) Hodge, J. E. Dehydrated Foods, Chemistry of Browning Reactions in Model Systems. *Journal of Agricultural and Food Chemistry* **1953**, *1* (15), 928–943. https://doi.org/10.1021/jf60015a004.

(2) Totlani, V.; food, D. P.-J. of agricultural and; 2007, undefined. Influence of Epicatechin Reactions on the Mechanisms of Maillard Product Formation in Low Moisture Model Systems. *ACS Publications* **2007**, *55* (2), 414–420. https://doi.org/10.1021/jf0617521.

(3) Morales, F. J. *Analytical, Nutritional and Clinical Methods Section Application of Capillary Zone Electrophoresis to the Study of Food and Food-Model Melanoidins*.

(4) Montavon, P.; Mauron, A.; and, E. D.-J. of A.; 2003, undefined. Changes in Green Coffee Protein Profiles during Roasting. *ACS Publications* **2003**, *51* (8), 2335–2343. https://doi.org/10.1021/jf020832b.

(5) Morales, F. J.; Somoza, V.; Fogliano, V. Physiological Relevance of Dietary Melanoidins. *Amino Acids*. April 2012, pp 1097–1109. https://doi.org/10.1007/s00726-010-0774-1.

(6) Fogliano, V.; Morales, F. J. Estimation of Dietary Intake of Melanoidins from Coffee and Bread. *Food and Function* **2011**, *2* (2), 117–123. https://doi.org/10.1039/c0fo00156b.

(7) Wrodnigg, T. M.; Eder, B. The Amadori and Heyns Rearrangements: Landmarks in the History of Carbohydrate Chemistry or Unrecognized Synthetic Opportunities? **2001**, 115–152. https://doi.org/10.1007/3-540-44422-X_6.

(8) Coca, M.; Garcı a, M.; Gonz lez, G.; Pe a, M.; chemistry, J. G.-F.; 2004, undefined. Study of Coloured Components Formed in Sugar Beet Processing. *Elsevier*.

(9) Bekedam, E. K.; Roos, E.; Schols, H. A.; van Boekel, M. A. J. S.; Smit, G. Low Molecular Weight Melanoidins in Coffee Brew. *Journal of Agricultural and Food Chemistry* **2008**, *56* (11), 4060–4067. https://doi.org/10.1021/JF8001894.

(10) nutrition, E. D.-T. A. journal of clinical; 1995, undefined. Functionality of Sugars: Physicochemical Interactions in Foods. *academic.oup.com* **1995**.

(11) Thomsen, M.; Lauridsen, L.; … L. S.-J. of A.; 2005, undefined. Temperature Effect on Lactose Crystallization, Maillard Reactions, and Lipid Oxidation in Whole Milk Powder. *ACS Publications* **2005**, *53* (18), 7082–7090. https://doi.org/10.1021/jf050862p.

(12) Oh, S.; Lee, Y.; Lee, J.; Kim, M.; Yook, H.; chemistry, M. B.-F.; 2005, undefined. The Effect of γ-Irradiation on the Non-Enzymatic Browning Reaction in the Aqueous Model Solutions. *Elsevier*.

(13) Noltes, A.; problems, C. C.-F. toxicology--real or imaginary; 1985, undefined. Toxicology of Caramel Colours: Current Status. *agris.fao.org*.

(14) Martins, S.; technology, W. J.-… food science &; 2000, undefined. A Review of Maillard Reaction in Food and Implications to Kinetic Modelling. *Elsevier*.

(15) Sherwin, C. P.; Labuza, T. P. Role of Moisture in Maillard Browning Reaction Rate in Intermediate Moisture Foods: Comparing Solvent Phase and Matrix Properties. *Journal of Food Science* **2003**, *68* (2), 588–593. https://doi.org/10.1111/J.1365-2621.2003.TB05715.X.

