Studies on non-enzymatic browning of glucosamine and glucosamine-amino acid solutions

by

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Abstract

Non-enzymatic browning reactions represent a complex phenomenon occurring during thermal processing of food, and are categorised as either the Maillard reaction or caramelization. Besides temperature, these reactions depend upon water activity (a_w), pH and the concentration of reactants. Non-enzymatic browning reactions give flavour and colour to foods, and are central in the production of caramel colours. On the other hand, these reactions also produce potential toxic compounds such as 4-methylimidazole (4-MEI), 5-hydroxymethylfurfural (5-HMF) and 2-acetyl-5-tetrahydroxybutyl imidazole (THI) which increase with temperature and are defined as neo-formed contaminants in caramel.

Glucosamine (GlcN) is an aminosugar capable of eliciting non-enzymatic browning reactions even at 25-37°C. The structure of GlcN is peculiar as it contains both an amino group and a carbonyl group in the same compound; this makes it an unstable molecule capable of forming degradative and self-condensation reactions generating plethora of compounds. One compound generated by the non-enzymatic browning of GlcN is diacetyl, and is known for its appealing butter-like aroma. Other compounds are fructosazine (FR) and deoxyfructosazine (DOFR), and are known for anti-diabetic and anti-inflammatory properties, and glyoxal, methylglyoxal, diacetyl and 3-deoxyglucosone for antimicrobial properties. Thus, GlcN can be used to produce a caramel containing interesting functional compounds with aromatic, antimicrobial and anti-inflammatory properties even at moderate temperatures. Hence, one of the main objectives of this thesis was to produce safe and flavourful GlcN-derived caramel under vacuum conditions ("sous-vide") or by combining with specific amino acids.

The first study was designed to study the sous-vide non-enzymatic browning of GlcN at 50, 60 and 70°C for 12 h. The physico-chemical properties, quantification of α-DCs, polyhydroxylalkyl pyrazines (FR and DOFR), alkylimidazoles (4-MEI and THI) and 5-HMF were determined in the sous-vide GlcN caramels as compared to GlcN caramels generated under the normal, ambient oxidative conditions. The results revealed that caramelization under vacuum generated significantly lower ($p < 0.05$) diacetyl concentrations compared to oxidative conditions at all incubation temperatures. Significantly greater concentrations of FR were found in the vacuum treatments. THI and 5-HMF concentrations in all caramels studied were well below the toxicity levels, while the potentially more toxic 4-MEI was not detected in any of the caramels produced. This study shows that sous-vide conditions did not improve the formation of butterscotch flavour but increased the amount of the biologically active polyhydroxylalkyl pyrazines.

The second study focused on investigating the effect of different amino acids on non-enzymatic browning of GlcN incubated at 70°C for 12 h. The resulting GlcN-amino acid "caramels" were analyzed for α-DCs, polyhydroxyalkyl pyrazines, heterocyclic compound and alkylimidazoles. The greatest ($p < 0.05$) amount of butterscotch aromatic compound diacetyl, and FR and DOFR were generated in GlcN-glycine caramels. GlcN-arginine caramels generated the greatest ($p < 0.05$) amount of HMF. The neo-formed contaminants alkylimidazoles (4-MEI and THI) were not present in any of the GlcN-amino acid caramels. The THI was present in the GlcN control, suggesting that the addition of the amino acids completely inhibited the formation of these contaminants. Principal component analysis categorised the majority of the GlcN-amino acid combinations where GlcN-glycine and GlcN-serine were the best discriminated. In general, the

addition of glycine to GlcN non-enzymatic browning produces a “caramel” solution with the greatest concentration of flavourful compounds.

Overall, this thesis demonstrated that different types of caramels produced depend on the presence or absence of vacuum, as well as the type of amino acids added during the non-enzymatic browning reaction of GlcN. These caramels have the potential to be used in different food applications such as simple colourants, as aromatic caramels, or even conferring beneficial health activities.

Preface

This thesis is an original work of Prinjiya Dhungel. The thesis is presented in manuscript format and consists of five chapters.

Chapter 1 is a brief introduction to the non-enzymatic browning reactions followed by the research problem, hypothesis and objectives of the experimental studies. Chapter 2 is the research background and contains the major aspects of non-enzymatic browning reactions. This chapter focuses on production and applications of different types of caramels in foods and extraction and uses of glucosamine and the bioactive compounds produced during glucosamine non-enzymatic browning. Chapters 3 and 4 constitute the main body of the thesis. Chapter 3 is based on a published manuscript (Dhungel et al., 2018. Sous-vide non-enzymatic browning of glucosamine at different temperatures. *Journal of Agricultural and Food Chemistry*, 66 (17), 4521–4530). The content of Chapter 4 corresponds to the second study of this research. Chapter 5 summarizes the outcomes of this research. Brief discussion regarding the implications of the study and suggestions for future work are also included in this chapter.

The present author was responsible for the experimental design, data collection, analyses and manuscripts preparation in the published and in preparation manuscripts. Dr. Y. Hrynets assisted with the technical support for the experiments and proof reading of the manuscripts. Dr. M. Betti was the supervisory author and was involved in providing advisory inputs in the project's design and work as it progressed, manuscript composition, editing and proof reading.

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Finally, I must express my profound gratitude to my parents and sister who have always been with me through thick and thin. It is their blessings that always kept me strong in life. Also, I cannot thank my boyfriend enough for his unfailing support and turning all my “I cannot do it” to “You can do it”.

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Abbreviations

3-DG - 3-deoxyglucosone
3,4-DGE - 3,4-dideoxyglucoson-3-ene
α-DC - alpha-dicarbonyl compound
*a**- redness
ANOVA – analysis of variance
*b**- yellowness
*C**- chroma
CE - collision energy
CXP - collision cell exit potential
DA - diacetyl
DOFR - deoxyfructosazine
DP - declustering potential
EP - entrance potential
ESI - electrospray ionization
FR - fructosazine
G - glucosone
GlcN - glucosamine
GlcNAc - *N*- acetyl-D-glucosamine
GO - glyoxal
H° - 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
PCA - principal component analyses
PVDF - poly(vinylidene fluoride)
SEM - standard error of the means
SPE - solid-phase extraction
THI - 2-acetyl-(4)5-tetrahydroxylbutyl imidazole

CHAPTER 1. General introduction and objectives

Non-enzymatic browning reactions represent a cascade of reactions where the browning of food occurs without involvement of enzymes. These reactions depend on factors such as pH, temperature, water activity, moisture content and chemical composition of the reactants (Gerrard, 2002; Morales & Van Boekel, 1997). It includes reactions such as the Maillard reaction and caramelization. The Maillard reaction is the condensation between a carbonyl group of a reducing sugar to any primary amino group on protein or peptide; whereas caramelization is the degradation of sugars in the absence of an amino compound. The Maillard reaction and caramelization yield similar products including organic acids, flavour compounds, reductones, brown pigments and classes of heterocyclic compounds. The Maillard reaction is usually categorized into 3 major stages, namely the initial, intermediate and final stages (Hodge, 1953). The initial stage involves the reducing sugar-amine condensation to form a Schiff base, which is subsequently rearranged into *N*-substituted glycosylamine, termed Amadori rearrangement products (ARPs for aldoses) or Heyns rearrangement products (HRPs for ketoses). At the intermediate stage, the ARPs and HRPs degrade to form reactive α-dicarbonyl compounds (α-DCs). The latter not only lead to the formation of colour (ie. caramel colour), but also give rise to the important volatile products which are typical for flavour. Subsequently, these α-DCs react with the other compounds including amino acids, amines, ammonia and aldehydes resulting in the formation of pyrazines, pyrroles, thiazoles, oxazoles and classes of heterocyclic compounds. Pigmented nitrogenous polymers termed melanoidins and flavour compounds, including pyrazines, pyrroles, furans and oxazoles, are produced in the final stage of the Maillard reaction (Hodge, 1953; Morales & Van Boekel, 1997).

Glucosamine (GlcN; 2-amino-2-deoxy-glucose) is an amino sugar which is widely used in over-the-counter health supplements in North America for the relief of osteoarthritis. Research has

shown that GlcN is capable of triggering non-enzymatic browning reactions at temperatures as low as 4°C (Betti et al., 2018, unpublished data). GlcN can generate self-condensation products (i.e. fructosazine and deoxyfructosazine), α-DCs, heterocyclic compounds and classes of imidazoles at moderate temperatures. Fructosazine (FR) and deoxyfructosazine (DOFR) are used in the prevention and treatment of osteoarthritis and is a popular anti-inflammatory agent (Giordani et al., 2006). These compounds also have gained attention as flavouring agents in food and beverage industries (Henry et al., 2012). α-DCs compounds such as glucosone (G), 1- and 3-deoxyglucosone (1-DG, 3-DG), glyoxal (GO), methylglyoxal (MGO) are precursors in flavour generation, whereas diacetyl (DA) is an important aroma compound in butter, margarine, sour cream, yogurt, and a number of cheeses (Clark and Winter, 2015). DA is usually added to foods and beverages to provide the buttery or butterscotch−like aroma (Smit et al., 2005).

Industrial manufacture of caramel colours involves a controlled heat treatment, where food grade carbohydrates (i.e. glucose, sucrose, invert sugar) are reacted in the presence of ammonium salts that promote colour formation (Moon and Shibamoto, 2010). One of the issues raised with these caramel colours is the generation of undesirable toxic compounds, namely 4(5)-methylimidazole (4-MEI), 5-(hydroxymethyl)furfural (HMF) and 2-acetyl-4(5)(tetrahydroxybutyl)imidazole (THI) (IARC 2012; EFSA 2011). In view of that, the concentration of these compounds is regularly monitored and for some of them the limits are set by regulatory agencies. Therefore, it was also important to identify and quantify these compounds in caramels resulting from GlcN non-enzymatic browning.

The general objectives of thesis are both theoretical and practical:

a) To understand the chemistry of glucosamine non-enzymatic browning in different conditions such as under vacuum (“sous-vide”) and in the presence of different amino acids.

b) To develop caramel solutions at moderate temperatures with the greatest amount of flavourful and bioactive compounds, while minimizing the generation of undesirable alkylimidazoles. These caramel solutions can be used in preparation of baked products, innovative meat products (i.e. meat snack), diary products, soft drinks and alcoholic beverages including dark beer.

The first study of this thesis (described in Chapter 2) was devoted to better understanding the chemistry behind the non-enzymatic browning of GlcN under under vacuum conditions ("sous-vide") as compared to oxidative condition at different temperatures. The specific objectives of the study one were:

a) To measure the physico-chemical changes of GlcN solutions incubated at 50, 60 and 70 ºC for 12 h;

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 monitor the presence of potential toxic compounds (4-MEI, HMF and THI) formed during non-enzymatic browning reaction and to quantify them.

In the second study (described in Chapter 3) GlcN non-enzymatic browning in the presence of amino acids was investigated. To identify the best combination of GlcN-amino acid solutions to generate caramels with the greatest concentration of butterscotch aromatic compound diacetyl and polyhydroxyalkyl pyrazines known for their bioactivities. Besides studying the flavour compounds or their precursors, this study also aimed to get better insight about quantities of the

potentially toxic compounds formed during non-enzymatic browning reaction of GlcN in combination with amino acids. The specific objectives of study two were:

a) To quantify the long and short chain α-DCs produced during non-enzymatic browning reaction of GlcN in the presence of amino acids incubated at 70 °C for 12 h;

b) To quantify the major polyhydroxyalkyl pyrazines (FR and DOFR) formed during non-enzymatic browning of GlcN - amino acids model reaction mixtures;

c) To monitor the presence of potentially toxic compounds formed during non-enzymatic browning of GlcN - amino acids model reaction mixtures.

CHAPTER 2. Research background

2.1. Chemistry of non-enzymatic browning reactions

Non-enzymatic browning reactions represent a complex phenomenon followed by a cascade of reactions. The browning in heat-processed food, mediated without enzymes is roughly divided into two types: caramelization and the Maillard reaction (Nursten, 2007). Different factors influence these two reactions such as initial pH, temperature, presence of oxygen, heating time, water activity (a_w), etc. (Wu et al., 2014). Although caramelization and the Maillard reaction differ from each other, they yield similar reaction products including reductones, brown pigments, flavour compounds, heterocyclic compounds, etc.

2.1.1. Caramelization

Caramelization is the process of degradation of sugar in the absence of an amino group. It is one of the oldest methods of providing colour and flavour in foods. This reaction is favourable at temperatures >120°C and 9 < pH <3 (Kroh, 1994). It is influenced by the pH of reaction, sugar concentration and its type (Clarke et al. 1997; Eggleston and Vercellotti, 2000). Caramelization is desirable in foods such as candies, jams and certain fruit juices. However, caramelization is undesirable during production of sucrose (Kroh, 1994). As proposed in the mechanisms shown in Figure 2.1., caramelization is a sequence of reactions initiated by enolization of a sugar molecule followed by the dehydration or β-elimination, dicarboxylic cleavage, retroaldolization, aldol condensation and a radical reaction (Kroh, 1994). Enolization plays a vital role because it gives rise to aliphatic sugar degradation products which then initiate a chain of reactions during caramelization. The resulting aliphatic sugar degradation products react further to produce heterocyclic and carboxylic compounds via aldol condensation. Alpha-dicarbonyl compounds are the key intermediates of caramelization and lead to the formation of colour with varying hues and

flavour (Kroh, 1994). Flavour compounds range from mild, sweet and caramel-like to bitter and are formed during the initial stage of the reaction (Luna and Aguilera, 2014).

