The effect of UV light and spray drying on glucosamine non-enzymatic browning

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Food Science and Technology

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Abstract

Non-enzymatic browning reactions, including Maillard and caramelization, are important and complex reactions occurring during food processing and storage. Many factors can affect non-enzymatic browning reactions, which include the concentration of reactants, initial pH, temperature, heating time, water activity, etc.

Glucosamine (GlcN) is an amino monosaccharide that contains both an amino group and a carbonyl group. It is capable of undergoing non-enzymatic browning at lower temperatures, generating a plethora of desirable compounds, including α-dicarbonyl compounds (α–DCs), fructosazine (FR) and deoxyfructosazine (DOFR), melanoidins, etc. Among the major α-DCs, 3-deoxyglucosone (3-DG), glucosone (G), methylglyoxal (MGO), glyoxal (GO) and diacetyl (DA) can be generated from GlcN at as low as 25 °C, while increasing the reaction temperature to 37 or 50 °C can speed up this degradation process. MGO, GO, DA, and 3-DG have been reported to have antibacterial activities. Apart from α-DCs, FR and DOFR are the self-condensation products of GlcN. The latter have been reported to have anti-inflammatory properties and are applied in therapeutic uses. At the same time, non-enzymatic browning reactions can generate potential toxic compounds, including 4-methylimidazole (4-MEI), 2-acetyl-4-tetrahydroxybutylimidazole (THI), and 5-hydroxymethylfurfural (5-HMF).

The first study aimed to examine the non-enzymatic browning of GlcN under UV-C radiation at 25 °C, referred as “cold caramelization”. Several UV-C exposure times were studied, including 0, 20, 60, 120 min at two GlcN concentrations of 15% and 30%. The physico-chemical properties, quantitation of α-DCs and polyhydroxylalkyl pyrazines (FR and DOFR), alkylimidazoles and 5-hydroxymethylfurfural were determined in all resulting GlcN caramels. The results revealed the possibility of generating GlcN caramel containing desirable compounds by

applying UV-C. Specifically, longer UV-C exposure time produced caramel with higher content of G and 3-DG, as well as the GlcN self-condensation products, FR and DOFR. The amount of glucosone was significantly higher compared to GlcN caramels produced under heat treatment. Additionally, none of the neo-formed contaminants were detected during the process. Thus, UV-C treatment may be a newly advantageous method for producing GlcN caramel.

The second study focused on the effect of spray-drying on the non-enzymatic browning of GlcN incubated at 50 °C and 90 °C for 12 h. The resulting spray-dried GlcN caramel powders were analyzed along with the non-spray-dried GlcN caramel solutions to compare their physico-chemical properties and concentrations of polyhydroxylalkyl pyrazines, alkylimidazoles, and heterocyclic compounds. Spray-dried GlcN caramels were found to have lower concentrations of both non-volatile FR and DOFR, as well as THI and 5-HMF.

In summary, this thesis examined the non-enzymatic browning of GlcN under the application of UV-light and spray-drying, which revealed the possibilities of creating desirable caramels containing bioactive compounds. The resulted caramels can be potentially applied in different food applications.

Preface

This thesis is an original work of Fuyao Zou, which is presented with five chapters.

Chapter 1 contains a brief introduction of non-enzymatic browning reactions, the research problem, hypothesis, and objectives of the experimental studies. Chapter 2 is the research background which consists of the major aspects of non-enzymatic browning of GlcN. Specifically, the production and application of different caramel colors as well as the production of GlcN and the produced desirable bioactive compounds presented in GlcN caramels were introduced. Chapter 3 and 4 are the experimental chapters, where two studies focusing on the effect of UV-C and spray-drying on the non-enzymatic browning of GlcN were presented. Chapter 5 is the summary of the research results along with the implications of this research and possible future directions.

The thesis author was responsible for the experimental design, sample analysis, data collection and thesis preparation. Dr. Yuliya Hrynets assisted with the technical support during the experiments and proof reading of the thesis. Dr. Mirko Betti was the supervisory author who was involved in providing advisory inputs in the experimental design and analysis, along with thesis composition and proof reading.

Acknowledgements

I would like to first give special thanks to my principal supervisor Dr. Mirko Betti for his continuous support and motivation. I'm very grateful for his trust in me even with my complicated health issues. Although the COVID-19 situation caused physical distance between us, he was always with me mentally and emotionally. I have learnt so much useful knowledge from him which I believe will change my future career and way of thinking.

I would like to acknowledge my supervisory committee members Dr. Roopesh Mohandas Syamaladevi and Dr. Zbigniew (Zeb) Pietrasik. They assisted me during my experiments and provided valuable comments on my work.

I would like to thank Dr. Chen for taking time evaluating my thesis and being the exam chair.

I would like to acknowledge Dr. Yuliya Hrynets for her long-term support on my experiments and thesis. She provided valuable advice throughout my study and made me feel warm and confident all the time.

Many thanks to my friend Tianzhi Yang for his support during thesis formatting. Our communication throughout my study helped me learn so much.

Last but not least, I would like to thank my friends and family for their support. My parents always encourage me no matter what happened. My husband always assists me throughout my daily life. I could not complete my work without him.

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Abbreviations

*a** – redness
α-DC – alpha-dicarbonyls
ANOVA – analysis of variance
*b** – yellowness
C^*_{ab} – chroma
DA – diacetyl
DFAs – di-D-fructose dianhydrides
3-DG – 3-deoxyglucosone
3,4- DGE – 3,4-dideoxyglucoson-3-ene
DOFR – deoxyfructosazine
FR – fructosazine
Fru – fructose
G - glucosone
GlcN – glucosamine
GlcNAc – *N*- acetyl-D-glucosamine
GO – glyoxal
h_{ab}– hue angle
HMF – 5-hydroxymethyl-2-furfural
HPA – hydroxypyruvaldehyde
HPLC – high-performance liquid chromatography
*L** – lightness
LOD – limit of detection
LOQ – limit of quantitation
4-MEI – 4-methylimidazole
MGO – methylglyoxal
MS – mass spectrometry
ND – not detected
OPD – *o*-phenylenediamine
PVDF – poly(vinylidene fluoride)
SEM – standard error of the means
THI – 2-acetyl-(4)5-tetrahydroxylbutyl imidazole
UV– ultraviolet light

CHAPTER 1. General introduction and objectives

Non-enzymatic browning reactions are important and complex reactions occurring during food processing and storage (Chen et al., 2019). One of the reactions, the Millard reaction, involves both a carbonyl compound, which is often a reducing sugar, and an amine, which is usually a peptide, an amino acid, or a protein. By contrast, caramelization is the degradation of sugar itself, where the amine is absent (Nursten, 2005). Many factors can affect non-enzymatic browning reactions, which include the concentration of reactants, initial pH, temperature, heating time, water activity, etc. (Wu et al., 2014). The Maillard reaction is divided into three stages: the initial stage, the intermediate stage, and the final stage (Hodge, 1953). The initial stage of the Maillard reaction occurs between a reducing sugar and a free amino group, which results in the formation of an unstable Schiff base. It then rearranges rapidly to form a more stable Amadori (for aldoses) or Heyns product (for ketoses).

The intermediate stage involves the degradation of the Amadori or Heyns products, producing reactive α-dicarbonyl compounds (α-DCs) that exert important color and flavor properties. They then react with other compounds including amino acids, amines, ammonia, and aldehydes to form pyrazines, pyrroles, thiazoles, oxazoles and classes of heterocyclic compounds (Zhang et al., 2009). The final stage forms polymeric compounds with higher molecular weight, including melanoidins, pyrazines, pyrroles, furans and oxazoles (Nursten, 2005).

Glucosamine (GlcN, 2-amino-2-deoxy-D-glucose) is an amino monosaccharide, which is widely known as a dietary supplement in the treatment of osteoarthritis, knee pain, and back pain (Benavente et al., 2015). The most common route of production for GlcN includes hydrolysis and deacetylation of chitin, which is a natural polysaccharide that is abundant in insect exoskeletons, crustacean shells, and fungal cell walls (Benavente et al., 2015). Another chemical way of

producing GlcN is through the reaction between fructose and ammonia, which involves both caramelization and the Maillard reaction (Lv et al., 2017). The nonenzymatic browning of GlcN generates caramel that has shown great bioactivity, including antioxidant (Hong and Betti, 2016) and antimicrobial (Hrynets et al., 2016) properties. Numerous desirable compounds are present in the GlcN caramel, for example, α-DCs, fructosazine (FR) and deoxyfructosazine (DOFR), melanoidins, etc. α-DCs not only act as the precursor to the color, aroma, and flavor of caramels, but also possess great bioactivity. For example, MGO, GO (Mavric et al., 2008), DA (Jay, 1984), and 3-DG (Hrynets et al., 2016) have been reported to have antibacterial activities. FR and DOFR are the major compounds found in the GlcN caramel, produced by the self-condensation reaction of GlcN. They are used as flavoring agents in many food systems, including caramel (Tsuchida, 1986), roasted peanuts (Magaletta, 1996), and soy sauce (Tsuchida et al., 1990). Apart from these, FR and DOFR are also shown to have anti-inflammatory properties, including their roles against type II diabetes (Zhu et al., 2007), as well as cartilage degradation (Giordani et al., 2006).

Conventionally, UV-C is applied in liquid and solid foods to reduce or eliminate undesirable microorganisms (Gurzadyan et al., 1995). However, recently, studies have shown that under UV-C treatment, proteins can undergo chemical modification due to the reaction between chromophores (e.g., tryptophan, tyrosine, phenylalanine, cysteine, flavins and heme) and other excited state species (Davies, 2003). As a result, both protein structure and their properties are modified where side-chain oxidation, backbone fragmentation and/or formation of cross-links and aggregates occur (Pattison et al., 2012). For example, UV-C treatment has shown to potentially improve the antioxidant activity in β-lactoglobulin (Wu et al., 2017). Additionally, in the case of egg white, UV-C treatment has shown to affect its foam stability, emulsifying activity, and immunoreactivity (Manzocco et al., 2012; Mendes de Souza et al., 2015). A study conducted by

Manzocco et al., in 2021 revealed the capability of egg white proteins and carbohydrates (i.e., glucose, maltose, trehalose and maltodextrin) undergoing non-enzymatic browning reactions during UV-C exposure. The results have shown that the UV-C induced glycation led to a decreased viscosity, a decreased gel forming temperature with a better foam stability. This study revealed the possibility of applying UV-C light to carbohydrates for the occurrence of non-enzymatic browning reactions.

In terms of GlcN, it can form self-condensation products (FR and DOFR) during non-enzymatic browning reactions which absorbs UV-light at 275 nm (Candiano et al., 1988; Horowitz, 1991). This wavelength falls into the wavelength range of UV-C, with a range from 100 to 280 nm. Thus, GlcN might be able to form caramels containing these compounds during the UV-C treatment.

Industrially, caramels are produced by controlled heat treatment, where food-grade acids, alkalis, and salts may be added to facilitate the process, where the reaction temperature is often greater than 120 °C (Kamuf et al., 2003; Kroh, 1994). One issue associated with the production of caramels at high temperatures is the formation of a series of toxic neo-formed contaminants, including 4-methylimidazole (4-MEI), 2-acetyl-4-tetrahydroxybutylimidazole (THI), and 5-hydroxymethylfurfural (5-HMF). The concentration of these compounds needs to be monitored, and limits are set by the regulatory agencies. By contrast, the degradation of GlcN can occur at a temperature as low as 4 °C, referred as “cold caramelization” (Zhao et al., 2020). Previous studies also targeted temperatures from 37 °C to 70 °C for generation of GlcN caramels with the application of heat (Hrynets et al., 2015a; Dhungel, 2018). As a result, GlcN is unstable and capable of undergoing non-enzymatic browning reactions at temperatures far lower than the conventional caramelization temperature. Similarly, by applying UV-C, the reaction temperature

can also be controlled at a fix temperature, for example, 25 °C, in order to reduce or ideally, eliminate the formation of neo-formed contaminants.

The general objectives of this thesis were:

a) To understand the chemistry of non-enzymatic browning of GlcN under UV-light;

b) To study the effect of spray drying at different conditions on composition of resulted GlcN caramels powders;

c) To manufacture GlcN caramel solutions with desirable bioactive compounds at the same time minimizing the formation of potential hazardous compounds, or to produce caramel powders with potentially longer shelf life that still contains desirable bioactive compound.

The first study of this thesis, described in Chapter 3, aimed to better understanding the chemistry of "cold caramelization" of GlcN under UV-C radiation. The specific objectives of this study were:

a) To measure the physico-chemical changes of GlcN caramels produced by application of UV-C with exposure times of 0, 20, 60, and 120 min at 25 °C.

b) To quantify the long and short chain α-DCs;

c) To quantify FR and DOFR, which are the major polyhydroxyalkyl pyrazines formed during self-condensation of GlcN;

d) To quantify neo-formed contaminants.

The second study of this thesis, described in Chapter 4, aimed to understand the effect of spray-drying on GlcN caramels. The specific objectives of this study were:

a) To measure the physico-chemical changes of non-spray-dried and spray-dried GlcN caramel incubated at 50 °C and 90 °C for 12 h;

b) To quantify FR and DOFR, which are the major polyhydroxyalkyl pyrazines formed during self-condensation of GlcN;

c) To quantify neo-formed contaminants.

CHAPTER 2. Research background

2.1. Chemistry of non-enzymatic browning reactions

Non-enzymatic browning reactions are important and complex reactions commonly occurring during food preparation and storage (Chen et al., 2019). They are divided into Maillard, caramelization and ascorbic acid browning reactions (Nursten, 2005). This review of the literature will focus on the chemistry of the Maillard and caramelization reactions.

The Millard reaction involves both a carbonyl compound, which is often a reducing sugar, and an amine, which is usually a peptide, an amino acid, or a protein. By contrast, caramelization is the degradation of sugar itself, where the amine is absent (Nursten, 2005).

Many factors can affect non-enzymatic browning reactions, which include the concentration of reactants, initial pH, temperature, heating time, water activity, etc. (Wu et al., 2014).

2.1.1. The Maillard reaction

The Maillard reaction was named after Louis-Camille Maillard (1878-1936), who first described it in 1912. Since then, the Maillard reaction was studied by scientists all over the world as it occurs every day in human's lives and is fundamental to the food science. Another scientist, Robert Ling (1861-1937) reported that amino compounds produced from proteins during kilning reacted with sugars (ie. glucose and maltose) at 120-140 °C. He hypothesized those were glucosamine-like compounds (Nursten, 2005).

The Maillard reaction is extremely complex due to the production of a wide range of compounds during its process. In 1953, a simplified scheme of the reactions was created by John Hodge, who divided the Maillard reaction into three stages: the initial, the intermediate, and the final stages (Nursten, 2005).

2.1.1.1. Initial stage

The initial stage of the Maillard reaction occurs between a reducing sugar and a free amino group, which results in the formation of a reversible *N*-substituted glycosylamine also known as a Schiff base (Figure 2.1) (Zhang et al., 2009). It is very unstable and rearranges rapidly to form a more stable Amadori (for aldoses) or Heyns (for ketoses) product (Wrodnigg & Eder, 2001). The products of the initial stage are colorless without absorption in the ultraviolet light at around 280 nm (Nursten, 2005) and possess no distinct aroma.

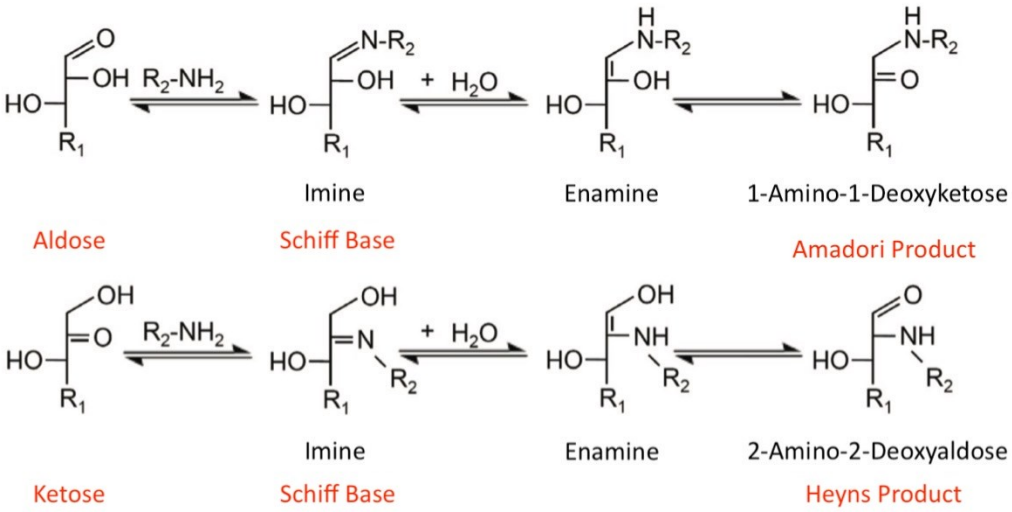


Figure 2.1. The initial stage of the Maillard reaction. Formation of Amadori and Heyns products from aldoses and ketoses. The figure is reproduced with permission from Grimm, Stefanie (2012): Advanced Glycation End Products (AGEs) in Säugerzellen: Abbau, Akkumulation und zelluläre Reaktionen. *Digitale Bibliothek Thüringen*, 1-115. Copyright (2012) DBT.

2.1.1.2. Intermediate stage

The intermediate stage (Figure 2.2) begins with the degradation of the Amadori or Heyns products in various ways including sugar dehydration, sugar fragmentation, and amino acid degradation (Strecker degradation) which produces furfurals, carbonyl, and hydroxycarbonyl compounds (Nursten, 2005). All of these compounds can be formed directly from sugar, without the presence of an amino compound (Zhang et al., 2009).

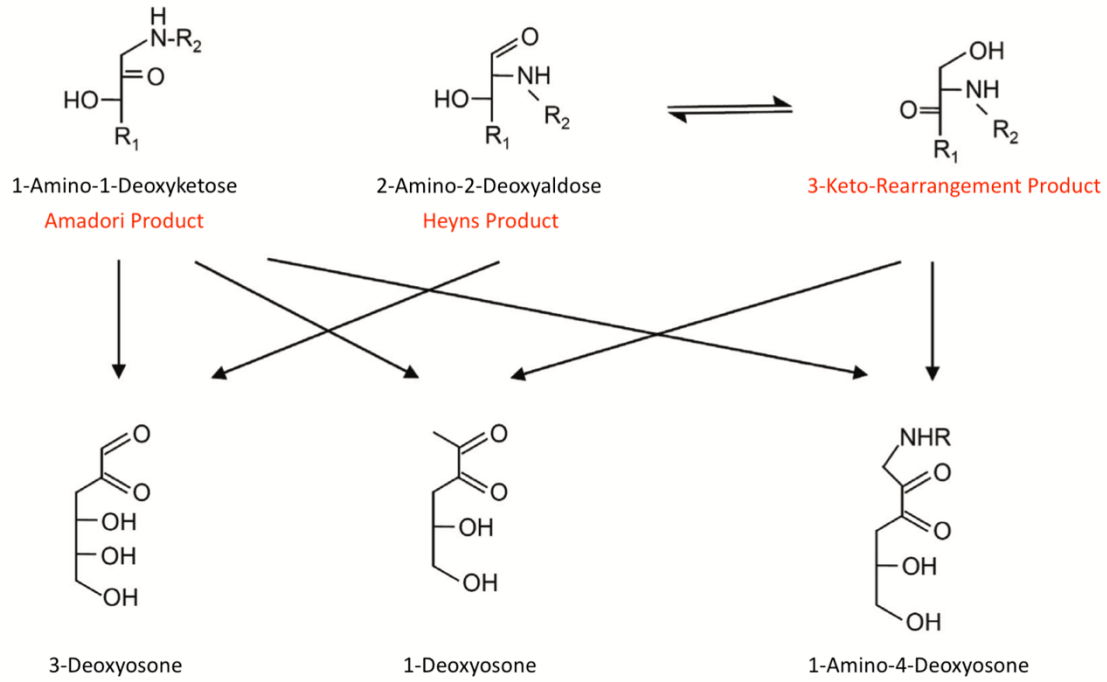


Figure 2.2. The intermediate stage of Maillard reaction. Formation of deoxyosones from Amadori and Heyns products. The figure is reproduced with permission from Grimm, Stefanie (2012): Advanced Glycation End Products (AGEs) in Säugerzellen: Abbau, Akkumulation und zelluläre Reaktionen. *Digitale Bibliothek Thüringen*, 1-115. Copyright (2012) DBT.

During sugar dehydration, furfurals are produced under acid conditions via 1,2-enolization and the 3-deoxyosone, whereas reductones are produced under neutral or alkaline conditions via 2,3-enolization and the 1-deoxy-2,3-dicarbonyl (Nursten, 2005). Sugar fragmentation occurs mostly by retroaldolisation with the cleavage of hexose derivatives. The produced α-dicarbonyl compounds such as butanedione, glyoxal, and methylglyoxal can react with amino acids during the Strecker degradation to produce subsequent imines, aldehydes, or ketones (Zhang et al., 2009).

The products of the intermediate stage are either colorless or yellow, with strong absorption in the ultraviolet light (Nursten, 2005). This is a crucial stage in terms of generating color, aroma, and flavor.

2.1.1.3. Final stage

The final stage involves aldol condensation and aldehyde-amine condensation, which forms polymeric compounds with higher molecular weight. Aldol condensation of the furfurals, reductones, and aldehydes produced in the intermediate stage occurs without the intervention of amino compounds (Zhang et al., 2009). This step can be effectively catalysed by amines. During aldehyde-amine condensation, polymeric, high molecular weight, and colored products called melanoidins are produced. Melanoidins contain heterocyclic ring systems, with 3-4% nitrogen (Nursten, 2005). Due to its complexity, very few structures of melanoidins have been described, and one proposed carbohydrate-based melanoidin structure is shown in Figure 2.3.

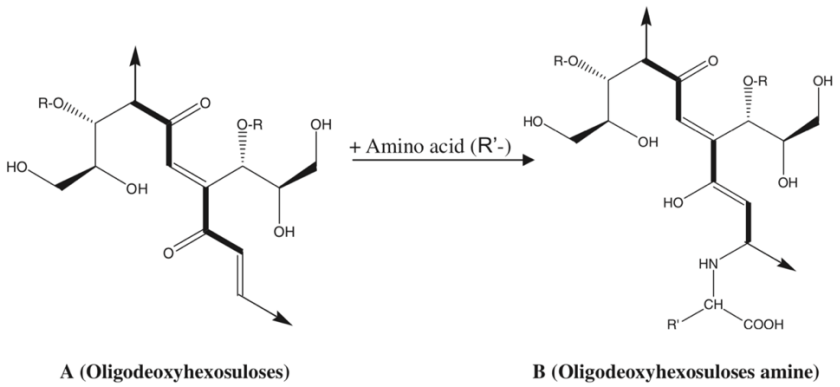


Figure 2.3. A proposed carbohydrate-based melanoidin structure. The figure is reprinted with permission from Wang, H. Y., Qian, H., & Yao, W. R. (2011). Melanoidins produced by the Maillard reaction: Structure and biological activity. *Food Chemistry*, *128,* 573-584. Copyright (2011) Elsevier Ltd.

Besides, the Amadori products can also form crosslinks between adjacent proteins or with other amino groups, resulting in polymeric aggregates known as advanced glycation end products (AGEs) (Friedman, 1996). The structure of some well-known AGEs is shown in Figure 2.4., including N^{ε}-carboxymethyl-lysine (CML) and N^{ε}-carboxyethyl-lysine (CEL) (Figure 2.4. A); N^{δ}-(5-hydro-5-methyl-4- imidazolon-2-yl)-ornithine (MG-H1) and pentosidine (Figure 2.4. B); S-carboxymethyl-cysteine (CMC) and S-(1-carboxyethyl)cysteine (CEC) (Figure 2.4. C), etc. AGEs

have lower molecular weight than melanoidins, and are considered as the reactive intermediates, which condenses to form melanoidins (Van Der Lugt et al., 2020). Compared to the other stages, the products of this final stage are highly colored, for example, melanoidins have dark brown to black colours.