(16) Hofmann, T. Studies on the Relationship between Molecular Weight and the Color Potency of Fractions Obtained by Thermal Treatment of Glucose/Amino Acid and Glucose/Protein Solutions by Using Ultracentrifugation and Color Dilution Techniques. *Journal of Agricultural and Food Chemistry* **1998**, *46* (10), 3891–3895. https://doi.org/10.1021/JF980397E.

(17) Crozier, A.; Ashihara, H.; Tom s-Barbéran, F. Teas, Cocoa and Coffee: Plant Secondary Metabolites and Health. **2011**.

(18) Borrelli, R.; Fogliano, V.; Monti, S.; Research, J. A.-E. F.; 2002, undefined. Characterization of Melanoidins from a Glucose-Glycine Model System. *Springer* **2002**, *215* (3), 210–215. https://doi.org/10.1007/s00217-002-0531-0.

(19) Nunes, F.; Food, M. C.-J. of A. and; 2007, undefined. Melanoidins from Coffee Infusions. Fractionation, Chemical Characterization, and Effect of the Degree of Roast. *ACS Publications* **2007**, *55* (10), 3967–3977. https://doi.org/10.1021/jf063735h.

(20) Tamanna, N.; Mahmood, N. Food Processing and Maillard Reaction Products: Effect on Human Health and Nutrition. *International Journal of Food Science*. Hindawi Publishing Corporation 2015. https://doi.org/10.1155/2015/526762.

(21) Research, V. S.-M. N. & F.; 2005, undefined. Five Years of Research on Health Risks and Benefits of Maillard Reaction Products: An Update. *Wiley Online Library* **2005**, *49* (7), 663–672. https://doi.org/10.1002/mnfr.200500034.

(22) LEE, T.-C.; CHICHESTER, C. O. Physiological, Toxicological, and Nutritional Aspects of Various Maillard Browned Proteins. **1983**, 379–408. https://doi.org/10.1021/BK-1983-0234.CH023.

(23) Borrelli, R. C.; Fogliano, V. Bread Crust Melanoidins as Potential Prebiotic Ingredients. In *Molecular Nutrition and Food Research*; 2005; Vol. 49, pp 673–678. https://doi.org/10.1002/mnfr.200500011.

(24) Lindenmeier, M.; Hofmann, T. Influence of Baking Conditions and Precursor Supplementation on the Amounts of the Antioxidant Pronyl-L-Lysine in Bakery Products. *Journal of Agricultural and Food Chemistry* **2004**, *52* (2), 350–354. https://doi.org/10.1021/jf0346657.

(25) Hayase, F.; Usui, T.; food, H. W.-M. nutrition &; 2006, undefined. Chemistry and Some Biological Effects of Model Melanoidins and Pigments as Maillard

Intermediates. *Wiley Online Library* **2006**, *50* (12), 1171–1179. https://doi.org/10.1002/mnfr.200600078.

(26) Hayase, F.; Usui, T.; Ono, Y.; Shirahashi, Y.; Machida, T.; Ito, T.; Nishitani, N.; Shimohira, K.; Watanabe, H. Formation Mechanisms of Melanoidins and Fluorescent Pyridinium Compounds as Advanced Glycation End Products. *Wiley Online Library* **2008**, *1126*, 53–58. https://doi.org/10.1196/annals.1433.009.

(27) Tressl, R.; Wondark, G. T.; Krüger, R. P.; Rewicki, D. New Melanoidin-like Maillard Polymers from 2-Deoxypentoses. *Journal of Agricultural and Food Chemistry* **1998**, *46* (1), 104–110. https://doi.org/10.1021/JF970657C.

(28) Cämmerer, B.; Jalyschko, W.; and, L. K.-J. of A.; 2002, undefined. Intact Carbohydrate Structures as Part of the Melanoidin Skeleton. *ACS Publications* **2002**, *50* (7), 2083–2087. https://doi.org/10.1021/jf011106w.

(29) Koen Bekedam, E.; Schols, H. A.; van Boekel, M. A. J. S.; Smit, G. Incorporation of Chlorogenic Acids in Coffee Brew Melanoidins. *ACS Publications* **2008**, *56* (6), 2055–2063. https://doi.org/10.1021/jf073157k.