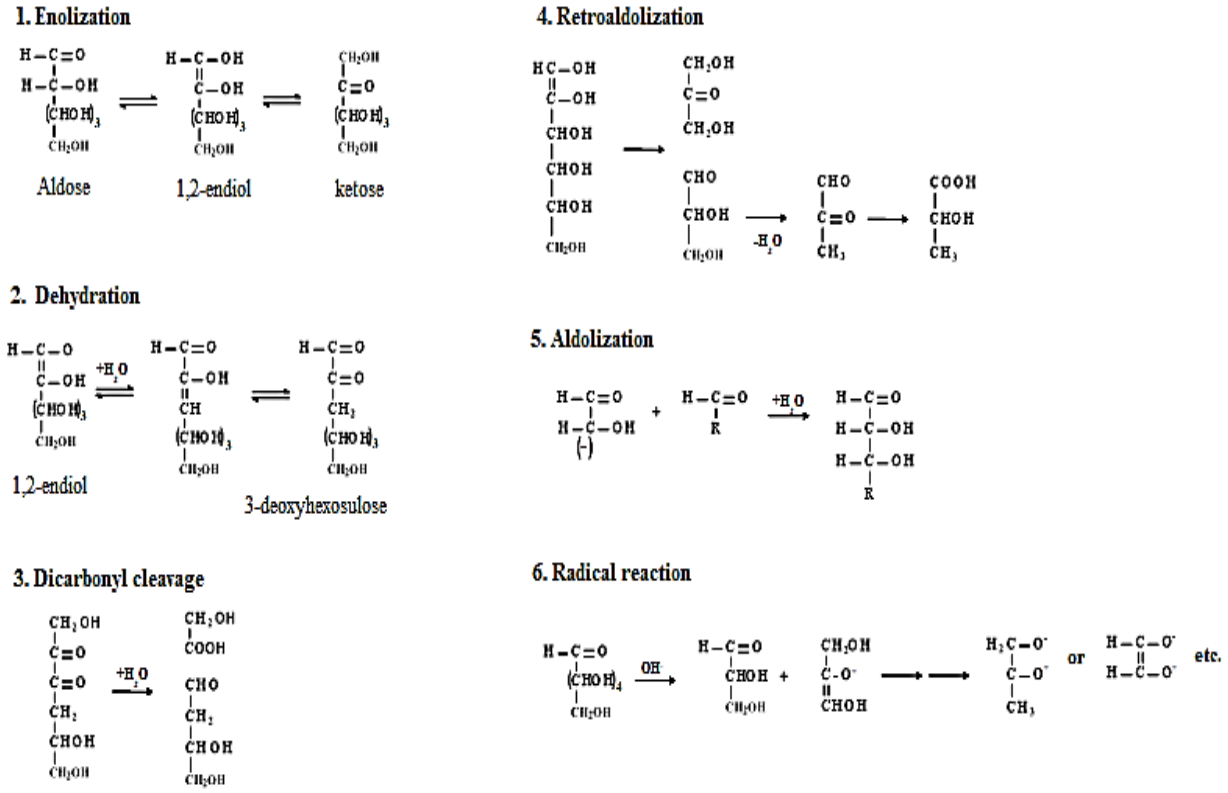


Figure 2. 1. Steps involved in caramelization reaction namely: enolization, dehydration, dicarbonyls cleavage, retro aldolization, aldolization and radical reaction. Figure is reproduced with permission from Kroh, L. W. Caramelisation in food and beverages. *Food Chem*. **1994**, *51*, 373-379. Copyright (1994) Elsevier Ltd.

The products of caramelization are a complex mixture of high molecular weight (HMW) and low molecular weight (LMW) compounds. HMW compounds are non-volatile components which represent the majority of coloured products in caramelization reaction (Myers and Howell, 1992). Caramelized compounds constitute of 90-95% HMW compounds, while LMW compounds accounts for remaining 5-10% (Paravisini et al., 2012). Separation techniques such as ultrafiltration, gel permeation and ion-exchange chromatography have been used to study and

isolate these compounds (Patey et al., 1985). However, no feasible methods have been developed to determine the structure of these macromolecules precisely. On the other hand, advances have been made in identifying LMW compounds and *N*-heterocyclic compounds in ammonia caramel (caramel colour III) colours, namely pyridines, pyrazines, imidazoles and pyrroles (Patey et al., 1987).

2.1.2. The Maillard reaction

In 1953 John Hodge subdivided non-enzymatic browning reaction into initial, intermediate and final stages (Figure 2.3.). In 1981, Jean Mauron named these three stages as early, advanced and final stages, respectively. This classification simply serves to systematize a complicated reaction such as the Maillard reaction.

2.1.2.1. Initial stage

The initial stage of the Maillard reaction is one series of reactions involves the condensation of the carbonyl and amine through the C-l of aldoses and the C-2 of ketoses to form a Schiff base. Schiff base is unstable and a reversible compound, and rapidly converts itself to Amadori rearrangement product (ARP) or Heyns rearrangement product (HRP) depending on whether the reaction involves aldose or ketose, respectively. The resulting products of initial stage are colourless.

2.1.2.2. Intermediate stage

The intermediate stage of the Maillard reaction begins with the degradation of ARP and HRP thought a variety of reactions including oxidation, fragmentation, enolization, dehydration, and amino acid degradation. For instance, HRP dehydrates to reductones and dehydro-reductones or short-chained α-dicarbonyls such as diacetyl and methylglyoxal (pyruvaldehyde). Furfural is

also produced during this stage. Sugar fragmentation normally involves retroaldolization where the hexose derivatives are cleaved to form corresponding sugar fragments. For instance, glyoxal, 2-oxopropanal and 2,3-pentanedione are generated from glucosone, fructose/glucose and 3-deoxyglucosone, respectively. Amino acid degradation also known as Strecker degradation is deamination and decarboxylation of amino acids in the presence of a carbonyl compound (α or β-dicarbonyl compound) to form subsequent imines, aldehydes or ketones. The products of intermediate stage are slightly coloured and their electromagnetic absorption spectra show large absorption in the UV range. This stage has crucial role in generation of colour, aroma and flavour in the Maillard reaction.

2.1.2.3. Final stage

The final stage of the Maillard reaction is comprised of aldol condensation and aldehyde-amine condensation. The high molecular weight, coloured polymeric pigments (as shown in Figure 2.2) are formed, called melanoidins. Melanoidins are heterocyclic compounds comprised of 3-4% nitrogen. In the final stage the Amadori products can also form crosslinks between adjacent proteins or with other amino groups forming polymeric aggregates known as advanced glycated end products (AGEs) (Friedman, 1996).

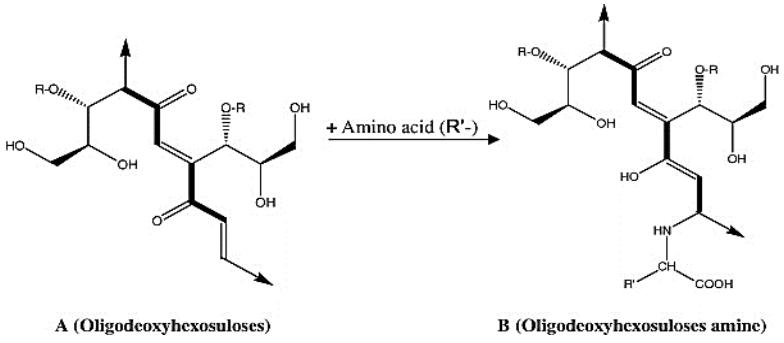


Figure 2.2. Structure of carbohydrate based melanoidin. Figure reprinted with permission from Wang, H. Y., Qian, H., & Yao, W. R. (2011). Melanoidins produced by the Maillard reaction:

Structure and biological activity. **2011.** Food Chemistry, *128,* 573-584. Copyright (2011) Elsevier Ltd.

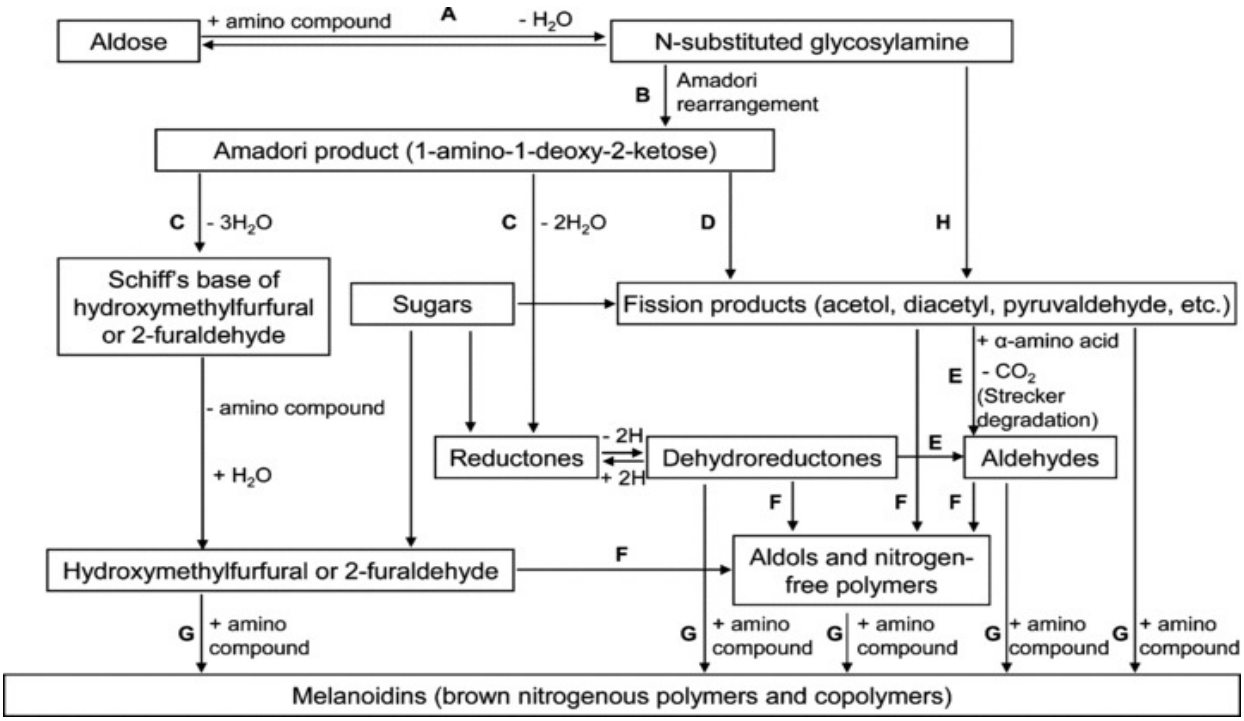


Figure 2. 3. Hodge diagram, a summary of the pathways of the Maillard reaction. Hodge summarized the reaction into 3 main stages that comprised of the initial stage (Reactions A & B), intermediate stage (Reactions C, D & E) and final stage (Reactions F & G). Free radical mediated degradation of the Maillard intermediates (Reaction H) was proposed by Namiki and Hayashi (1983). Figure is reprinted with permission from Zhang, Q., Ames, J.M., Smith, R.D., Baynes, J.W., and Metz, T. A perspective on the Maillard reaction and the analysis of protein glycation by mass spectrometry: probing the pathogenesis of chronic disease. *J. Proteome Res.* **2009**, *8*, 754-769.

2.1.3. Industrial production of caramel and its applications

2.1.3.1. Introduction

Caramel colours are water soluble, viscous liquid or hygroscopic powder with varying hue, from light yellows to red-browns to black, added to impart stable colours for use in foods and beverages (Vollmuth, 2017). Caramel colours have an odour of burnt sugar and a pleasant,

somewhat bitter taste (Sengar and Sharma, 2014). It has wide application in food and beverage industries. In contrast to its chemistry, the application of caramel is relatively uncomplicated. Therefore, ready-made caramel and caramel candies produced by Cargill and Kraft are very popular in North America. It is mostly used in carbonated beverages, pharmaceutical flavouring extract, candies, distilled liquor, bakery products and soups (Chappel & Howell, 1992; Licht et al., 1992). Although caramel colour has pivotal role in the beverage industry, it is also popular in meat products, baked goods, and texturized vegetable proteins (Shoberi, 2010). Apart from its use as a colour additive, it also imparts important functional activities in foods and beverages. For instance, it has emulsifying properties, facilitating flavour retention by stabilizing colloidal systems and preventing flocculation and haze formation (Delgado-Vargas and Paredes-López, 2002). Caramel colour also retards sunlight induced flavour changes that occur in some beverages (Chappel and Howell, 1992).

2.1.3.2. Types of caramel

Caramel colourants are very popular food additives, such as E150, due to its appealing colour and the good taste associated with it. This is the oldest colourant known to be added to foods and beverages. Nowadays, on a weight basis it is the most used food colourant in the world (Sengar and Sharma 2012; Goscinny et al. 2014). These caramel colours can be classified into 4 different types (Table 2.1) based on the reactants used for manufacturing and the application (JECFA, 1992; Codex, 1996).

a. Caramel Colour I (Plain caramel, Caustic caramel, spirit caramel): This caramel is prepared by heating the carbohydrates with or without acid or alkali. Ammonium or sulfite compounds are not used for preparing this caramel.

b. Caramel Colour II (Caustic sulfite caramel): This class of caramel is prepared by heat treatment of carbohydrates with or without acid or alkali in presence of sulfite-containing compounds. No ammonium compounds are used during the process.

c. Caramel Colour III (Ammonia caramel, bakers' caramel, confectioners' caramel or beer caramel): It is prepared by the heat treatment of carbohydrate with or without acid or alkali in presence of ammonium compounds but in absence of sulfite compounds.

d. Caramel Colour IV (Sulfite-ammonia caramel, soft drink caramel or acid-proof caramel): It is prepared by heat treatment of carbohydrates with or without acid or alkali in presence of both ammonium and sulfite compounds.

Hydroxides, carbonates, bicarbonates, phosphates, sulfates, sulfites, and bisulfites are the commonly used ammonium compounds, while the sulfite compounds are sulfurous acid, sulfites and bisulfites of potassium, sodium, and ammonium (Kamuf et al., 2003). Class II, III and IV caramels are the most widely used caramel colours in food and beverages (Nursten, 2007).

Table 2. 1. Codex classification of caramel. Adapted from Codex, 1996.

Parameters	Class I-E150 a	Class II-E150 b	Class III-E150 c	Class IV-E150 d
Colour 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

Class III and IV caramels have higher colour intensity (i.e. darkness) compared to Class I and II caramels due to the presence of ammonium compound. Ammonia caramel is formed in a Maillard-

type reaction where carbonyl compounds react with amino groups or ammonia. Sulfite caramel is also a Maillard-type polymer (Sengar and Sharma, 2014).

Besides the classification based on the method of production, the caramels can also be categorised according to their isoelectric points (pI) being positive (pI 5.0–7.0), negative (pI 4.0–6.0), and spirit (pI < 3.0) (Sengar and Sharma, 2014). At pHs above the pI the caramel is negatively charged, and below that pH is it positively charged. The information about the ionic charge of the particular caramel is very important when selecting for application in the specific formulation to avoid colour precipitation or flocculation. For instance, Classes I, II or IV are neutral or negatively charged and are "compatible", therefore their blending does not cause ionic haze formation or precipitation (Myers and Howell, 1992).