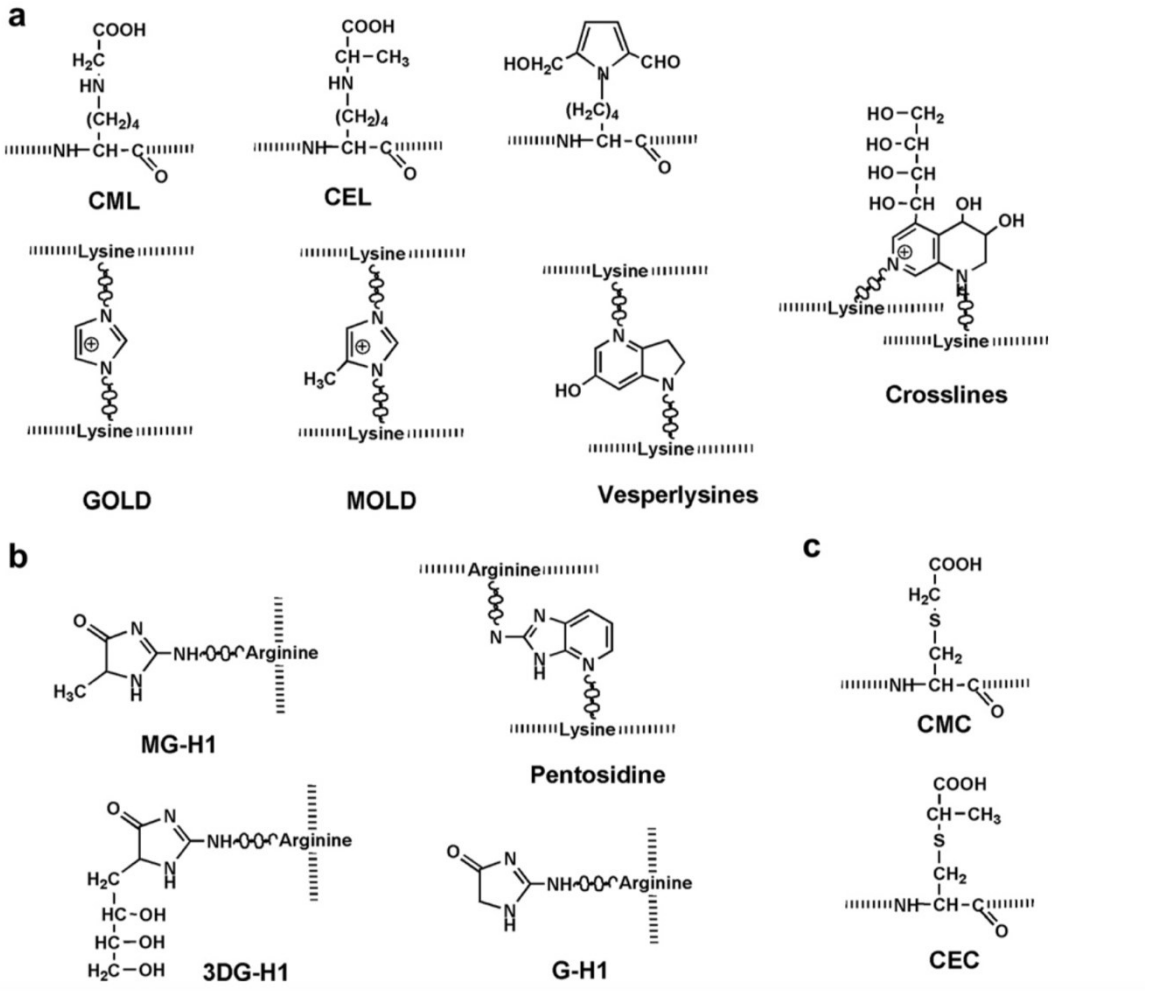


Figure 2.4. Structures of main AGE (a) derivatives of lysine residues; (b) derivatives of arginine residues; (c) derivatives of cysteine residues. CML: N^{ε}-carboxymethyl-lysine; CEL: N^{ε}-(1-carboxyethyl)lysine; GOLD: glyoxal-derived lysine dimmer; MOLD: bis(lysyl)imidazolium cross-links methylglyoxalderived lysine dimmer; MG-H1: N^{δ}-(5-hydro-5-methyl-4- imidazolon-2-yl)-ornithine; 3DG-H1: N^{δ}-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine; G-H1: N^{δ}-(5-hydro-4-imidazolon-2-yl)-ornithine; CMC: S-carboxymethyl-cysteine; CEC: S-(1-carboxyethyl)cysteine. The figure is reprinted with permission from Rondeau, P., & Bourdon, E. (2011). The glycation of albumin: Structural and functional impacts. *Biochimie*, *93*, 645–658. Copyright (2010) Elsevier Ltd.

2.1.2. Caramelization

Caramelization is one of the oldest methods to create color and flavor in foods. It occurs without the presence of an amino group, where sugar degrades by itself. The process of caramelization requires temperatures >120 °C or 9<pH<3 (Kroh, 1994). Its reaction rate is affected by pH, as well as the concentration and type of sugar (Eggleston and Vercellotti, 2000). Depending on the food product, caramelization can be either desirable or undesirable. For example, during the production of candies and bakery products, it is very important to generate the desired color, flavor, and aroma; however, it is undesirable during the production of sugar crystals, as the resulting color change will affect the appearance of the product (Kroh, 1994).

As shown in Figure 2.5., a sequence of sugar degradation reactions occurs during thermal caramelization, including enolization, dehydration, dicarbonyl cleavage, retroaldolisation, aldolization, and radical reaction. Enolization is of great importance as it initiates a chain of reactions and gives rise to aliphatic sugar degradation products that will react further via aldol condensation to generate oxygen heterocyclic and carbocyclic compounds (Kroh, 1994).

Based on these key steps, the key intermediates of thermal caramelization are α-dicarbonyl compounds such as 3-deoxyhexosulose and diacetyl. They contribute to the formation of caramel color and flavor (Kroh, 1994). Indeed the diacetyl is responsible for buttery or butterscotch flavor of caramels. Volatile compounds that contribute to caramel flavor are produced mostly during the initial steps during caramelization, and as the reactions continue, polymeric compounds that are brown-colored gradually form via radical polymerization, causing the bitter taste (Luna and Aguilera, 2014).

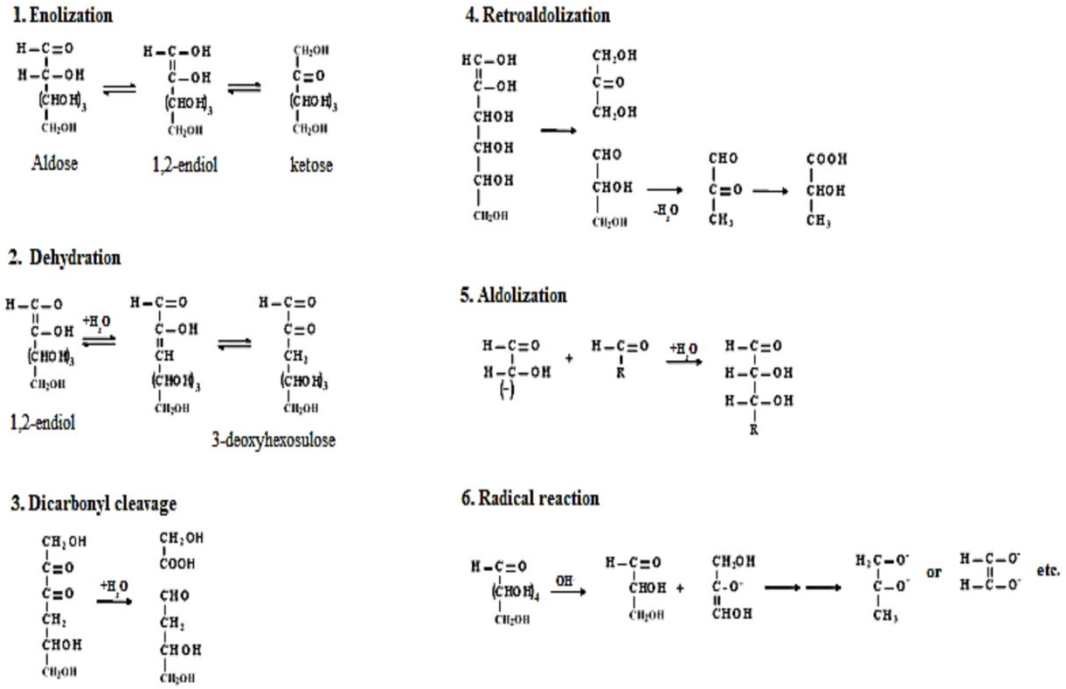


Figure 2.5. A proposed sequence of sugar degradation during thermal caramelization. The figure is reproduced with permission from Kroh, L. W. (1994). Caramelization in food and beverages. *Food Chem*istry, *51*, 373-379. Copyright (1994) Elsevier Ltd.

Low molecular weight (LMW) and high molecular weight (HMW) compounds are both produced during caramelization. HMW compounds are non-volatile, which represent 90-95% of the produced compounds in caramelization, whereas LMW compounds are volatile, which account for the remaining 5-10% (Paravisini et al., 2012). In order to study the characteristics of the major HMW compounds presented in caramel, numerous analytical techniques were used to separate them, including gel permeation, and cellulose ion-exchange chromatography. However, no precise structures of these complex macromolecules were reported. On the other hand, the identification of LMW compounds has shown considerable progress. For example, N-heterocyclic compounds including pyridines, pyrazines, and imidazoles have been identified in caramel colors, and because of their volatile nature, they can be directly identified by gas-liquid chromatography-mass spectrometry (Myers and Howell, 1992).

2.1.3. Production and applications of caramel

Caramel colors are distinctive yellow to red to dark-brown viscous liquids or hygroscopic powders that are applied to a wide range of products including foods and beverages, animal feeds, cosmetics, and pharmaceuticals. They have a burnt sugar odor, and a pleasant, slightly bitter taste (Vollmuth, 2018).

Caramel colors have been used in foods and beverages for over 150 years globally and are consumed at an estimated level of 50 Mt annually (Vollmuth, 2018; Golon and Kuhnert, 2012). In 1983, the first commercial caramel colors were produced in the United States. They can be found in many familiar products: carbonated beverages, bakery products, and even in jams and jellies, cereals, and soups. Apart from these common applications, they are also used in meats, sauces, and seasoning (Vollmuth, 2018).

In addition to their uses as color additives, caramel colors possess other valuable functional properties in foods. For instance, they can slowly absorb oxygen and stabilize colloidal systems, thus facilitating flavor retention and preventing hazes in beers. Additionally, their emulsifying properties can facilitate the dispersion of water-insoluble compounds, for example, flavoring oils, in an aqueous phase. They can also retard flavor changes caused by exposure to light during storage in some beverages. Some caramel colors with better foaming capacity are desired to be used in root beers (Chappel and Howell, 1992).

2.1.3.1. Production of caramel

Caramel colors are a complex mixture prepared by heating carbohydrates under controlled temperature and production conditions (pressure, pH, time, etc.), with or without the addition of chemical reactants.

Commercially available food-grade nutritive sweeteners are used as the raw material for caramel colors production, including monomers such as glucose and fructose, as well as polymers such as glucose syrups, sucrose, invert sugars, and dextrose (Kamuf et al., 2003). Of these, sucrose (i.e. table sugar), is most commonly used to produce caramel. It decomposes to fructose and glucose, and the dimerization of fructose result in D-fructose dianhydride (DFAs) (discussed further in 2.1.3.5.1). In the United States, high dextrose corn syrup is a commonly used as a starting material (Vollmuth, 2018).

In order to facilitate the process, food-grade acids, alkalis, and salts may be added based on regulations (Kamuf et al., 2003). Food-grade acids include, for example, sulfuric, citric, phosphoric, acetic, or carbonic acids. As for alkalis, sodium, potassium, calcium hydroxides, or mixtures of these are often used. Other catalysts such as ammonium compounds may be used depending on the process (Vollmuth, 2018). Other than these, on some occasions, food-grade polyglycerol esters of fatty acids with minimum amount may be used as processing aids to achieve antifoaming effects in the product (Kamuf et al., 2003).

Commercial caramel production is conducted by mixing and blending water, corn syrup, refined and/or brown sugar, dairy solids, milk fat or vegetable fat, and emulsifier (Mendenhall and Hartel, 2016). The resulting mixture is then heated at 190-250 °C without the use of catalyst or 130 °C with catalyst. Among the choices of catalysts, ammonia is superior as it can sufficiently reduce the temperature of caramelization (Tomasik, 2016).

The production process usually takes place batchwise in entirely stainless steel, including either open or pressure kettles, lines, agitators, fillers, and storage tanks. When using pressure kettles, the typical gauge pressure is 483 kPa (70 psi, 5 atm), at up to 160 °C. Continuous

monitoring of reaction temperature and time is required during the process to maintain a uniform product (Tomasik, 2016).

As for the production of caramel powder, spray-drying can be applied to remove water from the substrate. Spray-drying is an important and conventional food processing technology, which generates dry powder from a solution or slurry by applying hot gas. The liquid solution is sprayed into a hot air chamber, resulting in the evaporation of water or other solvents, producing products with generally consistent particle size (Li et al., 2019).

Various ingredients are added during the process to monitor the flavor, color, and texture of caramel. For example, adding milk can provide softness to the product; adding corn or maple syrup can improve the sweetness and prevent aggregation; adding fat can enrich the taste (Tomasik, 2016). Milk also contributes to a higher protein content of the product, where casein can create a firm and chewy texture, while whey proteins are essential for color and flavor. A study conducted by Mendenhall and Hartel (2016) revealed that increasing protein content during the production of caramel can increase the cooking rate and reduce the holding time. Therefore, caramels with increased protein content, lower production temperature, lighter color, and enriched taste become increasingly desirable.

2.1.3.2. Types of caramel

Caramel colors have been used as a food additive in a variety of foods for a very long time. It is the oldest colorant which accounts for more than 80% (by weight) of all colorants added to foods and beverages (Sengar and Sharma, 2014).

In 1965, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) first reviewed caramel colors (JECFA, 1965). With a more in-depth understanding of caramel color, JECFA ultimately recognized four classes of caramel color in 1980 based on the reactants used

during manufacture and their applications (JECFA, 1980). Likewise, the Food Chemicals Codex (FCC), which is a listing of standards for the identity, purity, and quality of food ingredients, classified caramel colors into four different types (Table 2.1.) (Codex, 1996).

a) Caramel Color I (plain or spirit caramel): This type of caramel is produced with or without acids and alkalis, with no ammonium or sulfite compounds being added during the process.

b) Caramel Color II (caustic sulfite caramel): This type of caramel is produced with or without acids and alkalis, where sulfite compounds are added but without any ammonium compounds.

c) Caramel Color III (ammonia or beer caramel, baker's and confectioner's caramel): This type of caramel is produced with or without acids and alkalis, where ammonium compounds are added but without any sulfite compounds.

d) Caramel Color IV (sulfite-ammonia, soft drink caramel, or acid-proof caramel): This type of caramel is produced with or without acids and alkalis, with both ammonium and sulfite compounds being added during the process.

Common ammonium compounds used are ammonium hydroxide, ammonium carbonate and ammonium hydrogen carbonate, ammonium phosphate, ammonium sulfate, ammonium sulfite and ammonium hydrogen sulfite (Vollmuth, 2018). Sulfite compounds used are sulfurous acid, hydrogen sulfites, and sulfites and bisulfites of potassium, sodium and ammonium. For all four types of caramel color, sulfuric and citric acid, sodium, potassium, and calcium hydroxide can be used during the manufacture (Kamuf et al., 2003). The most common caramel colors used in foods and beverages are class II, III, and IV caramels (Nursten, 2007).

Table 2.1. Codex classification of caramel. The table is reprinted from Codex, 1996.

Parameters	Class I – E150 a	Class II – E150 b	Class III – E150 c	Class IV – E150 d

Color intensity	0.01-0.12	0.06-0.10	0.08-0.36	0.10-0.60
Total nitrogen (%)	<0.1	<0.2	1.3-6.8	0.5-7.5
Total sulphur (%)	<0.3	1.0-3.5	<0.3	1.4-10.0

Other than the reactants being used during the production, other factors can also be considered in terms of caramel colors classification. Caramels can be roughly classified based on their isoelectric points (pIs): positive (pI 5.0–7.0), negative (pI 4.0– 6.0), and spirit (pI < 3.0). Caramel is negatively charged at pH above its pI, and positively charged below the pI. Using pI, the application of caramel can be determined accordingly. Other important properties include pH, aqueous solubility, specific gravity (usually 1.315– 1.345), color intensity or tinctorial strength, hue index, and flavor (Sengar and Sharma, 2014).

2.1.3.3. Colors of caramel and its applications

According to international standards, color intensity is defined as the absorbance of a 0.1% (w/v) solution of caramel color solids in water in a 1-cm cell at 610 nm. A common method to determine caramel color involves the use of a colorimeter or comparator to match it to different standardized colored glasses (Kamuf et al., 2003). Another parameter important for the determination of caramel color is the hue index (HI), which is estimated from absorbance values at 510 and 610 nm. As HI increases, the yellowness of the caramel color also increases. By contrast, a lower HI indicates a browner product (Sepe et al., 2008).

Before applying a caramel color to foods, it is very important to understand its color properties to best match its appearance and flavor to the specific food. For example, a Class IV caramel color is typically used in soda drinks as it can add the classic "cola" color to those beverages. By contrast, a Class I caramel color can be very effective when adding to coffee, as it can help enhance the color and flavor of coffee products. Table 2.2 shows the common applications of caramel color based on their HI (Sepe et al., 2008).

Table 2.2. Hue Index (HI) of four classes of caramel color. The table is reproduced with permission from Sepe, H. A., Parker, O. D., Nixon, A. R., & Kamuf, W. E. (2008). In colour quality of fresh and processed foods. Global colour quality of beverages utilizing caramel colour. *ACS Symposium Series*, *18*, 226–240. Copyright (2008) American Chemical Society.

	Class I	Class II	Class III	Class IV
Hue index	>7.0-6.3	5.5-<4.5	6.3-5.0	5.5 - < 4.5
Color	pale to bright yellow	amber to dark brown	golden yellow to reddish-brown	reddish-brown to dark brown
Major applications	flavoring	coloring	flavoring or coloring	ready-to-eat candies, fillings, toppings, etc. Soft drinks and confectionary
Typical use	alcoholic spirits, coffee, meat, bread, spice blends, desserts	liqueurs (i.e., vermouths and brandies), aromatic extracts	beers, vinegar, biscuits, sauces (i.e., gravies)	

Another crucial aspect to consider for caramel colors is its stability. The colloidal charge of each class of caramel color is different. Class I with the fewest reactants, carries a slightly negative charge, while Class II and IV with sulfites as the catalyst are strongly negative. By contrast, Class III with ammonium compounds as the catalyst, are strongly positive. The colloidal charge can be highly affected by pH, thus altering pH can change its charge (Kamuf et al., 2003).

Normally the shelf life of a caramel color under ambient storage conditions (<22 °C and <60 relative humidity) is either one or two years depending on its class (Sepe et al., 2008). As caramelization reaction continues at a slow rate during storage, the color and viscosity of caramel colors will gradually increase with time (Kamuf et al., 2003).

Caramel colors contain both volatile and non-volatile compounds (shown in Table 2.3.), where three non-volatile compounds named caramelan ($C_{24}H_{36}O_{18}$), caramelen ($C_{36}H_{50}O_{25}$), and caramelin ($C_{96}H_{102}O_{51}$) were first reported by the French chemist M. A. Gelis in 1858. They are found in sucrose caramels, with the latter two being polymeric (Gelis, 1858).

Table 2.3. Coloring and flavoring compounds of caramel. Table is reprinted with permission from Sengar, G., & Sharma, H. K. (2014). Food caramels: a review. *Journal of Food Science and Technology*, 51, 1686-1696. Copyright (2014) Springer India.

Coloring compounds of caramel (Non-volatile compounds)
$C_{24}H_{36}O_{18}$ (caramelan)
$C_{36}H_{50}O_{25}$ (caramelen)
$C_{96}H_{102}O_{51}$ (caramelin)
Flavoring compounds of caramel (Volatile compounds)
acetylfuran
furfurol
5-hydroxymethylfurfural
3-hydroxy- 2-acetylfuran
3-hydroxy-2(5H)-furanones
4-hydroxy-3(2H)-furanones
4-pyrone derivatives

2.1.3.4. Flavour/aromatic compounds associated with caramels

Numerous compounds are produced during the manufacture of caramel depending on the ingredients and reaction conditions. The Maillard reaction and caramelization generates the characteristic caramel flavor and the vocabulary of a typical lexicon of a caramel flavor is listed below in Table 2.4.

Table. 2.4. Lexicon of vocabulary used to describe the typical components of a caramel flavour. Information is adapted from Reineccius, G., & Heath, H. B. (1994). *Source book of flavors.* First edition. Boston: Springer

Descriptors	Chemical compound
rummy	ethyl butyrate, ethyl heptonoate
buttery	diacetyl, acetoin, acetyl propionyl
maple	fenugreek, ethyl cyclopenteolone
brown sugar	maltol, 3-hydroxyl-4-5-di-methyl-2(5h)-furanone
fatty	2,4-decadienal

marshmallow	heliotropin and ethyl vanillin
coffee	coffee extract
vanilla	vanillin
burnt	guaicol, smoke flavour
fruity	gamma and deca lactones

Caramels contain both non-volatile and volatile compounds. The non-volatile fraction with high molecular weight represents 90-95% of the compounds in caramel. Some examples of non-volatile compounds are fructose dianhydrides, oligosaccharides, and melanoidins. The rest 5-10% are volatile compounds with low molecular weight, for instance, maltol, cyclotene, furaneol, and furfural (Paravisini et al., 2012). Compared with non-volatile compounds, volatile compounds are easier to separate and identify and they are also considered to play a major role in determining the overall flavor of the caramel. The characteristic volatile profile is sensed as aroma by humans when the partition of volatile flavor molecules from the food phases into the air phase occurs (Taylor, 1998). A study conducted by Paravisini et al. (2012) identified 49 aroma compounds, with some of them being reported in caramel for the first time. The sensory descriptors related to these compounds range from sweet-like descriptors such as cooked-syrup, fruity, honey to burnt sugar descriptors including strong, pungent and roasted. For burnt sugars, heterocycles, carboxylic acids, and carboxylic compounds appeared to be the major contribution. The aromatic molecules combined to create the typical aroma of caramel, with a balance of caramel, fruity, nutty, vegetal, animal, toasted, floral, and spicy notes.

2.1.3.5. Bioactivity of caramel

Caramel not only contains compounds that contribute to its color, flavor, and aroma but also compounds that possess bioactivity. Examples of such compounds are di-D-fructose dianhydrides (DFAs) and melanoidins, with both of them being non-volatile.

2.1.3.5.1. Di-D-fructose dianhydrides (DFAs)

DFAs are unique disaccharides that have shown remarkable nutritional values in foods. They are low-calorie sweeteners, with beneficial prebiotic functions, promoting the growth of *Bifidobacterium* spp. (García-Moreno et al., 2008). Prebiotics are non-digestible oligosaccharides that beneficially affect the colonic microflora by selectively stimulating the growth or activity of certain beneficial bacteria (Floch, 2014).

DFAs are formed during caramelization upon thermal or protonic activation of fructose and fructose-containing disaccharides (sucrose) and oligosaccharides (levan and inulin) (García-Moreno et al, 2008). Some typical chemical structures of DFAs are shown in Figure 2.6. They are potentially present in any carbohydrate-rich foods that are subjected to cooking or foods that contain caramel or other cooked sugars as an additive (García-Moreno et al., 2008). These include, for example, honey, commercial syrups (Ruíz-Matute et al., 2007), natural- and sugar-roasted torrefacto coffee (Montilla et al., 2006), heat-dried fruits (Defaye and Garcia Fernandez, 2000), roasted chicory (Defaye and Garcia Fernandez, 1995), and traditional tequila (Waleckx et al., 2008).

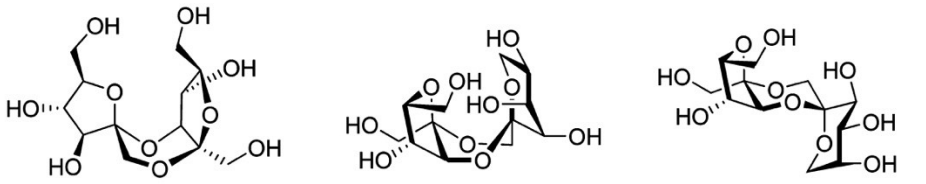


Figure 2.6. Chemical structures of selected di-D-fructose dianhydrides (1 and 2) and the D-fructose-D-glucose mixed dianhydride (3) present in caramel. Figure is reprinted with permission from Arribas, B., Suárez-Pereira, E., Ortiz Mellet, C., García Fernández, J.M., Buttersack, C., Rodríguez-Cabezas, M.E., Garrido-Mesa, N., Bailon, E., Guerra-Hernández, E., Zarzuelo, A. and Gálvez, J. (2010). Di-D-fructose dianhydride-enriched caramels: effect on colon microbiota, inflammation, and tissue damage in trinitrobenzenesulfonic acid-induced colitic rats. *Journal of Agricultural and Food Chemistry*, 58, 6476–6484. Copyright (2010) American Chemical Society.