(30) Koen Bekedam, E.; Schols, H. A.; van Boekel, M. A. J. S.; Smit, G. High Molecular Weight Melanoidins from Coffee Brew. *ACS Publications* **2006**, *54* (20), 7658–7666. https://doi.org/10.1021/jf0615449.

(31) Wang, H. Y.; Qian, H.; Yao, W. R. Melanoidins Produced by the Maillard Reaction: Structure and Biological Activity. *Food Chemistry*. October 1, 2011, pp 573–584. https://doi.org/10.1016/j.foodchem.2011.03.075.

(32) Šosoviš, B.; Vojvodiš, V.; Boškoviš, N.; … M. P.-O.; 2010, undefined. Characterization of Natural and Synthetic Humic Substances (Melanoidins) by Chemical Composition and Adsorption Measurements. *Elsevier*.

(33) Koen Bekedam, E.; de Laat, M. P. F. C.; Schols, H. A.; van Boekel, M. A. J. S.; Smit, G. Arabinogalactan Proteins Are Incorporated in Negatively Charged Coffee Brew Melanoidins. *ACS Publications* **2007**, *55* (3), 761–768. https://doi.org/10.1021/jf063010d.

(34) Verzelloni, E.; Tagliazucchi, D.; Toxicology, A. C.-F. and C.; 2010, undefined. From Balsamic to Healthy: Traditional Balsamic Vinegar Melanoidins Inhibit Lipid Peroxidation during Simulated Gastric Digestion of Meat. *Elsevier*.

(35) Delgado-Andrade, C.; Rufi n-Henares, J. A.; Morales, F. J. Fast Method to Determine Furosine in Breakfast Cereals by Capillary Zone Electrophoresis. *European Food Research and Technology* **2005**, *221* (5), 707–711. https://doi.org/10.1007/S00217-005-0030-1.

(36) Kuntcheva Tzvetan D Obretenov, M. J. *Isolation and Characterization of Melanoidins from Beer*; Springer-Verlag, 1996; Vol. 202.

(37) Nunes, F. M.; Coimbra, M. A. Role of Hydroxycinnamates in Coffee Melanoidin Formation. *Phytochemistry Reviews* **2010**, *9* (1), 171–185. https://doi.org/10.1007/S11101-009-9151-7.

(38) Hofmann, T.; Bors, W.; Stettmaier, K. Radical-Assisted Melanoidin Formation during Thermal Processing of Foods as Well as under Physiological Conditions. *Journal of Agricultural and Food Chemistry* **1999**, *47* (2), 391–396. https://doi.org/10.1021/JF980627P.

(39) Mesías, M.; Delgado-Andrade, C. Melanoidins as a Potential Functional Food Ingredient. *Current Opinion in Food Science*. Elsevier Ltd April 1, 2017, pp 37–42. https://doi.org/10.1016/j.cofs.2017.01.007.

(40) Silv n, J.; Lagemaat, J. van de; … A. O.-… of pharmaceutical and; 2006, undefined. Analysis and Biological Properties of Amino Acid Derivates Formed by Maillard Reaction in Foods. *Elsevier*.

(41) Ibarz, A.; Garvín, A.; Garza, S.; Toxicology, J. P.-F. and C.; 2009, undefined. Toxic Effect of Melanoidins from Glucose–Asparagine on Trypsin Activity. *Elsevier*.

(42) Langner, E.; Rzeski, W. Biological Properties of Melanoidins: A Review. *International Journal of Food Properties*. February 7, 2014, pp 344–353. https://doi.org/10.1080/10942912.2011.631253.

(43) Technologie, H. E.-L.-W. und; 1987, undefined. The Effect of Time, Temperature, PH and Reactants on the Formation of Antibacterial Compunds in the Maillard Reaction. *diva-portal.org*.