2.1.3.3. Production of caramel

Caramel colour is produced by a controlled heat treatment of carbohydrates at high temperature, with or without chemical reagents. Addition of food-grade acids, alkalis or salts catalyzes the caramelization reaction. The carbohydrate raw materials used are monomers of glucose and fructose, but glucose syrup, sucrose, invert sugars, dextrose, honey and molasses are also used as starting materials. For instance, in the U.S., high dextrose corn syrup is a commonly used carbohydrate for production of caramel colour (Vollmuth, 2017). Glucose and fructose caramelize readily, however cane sugar, molasses, lactose, starch hydrolysates, malt, lactose, and honey have also been used (Kamuf, 2003).

Commercially, caramel is manufactured by water, sugar, corn syrup, dairy solids, fat and emulsifier. Caramels can be produced with or without catalyst (Mendenhall and Hartel, 2016). The substrate is heated at the temperature 190-250°C without catalyst or 130°C in presence of a catalyst. Ammonia is the mostly used catalyst for caramelization as it can caramelize sugar at low

temperatures and shorter times. Commercially, the caramel is manufactured using an open or pressure kettle in stainless steel tank. The gauge pressure for manufacture under pressure kettle is 483 kPa (70 psi, 5 atm) at temperature up to 160°C. Various ingredients are added during reaction to provide the desired consistency and taste in caramels. For instance, milk is added to produce soft caramel, corn or maple syrup is added for sweetness and fat is added to enhance the taste (Tomasik, 2016). Milk also provides protein, whey protein in particular, which is essential to provide flavour via the Maillard reaction (Mohos, 2010). For instance, a recent study from Mendenhall and Hartel (2016) reported the increase in cooking rate and shorter holding time at 88°C with an increase in protein content. Therefore, increasing the protein content is favourable to produce caramels at low temperatures and hence lighter colour.

2.1.3.4. Flavour/ aromatic compounds associated with caramels

Depending upon the type of ingredients and method used to produce caramel, there are unlimited caramel flavour profiles. The Maillard reaction generates the characteristic caramel flavour and the vocabulary of a typical lexicon of a caramel flavour is shown in Table 2.2.

Table 2. 2. Lexicon of vocabulary used to describe the typical components of a caramel flavour.

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

(The information is adapted from Heath, H. B. Source Book of Flavours, 1981)

Caramel constitutes non-volatile fractions as well as volatile fractions. Non-volatile fraction represents 90-95% mass and constitute high molecular weight compounds (i.e. fructose dianhydrides, oligosaccharides and melanoidins) whereas volatile fraction (5-10%) is composed of low molecular weight compounds created by the degradation of sugars, including maltol, cyclotene, furaneol and furfural (Tomasik et al., 1989). The odorant characteristic of caramel is linked to the volatile fraction; however, not all volatile compounds contribute to odour generation (Taylor, 1998). Paravisini et al. (2012) identified 49 aroma compounds with some being reported in caramel for the first time. The sensory descriptors of caramels ranged from sweet-like (cooked-syrup, fruity, honey) to burnt sugar (strong, pungent, roasted). Heterocycles, carboxylic acids and carboxylic compounds are the major contributors of odorant properties. The aromatic molecules in the caramel do not necessarily smell individually, but their combination creates the typical aroma of caramel, with a balance of caramel, fruity, nutty, vegetal, animal, toasted, floral and spicy notes (Paravisini, 2013).

2.1.3.5. Colours of caramel

One of the primary characteristics of the caramel is its colour strength, so-called tinctorial power. According to international standards, tinctorial power is defined as absorbance of 0.1% (w/v) caramel colour solution in a 1-cm cell at 610 nm (Sengar & Sharma, 2014). For the purpose of colour standardization, the intensity of a caramel colouring is compared to the colour of standardized references using a spectrophotometer. Hue index (HI), which is estimated from absorbance values at 510 and 610 nm, is another method used to determine caramel colour (Clydesdale, 1993). A higher HI indicates more yellowness and lower indicates more brown colour (Linner, 1970). Different classes of caramel have different HI as shown in Table 2.3.

Before a caramel colour is offered for use, ensuring its colour consistency is crucial to match its appearance and flavour to the particular foodstuffs. For instance, Class IV caramel is mostly associated with cola-flavoured drinks (EFSA 2011), while Class III richer in flavour compounds is used to facilitate chocolate- or coffee-like flavour. The major applications and typical uses of caramel colours are presented in Table 2.3.

Table 2. 3. Hue Index of different classes of caramel colour. Table is adapted with modifications with permission from Sepe, H. A., Parker, O. D., Nixon, A. R., & Kamuf, W. E. 2008. Global colour quality of beverages utilizing caramel colour. 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
Colour	Pale to bright yellow	Amber to dark brown	Golden yellow to reddish brown	Reddish brown to dark brown
Major applications	Flavouring	Colouring	Flavouring or colouring	Ready-to- eat candies, fillings, toppings, etc.
Typical uses	Alcoholic spirits, coffee, meat, breads, spice blends, desserts	Liqueurs (i.e. vermouths and brandies), aromatic extracts	Beers, vinegars, biscuits, sauces (i.e. gravies)	Soft drinks and confectionary

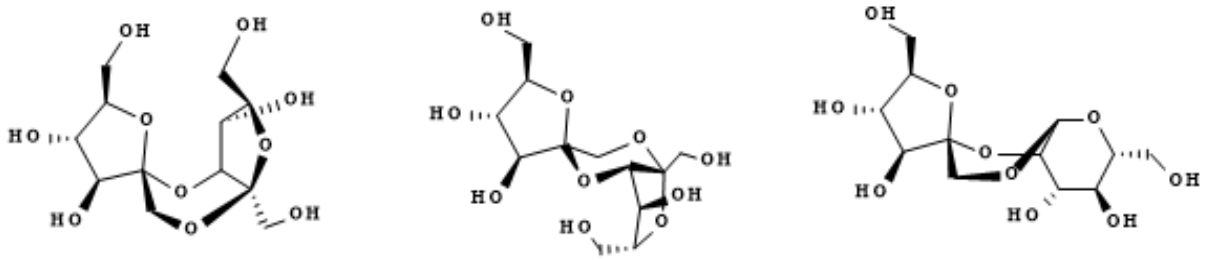
For the stability of caramel colour, it is recommended to store caramel inside closed container under conditions <22°C and <60% relative humidity, since storage time and temperature can cause dramatic effects. The concentrated form of caramel will gain colour with time, while the diluted caramel or the caramel used in finished product is susceptible to fade by UV light (Sengar & Sharma, 2014). Under ambient storage conditions, the shelf-life of caramel is normally one or two years depending on the class of caramel (Kamuf et al., 2003).

The applications of caramels depend upon the class of caramel. As shown in Table 2.3. Class I caramel is used in alcoholic spirits, coffee extracts, meat, breads, etc. Class II caramel finds

its use in different liqueurs. Beers, vinegars, sauces and biscuits use Class III caramel, while Class IV caramel is used in soft drinks and confectionary (Myers & Howell, 1992; Kamuf et al., 2003).

2.1.3.6. Bioactivity of caramels

Caramel production involves formation of multitude of compounds which results not only in colour, flavour and aroma generation, but also compounds that possess bioactivity. One of the recognized examples of such compounds is di-D-fructose dianhydrides (DFAs), a non-volatile cyclic fructodisaccharide which have prebiotic activities shown in animal trials (Orban et al., 1997). Some typical chemical structures of DFAs found in fructose and fructose-glucose derived caramels are shown in Figure 2.4. A prebiotic is an oligosaccharide, which, being indigestible by humans, passes into the colon, where it is fermented by certain beneficial species of the colonic microflora (Chrisitian and Manley-Harris, 2000). DFAs occur naturally in heat-dried fruits (Defaye and Garcia Fernandez, 2000), roasted chicory (Defaye and Garcia Fernandez, 1995), traditional tequila (Waleckx et al., 2008) and natural and sugar-roasted torrefacto coffee (Oosterveld et al., 2003; Montilla et al., 2006).



α–D-Fruf-1,2':2,3'-β-D-Fruf

1

β–D-Fruf-1,2':2,3'-β-D-Fruf

2

α–D-Fruf-1,2':2,3'-α-D-Glcp

3

Figure 2. 4. Chemical structures of selected di-d-fructose dianhydrides (1 and 2) and the D-fructose-D-glucose mixed dianhydride (3) present in caramel. The figure is adapted with a permission from Arribas, Suárez-Pereira, Ortiz Mellet, García Fernández, Buttersack, Rodríguez-Cabezas, Garrido-Mesa, Bailon, Guerra-Hernández, Zarzuelo, Gálvez. Di-D-fructose dianhydride-enriched caramels: effect on colon microbiota, inflammation, and tissue damage in

trinitrobenzenesulfonic acid-induced colitic rats. *J. Agric. Food Chem*. **2010**, *58*, 6476-6484. Copyright (2010) American Chemical Society.

Previous studies have shown the efficacy of glycosylated-DFAs derived from palatinose to promote the growth of *Bifidobacterium bifidum* in an *in vitro* model system (Kashimura et al., 1990). The media containing aromatic sucrose caramel was shown to promote the growth of certain bacterial strains like *Lactobacilli* and *Bifidobacteria* (Peinado et al., 2013). The studies in chicken have shown the stimulation in growth of *Bifidobacteria* in the caecum by the presence of DFAs in sucrose caramel supplementation (Orban et al., 1997). Furthermore, it is also found that the ingestion of fructose caramel with a large content of DFAs reduces *Enterobacteriaceae* and *Escherichia Shigella* and increases the number of prebiotic bacteria such as *Eusobacterium rectale* and *Clostridium coccoides*. The use of DFA as prebiotic has also been proposed for animal nutrition used as an alternative to antibiotics, especially in chickens (Peinado et al., 2013).

Some studies have reported the improvement of mineral absorption by DFAs. DFAs play beneficial role in the intestinal absorption of iron and magnesium in rat models (Afsana et al., 2003; Hara et al., 2010). They were found to prevent the inhibitory effect of tannins during iron uptake in rats (Hara et al., 2010). Kitts et al. (2006) examined the mutagenic activity of caramelized sucrose samples with *Salmonella typhimurium* strains TA-98 and TA-100, respectively. Caramelized sucrose expressed mutagenicity against *Salmonella typhimurium* strain TA-100, but not against strain TA-98.

Usually during caramelization, DFAs competes with unspecific intramolecular dehydration and condensation reactions of the starting sugar, which results in the generation of furanic derivatives, including 2- and 5-hydroxymethylfurfural, and also oligomeric colour compounds, melanoidins (Rasrendra et al., 2012; Audemar et al., 2017). Therefore, new processes have been proposed to enhance the proportion of DFAs in caramel. Among those is caramelization

of fructose at relatively low temperature (80−100°C) catalyzed by ion-exchange resin (García-Moreno et al., 2008; Idri et al., 2013).

Melanoidins, a high molecular weight, brown-coloured polymeric compounds generated at the final stage of caramelization and the Maillard reaction were also shown to have bioactive properties. These compounds are inevitable during thermal treatment of foods and provide colour and specific appearance in processed foods. Studies have shown its potential as antioxidant, as well as antimicrobial and prebiotic agents, making it a suitable candidate as a functional food ingredient. Coffee and bakery products are the major sources of melanoidins in western diets (Mesías and Delgado-Andrade., 2017). Melanoidin is anionic which enables to trap some positively charged electrophilic species (such as Fe, Zn and Cu), scavenge oxygen radicals and form inactive complexes via metal chelation (Echavarría et al., 2012; Delgado-Andrade et al., 2005; Martin et al., 2009). These properties make these compounds antioxidants, and have been demonstrated in foods like coffee, honey, beer, soy sauce etc. (Delgado-Andrade et al., 2005; Brudzynski and Miotto, 2011; Rivero et al., 2005; Wang et al., 2007). The metal chelating ability of melanoidins plays a crucial role in performing antimicrobial activity; the melanoidins trap cations which then hinder the growth and survival of pathogenic bacteria (Helou et al., 2015). Recent studies also demonstrated the prebiotic activity of melanoidins because they are used as carbon and nitrogen source by the hindgut microflora, promoting the growth of *Bifidobacteria*. This prebiotic activity has been reported in coffee and bread melanoidin (Jiménez-Zamora et al., 2015; Borrelli and Fogliano, 2005). Ammonia caramel colours are likely to contain greater concentrations of melanoidins (Nursten, 2007; Sengar and Sharma, 2014).

2.1.3.7. Toxic compounds associated with caramels

Caramel production leads to the formation of not only flavour compounds, but also some neo-formed toxic compounds possessing potential hazards to human health.

4-MEI, HMF and THI, shown in Figure 2.5 are among the most studied heterocyclic compounds in caramels due to their potential toxicity. 4-MEI and THI are the imidazoles present in caramel colour Classes III and IV (EFSA, 2011), in which MGO and NH_3 act as precursors for these compounds. Recent studies have reported the presence of THI in Class I caramel colourant as well (Elsinghorst et al., 2013; Wang et al., 2015). While these compounds belong to the same chemical group, their toxicological effects and occurrence differ (Goscinny et al. 2014). THI is an immunosuppressive compound (Houben et al. 1992; Bradbury et al. 1996; IARC, 2012; EFSA, 2011), whereas 4-MEI is identified as a carcinogen (National Toxicology Program, 2007) and classified as a group 2B compound, defined as possibly carcinogenic to humans, by the International Agency for Research on Cancer in 2011.

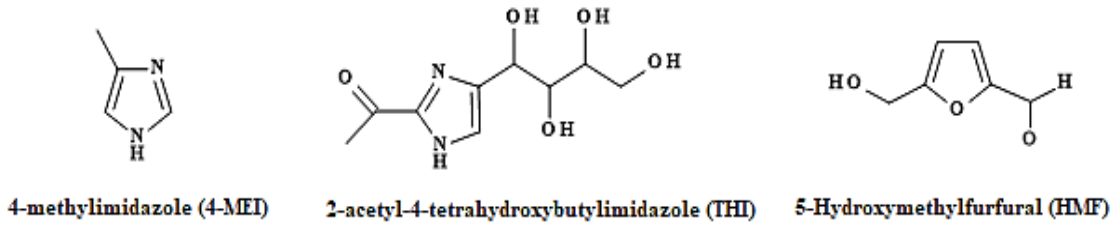


Figure 2. 5. Structures of 4-methylimidazole (4-MEI), 2-acetyl-tetrahydroxybutylimidazole (THI) and 5-hydroxymehtylfurfural (HMF).