In most of the studies on DFAs, they are reported to have bifidogenic, anticarcinogenic, and anti-tooth decaying effects (García-Moreno et al., 2008). Additionally, DFAs promote *in vitro* growth of Bifidobacteria (Kashimura et al., 1990). There are many beneficial effects associated with keeping a healthy *Bifidobacterium* spp. in the digestive tract of mammals, which include carcinogenesis inhibition (Reddy and Rivenson, 1993), decreasing blood pressure and blood cholesterol levels (Hidaka and Hirayama, 1991), vitamin B complex synthesis stimulation (Mitsuoka, 1990), inhibition of proliferation of undesirable bacteria such as *Clostridium perfringens* or *Escherichia coli* (Ibrahim and Bezkorovainy, 1993). Moreover, the use of DFAs-containing products in animal feeding (i.e., fowls) can protect them against digestive tract infections.

Several studies revealed that certain DFA isomers can improve intestinal mineral absorption. The ingestion of DFAs in daily diets was observed to increase the absorption of calcium, magnesium, and zinc in rats (Mineo et al., 2001). DFAs also prevent tannic acid-induced suppression of iron absorption, thus contributing to bone strength and preventing osteoporosis. These properties are stated to be exclusive to DFAs and are not observed in other tested oligosaccharides (Shiga et al., 2003).

During caramelization, DFAs often compete with unspecific intramolecular dehydration and condensation reactions of the starting sugar, generating furanic derivatives, such as 2- and 5-hydroxymethylfurfural, as well as oligomeric colour compounds and melanoidins (Rasrendra et al., 2012; Audemar et al., 2017). As a result, a new process of caramelizing fructose at a relatively lower temperature of 80−100°C with ion-exchange resin as a catalyst has been proposed (Idri et al., 2013).

2.1.3.5.2. Melanoidins

Melanoidins are nitrogen-containing, brown-colored polymeric compounds with high molecular weight. They are generated at the final stage of the Maillard reaction and caramelization and can absorb light at a wavelength as high as 420 nm. They are inevitable in heat-treated foods and are crucial for providing color to various food products. The structures of melanoidins are very complex and diverse, therefore it is difficult to definitely assign their positive or negative effects asserted to the human health. In the past the undesirable effects of melanoidins were associated with decreased nutritional value of foods due to the destruction of the essential amino acids, decreased digestibility, inactivation of the enzymes and others (Echavarría et al., 2012). In the recent years, more studies are focusing on the advantageous effects of the melanoidins. For instance, it has been reported that melanoidins can possess several beneficial biological effects, including antioxidative, antimicrobial, prebiotic activities, etc. (Wang et al., 2011).

Melanoidins were reported to effectively prevent oxidative damage related to free radicals. The antioxidant properties are partly associated with its metal chelating capacity. They are anionic, which enables them to chelate transition metals (Verzelloni et al., 2010). The other main mechanism that contributes to the antioxidative activity of melanoidins is their radical-scavenging property, where melanoidins in foods showed their ability to scavenge various reactive oxygen species (Michalska et al., 2008; Xu et al, 2007).

Melanoidins in foods, including coffee, beer, and sweet wine, have shown to have a greater antimicrobial activity towards gram-positive microorganisms (e.g. *Staphylococcus aureus*) compared to gram-negative microorganisms (e.g. *Escherichia coli*). The antimicrobial activity was described to be related to the metal-chelating properties of melanoidins, where they can disrupt both the outer and inner membranes of microorganisms (Rufian-Henares and de la Cueva, 2009).

Both *in vitro* and *in vivo* studies on melanoidins suggested that they can work as prebiotics in the gastrointestinal tract by escaping digestion and recovering in the feces (Faist and Erbersdobler, 2001; Borrelli and Fogliano, 2005). Moreover, they can promote the growth of Bifidobacteria in the gut, similar to that of dietary fibres (Borrelli and Fogliano, 2005).

The bioactivity of melanoidins is not limited to those stated above and as research continues on melanoidins, more understanding of these compounds will be gained. In terms of their content in caramel colors, the ammonia caramel colors are likely to contain higher concentrations (Sengar and Sharma, 2014).

2.1.3.6. Toxicity of Caramel

The production of caramel not only leads to the formation of beneficial compounds that exhibit color, flavor, and bioactive properties but also toxic compounds that are potentially hazardous to human health. Among these, three compounds that have been intensively studied are 4-methylimidazole (4-MEI), 2-acetyl-4-tetrahydroxybutylimidazole (THI), and 5-hydroxymethylfurfural (5-HMF).

2.1.3.6.1. 4-MEI and THI

4-MEI and THI are toxic imidazoles presented in caramel colors. 4-MEI is presented in Caramel Class III and IV, while THI is only found in Caramel Class III (Vollmuth, 2018). 4-MEI relies on methylglyoxal (MGO) as a product of carbohydrate pyrolysis, which condenses with nitrogen synthons to form the imidazole heterocycle. A proposed mechanism of its formation is shown in Figure 2.7. As for THI, D-glucose or D-fructose act as its precursor (Elsinghorst et al., 2013). A proposed mechanism of its formation is shown in Figure 2.8.

Although 4-MEI and THI belong to the same chemical group, their toxicity is different (Fierens et al. 2018). THI has been reported to affect the immune system in the short term, for

example, causing reduced circulation of lymphocytes in the blood, especially in organisms with vitamin B6 deficiency (Gobin and Phillips, 1991). By contrast, 4-MEI has been classified as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC, 2012).

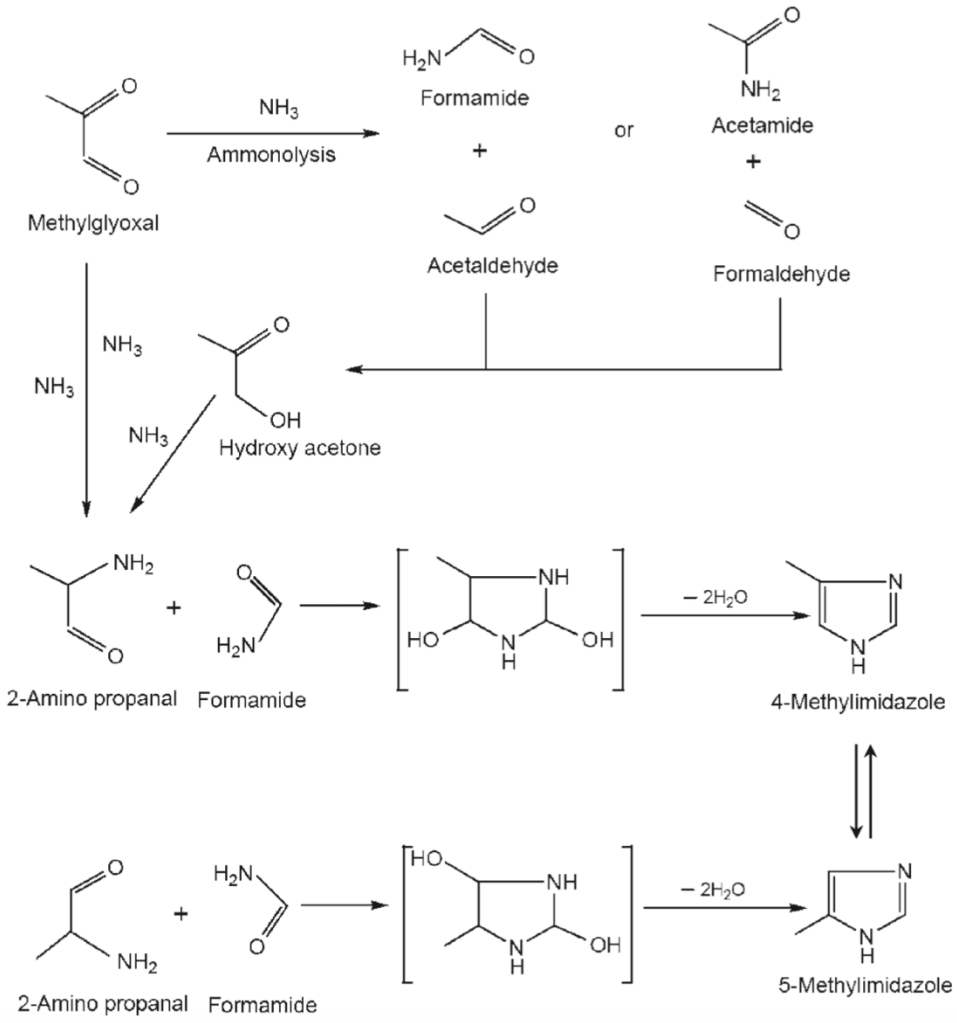


Figure 2.7. Proposed formation mechanism of 4-methylimidazole (4-MEI) from methylglyoxal. Figure is reprinted with permission from Moon, J. K., & Shibamoto, T. (2011). Formation of Carcinogenic 4(5)-Methylimidazole in Maillard Reaction Systems. *Journal of Agricultural and Food Chemistry*, *59*, 615–618. Copyright (2011) American Chemical Society.

Caramel Class III is often added to dark beers, while Caramel Class IV is typically used in producing cola soft drinks, both of them are very common in the human's diet. Research conducted

on the evaluation of these caramel colors has found the presence of both compounds in these beverages (MacKenzie et al., 1992a; MacKenzie et al., 1992b).

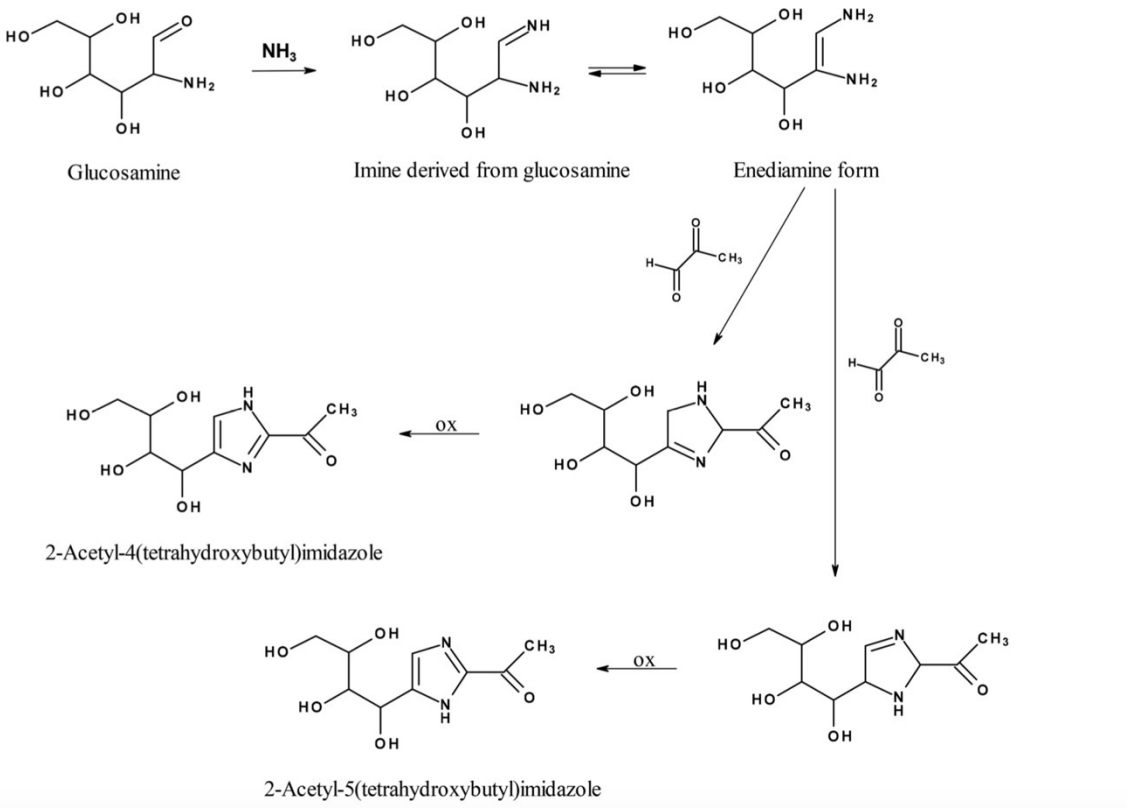


Figure 2.8. Proposed formation mechanism of 2-acetyl-4-tetrahydroxybutylimidazole (THI) from GlcN. Figure is reprinted with permission from Dhungel, P., Hrynets, Y., & Betti, M. (2018). Sous-vide nonenzymatic browning of glucosamine at different temperatures. *Journal of Agricultural and Food Chemistry*, *66,* 4521-4530. Copyright (2018) Elsevier Ltd.

Due to their potential toxicity, 4-MEI and THI are strictly regulated around the world. As shown in Table 2.5, The European Union has set the maximum concentration limits of 4-MEI and THI in Caramel Class III as 200 mg/kg and 10 mg/kg, respectively. For Caramel Class IV, the 4-MEI limit is set to 250 mg/kg. The United States has set the limit of 4-MEI in both Caramel Class III and IV to 250 mg/kg. However, in California, the Proposition 65 law sets the concentration of 4-MEI to be less than 12 mg/kg, and the daily intake of 4-MEI per person is limited to 29 μg.

Table 2.5. The concentration limit of 4-MEI and THI is set in different countries. The table is reprinted from Monograph Caramel & Health: What's today's situation?

	Europe	USA	Rest of the world
Regulation	Regulation (EC) No 231/2012	Food Chemicals Codex	The Joint FAO/WHO Expert Committee on Food Additives
4-MEI in Caramel Class III	200 mg/kg*	250 mg/kg	200 mg/kg
4-MEI in Caramel Class IV	250 mg/kg	250 mg/kg	250 mg/kg
THI in Caramel Class III	10 mg/kg	ND	25 mg/kg

*The values are given based on colour intensity. ND refers to not defined.

2.1.3.6.2. 5-Hydroxymethylfurfural

5-HMF is another neo-formed compound during the Maillard reaction and caramelization and is presented in various foods and beverages (Murkovic and Pichler, 2006). It is found in all four classes of caramel colors, with Class III caramel having the lowest concentrations overall (Licht et al., 1992). A proposed mechanism of its formation is shown in Figure 2.9.

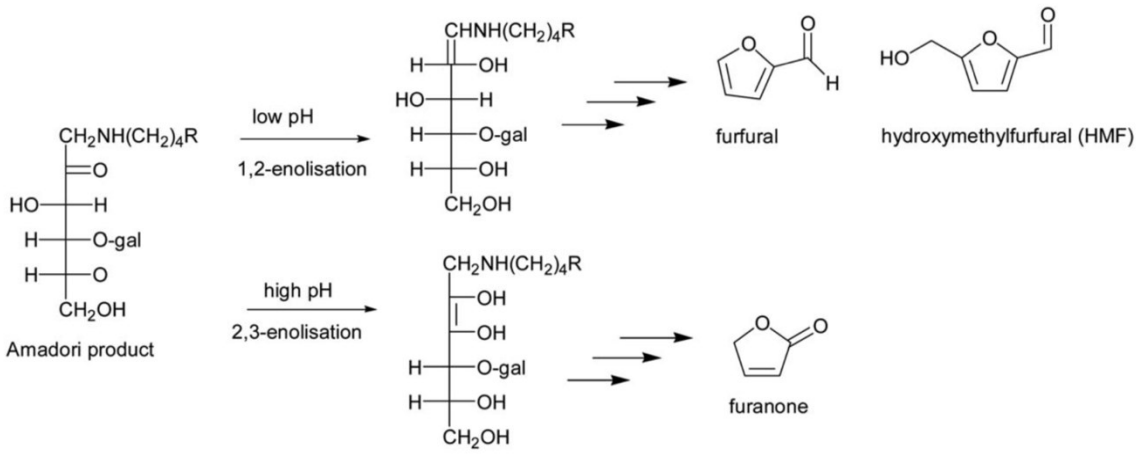


Figure 2.9. Proposed formation mechanism of 5-hydroxymethylfurfural (5-HMF) from Amadori product. Figure is reprinted with permission from Newton, A. E., Fairbanks, A. J., Gerrard, J. A., Andrewes, P., & Golding, M. (2012). The role of the Maillard reaction in the formation of flavour compounds in dairy products - Not only a deleterious reaction but also a rich source of flavour compounds. *Food and Function*, *3*, 1231–1241. Copyright (2013) Elsevier Ltd.

The concentration of 5-HMF increases as temperature and storage time increases (Kędzierska-Matysek, 2016). In addition to this, pH, water activity, and type of sugar used in the reaction also play a role in its formation (Lee and Nagy, 1990).

Since the correlation of exposure to 5-HMF and its adverse health effects is not conclusive, there are no regulatory limits set until now, except for honey (Janzowski et al. 2000; Abraham et al. 2011). However, it is still of great importance to analyse 5-HMF in foods, in order to study its potential risks and assess the quality of certain foods (Wang and Schnute, 2012). Numerous research have been conducted on 5-HMF. Its genotoxicity has been reported *in vitro*, but not *in vivo* (Vollmuth, 2018). The National Toxicology Program (NTP) studies reported some evidence of carcinogenic activity in female B6C3F1 mice based on their elevated incidences of liver tumours. However, no assessment of 5-HMF in caramel colors was conducted and these results were not specifically related to human risks (NTP, 2010; EFSA, 2011; Abraham et al., 2011).

2.2. Production and bioactivity of glucosamine (GlcN)

Glucosamine (2-amino-2-deoxy-D-glucose) is an amino monosaccharide with a molecular weight of 179.17, which is widely used as a dietary supplement in the treatment of osteoarthritis, knee pain, and back pain (Benavente et al., 2015).

2.2.1. Production of GlcN

Among its ways of production, the most two crucial routes include deacetylation of chitin as well as the reaction between fructose and ammonia.

2.2.1.1. Production of GlcN from the exoskeleton of shrimps

GlcN occurs in acetylated and polymerized forms of chitin, an β-(1–4)-poly-N-acetyl-D-glucosamine. Its chemical structure is shown in Figure 2.10 A. Chitin is the second most abundant biopolymer, which is found in the exoskeletons of crustaceans (especially crab, shrimp, and

lobster), insects, arthropods, as well as the cell wall of fungi (Zaeni, 2017). Due to its intermolecular hydrogen bonding, chitin is insoluble in all regular solvents, such as water, organic solvents, mildly acidic or basic solutions. By contrast, chitosan (fully or partly N-deacetylated chitin, Fig. 2.10. B) is more soluble in acidic conditions, due to the protonation of amine groups (Pillai et al., 2009).

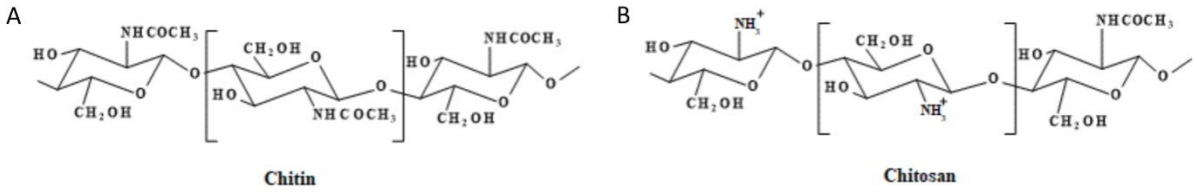


Figure 2.10. Chemical structures of chitin (A) and chitosan (B). The part of figure is reproduced with permission from Mojarrad, J.S., Nemati, M., Valizadeh, H., Ansarin, M. and Bourbour, S. (2007). Preparation of glucosamine from exoskeleton of shrimp and predicting production yield by response suf Bace methodology. *Journal of Agricultural and Food Chemistry*, *55*, 2246-2250. Copyright (2007) American Chemical Society.

The typical industrial production process of GlcN from crustacean shells is shown in Figure 2.11., with deproteinization, demineralization, chemical hydrolysis of chitin, crystallization, and drying steps (Benavente et al., 2015). Pre-treatments, including deproteinization, demineralization, depigmentation, and grinding are necessary as crustacean shells contain 30-40% proteins, 30-50% calcium carbonate, and lipid-soluble pigments (Aranaz et al., 2009).

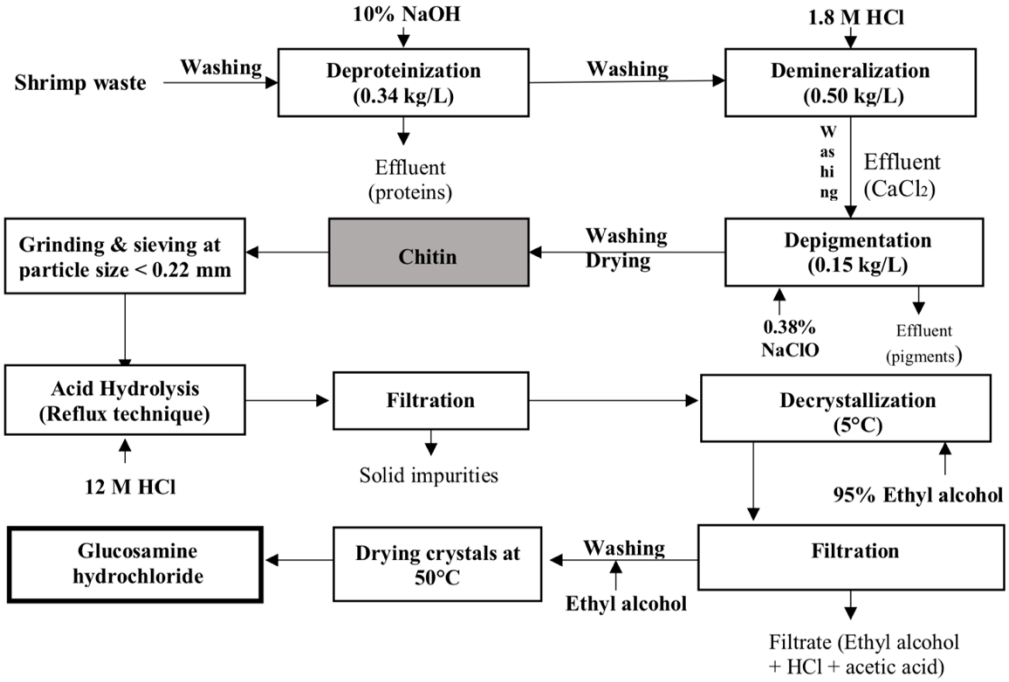


Figure 2.11. Block diagram showing the extraction of chitin from shrimp shells and production of glucosamine hydrochloride. The figure is reproduced with permission from Benavente, M., Arias, S., Moreno, L., & Martinez, J. (2015). Production of glucosamine hydrochloride from crustacean shell. *Journal of Pharmacy and Pharmacology*, 3, 20-26.

The acid hydrolysis procedure is conducted with the addition of hydrochloric acid, followed by heating at a temperature of 80-100°C in an electric furnace or hot plate. Next, filtration is conducted to separate hydrochloride from other solid particles. Crystallization occurs at room temperature for 25 days with the addition of ethyl alcohol to increase its rate. Finally, the resulting mixture is dried at 50°C in an oven for 12 h (Benavente, 2015).

2.2.1.2. Chemical Production of GlcN

Chemically, GlcN can be produced from the reaction of fructose and ammonia, shown in Figure 2.12.

This method can yield up to 30% GlcN with the formation of some coproducts (Lv et al., 2017). During this process, altering pH, incubation time and temperature can form various amounts

of GlcN. For example, using high fructose corn syrup and ammonium hydroxide as starting materials at 25-50 °C can produce GlcN and lesser amounts of mannosamine and galactosamine (approximately 8%) as the co-products (Hubbs, 2007).