(44) Wen, X.; Enokizo, A.; Hattori, H.; Kobayashi, S.; Murata, M.; Homma, S. Effect of Roasting on Properties of the Zinc-Chelating Substance in Coffee Brews. *Journal of Agricultural and Food Chemistry* **2005**, *53* (7), 2684–2689. https://doi.org/10.1021/JF048304I.

(45) Echavarría, A. P.; Pag n, J.; Ibarz, A. Melanoidins Formed by Maillard Reaction in Food and Their Biological Activity. *Food Engineering Reviews*. Springer Science and Business Media, LLC December 1, 2012, pp 203–223. https://doi.org/10.1007/s12393-012-9057-9.

(46) Wagner, K.; Derkits, S.; Herr, M.; Schuh, W.; Chemistry, I. E.-F.; 2002, undefined. Antioxidative Potential of Melanoidins Isolated from a Roasted Glucose–Glycine Model. *Elsevier*.

(47) Borrelli, R. C.; Visconti, A.; Mennella, C.; Anese, M.; Fogliano, V. Chemical Characterization and Antioxidant Properties of Coffee Melanoidins. *Journal of Agricultural and Food Chemistry* **2002**, *50* (22), 6527–6533. https://doi.org/10.1021/jf025686o.

(48) Shen, Y.; Chen, G.; Li, Y. Bread Characteristics and Antioxidant Activities of Maillard Reaction Products of White Pan Bread Containing Various Sugars. *LWT* **2018**, *95*, 308–315. https://doi.org/10.1016/j.lwt.2018.05.008.

(49) Rufi n-Henares, J. A.; Morales, F. J. Functional Properties of Melanoidins: In Vitro Antioxidant, Antimicrobial and Antihypertensive Activities. *Food Research International* **2007**, *40* (8), 995–1002. https://doi.org/10.1016/J.FOODRES.2007.05.002.

(50) Yen, G.-C.; Hsieh, P.-P. *Antioxidative Activity and Scavenging Effects on Active Oxygen of Xylose-Lysine Maillard Reaction Products*; 1995; Vol. 67.

(51) Brückner, H.; Justus, J.; Kirschbaum, J. Saccharide Induced Racemization of Amino Acids in the Course of the Maillard Reaction. *Amino Acids* **2001**, *21* (4), 429–433. https://doi.org/10.1007/S007260170007.

(52) Valls-Belí Es, V.; Torres, M. C.; Mu iz, P.; Mu iz, M.; Boix, L.; Gonz lezgonz´gonz lez-Sanjose, M. L.; Codõ Ner-Franch, P. The Protective Effects of Melanoidins in Adriamycin-induced Oxidative Stress in Isolated Rat Hepatocytes. *Wiley Online Library* **2004**, *84* (13), 1701–1707. https://doi.org/10.1002/jsfa.1859.

(53) Summa, C.; Mccourt, J.; Cämmerer, B.; Fiala, A.; Probst, M.; Kun, S.; Anklam, E.; Wagner, K.-H. Radical Scavenging Activity, Anti-bacterial and Mutagenic Effects of Cocoa Bean Maillard Reaction Products with Degree of Roasting. *Wiley Online Library* **2008**, *52* (3), 342–351. https://doi.org/10.1002/mnfr.200700403.

(54) Pérez-Burillo, S.; Rajakaruna, S.; Pastoriza, S.; Paliy, O.; Ángel Rufi n-Henares, J. Bioactivity of Food Melanoidins Is Mediated by Gut Microbiota. *Food Chemistry* **2020**, *316*. https://doi.org/10.1016/j.foodchem.2020.126309.

(55) Hiramoto, S.; Itoh, K.; Shizuuchi, S.; Kawachi, Y.; Morishita, Y.; Nagase, M.; Suzuki, Y.; Nobuta, Y.; Sudou, Y.; Nakamura, O.; Kagaya, I.; Goshima, H.; Kodama, Y.; Icatro, F. C.; Koizumi, W.; Saigenji, K.; Miura, S.; Sugiyama, T.; Kimura, N. Melanoidin, a Food Protein-Derived Advanced Maillard Reaction Product, Suppresses Helicobacter Pylori in Vitro and in Vivo. *Helicobacter* **2004**, *9* (5), 429–435. https://doi.org/10.1111/J.1083-4389.2004.00263.X.