4-MEI was identified in various caramel samples at levels ranging from 7 to 200 ppm (Mueller and Jork, 1993; Fernandes and Ferreira, 1997; Xiao and Liao, 2005). Due to the use of caramel colour, these chemical species have been found in several foods, beverages and were also identified in tobacco smoke (Moreetesta et al., 1984).

In caramel Classes III or IV, the condensation reaction between ammonium and MGO induces the formation of 4-MEI, usually at temperatures above 120°C (Karim and Smith, 2016). Formation of 4-MEI from a reaction of D-glucose and ammonia, a typical recipe for a caramel colour manufacture technology for beverages, was reported in the Maillard reaction system for the first time in the early 1960s (Komoto, 1962; Moon and Shibamoto, 2010).

Due to the potential toxicity the levels of 4-MEI and THI, they are monitored and strictly regulated with the limits shown in Table 2.5. The European Union (EU) has set a maximum residue limits for 4-MEI and THI in Class III caramel colourings, at 200 mg/kg and 10 mg/kg, respectively, and for 4-MEI in Class IV caramel colourings at 250 mg/kg. The USA sets the limits of 250 mg/kg for 4-MEI and THI at 250 mg/kg. According to California authorities, the "proposition 65" law the concentration of 4-MEI in caramel must be less than 12 mg/kg. The Office of Environmental Health Hazard Assessment (OEHHA) in California proposed no significant risk level (NSRL) at 29 μg of 4-MEI per person per day.

Table 2. 4. The concentration limit of 4-MEI and THI set in different countries. (The Table is adapted 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 (JECFA)
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 is Caramel Class III	10 mg/kg	ND	250 mg/kg

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

HMF is another neo-formed compound during the Maillard reaction as well as caramelization (Murkovic and Pichler, 2006). It is detected at temperature as low as 50°C and known as indicator of heat treatment in variety of processed foods. It has been detected in many types of foods including dried fruits, coffee, honey, breads, beverages, vinegar, treated milks,

roasted nuts and in all four classes of caramel (Capuano and Fogliano, 2011; Goscinny et al., 2014). Its concentration is reported to increase with increase in temperature or prolongation of storage period (Kędzierska-Matysek, 2016). Apart from temperature, its formation depends on pH, water activity and type of sugar involved in the reaction (Gökmen et al., 2007; Gökmen et al., 2008; Lee and Nagy, 1990). Although the correlation of adverse health effects and exposure to HMF is not conclusive (Janzowski et al. 2000; Abraham et al. 2011), no regulatory limits have been set, except for honey. The detection of HMF in foods is important for potential risk assessment of human exposure and for quality assessment of certain foods (Wang and Schnute, 2012). For instance, genotoxicity of HMF has been demonstrated *in vitro* under certain conditions, however it was not genotoxic when tested *in vivo*. In the earlier studies by Schoental et al. (1971) on the carcinogenic activity of HMF using a rodent model system, the development of lipomatous tumours in rat kidney by subcutaneous administration of 200 mg/kg body weight of HMF was reported. The National Toxicology Program (NTP) studies showed some evidence of carcinogenic activity in female B6C3F1 mice based on elevated incidences of liver tumours, whereas no carcinogenicity was found in rats (NTP, 2010; EFSA, 2011; Abraham et al., 2011). In their review of caramel colours in 2011 EFSA did not implement an exposure assessment for HMF in caramel colour or address the risk to humans (Vollmuth, 2017).

2.2. Production of GlcN and its bioactivity

2.2.1. Production of GlcN from exoskeleton of shrimps

Glucosamine (chitosamine) is an amino sugar that occurs in acetylated and polymerized forms in chitin, an β-(1–4)-poly-*N*-acetyl-D-glucosamine (Figure 2.6) (Mojarrad et al. 2007). Chitin is the second most abundant biopolymer after cellulose. It is found in the exoskeletons of

crustaceans (especially crab, shrimp and lobster), insects, arthropods and cell wall of fungi (Zaeni, 2017).

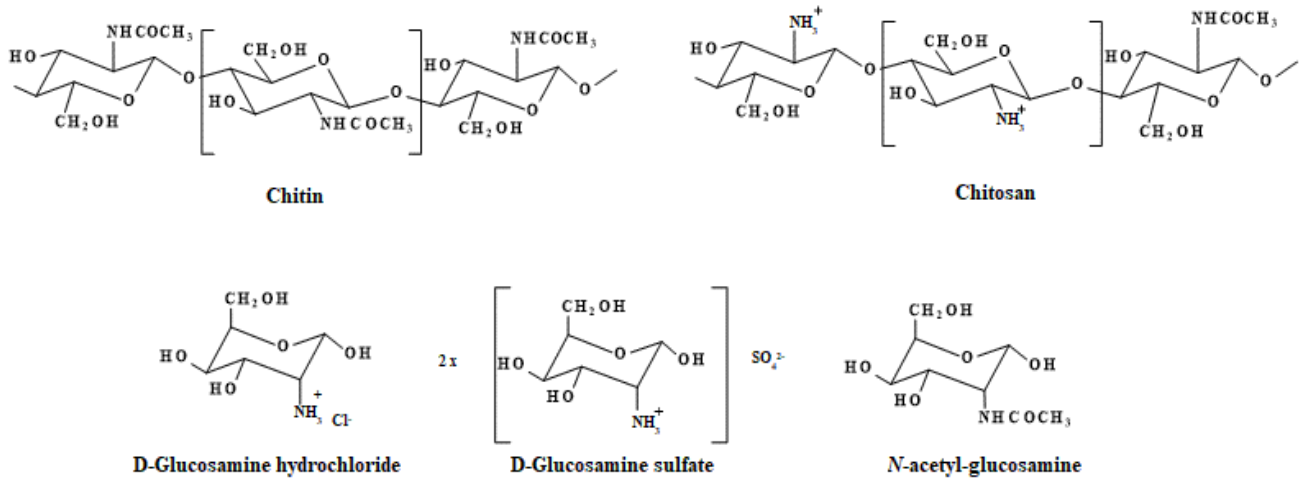


Figure 2. 6. Chemical structures of chitin, chitosan, glucosamine hydrochloride (GlcN·HCl), glucosamine sulfate (GlcN·SO4) and *N*-acetyl-glucosamine The figure is reproduced with permission from Mojarrad, J.S., Nemati, M., Valizadeh, H., Ansarin, M. and Bourbour, S. Preparation of glucosamine from exoskeleton of shrimp and predicting production yield by response surface methodology. *J. Agric. Food Chem*. **2007**, *55*, 2246-2250. Copyright (2007) American Chemical Society.

Exoskeletons (arthropod shells) are also a good source of chitin and contain 20-50% of chitin on dry weight basis. Chitin is insoluble due to intermolecular hydrogen bonding, whereas chitosan, a fully or partly *N*-deacetylated chitin, is more soluble in acidic conditions due to the protonation of amine groups (Pillai et al., 2009). The global production of crustacean shells is 6-8 million tonnes every year and are easily accessible from wastes of seafood processing industries (Yan and Chen, 2015). These waste shells are a low cost alternative to obtain chitin and chitosan (Benavente, 2015). The production of GlcN from exoskeleton of crustaceans has gained in popularity, different forms of GlcN extracted from chitin are available in the market, including GlcN·SO_4, GlcN·HCl, or *N*-acetyl-GlcN (Figure 2.6).

The typical process flow of production of GlcN from crustacean shells is shown in Figure 2.7 and involves following steps: deproteinization, demineralization, chemical hydrolysis of chitin,

crystallization and drying (Benavente, 2015). The crustacean shells require pre-treatments such as deproteinization, demineralization, depigmentation and grinding, because they contain 30-40% proteins, 30-50% calcium carbonate and lipid soluble pigments. The lipid soluble pigments comprise of astathin, lutein, β-carotene and astaxanthin (Aranaz et al., 2009). Conventionally, hydrochloric acid of given concentration is added for the acid hydrolysis of chitin followed by heating in an electric furnace or hot plate at temperature 80-100°C. Zaeni et al. (2017) reported the microwave assisted hydrolysis of shrimp shell waste for GlcN·HCl production and stated the significant difference in hydrolysis time using microwave exposure (5-20 min) as compared to conventional method (90 min).

The acid hydrolysis is followed by filtration to separate glucosamine hydrochloride from solid particles. The filtrate thus obtained is crystallized at room temperature for 25 days and ethyl alcohol is added to increase the crystallization rate. Finally, the resulting mixture is dried at 50°C in an oven for 12 h (Benavente, 2015). The major drawback of production of acid hydrolysis of chitin is high cost, low yield (below 65%) and generation of acidic waste (Sashiwa et al., 2001). Shellfish's seasonal dependence, as well as its unpredictable size and composition are also limiting factors to use this material for GlcN extraction (Rane and Hoover, 1993). In addition, GlcN produced from shellfish is not a suitable option for people with shellfish allergy (Deng et al., 2005). Taking this under consideration, companies like Cargill and Cyanotech have been producing non-shellfish based GlcN health supplements. Cargill's GlcN products are extracted from *Aspergillus niger* whereas Cyanotech's products are marketed as 100% vegetarian source GlcN products. These extraction methods will be discussed in the following paragraphs.

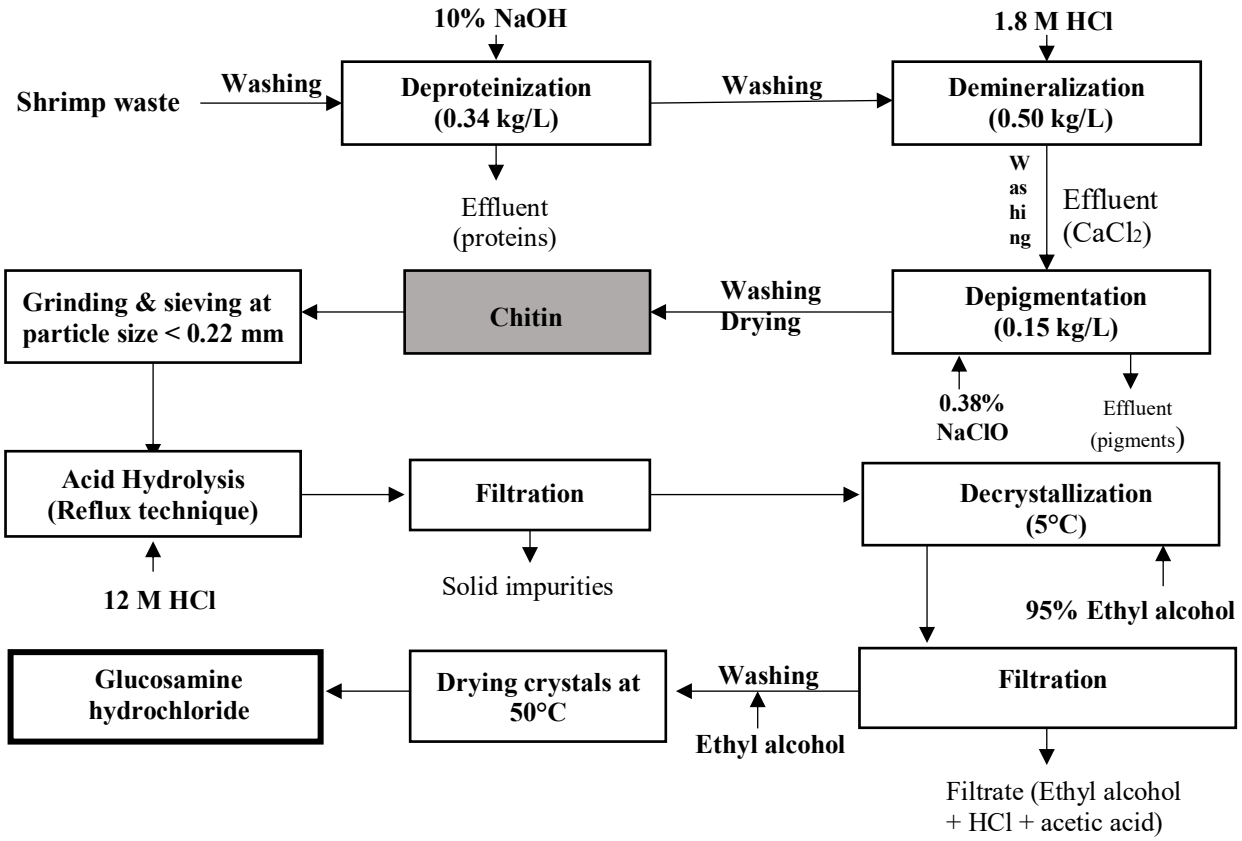


Figure 2. 7. Block diagram showing the extraction of chitin from shrimp shells and production of glucosamine hydrochloride. The figure is reproduced with permission from Benavente, M., Arias, S., Moreno, L., & Martínez, J. Production of glucosamine hydrochloride from crustacean shell. *J. Pharm. Pharmacol*. **2015**, *3*, 20-26.

2.2.2. Microbial production of GlcN

Due to the economical, environmental, and physiological drawbacks of using crustacean waste as a source of GlcN, the alternative source of GlcN production, from microorganisms are emerging (Sitanggang et al., 2012). In this section of the research background the production of GlcN using microorganisms is discussed with more detail.

2.2.2.1. Bacterial production of GlcN

Genetically engineered *E. coli* has been mainly developed to maximize the production of GlcN and GlcNAc. Deng et al. (2005) reported production of recombinant *E. coli* to overexpress GlcN-6-P acetyltransferase, GlcN-6-P synthase and GlcN-1-P acetyltransferase while supressing

GlcN-6-P and GlcNAc-1-P uridyltransferase. GlcNAc is a stable alternative fermentation product that is non-inhibitory to the host. Under acidic conditions, GlcNAc can be easily hydrolyzed to GlcN. Therefore, heterologous glucosamine-6-P *N*-acetyltransferase is overexpressed to extend the GlcN pathway for generation of GlcNAc. This method represents a high quality and low-cost system for GlcN and GlcNAc production, and can yield over 110g/L GlcNAc. Recently, *Bacillus subtilis* is also metabolically engineered for improved GlcNAc production (Liu et al., 2014).