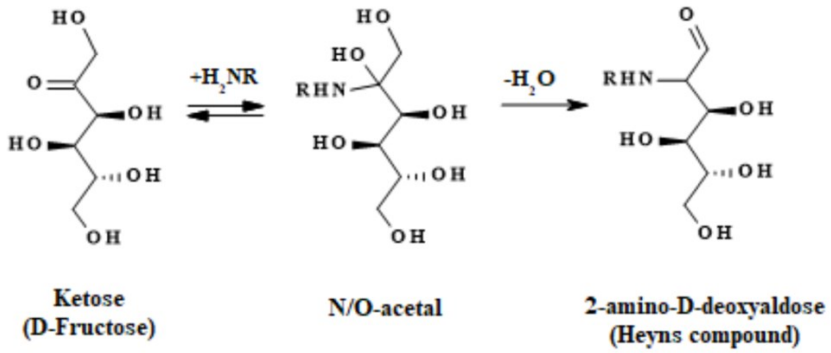


Figure 2.12. Production of GlcN from fructose and ammonia. Reproduced with permission from Bilova, T., Greifenhagen, U., Paudel, G., Lukasheva, E., Brauch, D., Osmolovskaya, N., Tarakhovskaya, E., Balcke, G.U., Tissier, A., Vogt, T. and Milkowski, C. (2016). Glycation of plant proteins under environmental stress-methodological approaches, potential mechanisms and biological role. *Abiotic and Biotic Stress in Plants-Recent Advances and Future Perspectives*, 295-316. Copyright (2016) InTech.

2.2.2. Bioactivity of GlcN

GlcN has been researched intensively upon its bioactivity. It is widely used as a dietary supplement for treating osteoarthritis. Additionally, GlcN was reported to possess antioxidant and anticancer activities. Its self-condensation products such as 2,5-bis-(D-arabino-1,2,3,4-tetrahydroxybutyl)pyrazine (FR) and 2-(D-*arabino*-1,2,3,4-tetrahydroxybutyl)-5-(D-*erythro*-2,3,4-trihydroxybutyl)pyrazinefructosazine (DOFR) were also studied for their therapeutic roles and discussed in the followed paragraphs.

2.2.2.1. GlcN in osteoarthritis treatment

Using GlcN as a dietary supplement has shown effective against osteoarthritis. It was reported to inhibit the gene expression of osteoarthritic cartilage *in vitro* (Uitterlinden et al., 2006).

GlcN produces synovial, a lubricant to the cartilage. Shortage of synovial fluid in the body will ultimately lead to osteoarthritis (Zaeni, 2017).

GlcN is orally administered in the form of GlcN·SO4, GlcN·HCl, GlcNAc, or in combination with chondroitin sulfate. Among these, GlcN·SO4 is the most effective, as it has great bioavailability (90%) (Anderson et al., 2005). Moreover, GlcN·SO4 is also a more potent inhibitor of gene expression compared to GlcN·HCl (Altman et al., 2006).

2.2.2.2. Antioxidant activity of GlcN

Both GlcN·SO4 and GlcN·HCl have been studied to possess antioxidant properties in a variety of *in vitro* systems, which include superoxide and hydroxyl radical scavenging activities, and pronounced reducing power. For example, as for GlcN·HCl, its superoxide radical scavenging activity was reported to be 72–84% at concentrations of 0.05–0.8 mg/mL (Xing et al., 2006).

2.2.2.3. Anticancer activity of GlcN

GlcN has been studied on its role in cancer treatment. In a mice model, it was found to have anti-tumor activity by oral administration of N-acetyl-D-glucosamine and GlcN oligomer against colon tumor. Their anticancer properties were associated with the apoptosis of tumour tissues and increasing the level of serum interleukin-12p70 and interferon-γ to enhance the immune system (Masuda et al., 2014). In addition to this, studies have also reported the antitumor activities of GlcN against prostate, lung, and breast cancers (Chesnokov et al., 2009; Brasky et al., 2011; Cooney, 2011).

2.2.2.3. Bioactivity of GlcN self-condensation products

Fructosazine (FR) and deoxyfructosazine (DOFR) are the self-condensation products of GlcN. The self-condensation of GlcN can form a cyclic compound named dihydrofructosazine, which is the precursor of FR and DOFR. Its structure is shown in Figure 2.13. FR and DOFR are

produced from dehydrogenation or dehydration of dihydrofructosazine, respectively (Hrynets et al., 2015b).

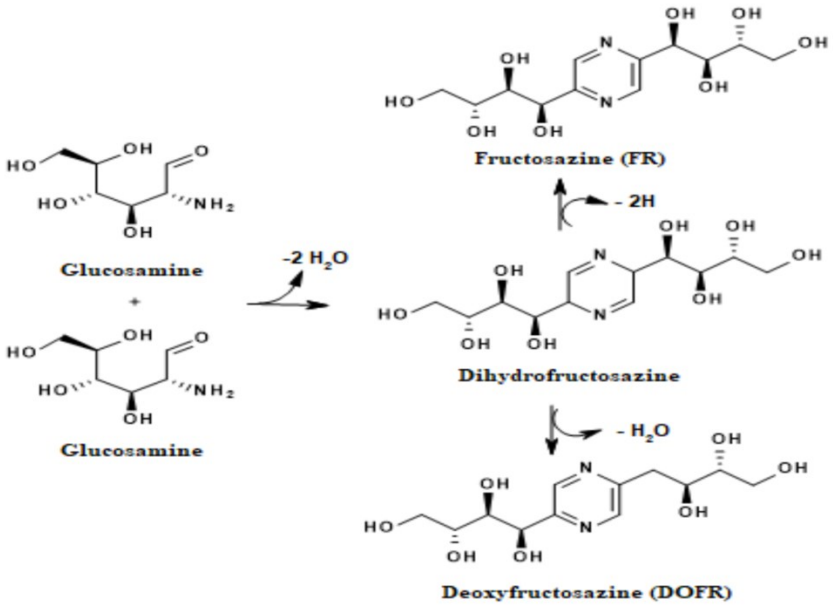


Figure 2.13. Mechanism of glucosamine self-condensation to dihydrofructosazine, fructosazine (FR), and deoxyfructosazine (DOFR). The figure is reproduced with permission from Hrynets, Y., Bhattacherjee, A., Ndagijimana, M., Hincapie Martinez, D.J. and Betti, M. (2016). Iron (Fe^{2+})-catalyzed glucosamine browning at 50°C: identification and quantification of major flavour compounds for antibacterial activity. *Journal of Agricultural and Food Chemistry*, *64*, 3266-3275. Copyright (2016) American Chemical Society.

The effect of GlcN and fructosazines on lymphocyte cytokine production was compared by Zhu et al. (2007). This study showed that GlcN is less effective in reducing IL-2 production than 29 fructosazines, therefore, the self-condensation products of GlcN are superior to native GlcN in terms of their anti-inflammatory properties. Apart from this, FR and DOFR are also reported to act therapeutically against osteoarthritis and rheumatoid arthritis by inhibiting IL-1β (Giordani et al., 2006). Also, they are shown to have efficacy as therapeutical agents against Type II diabetes (Bashiardes et al., 2002).

2.3. Recent advances in non-enzymatic browning of GlcN

Previous studies performed in our group have examined the non-enzymatic browning of GlcN in various aspects, including the products of the reaction, their applications in food systems, as well as their bioactivity.

2.3.1. Alpha-dicarbonyl compounds

Alpha-dicarbonyl compounds (α-DCs) generated from non-enzymatic browning reactions are the precursors of the aroma compounds found in their corresponding food systems. In 2015 (a), Hrynets et al. examined the non-enzymatic browning products of GlcN incubated at 37 °C, where the proposed pathways of the generation of α-DCs from GlcN were identified, shown in Figure 2.14.

There are five pathways involved in the reactions, which can be divided into oxidation (A1 in Figure 2.10.), enolization (A2 and A5 in Figure 2.10.), enolization/oxidation (A3 in Figure 2.10.), as well as interconversion. Glucosone (G) is most likely produced from the oxidative pathway (A1), whereas 3-deoxyglucosone (3DG) is produced from the 1,2-enolization pathway. Apart from these two major α-DCs, glyoxal (GO), methylglyoxal (MGO), and diacetyl (DA) are also formed in smaller amounts. They are the short-chain α-DCs. GO can be formed by the retro-aldol cleavage of G at C2-C3, while MGO can be produced by the retro-aldol cleavage of 3-DG at C3-C4 (Yaylayan and Keyhani, 2000; Weenen, 1998).

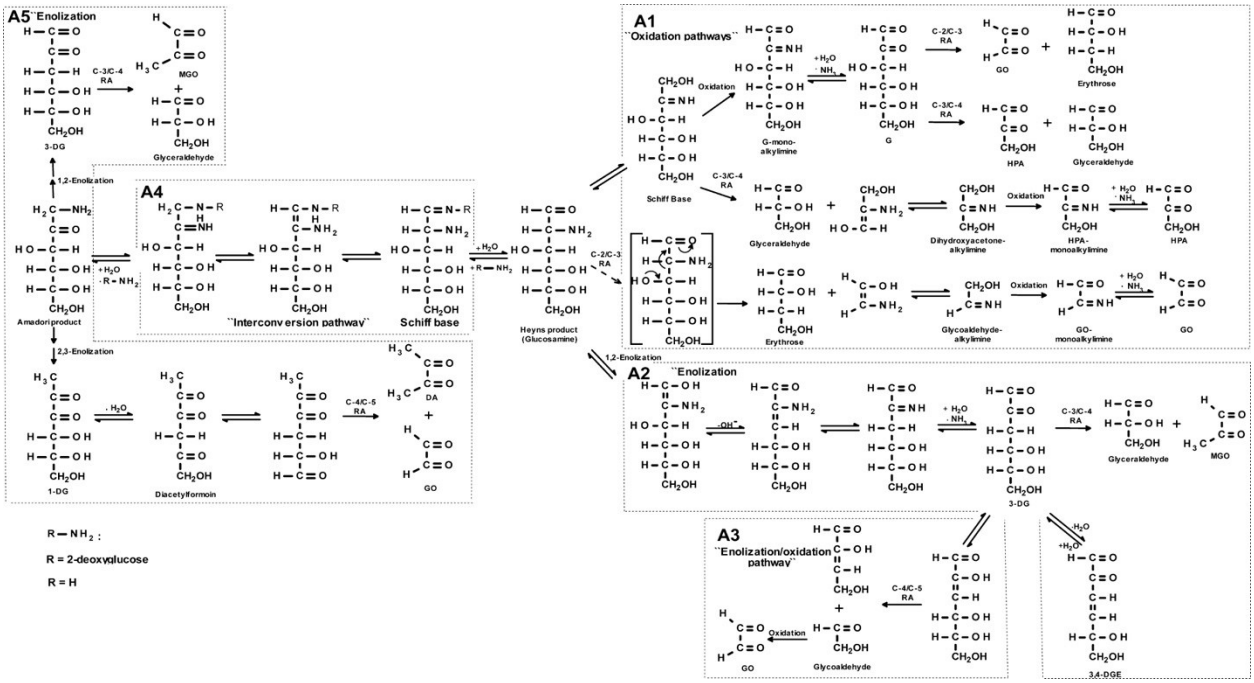


Figure 2.14. Proposed pathways of α-dicarbonyl formation from GlcN (a Heyns product) incubated at 37℃: A1= oxidation, A2, A5= enolization, A3= enolization and oxidation, and A4= interconversion. G, glucosone; 3-DG, 3-deoxyglucosone; 1-DG, 1- deoxyglucosone; 3,4-DGE, 3,4-dideoxyglucosone-3-ene; GO, glyoxal; MGO, methylglyoxal; HPA, hydroxypyruvaldehyde; RA, retroaldolization. The figure is reprinted with permission from Hrynets, Y., Ndagijimana, M., and Betti, M. (2015a). Studies on the formation of Maillard and caramelization products from glucosamine incubated at 37℃., *Journal of Agricultural and Food Chemistry*, *63*, 6249-6261. Copyright (2015) American Chemical Society.

GO and MGO act as the precursors of flavor and color formation during the non-enzymatic browning of GlcN. For example, they can react with amino acids to form heterocyclic aromatic compounds such as pyrazines, pyridines, and pyrroles. They can be found in various foods, such as coffee, honey, yogurt, vinegar, etc. (Hrynets et al., 2015a).

In terms of DA, it is volatile with a buttery or butter-scotch aroma, which occurs naturally in caramel, butter, yogurt, wine, and vinegar. It can also be added to foods that require such aroma, for example, popcorns (Zaccone et al., 2015).

2.3.2. FR and DOFR in Food Systems

FR and DOFR are used as flavoring agents in many food systems, including caramel, roasted peanuts, and soy sauce (Tsuchida, 1986; Magaletta, 1996; Tsuchida et al., 1990). Hrynets et al. in 2016 reported obtaining up to 37 g/L for FR and DOFR from 150 g/L GlcN/Fe^{2+} incubated for 48 h at 50 °C. Moreover, they were detected at greater amounts from GlcN/Fe^{2+} as compared to GlcN alone.

FR and DOFR can be very important in terms of suppressing the off-flavor in light-absorbing foods. Off-flavor is a common issue in the food industry, where it accelerates dramatically under the light with wavelengths below 500 nm. FR and DOFR were reported to absorb UV lights without generating any off-flavor (Van Der Ark et al., 2013). As a result, they can be used in generating a light-absorbing "colourless caramel". Apart from their applications in various foods, they also possess bioactivity, which has been discussed in 2.2.2.

2.3.3. "Cold" Caramelization of GlcN

Caramelization usually occurs at temperatures above 120 °C (Kroh, 1994). The use of high processing temperatures tends to generate neo-formed compounds that are potentially hazardous to human health.

By contrast, the degradation of GlcN can occur at a temperature as low as 25 °C (Dhungel, 2018). Previous studies on GlcN caramel have also targeted temperatures from 37 °C to 70 °C with the application of heat (Hrynets et al., 2015a; Dhungel, 2018). Another study conducted by Zhao et al., in 2020 examined GlcN and GlcN-myoglobin reaction systems incubated at 4 °C, where α-DCs, FR, and DOFR were produced. These studies all revealed the possibility of conducting non-enzymatic browning reactions of GlcN at low temperatures but still generating compounds of interest.

2.4. UV radiation

UV radiation is optical radiation, which is a form of electromagnetic energy that is invisible to the human eyes, with wavelengths between 100 and 400 nm (Radosevich, 2014). It can be further divided into different ranges depending on the wavelengths recommended by the International Organization of Standardization (ISO) standard 21348, including UV-A (315-400 nm), UV-B (280-315 nm), and UV-C (100-280 nm).

2.4.1. UV-A, UV-B, and UV-C

UV-A radiation is the most commonly encountered type of UV radiation. Exposure to UV-A can cause tanning, while excessive exposure can lead to skin toughening, immune system suppression, as well as cataract formation (Radosevich, 2014). UV-B radiation is essential for the synthesis of vitamin D in the human body but is only active at specific times of the day and of the year. It can be dangerous to cause erythema, cataracts, and skin cancer; nevertheless, thin glass or proper clothing can protect the skin against UV-B radiation (Hardy et al., 1997). UV-C radiation is mostly absorbed by the ozone layer of the atmosphere; however, accidental overexposure from artificial sources can cause corneal burns, snow blindness, as well as severe sunburn to the face (Radosevich, 2014).

A large fraction of UV radiation that reaches the surface of the earth is considered non-ionizing radiation. However, UV-C radiation that represents the higher energy region of UV, is ionizing. This means that UV-C contains enough kinetic energy to liberate an electron from an atom or a molecule, thereby ionizing it (Meulemans, 1987; Radosevich, 2014).

UV-C radiation is also considered as the “germicidal UV”, which has been reported to be effective against microorganisms, including fungi, yeast, bacteria, viruses, protozoa, and algae (Begum et al., 2009; Chaine et al., 2012; Thompson, 2009). It can inactivate microorganisms by

damaging the genetic material in the nucleus of the cell or nucleic acids in the virus through dimerization of pyrimidine molecules, thus preventing the replication of microorganisms or generating defects in their structures (Chang et al., 1985). The most lethal range of wavelengths for microorganisms is between 250 and 270 nm, known as the germicidal spectrum, where 262 nm is the peak germicidal wavelength (Gurzadyan et al., 1995).

UV-C has been applied in various fields, including air disinfection (Wells et al., 1942), water disinfection (Song et al., 2016), wound healing (Dai et al., 2012), etc. In the food industry, UV-C is widely used for air disinfection, contamination control on the surface of plant and packaging materials, or in post-harvest storage of fruits and vegetables (Begum et al., 2009). In bakery products, UV-C is applied to reduce mold contamination and growth (Guynot et al., 2002; Magan and Aldred, 2006). UV-C radiation is also used in liquid foods. Its efficacy is dependent on the type of liquid and the UV-absorbing capacity, where the increased number of solids, large suspended particles, or microbial populations will reduce the UV-C penetration intensity (Bintsis et al., 2000; Lopez-Malo and Palou, 2005).

2.4.2. Sources of UV

The most common artificial sources of UV radiation are mercury vapor lamps, including low-pressure mercury (LPM) and medium-pressure mercury (MPM) lamps. LPM lamps emit monochromatic light at 253.7 and 185 nm, with 85% of the total UV intensity being emitted at the 253.7 nm line (Bintsis et al., 2000). By contrast, MPM lamps emit more powerful polychromatic light between 250 and 600 nm with emission lines in the UV and visible light regions (Koutchma et al., 2009). However, they have a number of shortcomings. For example, most mercury lamps have fixed wavelengths and a short bulb lifetime. Moreover, they exhibit low energy efficiency,

limited cycling, large footprint, long warm-up time, environmental pollution due to mercury, and incapability of switching on and off with high frequency (Vilhunen and Sillanpää, 2010).

Compared with mercury vapor lamps, another UV emitting source that has gained much attention is called LED (light-emitting diode). It is advantageous because of the high energy efficiency, long service life, and environmentally friendly characteristics (Bae et al., 2015). UV-LEDs can emit UV radiation at diverse wavelengths and can be turned on and off with a high and adjustable frequency (Nyangaresi et al., 2019). Currently, UV-LEDs are applied to radiation curing, sterilization, water purification where traditional UV lamps are replaced (Bae et al., 2015).

UV radiation has been used in the manufacture of food products for over 50 years, and its uses are approved in both Canada and the United States (Health Canada, 2004; U.S. FDA, 2000). Many familiar food products, including juices and apple cider, grains, cheese, bakeries, frozen foods, fresh fruits and vegetables, and liquid egg products are treated with UV radiation, which may not be noticeable to some suppliers and consumers, as they are not required to be labeled (Ros-Polski et al., 2016).

2.4.3. Novel uses of UV in food science

Recently, UV-C has been applied in foods to study their interaction with food components. Within these, proteins have shown to be able to undergo chemical modification. The abundance of chromophores (e.g., tryptophan, tyrosine, phenylalanine, cysteine, flavins and heme) in proteins can react with other excited state species (Davies, 2003). The energy of the UV-C radiation is absorbed by chromophores and then transfers to protein parts where the structure and function are controlled from the initial oxidation sites (Manzocco, 2015). As a result, both protein structure and their properties are modified where side-chain oxidation, backbone fragmentation and/or formation of cross-links and aggregates occur (Pattison et al., 2012).

UV-C treatment has shown to potentially improve the antioxidant activity in β-lactoglobulin (Wu et al., 2017). In the case of egg white, UV-C treatment has shown to affect its foam stability, emulsifying activity, and immunoreactivity (Manzocco et al., 2012; Mendes de Souza et al., 2015). A study conducted by Manzocco et al., in 2021 revealed the capability of egg white proteins and carbohydrates (i.e., glucose, maltose, trehalose and maltodextrin) undergoing non-enzymatic browning reactions during UV-C exposure. The results have shown that the UV-C induced glycation led to a decreased viscosity, a decreased gel forming temperature with a better foam stability.

In the next chapter (study 1), the use of UV-C for the non-enzymatic browning of GlcN will be discussed in further detail with the support of experimental results.

CHAPTER 3. Cold caramelization of glucosamine under UV-C radiation

3.1. Introduction

Glucosamine (GlcN, 2-amino-2-deoxy-D-glucose) is an amino monosaccharide that naturally exists in acetylated form in proteoglycans found in articular cartilage, intervertebral disc, and synovial fluid (Henroitin et al., 2012). It is industrially produced by the deacetylation of chitin, an abundant material in the exoskeleton of crustaceans and insects as well as the cell walls of fungi (Zaeni, 2017). GlcN, in the form of sulfate or hydrochloride, is a widely used dietary supplement for the treatment of osteoarthritis, knee pain, and back pain (Benavente et al., 2015).

The studies from our research group have shown that upon heating GlcN produces a plethora of compounds, including the products of self-condensation namely fructosazine (FR) and deoxyfructosazine (DOFR), as well as α-dicarbonyl compounds (α-DCs). Among the major α-DCs, 3-deoxyglucosone (3-DG), glucosone (G), methylglyoxal (MGO), glyoxal (GO) and diacetyl (DA) are generated at 25°C and increasing the temperatures to 37°C, 50°C or 70°C can greatly speed up GlcN non-enzymatic browning (Hrynets 2015, 2016; Dhungel, 2018). GlcN incubated at as low as 4°C within several days self-condenses producing FR and DOFR and at the same time generates both long- and short-chain α-DCs as 3-DG, G, MGO and DA (Zhao et al. 2020).

Alpha-DCs are the precursor to the color, aroma, and flavor of caramels which also possess bioactivity. For example, MGO, GO (Mavric et al., 2008), DA (Jay, 1984), and 3-DG (Hrynets et al., 2016) have been reported to have antibacterial activities.

FR and DOFR were reported to be identified in peanuts, caramel, and soy sauce (Henry et al., 2012). FR is used as a flavor agent in many food products (Henry et al., 2012). It also has been reported to have antimicrobial activity against heat-resistant *E. coli* AW 1.7 in moderate acidic conditions (Bhattacherjee et al., 2016). Additionally, FR and DOFR have been researched

intensively on their therapeutic uses, including treating type II diabetes, preventing atherosclerosis, and pathological cartilage degradation (Zhu et al., 2007).

Previous studies showed that different conditions of GlcN non-enzymatic browning can either catalyze or inhibit the generation of specific self-condensation products and specific α-DCs, allowing to guide the reaction into the desired direction. For instance, using a sous-vide cooking under vacuum can maximize the generation of FR, while decrease the concentration of DA (Dhungel, 2018). Dhungel et al. (2019) reported that incubation of 15% GlcN in the presence of glycine at 70 °C for 12 h generated higher amount of self-condensation products and diacetyl, while the opposite effect on generation of FR and DOFR was observed upon the addition of alanine. Hrynets et al. (2015) reported that the presence of iron, Fe^{2+}, significantly increased the concentration of α-DCs at eartly incubation times of 3 h. The increase in concentration of α-DCs at earlier incubation times were observed in the presence of myoglobin during GlcN non-enzymatic browning at 37 °C (Hrynets et al. 2016). Zhao et al. 2020 found that myoglobin presence during GlcN incubation at 4 °C greatly increased the concentration of glucosone at each incubation time, while the concentration of 3-deoxyglucosone was significantly lower.

Generally, caramelization occurs at a temperature higher than 120 °C (Kroh, 1994). One issue associated with using this temperature is the formation of a series of undesired contaminants, including 4-methylimidazole (4-MEI), 2-acetyl-4-tetrahydroxybutylimidazole (THI), and 5-hydroxymethylfurfural (5-HMF). By contrast, the thermal degradation of GlcN occurs at much lower temperatures, as described above. Previous studies on GlcN caramel have also targeted temperatures from 37 to 70°C (Dhungel, 2018; Hrynets et al., 2015a). In order to minimize the formation of neo-formed contaminants, but still successfully produce compounds of interest in the

GlcN caramel, the one proposed way is to expose GlcN solution to UV-C radiation at low temperatures instead of applying heat.

UV radiation is optical radiation, which is a form of electromagnetic energy that is invisible to the human eyes, with wavelengths between 100 and 400 nm (Radosevich, 2014). It is further divided into different ranges including UV-A (315-400 nm), UV-B (280-315 nm), and UV-C (100-280 nm) (ISO, 2007). UV-A radiation is the most commonly encountered type of UV radiation. It causes tanning, while excessive exposure can lead to skin toughening, immune system suppression, as well as cataract formation (Radosevich, 2014). UV-B radiation is essential for the synthesis of vitamin D in the human body but is only active at specific times of the day and of the year (Hardy et al., 1997). UV-C radiation is mostly absorbed by the ozone layer of the atmosphere; however, accidental overexposure from artificial sources can cause corneal burns, snow blindness, as well as severe sunburn to the face (Radosevich, 2014).