(56) Jos´, J.; Rufi´an, J. A.; Rufi´an-Henares, R.; De, S. P.; Cueva, L. A. Antimicrobial Activity of Coffee Melanoidins□ A Study of Their Metal-Chelating Properties. *ACS Publications* **2009**, *57* (2), 432–438. https://doi.org/10.1021/jf8027842.

(57) Reichardt, N.; Gniechwitz, D.; Steinhart, H.; Bunzel, M.; Blaut, M. Characterization of High Molecular Weight Coffee Fractions and Their Fermentation by Human Intestinal Microbiota. *Wiley Online Library* **2009**, *53* (2), 287–299. https://doi.org/10.1002/mnfr.200700509.

(58) Borrelli, R. C.; Fogliano, V.; Monti, S. M.; Ames, J. M. Characterization of Melanoidins from a Glucose-Glycine Model System. *European Food Research and Technology* **2002**, *215* (3), 210–215. https://doi.org/10.1007/s00217-002-0531-0.

(59) Zhang, H.; Zhang, H.; Troise, A. D.; Fogliano, V. Melanoidins from Coffee, Cocoa, and Bread Are Able to Scavenge α-Dicarbonyl Compounds under Simulated Physiological Conditions. *Journal of Agricultural and Food Chemistry* **2019**, *67* (39), 10921–10929. https://doi.org/10.1021/acs.jafc.9b03744.

(60) Mohsin, G. F.; Schmitt, F. J.; Kanzler, C.; Dirk Epping, J.; Flemig, S.; Hornemann, A. Structural Characterization of Melanoidin Formed from D-Glucose and L-Alanine at Different Temperatures Applying FTIR, NMR, EPR, and MALDI-ToF-MS. *Food Chemistry* **2018**, *245*, 761–767. https://doi.org/10.1016/j.foodchem.2017.11.115.

(61) Horzum, N.; Hilal, M.; Chemistry, T. I.-N. J. of; 2018, undefined. Enhanced Bactericidal and Photocatalytic Activities of ZnO Nanostructures by Changing the Cooling Route. *pubs.rsc.org*.

(62) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. *Original Contribution ANTIOXIDANT ACTIVITY APPLYING AN IMPROVED ABTS RADICAL CATION DECOLORIZATION ASSAY*; 1999.

(63) Bayçin, D.; Altiok, E.; Ülkü, S.; Bayraktar, O. Adsorption of Olive Leaf (Olea Europaea L.) Antioxidants on Silk Fibroin. *Journal of Agricultural and Food Chemistry* **2007**, *55* (4), 1227–1236. https://doi.org/10.1021/jf062829o.

(64) Veiga, A.; Sinthuvanich, C.; Gaspar, D.; Biomaterials, H. F.-; 2012, undefined. Arginine-Rich Self-Assembling Peptides as Potent Antibacterial Gels. *Elsevier*.

(65) Jos´, J.; Rufi´an, J. A.; Rufi´an-Henares, R.; De, S. P.; Cueva, L. A. Antimicrobial Activity of Coffee MelanoidinssA Study of Their Metal-Chelating Properties. https://doi.org/10.1021/jf8027842.

(66) Hu, Q.; Shi, X.; Sun, K.; Cui, S.; … H. H.-J. of A. and; 2022, undefined. A Super-Stretchable and Thermally Stable Hydrogel Electrolyte for High Performance Supercapacitor with Wide Operation Temperature. *Elsevier*.

(67) Chieng, B.; Ibrahim, N.; Yunus, W. W.; Polymers, M. H.-; 2013, undefined. Poly (Lactic Acid)/Poly (Ethylene Glycol) Polymer Nanocomposites: Effects of Graphene Nanoplatelets. *mdpi.com*.