2.2.2.2. Fungal production of GlcN

GlcN is found in the cell wall of fungi as monomers of chitin and chitosan. Recently, fermentation of filamentous fungi has been widely studied for the production of GlcN. Among four subdivisions of fungi: Ascomycotina, Zygomycotina, Basidiomycotina and Deuteromycotina, only Ascomycotaina (e.g., *Aspergillus* sp.) and Zygomycotina (e.g., *Rhizopus* sp.) can be used for the production of GlcN due to the absence of chitin in the cell wall of Basidiomycotina and Deuteromycotina (Hsieh et al., 2007; Chen and Chiou, 1999; Schaechter, 2010). The fungal cell wall of Zygomycotina is predominantly comprised of chitin and chitosan; thus, extensively studied for chitosan production. The species of Zygomycotina (*Mucor rouxii*) has been explored due to a large proportion of chitosan in the cell well along with the ease of its cultivation (Bartnicki-Garcia, 1968). Chatterjee et al. (2005) reported that the production of chitosan from *Mucor rouxii* resulted in no significant difference in the concentrations of chitosan in different culture media such as molasses salt medium, potato dextrose broth, and yeast extract peptone glucose.

Rhizopus oryzae, Rhizopus pusillus, Gongronella butleri, Absidia coerulea, etc. are some other species under Zygomycotina class commonly studied for the production of chitosan (Sitanggang et al., 2012). Hsieh et al. (2007) reported that *Aspergillus* sp. BCRC31742 was the best fungi for the production of GlcN. Zhang et al. (2012) investigated the dissolved oxygen (DO)

shifting strategy to increase the productivity of GlcN. The authors reported the change in DO at 30% from 0 to 12 h to 50% at 12 to 60 h reached the greatest production of GlcN.

2.2.3. Chemical production of GlcN

GlcN can also be generated from fructose and ammonia as starting materials as shown in Figure 2.8. This method can yield up to 30% GlcN and results in the formation of several co-products such as mannosamine (Lv et al., 2017).

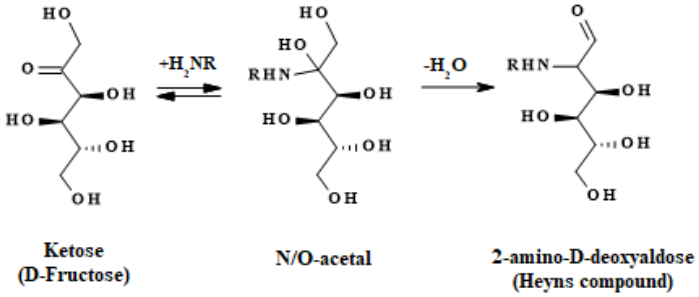


Figure 2. 8. Production of GlcN from fructose and ammonia. Reproduced with persmission from Bilova, T., Greifenhagen, U., Paudel, G., Lukasheva, E., Brauch, D., Osmolovskaya, N., Tarakhovskaya, E., Balcke, G.U., Tissier, A., Vogt, T. and Milkowski, C. 2016. Glycation of plant proteins under environmental stress-methodological approaches, potential mechanisms and biological role. In *Abiotic and Biotic Stress in Plants-Recent Advances and Future Perspectives*. Copyright (2016) InTech.

Schloss (1951) reported the variability in GlcN obtained by this method is a function of pH, incubation temperature and incubation time. For instance, the reaction between high fructose corn syrup and ammonium hydroxide at 25-50°C produces GlcN and lesser amounts of mannosamine and galactosamine (approximately 8%).

2.2.4. Glucosamine production from plants

The production of GlcN from plants has been studied recently because with plants less raw material is required to reach the active dose of GlcN (e.g. 1.5 g/day for treating osteoarthritis of the knee) (Rindone, 2000). The raw material used in this process is chicory root, carrots, Jerusalem artichoke tubers, and beets. Petiard et al. (2013) reported the production of GlcN from fresh chicory

roots treated with nitrogen-based fertilizer when subjected to heating at 70-110°C for more than 10 h to less than a week. This method generated 5 g GlcN/kg of dry matter of the plant material. Too low heating temperatures and/or too low heating times lead to a process which is not economically viable. On the contrary, too high heating temperatures and/or too high heating times can progressively degrade GlcN.

2.2.5. GlcN as a multifunctional therapeutic agent

2.2.5.1. GlcN in osteoarthiritis treatment

GlcN is non-vitamin, non-mineral over-the-counter dietary supplement which has shown to be effective in treating osteoarthritis. It is orally administered in the form of GlcN·SO_4, GlcN·HCl, GlcNAc or in combination with chondroitin sulfate. Oral administration of GlcN·SO_4 is more effective due to its greater bioavailability (90%) (Anderson et al., 2005). GlcN plays a vital role in the biosynthesis of glycosaminoglycan chains, aggrecan and proteoglycans in cartilage (Setnikar et al., 1991). Uitterlinden et al. (2006) reported the inhibition of gene expression of osteoarthritic cartilage by GlcN *in vitro*. GlcN·SO_4 is also a more potent inhibitor of gene expression compared to GlcN·HCl (Altman et al., 2006). In a study conducted with 252 patients with Stage I and III osteoarthritis of knee, those treated with 1.5 g/day GlcN·SO_4 for four weeks had a significantly greater decrease in the severity index than those administered with a placebo (Noack et al., 1994). However, it was observed that GlcN·HCl was not as effective as GlcN·SO_4 for the relief of knee osteoarthritis (Houpt et al., 1999).

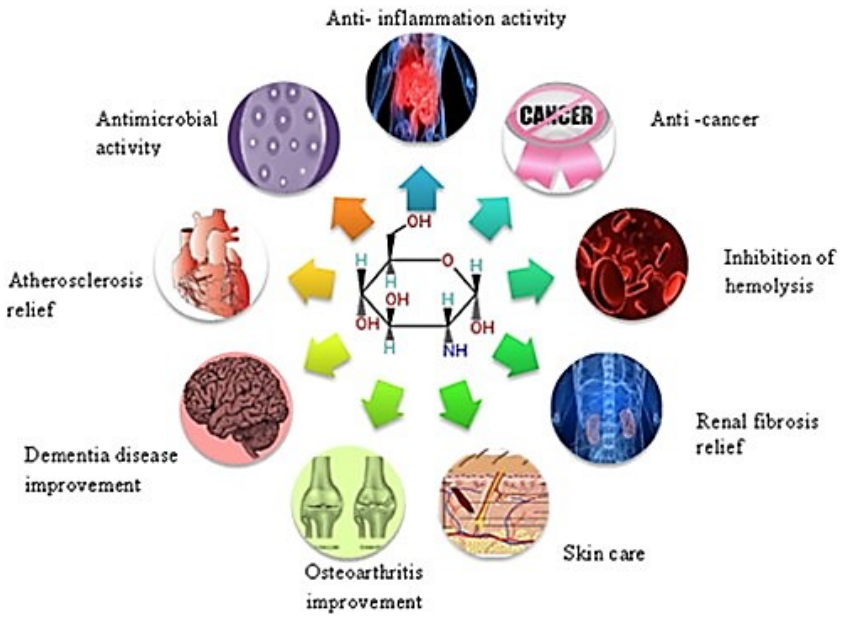


Figure 2. 9. Potential biological activities of glucosamine and its derivatives. Figure reprinted with permission from Dalirfardouei, R., Karimi, G., & Jamialahmadi, K. Molecular mechanisms and biomedical applications of glucosamine as a potential multifunctional therapeutic agent. **2016**. *Life Sci*, *152*, 21-29. Copyright (2016) Elsevier Ltd.

In a 6-week randomized, double-blind, placebo-controlled study with sixteen volunteers with knee pain each group were assigned to the dietary supplement or placebo group. The inflammation was significantly decreased in the dietary supplement treated group after 6 weeks as compared to placebo group (Katayoshi et al., 2007). A 1.5 g GlcN·SO_4 showed similar success rate as an anti-inflammatory agent as compared to 1.2 g ibuprofen. Besides, GlcN·SO_4 (6%) also showed significantly fewer side effects in patients than with ibuprofen (35%) (Rovati, 1997).

2.2.5.2. GlcN as an antioxidant

GlcN·HCl and GlcN·SO_4 have been investigated in various *in vitro* systems and shown to have antioxidant properties. GlcN·HCl has a pronounced superoxide radical scavenging activity (72–84%) at concentrations of 0.05–0.8 mg/mL. It is also reported to have hydroxyl radical scavenging activity in a deoxyribose system. The oxidative damage induced by Fe^{3+}/H_2O_2 was inhibited by 55% at the concentration of 3.2 mg/mL GlcN·HCl. These authors also reported the

reducing power of GlcN·HCl (Xing et al., 2006). GlcN·SO_4 also has superoxide and hydroxyl radical scavenging activity. For instance, the superoxide radical scavenging activity of GlcN·SO_4 was 92% at 0.8 mg/mL and hydroxyl scavenging activity was 50% at 3.2 mg/mL. Also, it has a strong reducing potential of 0.64 at 0.75 mg/mL. However, the ferrous ion-chelating activity of both compounds was weak (Xing et al., 2006).

2.2.5.3. GlcN as an anticancer agent

The anticancer activity of GlcN was first reported in 1953 and the application of GlcN in the treatment of cancer has been studied since (Quastel and Cantero, 1953). The effect of GlcN depends on the type of cancer. Studies have shown the anti-tumor activity by oral administration of *N*-acetyl-D-glucosamine and GlcN oligomer in a colon tumour in mice model. These oligomers exhibited anti-tumour action by apoptosis of tumour tissues and by increasing the level of serum interleukin-12p70 and interferon-γ to enhance immune system (Masuda et al., 2014). Furthermore, GlcN has shown anti-tumor efficacy against prostate, lung and breast cancers (Chesnokov et al., 2009; Brasky et al., 2011; Cooney, 2011). Therfore, GlcN is a multifunctional therapeutic agent as shown in Figure 2.9.

2.2.6. Glucosamine self-condensation product as a new therapeutic agent

Recently, the self-condensation products of GlcN, FR and DOFR have been studied for their therapeutic role. GlcN can undergo self-condensation to form a cyclic compound called dihydrofructosazine (Hrynets et al., 2015b). As shown in Figure 2.10 dihydrofructosazine is the precursor of both FR and DOFR. Dehydrogenation or dehydration of dihydrofructosazine generate FR or DOFR, respectively (Hrynets et al., 2015b).

Zhu et al. (2007) compared the effect of GlcN and fructosazines on lymphocyte cytokine production and noted that GlcN is substantially less effect in reducing IL-2 production than

fructosazines. Therefore, these GlcN self-condensation products possess superior anti-inflammatory activity than native GlcN.

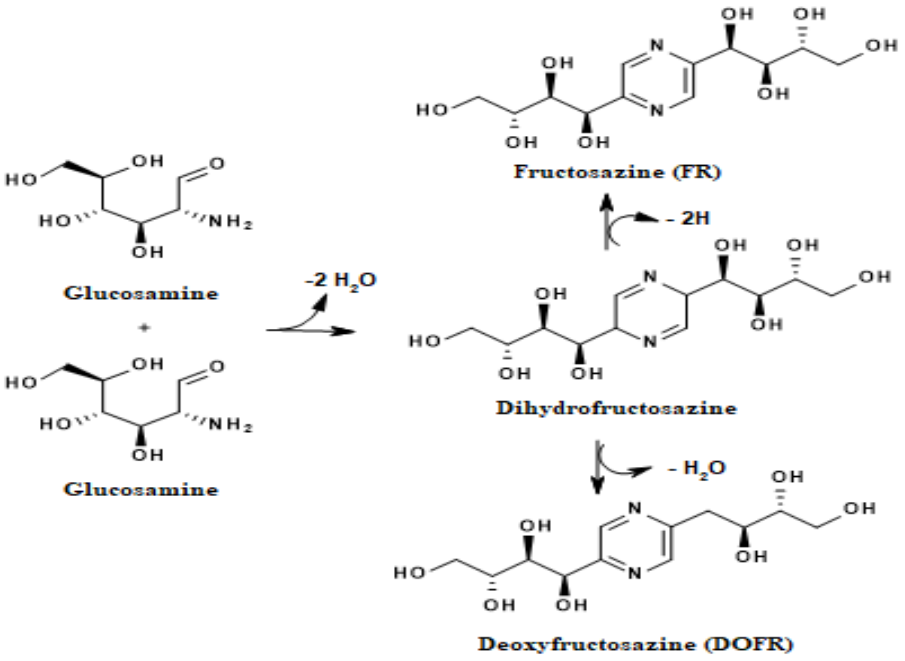


Figure 2. 10. Mechanism of glucosamine self-condensation to dihydrofructosazine, fructosazine (FR), and deoxyfructosazine (DOFR). Figure is reproduced with permission from Hrynets, Y., Bhattacherjee, A., Ndagijimana, M., Hincapie Martinez, D.J. and Betti, M. 2016. Iron (Fe^{2+})-catalyzed glucosamine browning at 50°C: identification and quantification of major flavour compounds for antibacterial activity. *J. Agric. Food Chem*. **2015**. *64*, 3266-3275. Copyright (2016) American Chemical Society.

These polyhydroxylalkyl pyrazines are also reported to act therapeutically in osteoarthiritis and rheumatoid arthiritis by inhibiting IL-1β (Giordani et al., 2006). Furthermore, FR and DOFR have efficacy as therapeutical agents against Type II diabetes (Bashiardes et al., 2002).

As the non-volatile polyhydroxyalkyl pyrazine derivatives, FR and DOFR, exhibit potential physiological and pharmacological activities, synthetic methods have been developed to produce these *N*-heterocyclic compounds. The main challenge of FR and DOFR synthesis is that

the ratio of target products cannot be selectively controlled, since the reaction is strongly dependent on the type of catalyst, pH and temperature (Jia et al, 2014; Jia et al., 2017).