UV-C radiation is also considered as the "germicidal UV", which has been reported to be effective against microorganisms, including fungi, yeast, bacteria, viruses, protozoa, and algae (Begum et al., 2009; Chaine et al., 2012; Thompson, 2009). It can inactivate microorganisms by damaging the genetic material in the nucleus of the cell, thus preventing the replication of microorganisms, or generating defects in their structures (Gayán et al., 2013). The most lethal range of wavelengths for microorganisms is between 250 and 270 nm, known as the germicidal spectrum (Gurzadyan et al., 1995).

UV radiation has been used in the manufacture of food products for over 50 years, and its uses are approved in both Canada and the United States (Health Canada, 2004; U.S. FDA, 2000). UV-C is widely used for air disinfection, contamination control on the surface of plant and packaging materials, or in post-harvest storage of fruits and vegetables (Begum et al., 2009). In

bakery products, UV-C is applied to reduce mold contamination and growth (Guynot et al., 2002; Magan and Aldred, 2006). UV-C radiation is also used in liquid foods (Ochoa-Velasco et al., 2020), where its efficacy depended on the type of liquid and the UV-absorbing capacity. Increased number of solids, large suspended particles, or microbial populations reduce the UV-C penetration intensity (Bintsis et al., 2000; Lopez-Malo and Palou, 2005). Many familiar food products, including juices and apple cider, grains, cheese, bakeries, frozen foods, fresh fruits and vegetables, and liquid egg products are treated with UV radiation, which may not be noticeable to some suppliers and consumers, as they are not required to be labeled (Ros-Polski et al., 2016).

The most common artificial sources of UV radiation are mercury vapor lamps, including low-pressure mercury (LPM). LPM lamps emit monochromatic light at 253.7 and 185 nm, with 85% of the total UV intensity being emitted at the 253.7 nm line (Bintsis et al., 2000). Another UV emitting source that has gained much attention is called LED (light-emitting diode). It is advantageous because of the high energy efficiency, long service life, and environmentally friendly characteristics (Bae et al., 2015). UV-LEDs can emit UV radiation at diverse wavelengths and can be turned on and off with a high and adjustable frequency (Nyangaresi et al., 2019). Currently, UV-LEDs are applied to radiation curing, sterilization, water purification where traditional UV lamps are replaced (Bae et al., 2015).

3.2. Hypothesis and objectives

This study was designed to use UV-C instead of heat for the non-enzymatic browning of GlcN. The experimental temperature was planned to be controlled at 25 °C compared with common caramelization temperature of higher than 120 °C (Kroh, 1994). It was hypothesized that the concentration of neo-formed contaminants in the UV-C treated GlcN caramel samples would be significantly lower than that of normal heat-induced GlcN caramels. Additionally, we

hypothesized that the UV-C treatment of GlcN would aid in maximizing the generation of the desired compounds as for example, FR and DOFR.

The objectives of this study were to measure the physico-chemical changes of GlcN caramels produced by application of UV-C with exposure times of 0, 20, 60, and 120 min at 25 °C, to quantify the long and short chain α-DCs; FR, DOFR and neo-formed contaminants.

3.3. Materials and methods

3.3.1. Chemicals

D-glucosamine hydrochloride (GlcN, ≥99%), fructose (≥99%), HPLC grade solvents (methanol, formic acid), *o*-phenylenediamine (OPD; 99.5%), glucosone (G, D-*arabino*-2-hexosulose; ≥98%), methylglyoxal (MGO, 2-oxopropanal, 40% in H_2O), and glyoxal (GO; ethanedial, 40% in H_2O), 4(5)-methylimidazole (4(5)-MEI, 98%), 5-(hydroxymethyl)furfural (HMF, ≥ 99%) and an ammonia assay kit were from Sigma-Aldrich (St. Louis, MO, USA). 3-deoxyglucosone (3-DG; 3- deoxy-D-*erythro*-2-hexosulose; ≥ 95%) and 2-acetyl-4(5)-tetrahydroxybutyl imidazole (THI, ≥ 95%) were from Cayman Chemical (Ann Arbor, MI, USA). Diacetyl (DA; 2,3-butanedione; 99%) was from Acros Organics (NJ, USA). Fructosazine (FR) and deoxyfructosazine (DOFR) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sodium 1-octanesulfonate (99%) was from Alfa Aesar (Ward Hill, MA, USA). Ammonium hydroxide and potassium dihydrogen phosphate (99.4%) were from Fisher Scientific (NJ, USA). SPE tC-18 Sep-Pak Vac 6 cc columns were from Waters (Milford, MA, USA). Polyvinylidene fluoride (PVDF) syringe filters (0.22 μm) and filtration membranes (0.1 μm) were from Millipore (Billerica, MA, USA). The buffers and solutions were prepared with Milli-Q purified distilled water (Millipore, Bedford, MA, USA).

3.3.2. Experimental design

3.3.2.1. Screening of UV-C exposure times

GlcN solutions at two concentrations, 15% and 30%, were subjected to UV-C radiation at 275 nm with a 20-minute interval at controlled temperature and humidity. The cups with GlcN solutions were treated for 0 (control), 20, 40, 60, 80, 100 and 120 min. Each treatment had three independent replicates treated on three consecutive days resulting in a total of 42 treatments. After the UV-C treatment, the absorbance spectrum of each sample was obtained from 200 nm to 450 nm. Additionally, the color, volume, and pH of each sample were measured. Based on observations and measured absorbance, four optimized exposure time durations and two concentrations were selected for further study.

There were the other two control solutions at two concentrations of 15% and 30%: 1. fructose solution in water and 2. fructose + ammonia (aqueous, NH_4OH). None of these two controls did show a color change after 120 min of UV-C treatment under the same experimental conditions as for GlcN (Appendix A, Figure A1).

3.3.2.2. Main analysis

A 4 × 2 factorial design was planned to study the effect of exposure time under UV radiation (0, 20, 60, 120 min) and GlcN concentration (15% and 30%) on the generation of α-DCs, GlcN self-condensation products, FR and DOFR, and neo-formed contaminants (5-HMF, THI, and 4-MEI) during GlcN non-enzymatic browning. GlcN solutions at two concentrations were treated at four different exposure times under the same experimental conditions as during screening. Each treatment had three independent replicates on three different days (a total of 24 treatments).

3.3.3. Preparation of GlcN solutions

The GlcN solutions were prepared at a 15% and 30% concentration by dissolving it in water. Prior to UV-C treatment, the pH of the solutions was adjusted to 7.0 ± 0.01 with 1 M KOH. Samples with a volume of 2 mL were pipetted into Aqualab sample cups with diameter of 4 cm (Meter Group, Pullman, WA, USA) and placed under the UV-C LED (275 nm) for a height of 4 cm. The UV-C LED along with the sample were placed inside a closed humidity chamber, in which the temperature and the relative humidity were set to 25 °C and 85 %, respectively. The pH was not adjusted during the UV-C treatments. After retrieval, an aliquot of each of the GlcN caramel solutions was immediately tested for respective analyses. None of the samples was refrigerated or frozen prior to the analyses.

3.3.4. Spectrophotometric measurements

UV-visible spectra of the experimental solutions in the range of 200 -450 nm were recorded on a Spectramax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA) using a 1-cm quartz cuvette. The 15% UV-C treated GlcN caramel solutions were diluted 8 times. By using the same dilution factor, the absorbance for 30% UV-C treated GlcN caramel solutions was off scale. To best present the absorbance profiles for 30% samples, they were diluted 32 times at the end.

3.3.5. Color measurements and pH

The color parameters corresponding to the uniform color space CIELAB (CIE, 1978) were determined using the colorimeter (Minolta CR-400, Konica Minolta Sensing Americas, Inc., Ramsey, NJ). Calibration was conducted using a white tile plate (L^* = 32.80; a^* = 14.51; b^* = 15.19). From the uniform color spaces, the chroma (C^*_{ab}) and the hue angle (h_{ab}) were calculated using the following formulas, $C^* = (a^*2 + b^*2)^{1/2}$ and h_{ab} = arctan (b^*/a^*), respectively.

A pH meter (UB-10, Ultra basic pH meter, Denver Instrument, Bohemia, NY, USA) was used to monitor the pH of the experimental solutions.

3.3.6. Analysis of alpha-dicarbonyl compounds

3.3.6.1. Identification by HPLC-ESI-MS/MS

The identification of α-DCs was based on the method described by Hrynets et al. (2015a; 2015b) with modification. Briefly, the aliquots of UV-C treated GlcN caramel solutions were derivatized with 0.006 g of OPD, followed by pH adjustment to 3.00 ± 0.02 with 4 M HCl. The mixtures were then incubated at 37 °C for 1 h in a water bath circulator (Haake SC100, Thermo Scientific, Waltham, MA, USA). After derivatization, the samples were filtered with a PVDF syringe filter (13 mm, 0.22 μm; Millipore, Billerica, MA, USA) and subjected to analyses. The standards of α-DCs were derivatized followed the same method.

The α-DCs were identified using a LC-MS/MS analyses conducted on a HPLC-DAD-ESI/MS instrument equipped with an electrospray ionization (ESI) source interfaced to a QTRAP 4000 mass spectrometer (AB Sciex, ON, Canada). Chromatographic separations of quinoxaline derivatives were performed with an Agilent 1200 HPLC system (Agilent, Palo Alto, CA, USA) with a degasser, a quaternary pump, a thermostated autosampler, a UV-visible detector and a fitted Ascentis Express ES-C18 column (150 mm × 4.6 mm, 27 μm, Sigma-Aldrich, MO, USA). The binary mobile phase consisted of (A) 0.1% formic acid in water and (B) methanol. The 120 min gradient is described as follows: 0−5 min, 90−85% A; 5−13 min, 85−80% A; 13−40 min, 80% A; 40−65 min, 80−70% A; 65−90 min, 70−50% A; 90−100 min, 50−0% A; 100−105 min, 0% A; and 105−110 min, 0− 90% A; the column was then re-equilibrated with the initial mobile phase for 15 min. The flow rate was 1.0 mL/min and the injection volume was 5 μL. The ionization conditions

as well as conditions of MS/MS fragmentation were those as described by Hrynets et al. (2015a; 2015b). The interpretation of the analysis was carried out in comparison to the retention times, mass, and MS/MS fragmentation spectra of derivatized authentic standards and previously published studies (Hrynets et al., 2015a; Hrynets et al., 2015b). Mass spectrometry analyses were performed in duplicates.

3.3.6.2. Quantitation by HPLC

Followed identification the samples were subjected to RP-HPLC analyses carried out on an Agilent 1100 assembly (Agilent Technologies, Inc., Santa Clara, CA, USA) consisted of a G-1312 binary pump, a G-42 1328A injector, a G-1322A degasser, and a G-1315A photodiode array detector and an Ascentis Express ES-C18 column. The conditions of HPLC separation were the same as those described in the identification by HPLC-ESI-MS/MS section. The injection volume was 5 µL. The quinoxaline derivatives of α-DCs and standards were detected at 314 nm.

The α-DCs were quantified using the external standard method. Standard curves were obtained using five different concentrations of the standards, all with correlation coefficients of $R^2 \geq 0.99$. The average limits of detection (LODs) were calculated as 0.1 mg/mL (G), 0.02 mg/mL (3-DG), 2.2 µg/mL (GO), 0.5 µg/mL (MGO) and 0.5 µg/mL (DA) and the average limits of quantitation (LOQs) were 0.3 mg/mL (G), 0.07 mg/mL (3-DG), 6.7 µg/mL (GO), 1.6 µg/mL (MGO) and 1.4 µg/mL (DA). The signal-to-noise ratios (S/N) were 3.3:1 and 10:1 for LOD and LOQ, respectively.

3.3.7. Analyses of GlcN self-condensation products, FR and DOFR

3.3.7.1. Identification by LC-MS/MS

FR ad DOFR were identified using the same liquid chromatography tandem mass spectrometry with instrumental settings as described in the section 3.3.6.1. The LC separation was

performed using the method of Hrynets et al. (2016). The binary mobile phase consisted of (A) 0.1% formic acid in water and (B) methanol. The 35-min gradient is described as follows: 0−15 min, 100-95% A, 15−25 min, 95−50% A; 25−35 min, 50-95% A.

3.3.7.2. Quantitation by HPLC

FR and DOFR were quantified using the method of Hrynets et al. (2016) employing the same HPLC apparatus as described in the section 3.3.6.2. The chromatographic separation was performed at the conditions described in the section 3.3.7.1. The injection volume was 10 μL. The UV spectra were recorded at 275 nm.

Quantitation was performed using the external standard method. Standard curves were obtained using five different concentrations of the standards, all with correlation coefficients of $R^2 \geq 0.99$. The LODs were calculated as 0.02 mg/mL (FR), 6.1 μg/mL (DOFR) and the LOQs were 0.05 mg/mL (FR), and 18.4 μg/mL (DOFR). The signal-to-noise ratios (S/N) were 3.3:1 and 10:1 for LOD and LOQ, respectively.

3.3.8. Analysis of neo-formed contaminants, 5-HMF, THI, and 4-MEI

The neo-formed contaminants, namely 5-HMF, THI, and 4-MEI were analyzed using the same HPLC apparatus described above based on the method described by Ciolino (1998). The binary mobile phase consisted of (A) 0.05 M potassium dihydrogen phosphate and 0.005 M sodium octane sulfonate, adjusted to a pH of 3.0 ± 0.01 in water, and (B) methanol. The mobile phase consisted of 92.5:7.5 A:B. The injection volume was 10 μL and the flow rate was 0.5 mL/min. The UV spectra were recorded at 285 nm. Each sample was filtered with a PVDF syringe filter (13 mm, 0.22 μm; Millipore, Billerica, MA, USA).

Since tested neo-formed contaminants were not detected or were below the level of detection in any of the UV-C treated GlcN caramel solutions, quantitation was not applicable.

3.3.9. Statistical analysis

The data were analyzed as a 4 × 2 factorial analysis of variance (ANOVA) using RStudio (v. 1.1.463, RStudio Inc., Boston, MA, USA). The model tested the interaction of GlcN concentration and exposure time and used the day of trial replication as a random variable. Tukey's honestly significant difference ($p < 0.05$) multiple-range test was conducted to determine differences between the means.

3.4. Results and discussion

3.4.1. Chemico-physical characteristics of UV-C treated GlcN caramel solutions

As shown in Figure 3.1. (A) and (B), as the exposure time increased, absorbance in the range of 200 to 450 nm also increased for both 15% and 30% concentrations. The peaks for all GlcN caramel solutions appeared at the wavelength of around 275 nm, with bigger shoulders toward the higher wavelengths as the exposure time increased. The 15% GlcN caramel solutions were diluted 8 times. However, with the same dilution factor, the absorbance profiles of 30% samples were off scale, indicating higher absorbance than that of 15% samples. These samples were finally diluted 32 times, and as a result, (A) and (B) cannot be compared directly. Compared to previously published studies (Hrynets et al., 2015), the role of exposure time was similar to that of incubation duration in heat-induced caramelization of GlcN, with longer incubation times resulting in higher peaks of the resulting caramel solution. Based on (C) and (D), it is clear that the absorbance at 275 nm increased as exposure time increased, and this is true for both 15% and 30% concentrations. The formation of pyrazine compounds from the cyclo-condensation between GlcN molecules during the caramelization process caused the increase in absorbance at 275 nm (Candiano et al., 1988; Horowitz, 1991).

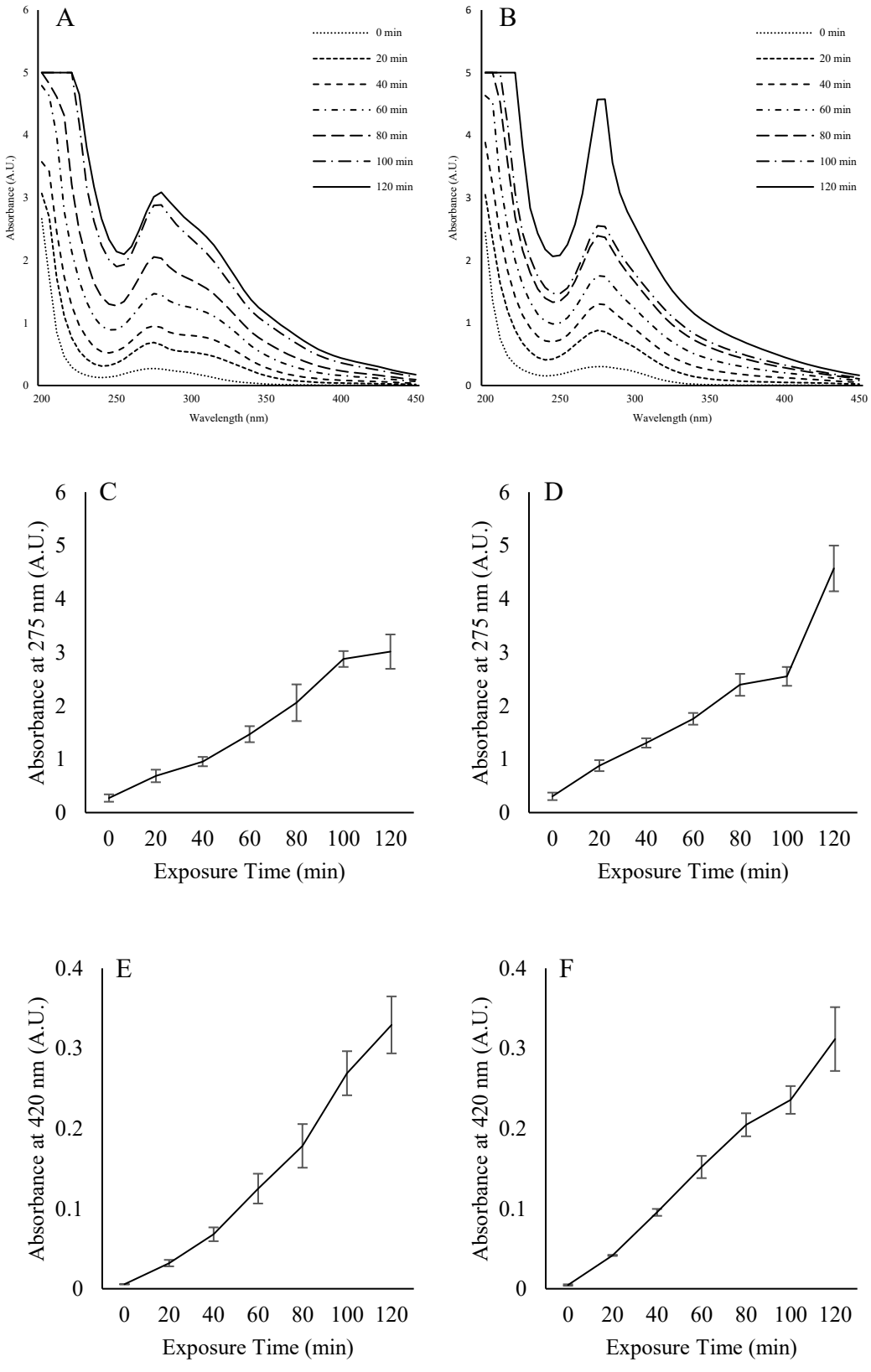

A
B
C
D
E
F
0 min
20 min
40 min
60 min
80 min
100 min
120 min
Absorbance (A.U.)
Wavelength (nm)
Absorbance at 275 nm (A.U.)
Absorbance at 420 nm (A.U.)
Exposure Time (min)

Figure 3.1. Absorbance profiles of GlcN 15% (A) and GlcN 30% (B) as a function of UV-C exposure time from 0 to 120 minutes. Absorbance at 275 nm of GlcN 15% (C) and GlcN 30% (D) as a function of UV-C exposure time from 0 to 120 minutes. Absorbance at 420 nm of GlcN 15% (E) and GlcN 30% (F) as a function of UV-C exposure time from 0 to 120 minutes. The values are expressed in arbitrary units (A.U.)

The increase of absorbance at higher wavelengths could indicate the formation of compounds with higher molecular weight. For example, the soluble pre-melanoidins were reported to have an absorption at the wavelength of 320 nm (Fogliano et al., 1999); whereas melanoidins absorb at 420 nm (Adams et al., 2005). Based on (E) and (F), absorbance at 420 nm increased for both concentrations with increasing exposure time, indicating that the formation of melanoidins continued as exposure time increased.

3.4.2. pH and color changes

As reported in Table 3.1., a significant drop in pH of GlcN solutions was found with longer UV-C treatment times for both 15% and 30% concentrations, being 0.5 and 0.8, respectively, after 120 min. The reduction of pH values during GlcN thermal degradation was reported before (Hrynets et al., 2015, 2016; Dhungel, 2018), and was attributed to the degradation of 1- and 3-deoxyglucosone forming acetic and formic acids, respectively. Both factors including the concentration of GlcN and UV-C exposure time as well as their interaction significantly affect the pH of GlcN samples.

The color was also expressed in Chroma (C^*_{ab}, metric chroma), and hue angle (h_{ab}, chromatic tonality). Chroma is defined as the magnitude of the vector at each point designating the departure from dull to more vivid chromatic color ("–" to "+" values), which indicates color saturation and intensity (Serratosa et al., 2008). By contrast, hue angle measures redness at values near 0° and yellowness near 90° (Jing & Kitts, 2004). The significant effects of GlcN concentration and exposure time as well as their interaction were observed for both C^*_{ab} and h_{ab}. The least ($p <$

0.05) hue values of 85.0° and 86.4° were observed in 15% and 30% GlcN caramel samples with an exposure time of 0 min, which indicated its reddish tonality.

Based on color characteristics, it could be seen that longer UV-C exposure led to darker, more yellow, less red, and more saturated color (Figure 3.2.). Thus, for the identification and quantification of these compounds, only four exposure times were selected, namely, 0, 20, 60, and 120 min.

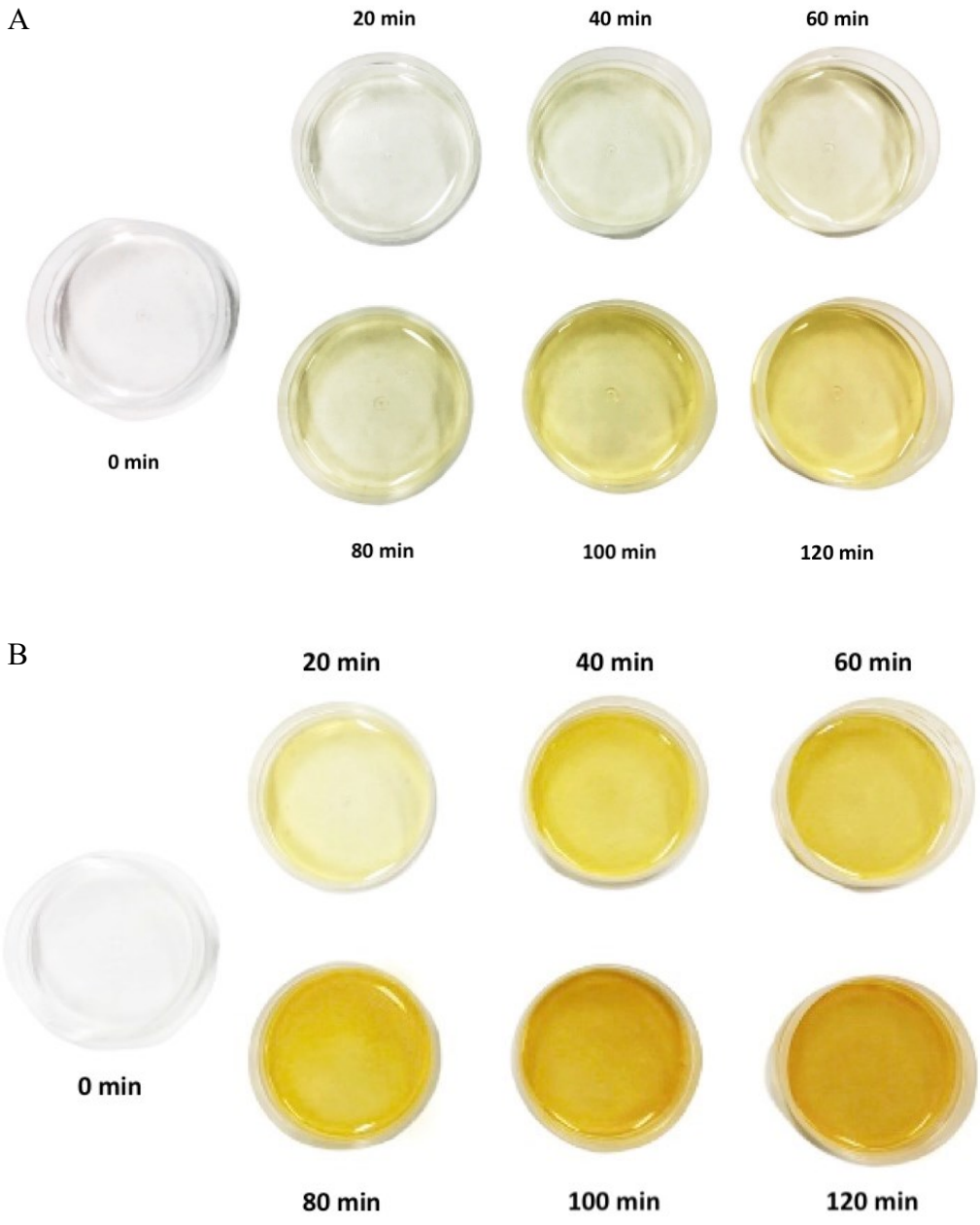


Figure 3.2. Visual appearance of (A) 15% and (B) 30% GlcN solutions after exposure to UV-C light for 0 to 120 min with 20-min interval.