(68) Liu, C.; Jiang, T. T.; Yuan, Z. X.; Lu, Y. Self-Assembled Casein Nanoparticles Loading Triptolide for the Enhancement of Oral Bioavailability. *Natural Product Communications* **2020**, *15* (8). https://doi.org/10.1177/1934578X20948352.

(69) Carlos, J.; García, J.; Lopez, P.; Rojas De G scue, B.; Linares, A. B.; Carlos Jiménez, J.; López, P. Biodegradability Study by FTIR and DSC of Polymers Films Based on Polypropylene and Cassava Starch. **2019**. https://doi.org/10.17807/orbital.v11i2.1360.

(70) Wood, B. R.; Quinn, M. A.; Tait, B.; Ashdown, M.; Hislop, T.; Romeo, M.; Mcnaughton, D. FTIR Microspectroscopic Study of Cell Types and Potential Confounding Variables in Screening for Cervical Malignancies. *John Wiley & Sons, Inc. Biospectroscopy* **1998**, *4*, 75–91. https://doi.org/10.1002/(SICI)1520-6343(1998)4:2.

(71) Sadat, A.; Sciences, I. J.-A.; 2020, undefined. Peak Fitting Applied to Fourier Transform Infrared and Raman Spectroscopic Analysis of Proteins. *mdpi.com*.

(72) Fung, M.; Fung, K.; Senterman,', M. K.; Mikhael,', N. Z.; Lacelle, S.; Wong3, P. 1 1. Pressure-Tuning Fourier Transform Infrared Spectroscopic Study of Carcinogenesis in Human Endometrium. https://doi.org/10.1002/(SICI)1520-6343(1996)2:3.

(73) Yoshida, S.; Miyazaki, M.; Sakai, K.; Takeshita, M.; Yuasa, S.; Sato, A.; Kobayashi, T.; Watanabe, S.; Okuyama, H. Fourier Transform Infrared Spectroscopic Analysis of Rat Brain Microsomal Membranes Modified by Dietary Fatty Acids: Possible Correlation with Altered Learning Behavior. *Biospectroscopy* **1997**, *3*, 281–290. https://doi.org/10.1002/(SICI)1520-6343(1997)3:4.

(74) Tan, C.; Leow, S.; Ying, C.; Tan, C.; … T. Y.-M.; 2021, undefined. Comparison of FTIR Spectrum with Chemometric and Machine Learning Classifying Analysis for Differentiating Guan-Mutong a Nephrotoxic and Carcinogenic. *Elsevier*.

(75) Li, X.; Lin, J.; Ding, J.; Wang, S.; … Q. L.-T. 26th annual; 2004, undefined. Raman Spectroscopy and Fluorescence for the Detection of Liver Cancer and Abnormal Liver Tissue. *ieeexplore.ieee.org*.

(76) Schulz, H.; Spectroscopy, M. B.-V.; 2007, undefined. Identification and Quantification of Valuable Plant Substances by IR and Raman Spectroscopy. *Elsevier*.

(77) Jiang, Z.; Zhang, X.; Wu, L.; Li, H.; Chen, Y.; Li, L.; … H. N.-C.; 2021, undefined. Exolytic Products of Alginate by the Immobilized Alginate Lyase Confer Antioxidant and Antiapoptotic Bioactivities in Human Umbilical Vein Endothelial Cells. *Elsevier*.

(78) Schulz, H.; Spectroscopy, M. B.-V.; 2007, undefined. Identification and Quantification of Valuable Plant Substances by IR and Raman Spectroscopy. *Elsevier*.

(79) Meyns, M.; Primpke, S.; Methods, G. G.-A.; 2019, undefined. Library Based Identification and Characterisation of Polymers with Nano-FTIR and IR-SSNOM Imaging. *pubs.rsc.org*.