Rohovec et al. (2001) reported that GlcN can undergo self-condensation to produce DOFR in presence of phenylboronic acid and sodium hydroxide as catalysts, while the condensation of two moles of GlcN in hot methanol yields FR. However, as mentioned above. The reaction yields side products such as furfurals and other volatile and non-volatile pyrazine derivatives. Jia et al. (2014) reported the production of FR and DOFR by dehydration of GlcN in presence of the environmentally friendly catalyst 1-butyl-3-methylimidazolium hydroxide at 120°C for 3 h. Unlike traditional bases such as KOH, NaOH, $NaHCO_3$, triethylamine; basic ionic liquid, 1-butyl-3-methylimidazolium hydroxide, does not undergo corrosion, waste generation and other environmental problems. The maximum yield of FR and DOFR achieved by this method was 49%. The basic ionic liquid 1-ethyl-3-methylimidazolium acetate was also shown to efficiently catalyze the conversion GlcN into FR and DOFR (Jia et al., 2015). Jia et al. (2017) were able to achieve a significantly enhanced selective yield of DOFR, up to 40.2% by using noncorrosive, and nontoxic acid boric acid as the additive; whereas FR was gradually becoming the main product, up to 25.3%, with hydrogen peroxide as the oxidant.

2.3. Recent advances in non-enzymatic browning of glucosamine

2.3.1. Alpha-dicarbonyl compounds as precursors of flavour

α-DCs generated during non-enzymatic browning reactions are the precursors of the aroma compounds. As shown in Figure 2.11, during GlcN non-enzymatic browning, glucosone (G) may be formed via an oxidative pathway of Schiff base formed and 3-deoxyglucosone (3-DG) produced from a 1,2-enolization pathway. G and 3-DG are the major α-dicarbonyl compounds formed during the non-enzymatic degradation of GlcN, while GO, MGO and DA are the short chain α-DCs

formed in smaller amounts compared to long chain α-DCs. GO may be formed by the retro-aldol cleavage of G at C2-C3. MGO is generated by the retro-aldol cleavage of 3-DG at C3-C4 (Yaylayan and Keyhani, 2000; Weenen, 1998).

GO and MGO are important intermediates formed during non-enzymatic browning of GlcN; these short chain α-DCs are important precursors of flavour and colour formation. During Strecker degradation, GO and MGO react with amino acids and lead to the formation of heterocyclic aromatic compounds such as pyrazines, pyridines and pyrroles via series of reactions such as decarboxylation and α-aminoketone condensation. GO and MGO have been detected in variety of foods including coffee, honey, yogurt, vinegar etc. In honey, the concentration of GO and MGO ranges from 0.3–1.3 mg/kg and 0.8–33 mg/kg, respectively (Marceau and Yaylayan, 2009). MGO is reported to be the source of antibacterial activity of Manuka honey (Majtan, 2011). The three types of vinegar (wine, balsamic and white balsamic) showed MGO in a similar range of 1.7-53 mg/L (Degen et al., 2012).

Diacetyl is a volatile vicinal diketone known for buttery or butter-scotch aroma in foods. It occurs naturally in caramel, butter, yogurt, wine, vinegar. On the other hand, it is added to microwave popcorn (Zaccone et al., 2015), and is used in dry or liquid form while manufacturing this snack food (Rigler and Longo, 2010). The aroma threshold for diacetyl depends on the type of food matrix and is reported in the range of 0.001 to 0.55 ppm with least threshold in cheese (Smit, 2005; Milesi, 2010).

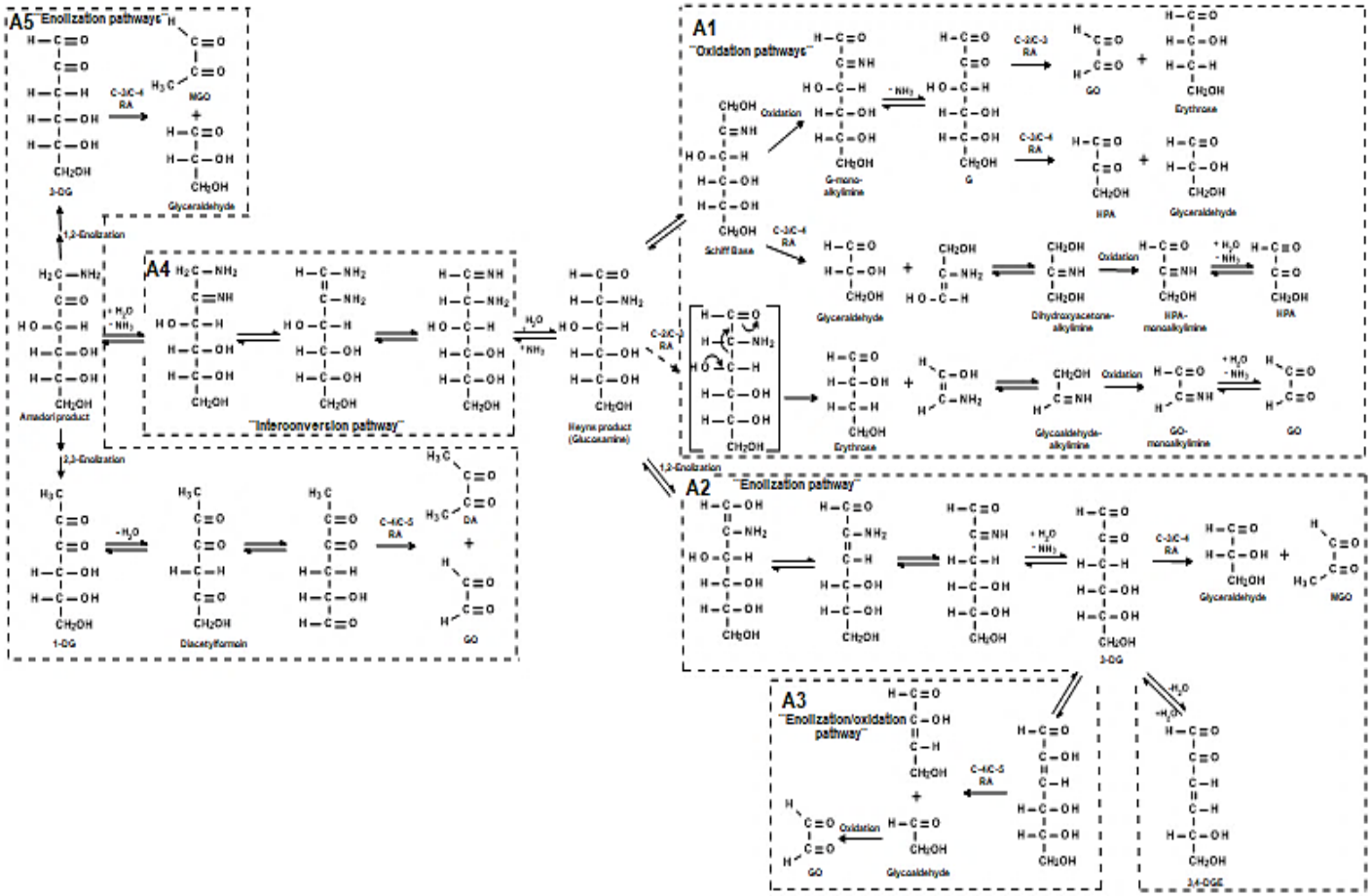


Figure 2. 11. Proposed five pathways of α-dicarbonyl formation from GlcN (a Heyns product) incubated at 37˚C: A1= oxidation, A2, A5= enolization, A3= enolization and oxidation, and A4= interconversion. G, glucosone; 3-DG, 3-deoxyglucosone; 1-DG, 1-deoxyglucosone; 3,4-DGE, 3,4-dideoxyglucosone-3-ene; GO, glyoxal; MGO, methylglyoxal; HPA, hydroxypyruvaldehyde; RA, retroaldolization. Figure reprinted with permission from Hrynets, Y., Ndagijimana, M., and Betti, M. Studies on the formation of Maillard and caramelization products from glucosamine incubated at 37˚C. *J. Agric. Food Chem*. **2015**a, *63*, 6249-6261. Copyright (2015) American Chemical Society.

2.3.2. Fructosazine and deoxyfructosazine in food applications

FR (2,5-bis-(D-*arabino*-1,2,3,4-tetrahydroxybutyl)pyrazine) and DOFR (2-(D-*arabino*-1,2,3,4-tetrahydroxybutyl)-5-(D-*erythro*-2,3,4-trihydroxybutyl)pyrazinefructosazine) are identified as flavouring agents in variety of foods. These polyhydroxylalkyl pyrazines have been detected in tobacco, caramel, roasted peanuts and soy sauce (Cheng et al., 2012; Tsuchida, 1986; Magaletta, 1996; Tsuchida et al., 1990). Recently Hrynets et al. (2016) reported their values up to about 37 g/L for FR and DOFR from 150 g/L GlcN/Fe^{+} at 50°C incubated for 48 h. The authors also detected the greater generation of FR and DOFR from GlcN/Fe^{+} as compared to GlcN alone. Previous studies have identified the roasty aroma formed by pyrolysis of GlcN as a result of major sugar degradation products, i.e. FR and DOFR (Chen and Ho, 1998).

FR and DOFR are pale yellow to yellow liquid with versatile functions. They provide the characteristic flavour in roasted peanut and soy sauce (Magaletta and Ho, 1996; Tsuchida et al., 1990). One of the issues in food and beverage industries can be the formation of a slight off-flavour. The off-flavour generation accelerates dramatically by the exposure to light below 500 nm. FR and DOFR absorbs UV light without generating any off-flavour substances (van der Ark et al., 2013). Therefore, caramel containing these polyhydroxyalkyl pyrazines can be used to develop light absorbing “colourless caramels”. Bhattacherjee et al., (2016) reported that FR possesses antimicrobial activity against extremely heat resistant *E. coli* AW 1.7 at moderate acidic pH. FR and DOFR have also shown efficacy in prevention and treatment of Type II diabetes and atherosclerosis. For instance, Aventis Pharma patented a combination of FR and DOFR as therapeutical agents against Type II diabetes (Bashiardes et al., 2002). Therefore, FR and DOFR are versatile compounds with a wide range of applications in food and pharmacology.

2.3.3. Antioxidant and antimicrobial activities of glucosamine-derived caramel

The pioneer work on GlcN browning conducted by Oyaizu in 1986 showed that GlcN powder incubated at 37°C for up to 30 d started to develop a brown colour after 12 d. Browning continued to increase up to 30 d and its intensity was proportional to the antioxidant activity. Recently, Hong et al. (2016) have demonstrated that GlcN non-enzymatic browning can generate caramel solutions with radical scavenging activity. Specifically, the study evaluated the radical scavenging activity (DPPH and ABTS) of several caramel solutions in relation to temperatures (40 and 60°C), time of incubation (0, 3, 6, 12, 24 and 48 h) and concentration (0.05–2.00 g/L). In general, higher caramel concentration produced at longer the incubation and higher temperatures, resulted in an increase of radical scavenging activity. Similarly, to the work conducted in dry condition by Oyaizu (1986), radical scavenging activity depends on the browning intensity and solution conditions.

Hrynets et al. (2016) also demonstrated that GlcN caramel solution produced at 50°C for 48 h in the presence of iron (Fe^{2+}) possessed inhibitory activity against extremely heat resistant *E. Coli* AW 1.7 at 5% (w/v). The authors tried to identify and quantify known and new possible antimicrobial compounds in GlcN-derived caramel. Among the known antimicrobial compound found were: MGO, GO, acetic acid and H_2O_2. New antimicrobial compounds identified were 3-DG and FR. However, only the level 3-DG and FR along with acidity were at the level that exceeded the concentration required for MIC activity.

In conclusion, GlcN-derived caramels have the potential to be used in food applications due to interesting compounds generated during the non-enzymatic browning process. However, more research is necessary to find appropriate processing conditions for developing caramels with heath benefits. In vivo animal models will be necessary to complete this task.

CHAPTER 3. Sous-vide non-enzymatic browning of glucosamine at different temperatures

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3.1. Introduction

Glucosamine (GlcN, 2-amino-2-deoxy-D-glucose) is a monosaccharide obtained by the tandem hydrolysis–deacetylation of chitin, an abundant biopolymer found in the exoskeletons of crustaceans, insects and the cell walls of fungi. GlcN also can be classified as a Heyns product resulting from the reaction between fructose and ammonia or amino acid, however in much lower yields as compared to the thermochemical degradation of chitin. For example, by using optimized extraction conditions a 96−98% yield of glucosamine hydrochloride can be achieved from marine shrimps' chitin within 4 h (Mojarrad et al, 2007).

In previous studies (Hrynets et al., 2015a; Hrynets et al., 2016), GlcN is shown to be an unstable amino sugar which rapidly degrades generating a plethora of compounds, including α-DCs. Among the major α-DCs, 3-DG, G, MGO, GO and DA can be generated from GlcN at as low as 25°C. Increasing the temperature to 37 or 50°C can speed up the degradation process; one kilogram of GlcN can generate up to 5.7 gram of α-DCs by heating at 37°C for 12 days. Besides being pivotal precursors of colour, flavour and aroma in foods, some of these α-DCs have some other important activities, such as "reductone" glucosone (Kanzler, Haase & Kroh, 2014) or antibacterial MGO, GO (Mavric et al., 2008), DA (Jay, 1984) and 3-DG (Hrynets et al., 2016). From the health point of view, endogenously formed α-DCs (ie.MGO, GO and 3-DG) resulting from glucose-derived modification of proteins have been shown to induce diabetes (Vlassara & Bucala, 1996). The mechanism to generate these α-DCs from GlcN has been proposed (Hrynets et al., 2015a). Glucosone, for instance, may be generated via an oxidative mechanism, while 3-DG

is formed through the 1,2-enolization of GlcN. Isomerization of GlcN to Amadori compounds through an interconversion reaction provides another several mechanisms of some α-DCs formation. Along with α-DCs, GlcN generates non-volatile polyhydroxyalkyl pyrazines, namely fructosazine (FR) (2,5-bis(D-*arabino*-tetrahydroxybutyl)pyrazine) and deoxyfructosazine (DOFR) (2-(D-*arabino*-tetrahydroxybutyl)-5-(D-*erythro*-2,3,4-trihydroxybutyl) pyrazine); one kilogram of GlcN can generate up to 370 g of these pyrazines when heated at 50°C for 2 days. These pyrazines have been recognized as flavouring agents and have been identified in roasted peanuts, caramel and soy sauce (Henry et al., 2012). Fructosazine also possesses some antimicrobial activity against heat-resistant *E. coli* AW 1.7 in moderate acidic conditions (Bhattacherjee et al., 2016). These molecules are formed as a result of the symmetric cyclocondensation of two GlcN molecules which follow dehydration form dihydrofructosazine [2,5-bis(D-*arabino*-tetrahydroxybutyl)dihydropyrazine)]. The latter oxidizes to form fructosazine or dehydrates to generate a deoxyfructosazine (Hrynets et al., 2015a). In addition to their application as food ingredients, these molecules are gaining interest for their uses in human therapeutics such as in the treatment of type II diabetes, the prevention of atherosclerosis, and in the prevention of the pathological cartilage degradation and other inflammatory diseases (Giordani et al., 2006; Zhu et al., 2007). Hence, GlcN can not only develop flavourful caramel solutions due to diacetyl and pyrazines production, but also has the potential to become a functional food/ingredient due to the bioactivity of FR and DOFR.