Table 3.1. Changes in pH, lightness (L^*), redness (a^*), yellowness (b^*), hue angle (h_{ab}), and chroma (C^*_{ab}) during UV-C treatment of 15% and 30% GlcN solutions for different exposure times.

		pH	L^*	a^*	b^*	h_{ab}	C^*_{ab}
Concentration of GlcN (%)							
15		6.7 a	48.6 a	-1.6 a	6.8 b	101 a	7.0 b
30		6.5 b	46.5 b	-2.1 b	18.7 a	95.9 b	18.8 a
SEM		0.01	0.1	0.07	0.3	0.2	0.3
Exposure time (min)							
0		7.0 a	49.3 a	0.1 a	1.4 e	85.7 f	1.4 e
20		6.8 b	48.9 a	-1.6 b	6.7 d	103 a	6.9 d
40		6.7 c	48.8 a	-2.5 d	11.8 c	102 ab	12.0 c
60		6.6 d	47.7 b	-2.6 d	14.6 b	101 bc	14.9 b
80		6.5 e	46.2 c	-2.3 cd	17.2 a	99.7 cd	17.4 a
100		6.4 ef	46.1 c	-2.1 bcd	18.4 a	98.6 e	18.5 a
120		6.3 f	45.8 c	-1.7 bc	19.2 a	97.6 e	19.3 a
SEM		0.02	0.2	0.1	0.5	0.4	0.5
Interaction (concentration of GlcN × exposure time)							
15	0	7.0 a	49.6 a	0.1 a	1.3 h	85.0 e	1.3 h
	20	6.9 b	49.4 ab	-0.7 ab	3.2 gh	103 a	3.3 gh
	40	6.8 bc	49.0 abc	-1.3 bc	5.3 fg	103 a	5.5 fg
	60	6.7 cd	48.5 abc	-1.8 cd	7.2 ef	104 a	7.4 ef
	80	6.6 de	48.0 cd	-2.3 cd	9.3 de	104 a	9.5 de
	100	6.5 e	47.8 cd	-2.5 de	10.5 de	103 a	10.8 de
	120	6.5 e	48.1 bcd	-2.6 de	10.9 d	103 a	11.2 d
30	0	7.0 a	48.9 abc	0.1 a	1.4 h	86.4 e	1.4 h
	20	6.8 bc	48.2 abcd	-2.5 de	10.1 de	104 a	10.4 de

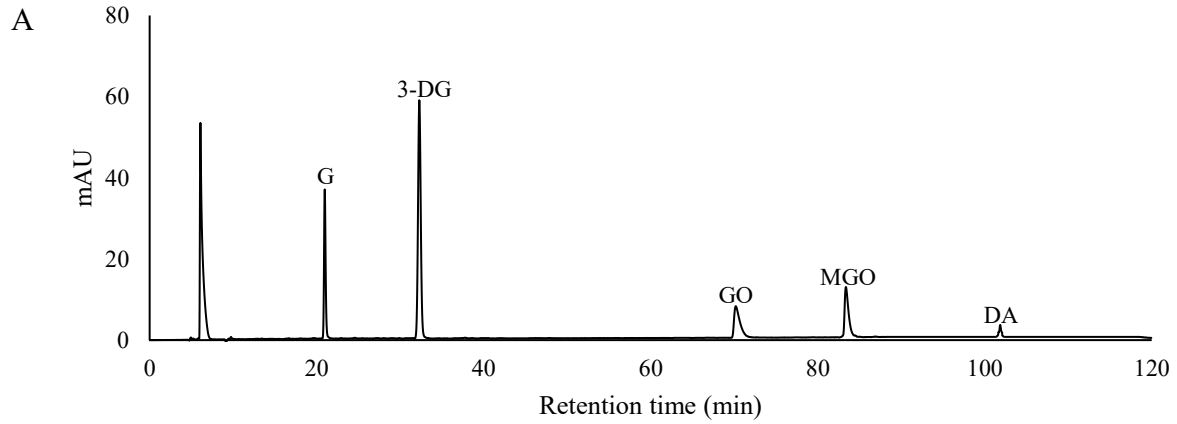
	40	6.6 de	48.7 abc	-3.7 f	18.2 c	101 a	18.6 c
	60	6.5 e	47.0 d	-3.3 ef	22.2 b	98.4 b	22.4 b
	80	6.3 f	44.4 e	-2.4 de	25.2 ab	95.5 c	25.3 ab
	100	6.3 fg	44.4 e	-1.8 cd	26.2 a	93.9 cd	26.2 a
	120	6.2 g	43.6 e	-0.9 bc	27.4 a	91.9 d	27.4 a
SEM		0.02	0.3	0.2	0.7	0.6	0.7
Sources of variation		*p*-values					
Concentration of GlcN		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Exposure time		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Interaction		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Means within the same column with no common superscript differ significantly ($p < 0.05$). SEM, pooled standard error of the means.

3.4.3. Formation of alpha-dicarbonyl compounds

The formation of α-DC compounds occurred during the degradation of the GlcN solutions exposed to UV-C radiation. The representative HPLC chromatograms, Figure 3.3, showed the elution pattern of major α-DCs using the *o*-OPD as a trapping agent. To confirm the correct identification, the mass spectrometry analyses were performed. The LC-MS/MS chromatograms of identified α-DCs are presented in Figure 3.4. By comparing chromatographic retention time and mass spectrometry data to those of authentic standards and previously published studies (Hrynets et al., 2015a; Hrynets et al., 2016 Dhungel et al., 2018; Zhao et al. 2019), G and 3-DG were identified in experimental solutions. Their concentrations are shown in Table 3.2.

Compared to the heat-induced caramelization (Hrynets et al., 2015a; Hrynets et al., 2016; Dhungel et al., 2018), GO, MGO and DA were not detected in the UV-C treated GlcN caramel solutions. Interestingly, G was found to be around 3-times greater than 3-DG in the 15% GlcN caramel solutions; however, 3-DG was found to be greater than G in the 30% GlcN caramel solutions. The significant effects of GlcN concentration and exposure time as well as their interaction were observed for both G and 3-DG. The GlcN concentration and UV-C exposure time both had significant effects on the formation of G and 3-DG, with an increase in both parameters resulting in increased amounts of G and 3-DG.



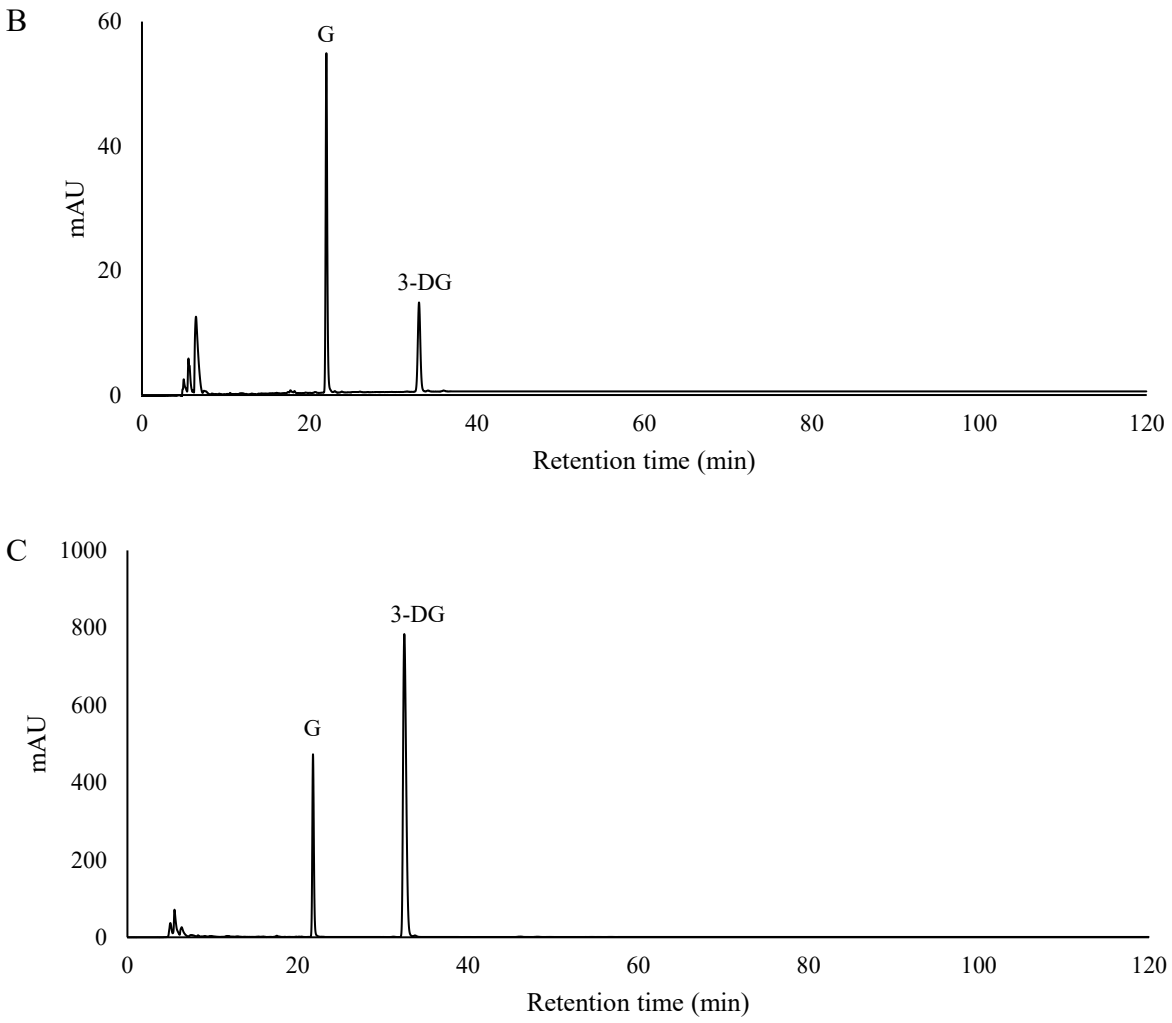
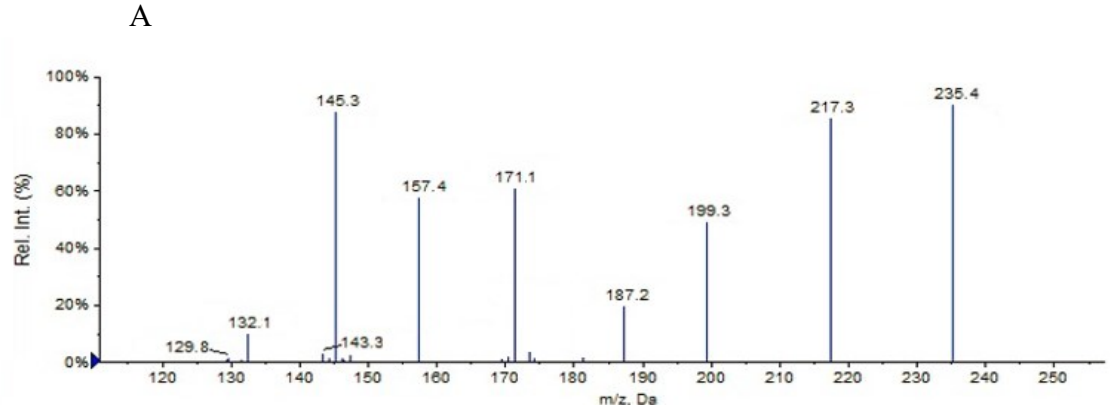


Figure 3.3. HPLC analysis of α-dicarbonyl compounds. (I) Chromatogram of (A) α-dicarbonyl compounds standards mixture. (II) Representative HPLC chromatograms showing the separation of quinoxaline derivatives of α-DCs in (B) 15 and (C) 30 % GlcN solutions exposed to UV-C light after 20 mins of UV-C treatment.



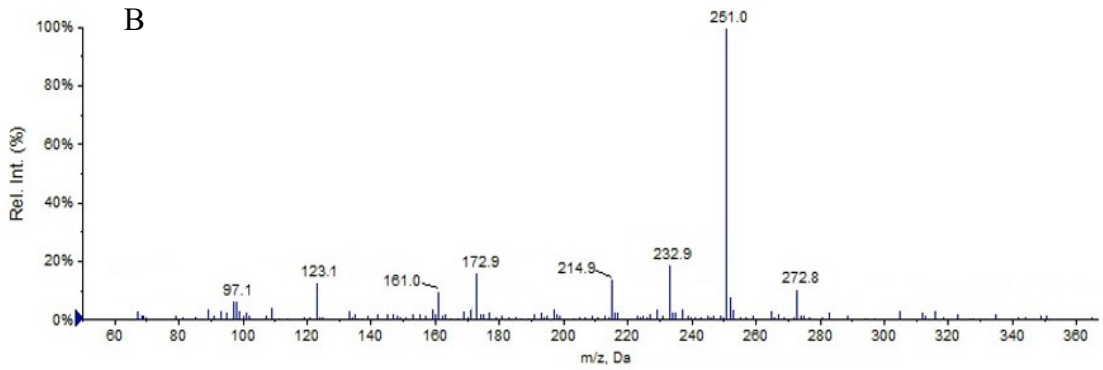


Figure 3.4. LC-MS/MS chromatogram of quinoxaline derivatives of (A) glucosone and (B) 3-deoxyglucosone identified in GlcN caramels exposed to UV-C light.

The results indicated that UV-C radiation favored the oxidative pathway during the caramelization of 15% GlcN to form G; in addition, the 1,2-enolization pathway forming 3-DG was favored at 30% GlcN. The processes of generating MGO, GO, and HMF, including C3-C4 or C4-C5 cleavage and dehydration, seemed not to occur under the exposure of UV-C radiation, in which 5-HMF was not detected in the resulting GlcN caramel solutions.

Table 3.2. Concentration (mg/L) of free α-dicarbonyl compounds, fructosazine (FR) and deoxyfructosazine (DOFR) during UV-C treatment of 15% and 30% GlcN solutions for different exposure times.

		G	3-DG	GO	MGO	DA	FR	DOFR
Concentration of GlcN (%)								
15		2676 b	895 b				121 b	13.7 b
30		3508 a	5781 a				347 a	195 a
SEM		230	102				11.0	4.9
Exposure time (min)								
0		336 d	341 c				112 c	27.1 c
20		2347 c	3403 b				213 b	83.6 b
60		3842 b	3646 b				298 a	104 b
120		5842 a	5959 a				314 a	203 a
SEM		325	145	ND	ND	ND	15.6	7.0
Interaction (concentration of GlcN × exposure time)								
15	0	0.0 e	50.8 d				25.5 d	2.7 d

	20	2205 cde	899 cd				84.4 cd	9.6 cd
	60	3947 bc	912 cd				185 bc	27.4 cd
	120	4550 b	1717 c				188 bc	15.0 cd
30	0	673 de	632 d				198 b	51.4 c
	20	2488 bcd	5908 b				341 a	158 b
	60	3737 bc	6381 b				410 a	182 b
	120	7133 a	10202 a				440 a	391 a
SEM		459	205.0				22.0	9.8
Sources of variation		*p*-values						
Concentration of GlcN		< 0.05	< 0.0001				< 0.0001	< 0.0001
Exposure time		< 0.0001	< 0.0001				< 0.0001	< 0.0001
Interaction		< 0.05	< 0.0001				> 0.05	< 0.0001

Means within the same column with no common superscript differ significantly ($p < 0.05$). SEM, pooled standard error of the means.

Glucosone is very important as it acts as a reductone and a radical scavenger (Kanzler et al., 2014). As shown in Table 3.2., double the concentration of GlcN resulted in approximately 1.3-time greater amount of G generated. Also, increasing the exposure time significantly increased the amount of G produced; for example, 15% GlcN treated for 20 min had 2347 mg/L of G, while 120 min gave rise to 4550 mg/L of G. Compared to a previous study conducted by Dhungel et al. (2018), the amount of G generated under UV-C treatment was significantly higher.

3.4.4. Analysis of FR and DOFR

FR and DOFR are the GlcN self-condensation products. They not only play a crucial role in food systems but also possess great bioactivity. FR and DOFR were reported to suppress the off-flavor in light-absorbing foods, and as a result, they can be used to develop a functional "colorless caramel" (Van Der Ark et al., 2013). The representative chromatograms, Figure 3.5., showed the elution pattern of FR and DOFR. The mass spectrometric analyses, presented in Figure 3.6, confirmed the FR and DOFR identification.

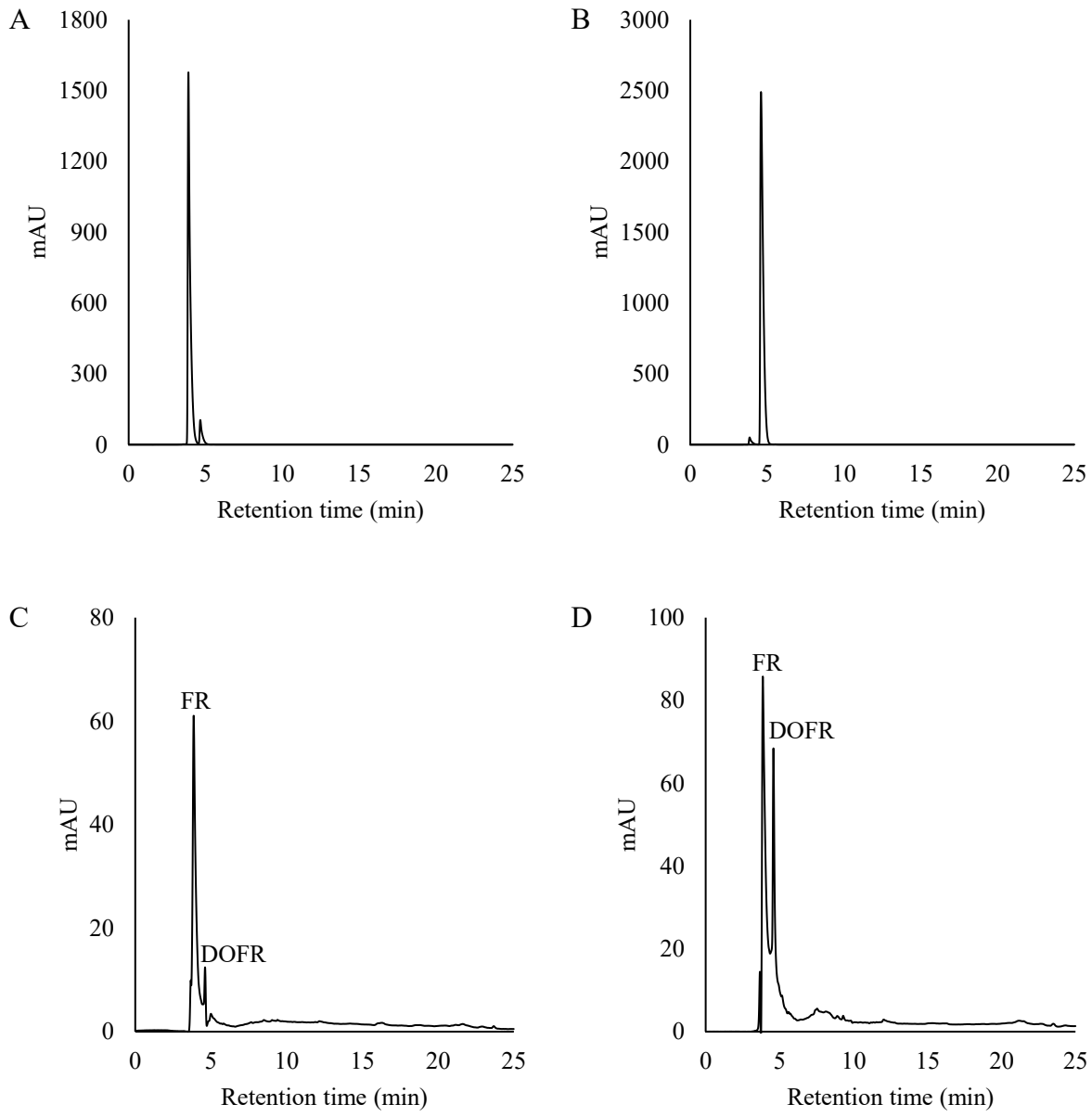


Figure 3.5. HPLC analysis of non-volatile fructosazine (FR) and deoxyfructosazine (DOFR). (I) Chromatograms of (A) FR and (B) DOFR standards. (II) Representative HPLC chromatograms of fructosazine and deoxyfructosazine in (C) 15 and (D) 30 % GlcN solutions exposed to UV-C light after 60 mins of UV-C treatment.

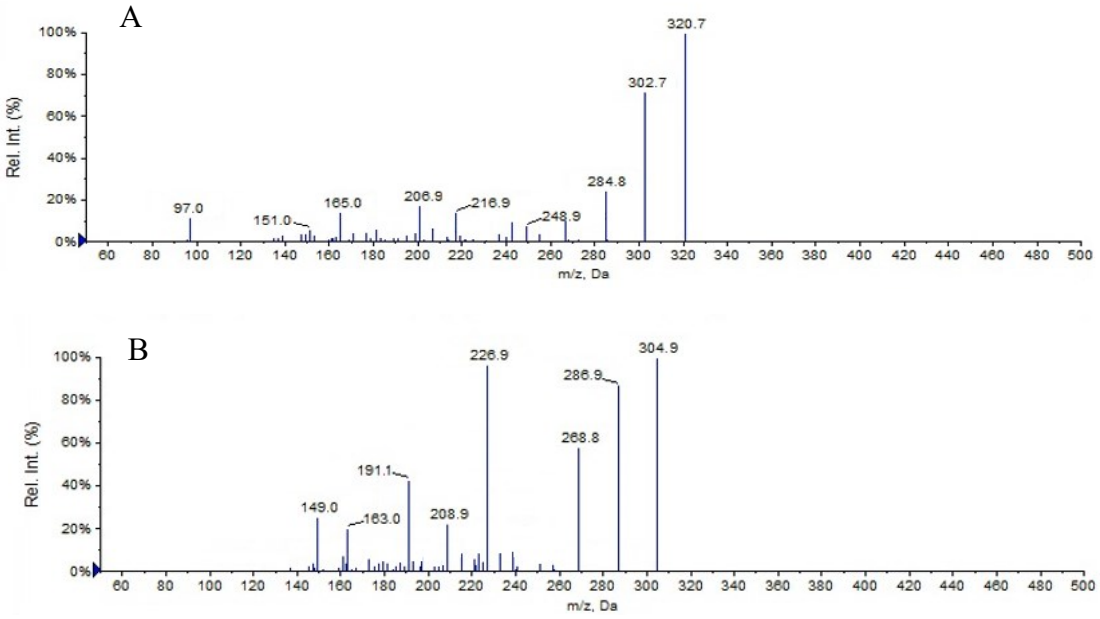


Figure 3.6. Representative LC-MS/MS chromatogram of (A) fructosazine and (B) deoxyfructosazine identified in GlcN caramels exposed to UV-C light.

Results shown in Table 3.2 indicated that GlcN concentration, exposure time, and their interaction significantly affect the concentration of DOFR, all increased in values along with the parameters. As for the concentration of FR, both GlcN concentration and exposure time significantly affect it. Noticeably, the concentration of DOFR significantly increased concerning the increase in GlcN concentration and UV-C exposure time, with around 14 times and 7 times increase in values, respectively.