(80) Liu, W.; Lin, C.; Hsieh, Y.; Molecules, Y. W.-; 2021, undefined. Nanoformulation Development to Improve the Biopharmaceutical Properties of Fisetin Using Design of Experiment Approach. *mdpi.com*.

(81) Besleaga, C.; Galca, A. C.; Miclea, C. F.; Mercioniu, I.; Enculescu, M.; Stan, G. E.; Mateescu, A. O.; Dumitru, V.; Costea, S. Physical Properties of AlxIn1-XN Thin Film Alloys Sputtered at Low Temperature. *Journal of Applied Physics* **2014**, *116* (15). https://doi.org/10.1063/1.4898565.

(82) Guleken, Z.; Tok, Y.; Jakubczyk, P.; Paja, W.; Measurement, K. P.-; 2022, undefined. Development of Novel Spectroscopic and Machine Learning Methods for the Measurement of Periodic Changes in COVID-19 Antibody Level. *Elsevier*.

(83) Richter, T.; Steiner, G.; Abu-Id, M.; … R. S.-V.; 2002, undefined. Identification of Tumor Tissue by FTIR Spectroscopy in Combination with Positron Emission Tomography. *Elsevier*.

(84) *Synthesis and Characterization of Chitosan Linked by Methylene Bridge and Schiff Base of 4,4-Diaminodiphenyl Ether-Vanillin - Sriwijaya University Repository*. https://repository.unsri.ac.id/54310/ (accessed 2022-06-24).

(85) Dovbeshko, G. I.; Chegel, V. I.; Gridina, N. Y.; Repnytska, O. P.; Shirshov, Y. M.; Tryndiak, V. P.; Todor, I. M.; Solyanik, G. I.; Romodanov, A. P. Surface Enhanced IR Absorption of Nucleic Acids from Tumor Cells: FTIR Reflectance Study. *Wiley Online Library* **2000**, *67* (6), 470–486. https://doi.org/10.1002/bip.10165.

(86) Dovbeshko, G.; Gridina, N.; Kruglova, E.; Talanta, O. P.-; 2000, undefined. FTIR Spectroscopy Studies of Nucleic Acid Damage. *Elsevier*.

(87) Dovbeshko, G.; Gridina, N.; Kruglova, E.; Talanta, O. P.-; 2000, undefined. FTIR Spectroscopy Studies of Nucleic Acid Damage. *Elsevier*.

(88) Hoang, L.; Chinh, N.; Trung, V.; Science, T. H.-V. J. of; 2018, undefined. Alginate/Chitosan Film Loading Golden Flower Tea (Camellia Chrysantha) Extract: Preparation and Characterization. *vjs.ac.vn*.

(89) Paluszkiewicz, C.; Structure, W. K.-J. of M.; 2001, undefined. Analysis of Human Cancer Prostate Tissues Using FTIR Microspectroscopy and SRIXE Techniques. *Elsevier*.

(90) Muthuselvi, C.; Pandiaraja, S. S.; Ravikumar, B.; Athimoolam, S.; Srinivasan, N.; Krishnakum, R. V. FT-IR and FT-Raman Spectroscopic Analyzes of Indeno Quinoxaline Derivative Crystal. *Asian Journal of Applied Sciences* **2018**, *11* (2), 83–91. https://doi.org/10.3923/AJAPS.2018.83.91.

(91) Chiang, H.; Song, R.; Mou, B.; Li, K. P.; Chiang, P.; Wang, D.; Tse, W. S.; Ho, L. T. Fourier Transform Raman Spectroscopy of Carcinogenic Polycyclic Aromatic Hydrocarbons in Biological Systems: Binding to Heme Proteins. *Wiley Online Library* **1999**, *30*, 551–555.

(92) Rodríguez, A.; Lema, P.; Bessio, M. I.; Moyna, G.; Panizzolo, L. A.; Ferreira, F. Isolation and Characterization of Melanoidins from Dulce de Leche, a Confectionary Dairy Product. *Molecules* **2019**, *24* (22). https://doi.org/10.3390/molecules24224163.