Since GlcN produces flavour, antimicrobial, antioxidant and bioactive health compounds (Zhu et al., 2007) in addition to the fact that it can be purified by the uncomplicated deacetylation of chitin, gives us opportunities to research its various applications and to carefully study the non-enzymatic browning mechanism of this compound under different conditions. Depending on the

reactants used in the manufacturing process, the industrial caramel colour is classified into four classes. Among these, Caramel Colours III and IV are produced by heating reducing sugars in the presence of ammonium compounds (III) and, additionally, sulfite (IV) (Elsinghorst et al., 2013). During caramelization these ammonium compounds serve as a source of nitrogen for a series of undesired neo-formed food contaminants, including a group of toxic imidazoles, such as 4-MEI and THI. HMF, is another characteristic heterocyclic product of non-enzymatic browning and is a ubiquitous food contaminant. The formation of HMF from sugar dehydration or by caramel colour addition is a potential issue. GlcN can be easily deaminated (Hrynets et al., 2015a), and released ammonia can react with the α-DCs produced during enolization and retroaldolization reactions, which in turn can possibly generate 4-MEI and THI. Our intention, so far, has been the production of GlcN caramel solution using moderate temperatures (50-70°C) so that the production of these toxicants can be minimized.

Sous-vide, is the increasingly popular method of cooking using vacuumized pouches at mild temperatures and long time in a circulated water bath (Chiavaro et al., 2012). Oxygen-free atmosphere is intended to prevent the oxidation processes and thus help preserving not only the nutritional quality of food but also to improve its sensory qualities in terms of aroma, flavour, and texture (Chiavaro et al., 2012; Creed, 1995). An oxygen-free environment would also reduce the reaction between triplet oxygen ($^3O_2$) and other excited molecules in the triplet state thus reducing the browning (Kanner & Shapira, 1989). Up to now there is no study that has examined the non-enzymatic reaction of Heyns compound in a vacuum. Therefore, the objective of this research was to study the chemistry of non-enzymatic browning of GlcN under vacuum condition (sous-vide technology) at different temperatures (50, 60 and 70°C) and evaluate the physico-chemical properties, and the generation of α-DCs, hydroxylalkylpyrazines and alkylimidazoles. From a

practical point of view, it is of interest to understand if mild temperatures under vacuum conditions increases or decreases the production of certain aromatic molecules (i.e. the butter- and caramel-like diacetyl odorant), while minimizing the production of the undesirable heterocyclic compounds (i.e. 4-MEI, THI and HMF).

3.2. Materials and methods

3.2.1. Chemicals

D-glucosamine hydrochloride (GlcN, ≥99%), HPLC grade solvents (methanol, formic acid), *o*-phenylenediamine (OPD; 99.5%), glucosone (G, 2-keto-D-glucose; ≥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-erythrohexosulose; ≥ 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). Poly(vinylidene 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).

3.2.2. Experimental design

A 3 × 2 factorial design was planned to study the influence of the temperature (50, 60, 70°C), "level of oxygen" (vacuum vs. non-vacuum) during 12 h incubation on physico-chemical

properties, generation of α-DCs, and heterocyclic compounds (non-volatile polyhydroxylalkyl pyrazines, HMF, THI and 4-MEI) production during GlcN non-enzymatic browning. For each treatment, 3 vacuum bags containing GlcN solutions were incubated in the water bath circulator at the three different temperatures. Three independent trials were conducted at three different days, resulting in a total number of observation of 54 (9 observations per treatment; n = 9).

3.2.3. Preparation of GlcN solutions

GlcN caramels were obtained by heating aqueous solutions of GlcN (150 g/L) for 12 h. Prior to incubation the pH of the solutions was adjusted to 7.0 ± 0.01 with 1 M NaOH. Ten mililiters of the solutions were transferred to the vacuum sealing pouches with oxygen barrier (FoodSaver, Brampton, ON, Canada) and heat-sealed (FoodSaver Vacuum Sealer V4420, Brampton, ON, Canada) to make vacuum condition, whereas GlcN solutions were sealed without vacuum in vacuum sealing plastic bags to make non-vacuum samples. The bags were completely submerged and randomly placed in the water bath circulator (Haake SC100, Thermo Scientific, Waltham, MA, USA) and incubated at 50, 60 and 70°C. The pH was not adjusted during incubation. After retrieval, the bags were cooled on ice and transferred to screw cap tubes. An aliquot of each of the GlcN caramel solutions were immediately tested for respective analyses.

3.2.4. Colour measurements and pH

The colour of GlcN caramel solutions was determined using a tristimulus colorimeter (Minolta CR-400, Konica Minolta Sensing Americas, Inc., Ramsey, NJ) according to Hong & Betti (2016). The instrument was calibrated before each series of measurements using a white tile plate (L^* = 32.80; a^* = 14.51; b^* = 15.19). Chromaticity results are expressed in L^*, a^* and b^* coordinates. Chroma (C^*) and hue angle ($H°$) were calculated using the following formulas, $C^* = (a^{*2} + b^{*2})^{1/2}$ and $H° = \arctan(b^*/a^*)$, respectively.

Spectrophotometric measurements were conducted with the absorbance recorded using a 1 cm quartz cuvette at 420 nm on a Spectramax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA).

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

3.2.5. HPLC and mass spectrometric analysis of free α-dicarbonyl compounds

For solid phase extraction, pre-column derivatization, MS identification and HPLC quantitation of G, 3-DG, MGO, GO and DA the previously published method was used (Hrynets et al., 2015a; Hrynets et al., 2015). The SPE cartridge (tC-18 Sep-Pak, Waters, Milford, MA, USA) was pre-conditioned with 10 mL methanol and 20 mL water. The GlcN samples obtained after retrieval from incubation were passed through a pre-conditioned column at a flow rate of 2 mL/min. The column was washed with 2 mL of water, which was added to previously eluted polar compounds (SPE 1). The aliquots from the SPE 1 were spiked with 0.006 g of 1,2-diaminobenzene (*o*-phenylenediamine (OPD)), followed by adjustment of pH to 3.00 ± 0.02 with 4 M HCl. The mixture was derivatized at 37°C for 1 h prior to passing through a pre-conditioned SPE cartridge (SPE 2). The cartridge was washed with 2 mL of water, and the quinoxalines were eluted with 4 mL of a MeOH/H_2O mixture (90:10, v/v) at a flow rate close to 2 mL/min. Concentrations of individual α-DC were determined by the external standard method. Standard curves were constructed using five different concentrations of the standards. The correlation coefficients for all calibration curves were $R^2 \geq 0.99$. The average limits of detection (LODs) were calculated as 3.6 ± 0.4(G), 1.8 ± 0.3(3-DG), 1.3 ± 0.08(GO), 0.5 ± 0.0 (MGO) and 0.6 ± 0.0 μg/mL (DA) and the average limits of quantitation (LOQs) were 10.9 ± 1.3(G), 5.5 ± 1.3(3-DG), 4.2 ± 0.7 (GO), 1.6 ± 0.1 (MGO) and 1.8 ± 0.1 μg/mL (DA) where signal-to-noise ratios (S/N) were 3.3:1 and 10:1 for LOD and LOQ, respectively.

3.2.6. Analysis of heterocyclic compounds

Fructosazine (FR) and Deoxyfructosazine (DOFR). HPLC and MS/MS. Analysis of non-volatile FR and DOFR were performed using the same method as described before (Hrynets et al., 2015a). To quantify FR and DOFR the standard curves (five points) were constructed with an $R^2 \geq 0.99$. The LODs and LOQs for FR were 1.40 ± 0.00 and 4.24 ± 0.03 μg/mL, respectively and for DOFR were 0.05 ± 0.01 and 0.15 ± 0.08 μg/mL, respectively.

HMF, THI and 4-MEI. Identification. HPLC-MS/MS analyses were used to identify the presence of HMF, THI and 4-MEI in GlcN caramels. HPLC with tandem mass spectrometric (MS) detection was 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). LC was run on an Agilent 1200 HPLC system (Agilent, Palo Alto, CA, USA) with a degasser, a quaternary pump, a thermostated autosampler, and a UV-visible detector. The MS procedures were followed as described by Wang et al. (2015) and were performed using the reversed-phase column Ascentis Express Peptide ES-C18 (150 × 4.6 mm, 2.7 μm particle size; Sigma-Aldrich). The samples were eluted with (A) 0.05% ammonia in water and (B) 5% acetonitrile with a gradient programmed as: 5% B (0 min), 5% B (3 min), 40% B (5 min), 5% B (5.1 min) and 5% B (6.5 min) at flow rate of 0.3 mL/ min. The sample injection volume was 10 μL. The mass spectrometer was operated in a selected reaction monitoring (SRM) mode. The effluent from the LC was directly introduced with a heated ESI probe operated in the positive mode into the mass spectrometer. The acquisition was performed at spray voltage 3000 V, capillary temperature 350 ºC, sheath and aux gas pressure 30 and 10, respectively.

Quantitation. The concentrations of HMF and THI were determined as described by Ciolino (1998). The chromatographic separations were performed using an Agilent 1100 system (Agilent Technologies, Inc., Santa Clara, CA, USA) consisting of a G-1312 binary pump, a G-

1328A injector, a G-1322A degasser, and a G-1315A photodiode array detector (PDA), equipped with an Ascentis Express ES-C18 column. The mobile phase was a binary mixture of (A) 0.05 M potassium dihydrogen phosphate and 0.005 M sodium octane sulfonate, adjusted to a pH of 3.0 ± 0.01, and (B) 100% methanol. The mobile phase consisted of 92.5:7.5 A:B and detection was performed at 285 nm. The injection volume was 10 µL and flow rate 0.5 mL/min. The analytes were filtered with a PVDF syringe filter (13 mm, 0.22 µm; Millipore Millex, Billerica, MA, USA). The quantitation was achieved using a 5-points standard curves with an $R^2 \geq 0.99$. The LODs were determined as 1.4 ± 0.06 µg/mL (THI), 0.7 ± 0.03 µg/mL (HMF) and the LOQs were 4.2 ± 0.2 µg/mL (THI) and 2.1 ± 0.17 µg/mL (HMF). Data acquisition and processing were performed with Agilent ChemStation software.

3.2.7. Ammonia detection assay

The ammonia was detected using commercially available kit (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions. The assay is based on the reaction of ammonia with α-ketoglutaric acid and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of L-glutamate dehydrogenase to form L-glutamate and oxidised $NADP^+$. The oxidation of NADPH to $NADP^+$ results in a decrease in the absorbance at 340 nm that is proportional to the concentration of ammonia.

3.2.8. Statistical analysis

The data was analysed as a 3 × 2 factorial analysis of variance (ANOVA) using the PROC MIXED procedure of SAS (v. 9.3, SAS Institute Inc., Cary, NC, USA). The model tested the interaction of vacuum and temperature 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. A principal component analysis (PCA) was conducted using OriginPro 8.6 software (OriginLab Corporation, MA, USA, 2012).

3.3. Results and discussion

3.3.1. Chemico-physical characteristics of GlcN caramel solutions

As shown in Table 3.1 a higher temperature, in general, resulted in a greater drop in pH. However, GlcN caramels produced under vacuum were significantly more acidic as compared to the treatments under non-vacuum, and this was more evident at 50°C (4.2 vs. 4.5 in vacuum vs non-vacuum, respectively). Generation of formic and acetic acids during GlcN incubation in the presence of oxygen, causing a decrease in pH, has been reported before (Hrynets et al., 2015a). Greater acidity of vacuum-treated samples is most likely due to the different degradation pathways leading to the formation of different types and/or concentrations of the major degradation products, which will be discussed in the following parts.

Tristimulus colorimetry was used to visualize and integrate different dimensions of the colour space. The colorimetric parameters, L^* (lightness, black (0)-white (100)), a^* and b^* representing red-green and yellow-blue, respectively were determined. The a^* and b^* values are reported in the Appendix A. The CIE values of a^* and b^* were then transformed into the $H°$ and C^*. With regard to the main effect of temperature (Table 3.1), the lightness of GlcN caramels significantly decreased with increased incubation temperatures. This is expected since higher temperatures usually produce darker caramels or Maillard reaction systems (Jing & Kitts, 2004). However, changes in L^* values not necessarily correlate with the visually observed browning (Rufian-Henares et al., 2004). Therefore, the colour was also expressed by means of the chroma (C^*, metric chroma) and hue angle ($H°$, chromatic tonality). The latter acquires measures of redness at values near 0° and yellowness near 90° (Jing & Kitts, 2004). The significant effect of vacuum, temperature and their interactions was found for hue angle values. In general, for all treatments the values were in the range between 13.9 to 53°, indicating orange-red to yellow hue

of GlcN caramels. The least ($p < 0.05$) hue value of 13.9° was observed in non-vacuum GlcN caramels incubated at 50°C followed by the vacuum 60°C treatments (14.5°) representing reddish tonality of these treatments. GlcN solutions incubated under vacuum at 50 and 70°C were also reddish with the hues of 20.1 and 21.4°, respectively. The non-vacuum 70°C GlcN caramels had a hue of 35.5°, denoting orange tonality. The greatest value of hue ($p < 0.05$) was observed in 60°C non-vacuum samples representing an orange-yellowish tonality.