However, compared with previously published results from heat-induced caramelization of GlcN, the resulted FR and DOFR concentration were a lot lower (Dhungel et al., 2018). For example, the lowest value of FR was found in the GlcN sample treated at 70 °C under non-vacuum condition, but it was still approximately 54 times greater than the lowest concentration of FR reported in the GlcN sample treated for 20 mins under UV-C radiation. An increase in the reaction temperature was reported to decrease the concentration of FR and DOFR (Dhungel et al., 2018).

By contrast, longer exposure time did not affect their concentration in the same way; instead, the concentration continued to increase with an increase in exposure time. The yield of FR and DOFR is favorable at pH 6-8 (Wu et al., 2011), which agrees with our previous recorded pH of the GlcN caramel solutions.

3.4.5. Analysis of Neo-formed Contaminants

5-HMF, THI, and 4-MEI are the neo-formed contaminants presented in caramels that are potentially hazardous to human health. Among these, THI and 4-MEI are carefully regulated in food products around the world, as they possess immunosuppressive and possibly carcinogenic effects in the body, respectively.

The initial approach of utilizing UV-C radiation was intended to minimize the formation of these compounds, as UV-C treatment occurs at a relatively lower temperature compared with traditional heat treatment of caramelization. In this experiment, the temperature of the reacting GlcN solution was controlled at 25°C during the entire reaction process. As a result, none of the three tested neo-formed contaminants was detected. This was expected as during the detection of α-DCs, MGO and GO were not detected, indicating the pathway of generating these compounds as well as 5-HMF was not activated. A study conducted by Ros-Polski et al. in 2016 also reported that 5-HMF content was reduced by 43-62% in fructose syrup under UV-C treatment at a dose of 250 mJ cm^{-2}. These indicates that the mechanisms of UV-induced caramelization possibly do not favour the pathway for 5-HMF formation.

3.5. Conclusion

In conclusion, this study revealed that the concentration of GlcN and UV-C exposure time both significantly affected the chemico-physical properties and the composition of the resulting GlcN caramel solutions. Combinations of different GlcN concentrations and exposure time

generated unique GlcN caramels that had different acidity, brownness, and concentration of volatile and non-volatile compounds.

Generally, longer UV-C exposure time can produce a more acidic and darker caramel with G and 3DG as the α-DCs, as well as the GlcN self-condensation products of FR and DOFR. The amount of G is significantly higher compared to GlcN caramels produced under heat treatment. Since none of the neo-formed contaminants were detected during the process, UV-C treatment may be a newly advantageous method of producing caramel, where the temperature of the reaction can be precisely monitored at a low degree.

CHAPTER 4. The Effect of spray-drying on the chemical composition of glucosamine caramel

4.1. Introduction

Glucosamine is an amino monosaccharide and the basic structural unit of chitin and chitosan. It is used as a dietary supplement for the treatment of osteoarthritis (Benavente et al., 2015). The industrial route of production for GlcN includes hydrolysis and deacetylation of chitin, which is a natural polysaccharide that is abundant in insect exoskeletons, crustacean shells, and fungal cell walls (Benavente et al., 2015). Another chemical way of producing GlcN is through the reaction between fructose and ammonia, which involves both caramelization and the Maillard reaction (Lv et al., 2017).

Our research group has performed numerous studies on the non-enzymatic browning of GlcN, where the produced GlcN caramel has shown bioactivity, including antioxidant (Hong and Betti, 2016) and antimicrobial (Hrynets et al., 2016) properties. Desirable compounds were found to be present in the GlcN caramel, including, α-DCs, fructosazine (FR) and deoxyfructosazine (DOFR). The latter, FR and DOFR, are the major compounds found in the GlcN caramel, produced by the self-cyclo-condensation reaction of GlcN. A study conducted by our group reported obtaining up to 37 g/L for FR and DOFR from 150 g/L GlcN/Fe^{2+} incubated for 48 h at 50 °C (Hrynets et al., 2016). FR and DOFR have been identified in caramel (Tsuchida, 1986), roasted peanuts (Magaletta, 1996), and soy sauce (Tsuchida et al., 1990). They were also reported to be able to suppress off-flavor by absorbing UV-lights, which can be used to create a light-absorbing "colorless caramel" (Van Der Ark et al., 2013). Apart from these, FR and DOFR are also shown to have anti-inflammatory properties, including their roles against type II diabetes (Zhu et al., 2007), as well as cartilage degradation (Giordani et al., 2006). As a result, studying these compounds is beneficial to the food industry to gain a deeper understanding of their production.

Since caramelization reaction occurs at temperatures higher than 120 °C (Kroh, 1994), a series of neo-formed contaminants are generated during the process, including 4-methylimidazole (4-MEI), 2-acetyl-4-tetrahydroxybutylimidazole (THI), and 5-hydroxymethylfurfural (5-HMF). These compounds have been reported to have potentially toxic effects on the human body, where 4-MEI is possibly carcinogenic (Hengel and Shibamoto, 2013), and THI affects the immune system in the short term (Gobin and Phillips, 1991). The toxicity of 5-HMF is not conclusive until now (Janzowski et al. 2000), but analyses of the concentration of 5-HMF is crucial to better understand its potential risks (Wang and Schnute, 2012).

Spray-drying is the most widely used technique to generate dry powder from a solution or slurry by applying hot gas (Huang et al., 2015). During the process, the liquid solution is sprayed into a hot air chamber, where water or other solvents are evaporated, producing products with generally consistent particle size (Li et al., 2019). Generally, the sprayed particles pass through the drying zone for only 15-30 seconds, which is vital for handling heat-sensitive products (Haggag and Faheem, 2015).

Spray-drying is a preferred method to produce stable powders that are made from liquid mixtures containing high concentration of sugars. This is due to reduced time of production, scale-up benefits, as well as better quality of the product (batch to batch consistency). Adequate monitoring of operating conditions is of great importance during the process, where inlet and outlet temperatures, liquid flow rate, the concentration of solids in the feed, etc. are adjusted according to the need (Vidović et al., 2019).

One of the issues associated with spray-drying of high sugar content material is stickiness, which refers to cohesion and adhesion of extracts on the drying chamber walls (Souza and Oliveira, 2006). Glass transition temperature (t_g) plays a crucial role in this issue, where it is reported that

when t_g is lower than the inlet temperature of the process for at least 10-20 °C, drying cannot be performed successfully (Vidović et al., 2019).

In the following experiment, GlcN caramel was prepared at two different incubation temperatures: 50 °C and 90 °C, followed by spray-drying to generate stable powders. The GlcN powders were analyzed to understand the effect of both incubation temperature and spray-drying on the concentration of targeted compounds, FR and DOFR, at the same time to monitor the concentration of neo-formed contaminants.

4.2. Materials and methods

4.2.1. Chemicals

D-glucosamine hydrochloride (GlcN, ≥99%) was from PureBulk (Roseburg, Oregon). HPLC grade solvents (methanol, formic acid), 4(5)-methylimidazole (4(5)-MEI, 98%), and 5-(hydroxymethyl)furfural (HMF ≥ 99%) were from Sigma-Aldrich (St. Louis, MO, USA). 2-acetyl-4(5)-tetrahydroxybutyl imidazole (THI, ≥ 95%) was from Cayman Chemical (Ann Arbor, MI, USA). Fructosazine (FR) and deoxyfructosazine (DOFR) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sodium 1-octanesulfonate (99%) was from Alfa Aesar (Ward Hill, MA, USA). Potassium dihydrogen phosphate (99.4%) was from Fisher Scientific (NJ, USA). SPE tC-18 Sep-Pak Vac 6 cc columns were from Waters (Milford, MA, USA). Polyvinylidene fluoride (PVDF) syringe filters (0.22 μm) and filtration membranes (0.1 μm) were from Millipore (Billerica, MA, USA). The buffers and solutions were prepared with Milli-Q purified distilled water (Millipore, Bedford, MA, USA).

4.2.2. Experimental design

4.2.2.1. Preliminary trials

The preliminary trials were conducted to find the best inlet/outlet temperature combination that can produce the best quality of GlcN caramel powder. GlcN experimental solutions/caramels were spray-dried using a pilot scale Anhydro spray dryer pilot 55 (SPX FLOW, Charlotte, NC, USA). Three inlet/outlet temperature combinations were tested three times on three different days, including 140 °C/70 °C, 150 °C/75 °C, and 160 °C/75 °C. Water activity and absorbance of all resulting GlcN caramel powders were then analyzed and compared. The water activity of samples obtained from different inlet/outlet temperature combinations was almost the same. In addition, the difference in absorbance among the samples was also unnoticeable (Appendix B). With these, the texture of the resulting powder was then considered to be the most important factor for identifying the best temperature combination (140 °C/70 °C), where the powder was finest and not sticky.

4.2.2.2. Main analysis

A 2 × 2 factorial design was planned to study the effect of incubation temperature (50 °C and 90 °C) and spray-drying (non-spray-dried and spray-dried) on the generation of FR and DOFR, as well as neo-formed contaminants in the GlcN caramel. Each treatment had three independent replicates on three different days (a total of 12 treatments).

4.2.3. Preparation of GlcN solutions

GlcN caramels were obtained by incubation with heat at two temperatures: 50°C and 90 °C for 12 hours. The GlcN solutions before incubation were prepared at a concentration of 15% by dissolving them in water. Prior to the incubation, the pH of the solution was adjusted to 7.0 ± 0.01 with 50% NaOH.

All spray-dried GlcN caramel powder samples were prepared into a 15% solution for following analysis by dissolving 0.75 g powder in 5 g of water. The non-spray-dried GlcN caramel samples were in liquid form with a concentration of 15%.

4.2.4. Water activity measurements

The water activity (a_w) of the spray-dried GlcN powders was measured at the ambient temperature using an AquaLab 4TE water activity meter (METER Group; Pullman, WA, USA). The sample container was filled with precisely weighted quantity of the caramel powder and placed in the chamber. The displayed results were recorded when the equilibrium of the reading was reached.

4.2.5. Color measurements and pH

The color of GlcN caramel samples was then measured using a tristimulus colorimeter (Minolta CR-400, Konica Minolta Sensing Americas, Inc., Ramsey, NJ), based on previously published method (Dhungel, 2018). Calibration was conducted before each measurement using a white tile plate (L^* = 32.80; a^* = 14.51; b^* = 15.19). Chromaticity results were expressed in L^*, a^*, and b^* coordinates. Chroma (C^*_{ab}) and hue angle (h_{ab}) were calculated using the following formulas, $C^*_{ab} = (a^*2 + b^*2)1/2$ and h_{ab}= arctan (b^*/a^*), respectively.

Spectrophotometric measurements were conducted for each sample with the absorbance recorded using a 1-cm quartz cuvette at 200 nm to 450 nm on a Spectramax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA). The 50 °C GlcN caramel samples were diluted 100 times. By using the same dilution factor, the absorbance for 90 °C GlcN caramel samples was off scale. To best present the absorbance profiles for 90 °C samples, they were diluted 250 times at the end.

A pH meter (UB-10, Ultra basic pH meter, Denver Instrument, Bohemia, NY, USA) was used to measure the pH of the GlcN caramel samples. Calibration was conducted before each series of measurements.

4.2.6. Quantitation of Heterocyclic Compounds

4.2.6.1. FR and DOFR

Fructosazine (FR) and deoxyfructosazine (DOFR) were analyzed using an Agilent 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA). This apparatus consists of a G-1312 binary pump, a G- 42 1328A injector, a G-1322A degasser, and a G-1315A photodiode array detector (PDA), equipped with an Ascentis Express ES-C18 column. Before injection, all samples were filtered with a PVDF syringe filter (13mm, 0.22 μm; Millipore, Billerica, MA, USA), where the injection volume was 10 μL. The binary mobile phase consisted of (A) 0.1% formic acid in water and (B) methanol. Then 35 min gradient is described as follows: 0−15 min, 100-95% A, 15−25 min, 95−50% A; 25−35 min, 50-95% A; the column was then re-equilibrated with the initial mobile phase for 10 min. The UV spectra were recorded at 275 nm.

Quantitation was performed using the external standard method. Standard curves were obtained using five different concentrations of the standards, all with correlation coefficients of $R^2 \geq 0.99$. Results were expressed in g/L for both non-spray-dried (prepared as 15% solution) and spray-dried GlcN caramel samples for keeping consistency and ease of comparison with previous published results. The LODs were calculated as 0.02 mg/mL (FR), 6.1 μg/mL (DOFR) and the LOQs were 0.05 mg/mL (FR), and 18.4 μg/mL (DOFR). The signal-to-noise ratios (S/N) were 3.3:1 and 10:1 for LOD and LOQ, respectively.

4.2.6.2. 5-HMF, THI, and 4-MEI

The neo-formed contaminants, including 5-HMF, THI, and 4-MEI were analyzed using the same HPLC apparatus described above based on the method described by Ciolino (1998). Before injection, all samples were filtered with a PVDF syringe filter (13mm, 0.22 μm; Millipore, Billerica, MA, USA), where the injection volume was 10 μL. The binary mobile phase consisted of (A) 0.05 M potassium dihydrogen phosphate and 0.005 M sodium octane sulfonate, adjusted to a pH of 3.0 ± 0.01 in water, and (B) methanol. The mobile phase consisted of 92.5:7.5 A: B. The flow rate was 0.5 mL/min. The UV spectra were recorded at 285 nm.

Quantitation was performed using the external standard method. Standard curves were obtained using four different concentrations of the standards, all with correlation coefficients of $R^2 \geq 0.99$. Results were expressed in g/L for both non-spray-dried (prepared as 15% solution) and spray-dried GlcN caramel samples for keeping consistency and ease of comparison with previous published results. Since no 4-MEI was detected in any of the GlcN caramel samples, quantification was not needed for this particular compound. For the rest two compounds, The LODs were calculated as 1.1 μg/mL (5-HMF), 1.6μg/mL (THI), and the LOQs were 3.4 μg/mL (5-HMF), and 4.8 μg/mL (THI). The signal-to-noise ratios (S/N) were 3.3:1 and 10:1 for LOD and LOQ, respectively.

4.2.7. Statistical Analysis

All data were analyzed as a 2 × 2 factorial analysis of variance (ANOVA) using RStudio (v. 1.1.463, RStudio Inc., Boston, MA, USA). The model tested the interaction of incubation temperature and spray-drying, where the day of trial replication was used as a random variable. Tukey's honestly significant difference ($p < 0.05$) multiple-range test was conducted to determine differences between the means.

4.3. Results and Discussion

4.3.1. Absorbance Profiles of Preliminary Trials

The preliminary trials were conducted to determine the inlet/outlet temperature combination that would be used in the main analysis, which was crucial in the process of carrying out the following experiments. Absorbance profiles of these samples were analyzed to examine and compare the generation of desired compounds, where the inlet/outlet temperature combination with the highest peak would be selected, indicating the formation of desired compounds, FR and DOFR with the highest concentration. The results are shown below in Figures 4.1 and 4.2.

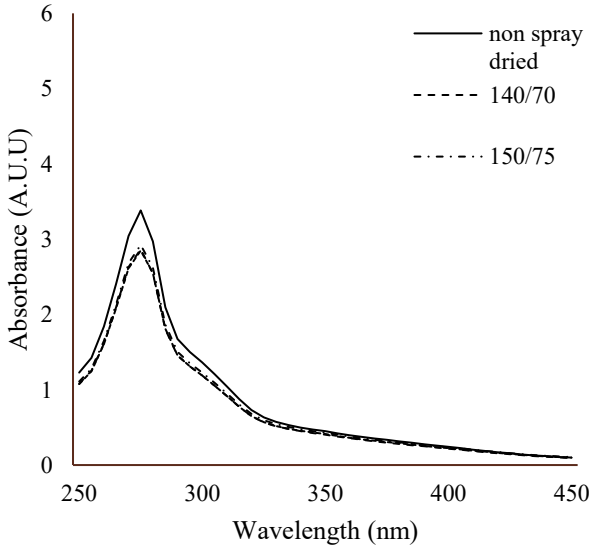


Figure 4.1. Absorbance profiles of non-spray-dried and spray-dried GlcN 15% caramels incubated at 50 °C for 12 h. Different inlet/outlet temperature combinations (expressed in °C) were used during spray-drying. The values are expressed in arbitrary units (A.U.).

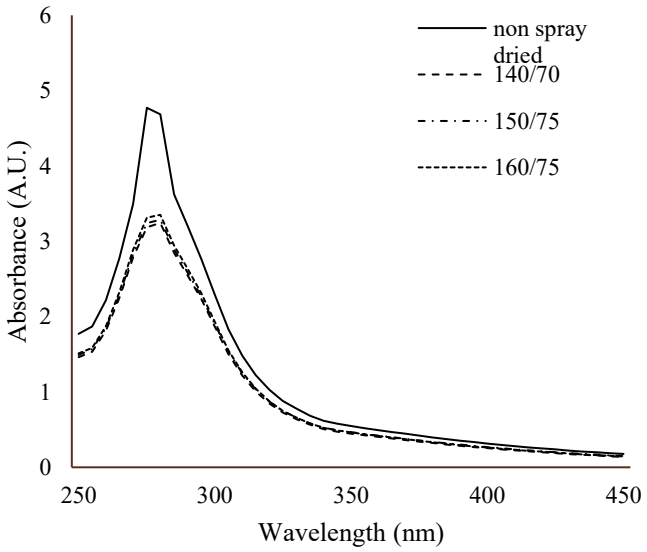


Figure 4.2. Absorbance profiles of non-spray-dried and spray-dried GlcN 15% caramels incubated at 90 °C for 12 h. Different inlet/outlet temperature combinations (expressed in °C) were used during spray-drying. The values are expressed in arbitrary units (A.U.).

Based on Figures 4.1 and 4.2, the GlcN caramels incubated at both 50 °C and 90 °C with different inlet/outlet temperature combinations showed peaks at 275 nm, indicating the formation of pyrazine compounds from the cyclo-condensation between GlcN molecules during the caramelization process (Candiano et al., 1988; Horowitz, 1991). However, these peaks were very similar to each other, with almost overlapping curves along the measured wavelengths. As a result, the texture of the spray-dried GlcN caramel powder was compared to determine the best inlet/outlet temperature combination. GlcN caramel powder that was the finest and least sticky was considered the best and based on this standard, 140 °C/70 °C was selected and used in the main analysis. This temperature combination also led to the highest yield as most of the powder after the spray-drying process can be successfully harvested.

4.3.2. Chemico-physical Characteristics of GlcN Caramel Samples

As shown in Figures 4.3 and 4.4, all GlcN caramel samples showed the highest peaks at 275 nm with bigger shoulders toward the higher wavelengths, indicating the formation of

fructosazines (Candiano et al., 1988; Horowitz, 1991). The increase of absorbance at higher wavelengths indicated the formation of compounds with higher molecular weight, including soluble pre-melanoidins absorbing at the wavelength of 320 nm (Fogliano et al., 1999), and melanoidins absorbing at 420 nm (Adams et al., 2005).

Specifically, the non-spray-dried GlcN caramel samples showed higher peaks than the spray-dried samples at 275 nm. This was true for both incubation temperatures, revealing that the spray-drying process decreased the concentration of fructosazines in the GlcN caramels. Likewise, the spray-dried GlcN caramels also showed a smaller shoulder toward the higher wavelengths compared to non-spray-dried samples for both incubation temperatures, where the amount of high molecular weight compounds seemed to decrease as well.

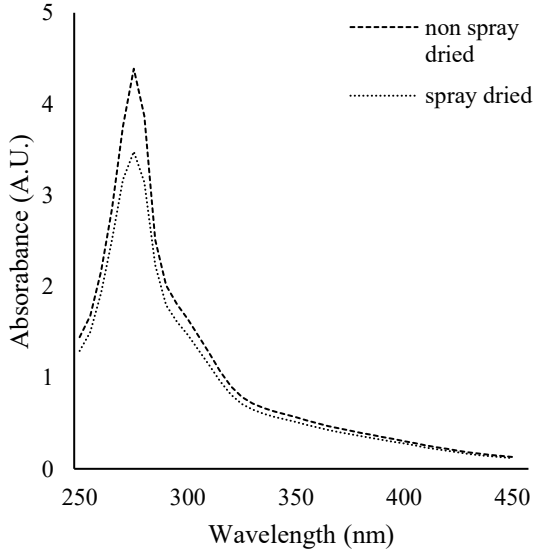


Figure 4.3. Absorbance profiles of non-spray-dried and spray-dried GlcN 15% caramels (incubated at 50 °C for 12 h). The values are expressed in arbitrary units (A.U.).

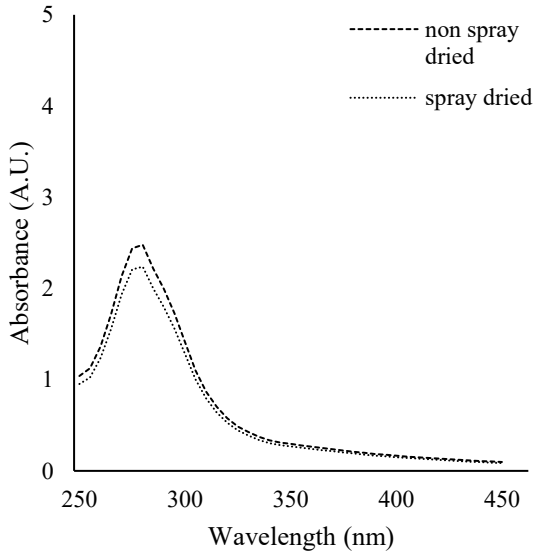


Figure 4.4. Absorbance profiles of non-spray-dried and spray-dried GlcN 15% caramels (incubated at 90 °C for 12 h). The values are expressed in arbitrary units (A.U.).

Due to the difference in dilution factors (100 times for 50 °C samples and 250 times for 90 °C), the two figures cannot be compared directly in their value of absorbance to analyze the effect of incubation temperature on the formation of desired compounds. HPLC analysis was performed to determine their concentrations.

As presented in Table 4.1., the pH of GlcN caramels decreased after the heat treatment. This is most likely due to the formation of organic acids including formic acid, acetic acid and levulinic acid in the solution during the caramelization of GlcN (Kroh, 1994). An increase in incubation temperature caused a significant decrease ($p < 0.05$) in pH, with a difference of 2.4 between 50 °C and 90 °C samples. An increase in temperature can accelerate the reaction, causing a higher concentration of organic acids to be formed in the solution, which leads to a lower pH (Wu et al., 2014). Spray-drying also significantly affects the pH of GlcN caramels, where the spray-dried GlcN caramels were significantly more acidic compared to the non-spray-dried

samples. This was more evident at 50 °C, with a pH value of 4.5 for non-spray-dried GlcN caramels and 4.2 for spray-dried GlcN caramels.

Tristimulus colorimetry was used to integrate different dimensions of the color space. The colorimetric parameters, L^* (lightness, black (0) - white (100)), a^* (red-green) and b^* (yellow-blue) were measured and recorded.

Table 4.1. Changes in pH, lightness (L^*), redness (a^*), yellowness (b^*), hue angle (h_{ab}), and chroma (C^*_{ab}) during non-spray-drying and spray-drying of 15% GlcN solutions incubated at 50 °C and 90 °C for 12h.

		pH	***L****	***a****	***b****	h_{ab}	C^*_{ab}
incubation temperature							
50 °C		4.3 a	33.7 a	4.8 a	12.7 a	52.9 b	14.1 a
90 °C		1.9 b	28.3 b	2.6 b	9.0 b	74.2 a	9.3 b
SEM		0.01	1.1	0.1	0.2	0.9	0.2
spray-drying							
non-spray-dried		3.2 a	20.0 b	1.8 b	1.3 b	52.5 b	2.3 b
spray-dried		3.1 b	42.0 a	5.6 a	20.4 a	74.5 a	21.1 a
SEM		0.01	1.1	0.1	0.2	0.9	0.2
interaction (incubation temperature × spray-drying)							
50 °C	non-spray-dried	4.5 a	20.6 c	3.4 c	2.0 c	30.8 b	3.9 c
	spray-dried	4.2 b	46.8 a	6.3 a	23.4 a	75.0 a	24.2 a
90 °C	non-spray-dried	2.0 c	19.4 c	0.2 d	0.6 d	74.3 a	0.7 d
	spray-dried	1.9 d	37.1 b	4.9 b	17.3 b	74.1 a	18.0 b
SEM		0.01	1.5	0.1	0.3	1.3	0.3
sources of variation		*p*-values					
incubation temperature		< 0.0001	< 0.01	< 0.0001	< 0.0001	< 0.0001	< 0.0001
spray-drying		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
interaction		< 0.001	< 0.05	< 0.001	< 0.0001	< 0.0001	< 0.001

Means within the same column with no common superscript differ significantly ($p < 0.05$). SEM, pooled standard error of the means.

Based on Table 4.1, the lightness of GlcN caramel solutions significantly decreased with increasing incubation temperature, indicating a darker solution at 90 °C. This was expected as higher temperatures during the processing often led to darker caramels (Jing & Kitts, 2004). Likewise, an increase in incubation temperature also caused a significant decrease in redness (a^*) and yellowness (b^*). However, the spray-dried caramel samples were significantly lighter in color, with significantly higher redness (a^*) and yellowness (b^*).

Chroma (C^*, metric chroma) and hue angle (h_{ab}, chromatic tonality) were also calculated in Table 4.1 to express color. Chroma is defined as the magnitude of the vector at each point designating the departure from dull to more vivid chromatic color ("–“ to “+” values), which indicates color saturation and intensity (Serratosa et al., 2008). By contrast, hue angle measures redness at values near 0° and yellowness near 90° (Jing & Kitts, 2004). The significant effects of incubation temperature and spray-drying as well as their interaction was observed for both C^* and h_{ab}. Specifically, the least ($p < 0.05$) hue value was found in the GlcN caramel sample that was incubated at 50 °C without spray-drying.

4.3.3. Analysis of FR and DOFR

Fructosazine (FR) and deoxyfructosazine (DOFR) are the self-condensation products of GlcN. They are important compounds in food systems that also possess great bioactivity, for example, they have anti-inflammatory properties (Zhu et al., 2007). Additionally, FR and DOFR have been reported to act therapeutically against osteoarthritis (Giordani et al., 2006) and Type II diabetes (Bashiardes et al., 2002).

According to the results presented in Table 4.2, incubation temperature and spray-drying significantly affected the concentrations of FR and DOFR. In terms of incubation temperature, the concentration of FR and DOFR significantly ($p<0.05$) decreased as incubation temperature

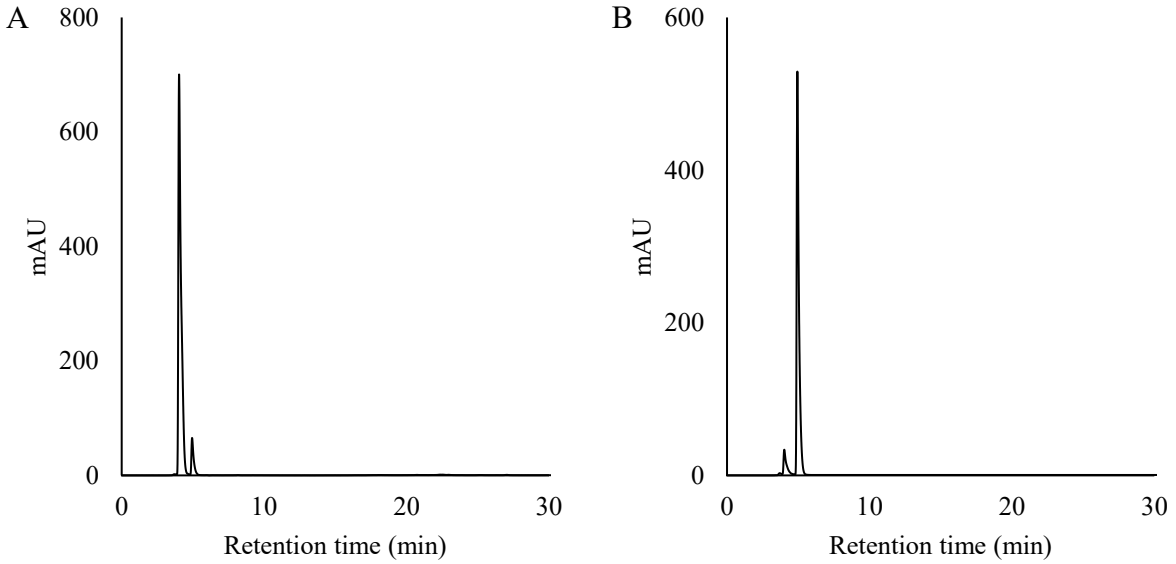
increased for both non-spray-dried and spray-dried GlcN caramel samples. This result is consistent with previously published results from our research group (Dhungel, 2018) where the concentration of FR and DOFR continued to decrease with an increase of the incubation temperature from 50 °C to 70 °C. This is potentially due to the acidification of GlcN caramel solutions at higher incubation temperature, shown in Table 4.1. The GlcN caramel solution obtained from 90 °C incubation was significantly more acidic than the 50 °C GlcN caramel and based on the study conducted by Wu et al. (2001), a large yield of FR and DOFR are favorable at pH 6-8. These results indicate that 50 °C incubation temperature was more favorable for the formation of FR and DOFR with a significantly ($p < 0.05$) higher pH.

Table 4.2. The concentration of non-volatile fructosazine (FR) and deoxyfructosazine (DOFR) in non-spray-dried and spray-dried 15% GlcN caramels produced at 50 °C and 90 °C for 12h.

		FR (g/L)	**DOFR** (g/L)
incubation temperature			
50 °C		1.6 a	18.1 a
90 °C		0.7 b	15.7 b
SEM		0.04	0.3
spray-drying			
non-spray-dried		1.3 a	18.3 a
spray-dried		1.0 b	15.5 b
SEM		0.04	0.3
interaction (incubation temperature × spray-drying)			
50 °C	non-spray-dried	1.8 a	20.0 a
	spray-dried	1.5 b	16.6 b
90 °C	non-spray-dried	0.8 c	16.2 b
	spray-dried	0.5 d	14.7 b
SEM		0.06	0.5
sources of variation		*p*-values	
incubation temperature		< 0.0001	< 0.001
spray-drying		< 0.01	< 0.001
interaction		> 0.05	> 0.05

Means within the same column with no common superscript differ significantly ($p < 0.05$). SEM, pooled standard error of the means.

As shown in Table 4.2, the non-spray-dried GlcN caramel solutions contained significantly higher concentrations of FR and DOFR compared with spray-dried solutions at two incubation temperatures, which is consistent with the absorbance profiles shown in Figure 4.3 and 4.4. Spray-drying can generate amorphous structures influencing the rate of non-enzymatic browning reactions (Luna and Aguilera, 2014). Song and Yrjö (2004) reported enhanced non-enzymatic browning reaction rates in spray-dried food systems. This is because the reactants are located more closely to each other during the rapid removal of water. Also, the concentration of reactants increases due to lower water sorption of spray-dried materials, thus enhancing the browning reaction. Similar to the effect of increased incubation temperature, spray-drying facilitated the reaction rate, causing an increase in the acidity of the GlcN caramel solutions (Table 4.1). In this case, the formation of FR and DOFR was not favorable, as a reduction in their concentrations was observed. The representative chromatograms, Figure 4.5., showed the elution pattern of FR and DOFR.



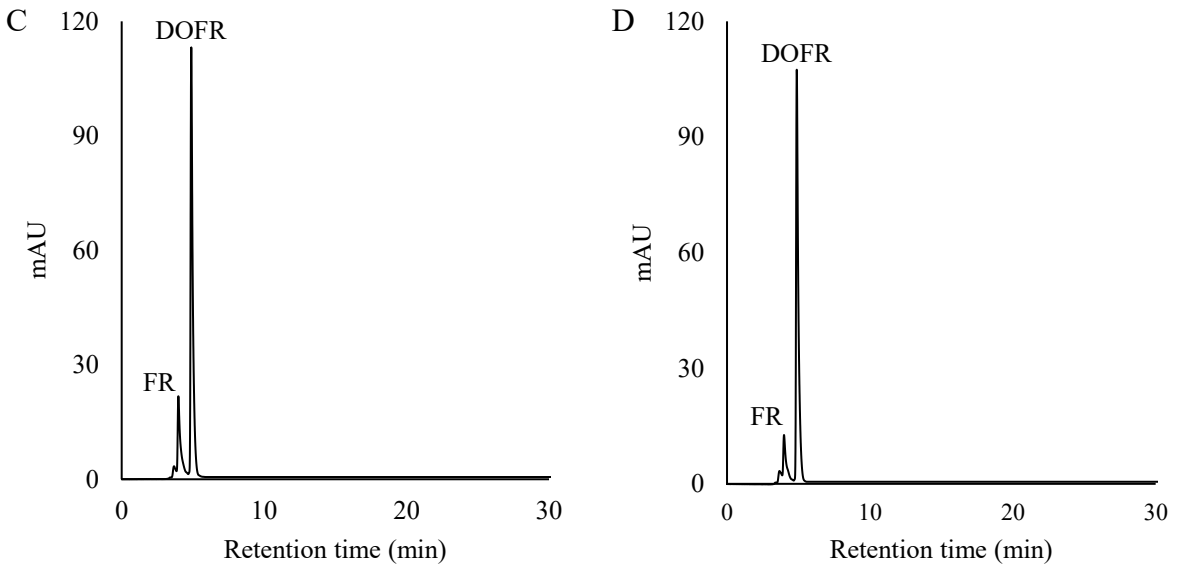


Figure 4.5. HPLC analysis of non-volatile fructosazine (FR) and deoxyfructosazine (DOFR). (I) Chromatograms of (A) FR and (B) DOFR standards. (II) Representative chromatograms of FR and DOFR in non-spray-dried (C) and spray-dried (D) GlcN 15% solutions incubated at 50 °C for 12 h.

4.3.4. Analysis of Neo-formed Contaminants

The neo-formed contaminants presented in industrially produced caramels are 5-HMF, THI, and 4-MEI (Vollmuth, 2018; Murkovic and Pichler, 2006). They are potentially hazardous to human health. Among these, THI and 4-MEI are carefully regulated in food products around the world, with the concentration limit of THI set at 25 mg/kg, and that of 4-MEI set at 200 mg/kg. They have been reported to be immunosuppressive and possibly carcinogenic, respectively.

Based on Table 4.3., the concentration of THI was significantly higher in the 90 °C incubated GlcN caramel solutions than in the 50 °C solutions. This result is consistent with the findings in the previously studies of our research group, where the highest concentration of THI was found in GlcN caramels incubated at 70 °C as compared to those incubated at 50 °C and 60 °C (Dhungel et al., 2018). However, spray-drying significantly (p<0.05) reduced the concentration of THI. On average, 115 mg/L of THI was formed in non-spray-drying conditions, whereas 100 mg/L

of THI was found in spray-drying conditions. Compared to the GlcN caramels incubated at 90 °C, the 50 °C GlcN caramel showed significantly lower concentrations of THI, with a value of 2.3 mg/L. This was far below the regulated amount of 25 mg/L. However, increasing the incubation temperature to 90 °C elevated the formation of THI, thus a relatively lower incubation temperature is favored to limit the concentration of THI being formed.

Table 4.3. Concentration of 5-hydroxymethyl-2-furfural (HMF), 2-acetyl-4(5)-(tetrahydroxybutyl)imidazole (THI), and 4-methylimidazole (4-MEI) during non-spray-drying and spray-drying of 15% GlcN solutions incubated at 50 °C and 90 °C for 12 h.

		HMF (mg/L)	**THI** (mg/L)	**4-MEI** (mg/L)
incubation temperature				
50 °C		2.4 b	2.3 b	
90 °C		1975 a	213 a	
SEM		56.7	1.6	
spray-drying				
non-spray-dried		1028	115 a	
spray-dried		950	100 b	
SEM		56.7	1.6	
interaction (incubation temperature × spray-drying)				ND
50 °C	non-spray-dried	4.5 b	3.4 c	
	spray-dried	0.2 b	1.2 c	
90 °C	non-spray-dried	2051 a	227 a	
	spray-dried	1899 a	199 b	
SEM		80.2	2.3	
sources of variation		*p*-values		
incubation temperature		< 0.0001	< 0.0001	
spray-drying		> 0.05	< 0.001	
interaction		> 0.05	< 0.001	

Means within the same column with no common superscript differ significantly ($p < 0.05$). SEM, pooled standard error of the means.

In terms of 5-HMF, a similar trend was observed as for THI. The concentration of 5-HMF elevated at GlcN caramel solutions incubated at 90 °C, with a value of 1975 mg/L. This concentration was significantly higher that of the 50 °C incubated GlcN caramel samples, with a

value of 2.4 mg/L. The level of 5-HMF in commercial food products is not regulated, and its concentration can vary considerably with different types of caramel. For example, the concentration of 5-HMF in Caramel Type I was reported to range from 700-2700 mg/kg (Aguilar et al., 2011). The highest concentration reported in Table 4.3 was still below the level found in commercial caramel products. The representative chromatograms, Figure 4.6., showed the elution pattern of THI and 5-HMF.

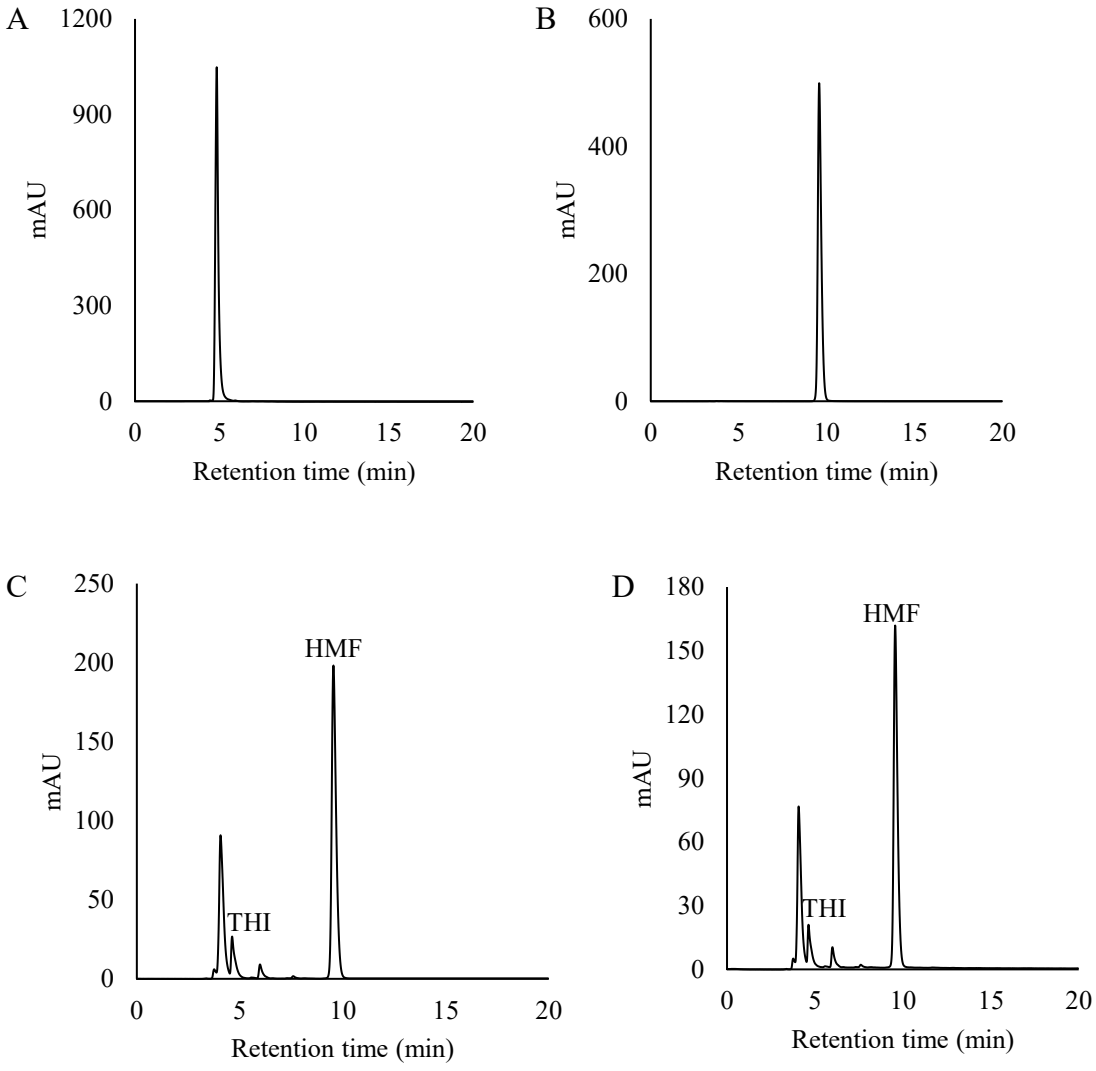


Figure 4.6. HPLC analysis of 2-acetyl-4(5)-(tetrahydroxybutyl)imidazole (THI) and 5-hydroxymethyl-2-furfural (HMF). (I) Chromatograms of (A) THI and (B) HMF standards. (II) Representative chromatograms of THI and HMF in non-spray-dried (C) and spray-dried (D) GlcN 15% solutions incubated at 90 °C for 12 h.

There was no 4-MEI detected in any of the GlcN caramel samples, which is very important since it has been reported to be possibly carcinogenic and causes a major concern in caramel food systems.

To limit neo-formed contaminants, a lower incubation temperature is preferred. Moreover, spray-drying can also lower the amount of THI and 5-HMF. These results are valuable in designing caramel products with limited levels of these potentially hazardous compounds.

4.4. Conclusion

In conclusion, this study revealed that both incubation temperature and spray-drying significantly affected the chemico-physical properties and the composition of the resulting GlcN caramel solutions. Combinations of different incubation temperatures and spray-drying generated unique GlcN caramels that had different acidity, color, and concentration of volatile and non-volatile compounds.

Briefly, higher incubation temperature generated GlcN caramels with higher acidity, darker color, and lower concentrations of FR and DOFR, but far more concentrations of THI and 5-HMF. Spray-dried GlcN caramels were found to have a higher acidity, lighter in color, and lower concentrations of both non-volatile FR and DOFR, as well as THI and 5-HMF. To limit the formation of neo-formed contaminants, a lower incubation temperature can be selected with spray-drying treatment. The resulting product is not only favorable in this aspect but also has a very low water content and is easier for transportation. The powder form also made it suitable for adding to animal feed,

CHAPTER 5. Conclusions, implications, and future research

This thesis provided an overview of the non-enzymatic browning of GlcN under UV-C radiation and spray-drying. To the best of my knowledge this is the first research study investigating the non-enzymatic browning of GlcN under UV-C radiation compared to conventional heat-induced caramelization of GlcN. Although heat-induced non-enzymatic browning of GlcN was studied previously (under vacuum condition etc.), no research has been performed comparing the effect of spray-drying on the non-enzymatic browning of GlcN. Overall, this research provided knowledge on the following aspects:

a) Chemistry of non-enzymatic browning of GlcN under UV-C radiation. Compared to conventional heat-induced caramelization which uses high temperature (>120 °C), temperature during the UV-C treatment was set to 25 °C. As a result, none of the neo-formed contaminants including 4-MEI, THI and 5-HMF was detected in the GlcN caramel samples. The amount of glucosone, a reductone compound, was found to be significantly higher compared to those in heat-induced GlcN caramels;

b) Chemistry of spray-dried GlcN caramel powders compared to non-spray-dried GlcN caramel solutions incubated at 50 °C and 90 °C;

c) Quantitation of bioactive compounds: bioactive FR and DOFR were quantified for all UV-C treated and non-spray-dried and spray-dried samples;

d) Quantitation of potentially hazardous compounds: 4-MEI, THI, and 5-HMF that are found in caramels have shown to be potentially toxic, thus temperature was controlled at 25 °C during the UV-C treatment and none of these compounds were detected in the GlcN caramel samples.

Overall, this study showed that altering the reaction condition can affect the chemistry of the resulting caramels. For food products that need caramels have certain traits, different methods of production can be used. The UV-C treated and spray-dried GlcN caramels contains bioactive FR and DOFR which can be added to certain foods where bioactivity is targeted. Additionally, the UV-C treated GlcN caramels contain none of the potentially toxic compounds that are important in any food applications. The spray-dried GlcN caramel powders is easier to transport in the food supply chain. The powder form is also convenient for different food applications, whether in industry or at-home settings.

Since the application of UV-C has shown to be able to generate caramels with desirable compounds without hazardous compounds, it can be further studied in the future for potentially longer exposure time. The spray-drying method may be applied for generating caramel powders that can be added to animal feed to study the bioactive compounds and their effect on animal nutrition.

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Appendix A

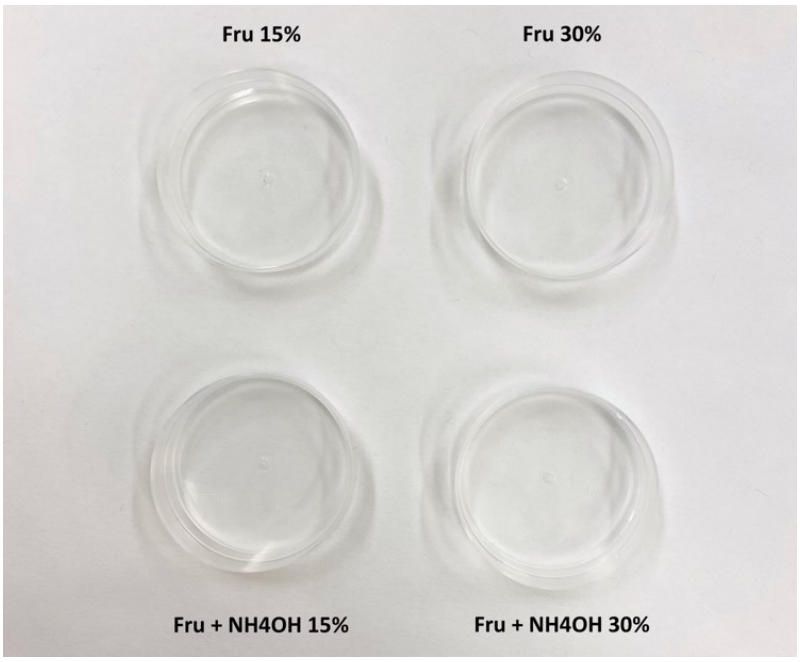

Figure A1. Fru and Fru + NH_4OH control solutions at 15% and 30% after exposing to UV-C light for 120 min.

Appendix B

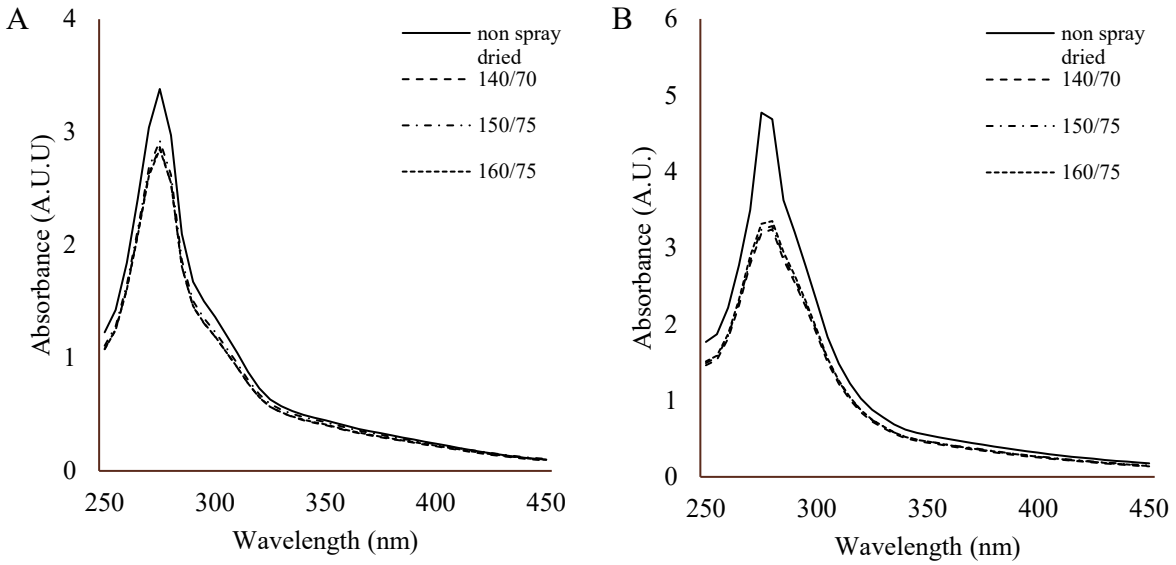

Figure B1. Absorbance profiles of non-spray-dried and spray-dried GlcN 15% caramels incubated at 50 °C (A) and 90 °C (B) for 12 h. Different inlet/outlet temperature combinations (expressed in °C) were used during spray-drying. The values are expressed in arbitrary units (A.U.).