Chroma is a measure of colour saturation or intensity, and is defined by the magnitude of the vector at each point designating the departure from dull to more vivid chromatic colour ("–" to "+" values) (Serratosa et al., 2008). Non-vacuum GlcN caramels had significantly smaller chroma values, indicating their lower vividness or colour intensity as compared to vacuum-produced caramels. Temperature also significantly affected chroma, where increasing temperature significantly decreased chroma values for both vacuum and non-vacuum caramels. These results indicate that vacuum conditions and lower incubation temperature generate caramels with the greatest colour vividness. Both caramelization and the Maillard reaction are responsible for the formation of browning compounds (ie. melanoidins) absorbing at 420 nm (Adams et al., 2005). Results outlined in Table 1 show that caramels produced under vacuum had significantly less absorbance at 420 nm as compared to non-vacuum samples, indicating less browning intensity of vacuum caramels. Melanoidin production occurs with consumption of oxygen (Oliver & Colicchio, 2011), therefore it is expected that caramel solutions prepared in the vacuum condition absorbed less at 420 nm. Interestingly, that an increase in temperature did not affect the absorbance at 420 nm in vacuum treatments; whereas temperature affected non-vacuum samples only between 50 and 70°C. These results agree with the study of Kanner and Shapira (1989) who found less non-enzymatic browning of grape fruit juice packaged with less oxygen.

Table 3. 1. Changes in pH, lightness (*L**), hue angle (*H°*), chroma (*C**) and absorbance at 420 nm during incubation of GlcN solutions at 50, 60 and 70°C under non-vacuum and vacuum conditions for 12 h.

		pH	*L**	*H°*	*C**	Absorbance at 420 nm
Treatment						
Non-vacuum		3.4^{a}	24.6^{b}	34.1^{a}	2.6^{b}	0.13^{a}
Vacuum		3.3^{b}	25.3^{a}	18.7^{b}	7.8^{a}	0.10^{b}
SEM		0.2	0.3	1.9	0.8	0.002
Temperature						
50°C		4.4^{a}	27.1^{a}	17.0^{b}	10.5^{a}	0.11^{b}
60°C		3.1^{b}	24.3^{b}	33.8^{a}	4.0^{b}	0.12^{a}
70°C		2.5^{c}	23.6^{c}	28.5^{a}	1.1^{c}	0.12^{a}
SEM		0.02	0.2	2.5	0.6	0.004
Interaction (Treatment*Temperature)						
Non-vacuum	50°C	4.5^{a}	26.4^{b}	13.9^{d}	6.0^{c}	0.12^{bc}
	60°C	3.1^{c}	24.1^{cd}	53.0^{a}	1.1^{e}	0.13^{ab}
	70°C	2.6^{e}	23.4^{d}	35.5^{b}	0.6^{f}	0.14^{a}
Vacuum	50°C	4.2^{b}	27.7^{a}	20.1^{c}	15.0^{a}	0.10^{d}
	60°C	3.0^{d}	24.4^{c}	14.5^{d}	6.9^{b}	0.11^{cd}
	70°C	2.5^{f}	23.7^{cd}	21.4^{c}	1.6^{d}	0.10^{d}
SEM		0.01	0.2	0.7	0.07	0.003
Sources of variation		***p-values***				
Treatment		<0.001	<0.05	<0.001	<0.001	<0.001
Temperature		<0.001	<0.001	<0.001	<0.001	<0.05
Interaction		<0.001	<0.05	<0.001	<0.001	<0.05

[a–g]Means within the same column with no common superscript differ significantly (p<0.05). n=9 for each treatment within each experiment. Results are reported as least-square means (LSMeans). SEM=Standard error of the LSMeans.

3.3.2. Analysis of α-dicarbonyl compounds

Analysis of the α-DCs content of GlcN yielded five major compounds, supporting previous findings on GlcN browning (Hrynets et al., 2015a; Hrynets et al., 2016). Representative HPLC-UV chromatograms and MS/MS identification of α-DC are shown in the Appendix B and C. The upper part of the Table 3.2 shows the main effect of treatment condition and temperature on dependent variables G, 3-DG, GO, MGO and DA. 3-DG was found to be the dominating α-DC and its concentration was 1.7-times greater in non-vacuum samples (Table 3.2). The temperature

also had a significant effect on 3-DG, where under non-vacuum conditions higher temperatures resulted in significantly less 3-DG concentration. Under vacuum no significant effect was found between treatments at 50 and 70°C. The non-oxidative mechanism of 3-DG formation from GlcN through 1,2-enolization has been previously proposed (Hrynets et al., 2015a). A significant decrease of 3-DG with higher temperatures under non-vacuum is most likely due to the progress of the reaction including C3-C4 or C4-C5 cleavage and dehydration generating MGO, GO, HMF and/or 3,4-dideoxyglucosone-3-ene (3,4-DGE) (Hrynets et al., 2015a). Indeed, the presence of not only MGO, GO and HMF, but also 3,4-DGE was identified in GlcN caramels (refer to Appendix C). G is an important α-DC as it acts as a reductone and as a radical scavenger (Kanzler, Haase & Kroh, 2014). It is therefore important to monitor the level of G in caramel solutions. The concentration of G was significantly greater in vacuum treatments in comparison to non-vacuum (512 vs 264 mg/L, respectively) and under both conditions its concentration significantly decreased as a function of temperature (Table 3.2). G was proposed to be generated oxidatively from GlcN (Hrynets et al., 2015a), therefore finding its greater concentration under vacuum conditions was initially surprising. However, it has been reported that reductone molecules like G consume oxygen during non-enzymatic browning reaction possibly forming melanoidins (Serban & Nissenbaum, 1981); therefore, in a more oxidative environment (i.e. non-vacuum treatment) G would consume oxygen forming more melanoidins. The results of absorbance at 420 nm (Table 3.1) supports this hypothesis. However, Gobert and Glomb (2009) while studying the degradation of glucose in the presence of lysine at 50°C, found greater amount of G under aerated condition. Smuda and Glomb (2011) also reported greater concentrations of G during incubation of maltose in the presence of lysine under aerated conditions. Possible reasons for discrepancies between these studies might be due to the fact that the glucose/maltose-lysine reaction systems would yield

lower quantity of Amadori compound compared to an already formed Heyns compound obtained from chitin hydrolysis and deacetylation. The other reason could be due to another mechanism involved in GlcN degradation under vacuum. In addition, vacuum conditions were achieved differently in the previous studies (Gobert & Glomb, 2009; Smuda & Glomb, 2011) compared to the current. The significant decrease of G with increased incubation temperatures was presumably the result of its cleavage at C2-C3 or C3-C4 generating GO or hydroxypyruvaldehyde, respectively. GO concentration was significantly greater under vacuum, the same as one of its precursors, G. Its concentration significantly increased with increased incubation temperature in both vacuum and non-vacuum treatments. MGO and DA were found in significantly greater concentrations in non-vacuum treatments, being on average 1.9 and 1.3-times greater respectively to those found in caramels produced under vacuum. As in the case of GO, the concentrations of MGO and DA significantly increased with higher incubation temperatures (Table 3.2); suggesting that higher temperatures facilitate the degradation of a long chain α-DCs 3-DG and G into the short chain GO, MGO and DA. A more oxidative environment (non-vacuum) promotes the formation of odorant molecules like diacetyl at a level of 14 ppm and the brown melanoidins.

Table 3. 2. Concentration (mg/L) of free α-dicarbonyl compounds during incubation of GlcN solutions at 50, 60 and 70°C under non-vacuum and vacuum conditions for 12 h.

		G	3-DG	GO	MGO	DA
Treatment						
Non-vacuum		264^{b}	742^{a}	8.3^{b}	3.8^{a}	14^{a}
Vacuum		512^{a}	440^{b}	10.8^{a}	2.0^{b}	11^{b}
SEM		40.7	18.9	0.3	0.2	0.8
Temperature						
50°C		619^{a}	699^{a}	7.7^{b}	2.4^{b}	7.6^{c}
60°C		424^{b}	512^{b}	9.9^{a}	2.9^{ab}	12.1^{b}
70°C		122^{c}	562^{b}	11.1^{a}	3.5^{a}	17.7^{a}
SEM		30.2	36.7	0.3	0.2	0.4
Interaction (Treatment*Temperature)						
Non-vacuum	50°C	384^{c}	902^{a}	7.1^{f}	3.2^{c}	8.9^{e}
	60°C	336^{d}	693^{b}	8.6^{d}	3.7^{b}	14.1^{c}
	70°C	74^{g}	632^{c}	9.2^{c}	4.5^{a}	19.0^{a}
Vacuum	50°C	854^{a}	496^{d}	8.2^{e}	1.5^{f}	6.4^{f}
	60°C	512^{b}	332^{e}	11.3^{b}	2.0^{e}	10.2^{d}
	70°C	171^{e}	492^{d}	12.9^{a}	2.5^{d}	16.4^{b}
SEM		3.0	2.7	0.08	0.05	0.1
Sources of variation		***p-values***				
Treatment		<0.001	<0.001	<0.001	<0.001	<0.001
Temperature		<0.001	<0.001	<0.001	<0.001	<0.001
Interaction		<0.001	<0.001	<0.001	<0.05	<0.001

$^{a-f}$Means within the same column with no common superscript differ significantly (p<0.05).
n=9 for each treatment within each α-dicarbonyl compound. Results are reported as least-square means (LSMeans). G – glucosone, 3-DG – 3-deoxyglucosone, GO – glyoxal, MGO – methylglyoxal, DA – diacetyl, SEM=Standard error of the LSMeans.

3.3.3. Effect of vacuum vs non-vacuum condition on FR and DOFR levels

Fructosazine and deoxyfructosazine are the major products of GlcN autocondensation. These molecules posses both bioactive and functional properties. For instance, they possess anti-inflammatory activity against diabetes and cartilage degradation (Giordani et al., 2006). At the same time, the so-called "colourless caramel" which is a caramel extract containing a relatively large amount of FR and DOFR, can also be used to protect beer from the phenomenon of UV-light induced off-flavour generation (Van der Ark et al., 2013). The representative HPLC-UV

chromatograms used for FR and DOFR quantitation and the MS/MS spectra that was used to verify the identification of these non-volatile pyrazines are shown in the Appendix D and E. Results reported in Table 3.3 show that GlcN caramel solutions generated under vacuum contained significantly more FR as compared to those produced with no vacuum, however DOFR showed significant interaction between vacuum condition and temperature although main effect of vacuum treatment was not evident. The first row of the Table 3.3 represents the main effect of treatment condition and temperature on FR, DOFR, HMF and THI.

Table 3. 3. Concentration of fructosazine (FR), deoxyfructosazine (DOFR), 5-hydroxymethyl-2-furfural (HMF) and 2-acetyl-(4)5-tetrahydroxylbutyl imidazole (THI) during incubation of GlcN solutions at 50, 60 and 70°C under non-vacuum and vacuum conditions for 12 h.

		FR	DOFR	HMF	THI
		g/L		mg/L	
Treatment					
Non-vacuum		11.6^{b}	23.4	5.1^{a}	1.3^{b}
Vacuum		35.2^{a}	23.2	2.8^{b}	3.3^{a}
SEM		2.4	1.1	0.2	0.2
Temperature					
50°C		39.6^{a}	30.8^{a}	2.9^{a}	1.3^{b}
60°C		20.5^{b}	20.2^{b}	3.9^{ab}	2.1^{b}
70°C		10.1^{b}	19.1^{b}	5.0^{b}	3.6^{a}
SEM		2.9	0.3	0.3	0.3
Interaction (Treatment*Temperature)					
Non-vacuum	50°C	19.1^{c}	29.8^{b}	3.9^{c}	0.5^{f}
	60°C	11.0^{e}	20.3^{c}	5.1^{b}	0.9^{e}
	70°C	4.6^{f}	20.1^{c}	6.2^{a}	2.5^{c}
Vacuum	50°C	60.1^{a}	31.7^{a}	2.1^{f}	2.1^{d}
	60°C	30.0^{b}	20.0^{c}	2.6^{e}	3.2^{b}
	70°C	15.5^{d}	18.0^{d}	3.7^{d}	4.7^{a}
SEM		0.4	0.4	0.02	0.05
Sources of variation		***p-values***			
Treatment		<0.001	0.604	<0.001	<0.001
Temperature		<0.001	<0.001	<0.001	<0.001
Interaction		<0.001	<0.001	<0.001	<0.001

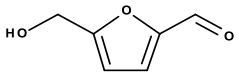
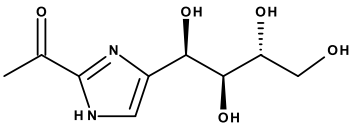
$^{a–f}$Means within the same column with no common superscript differ significantly (p<0.05).
n=9 for each treatment within each experiment. Results are reported as least-square means (LSMeans). SEM=Standard error of the LSMeans.

An increase in temperature significantly decreased the concentrations of both FR and DOFR in vacuum and non-vacuum treatments. FR is generated during GlcN's double dehydration followed by oxidation, therefore its greater concentration in vacuum samples is surprising. It is possible that FR is degraded in a more oxidative environment, forming new derived pyrazines. A decrease in FR and DOFR concentration with higher temperatures is most likely due to acidification of GlcN caramel solutions (refer to Table 3.1). Wu et al. (2011) showed that pH 6-8 are favorable for larger FR and DOFR yields; this agrees with our results where the formation of FR and DOFR was greater at 50°C treatments, which were significantly less acidic than treatments at 60 and 70°C. In summary, a lower temperature (50°C) and vacuum condition promote the formation of these bioactive compounds.

3.3.4. Changes in concentration of HMF

The generation of HMF during the Maillard reaction and caramelization (i.e. fructose) is well-established; however, HMF was not identified and quantified before during caramelization of GlcN. Representative HPLC-UV chromatograms of HMF are shown in Figure 3.1, where a well resolved peak of HMF was observed and was eluted at the exactly the same time as the HMF standard. Additional HPLC-UV chromatograms for the other treatments can be found in the Appendix F. For the unambiguous identification of HMF in GlcN caramels, HPLC-MS/MS was performed, where HMF yielded protonated molecular ions $[M+H]^+$ at *m/z* 127.1 and the major fragment ions at *m/z* 109.1 and 81.1 (Table 3.4, Figure 3.1). This fragmentation pattern corresponded to the one obtained from HMF standard and consistent to the previously reported elsewhere (Serra-Cayuela, 2013), allowing for correct identification. The concentration of HMF was significantly affected by absence of oxygen during GlcN caramelization and was on average 1.8-times less in vacuum conditions (Table 3.3).

Table 3. 4. Selected Reaction Monitoring Conditions in ESI (+) Mode

Analyte	Parent ion (*m/z*)	Daughter ion (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)
HMF 	127.1	109.1 81.1	50	11	17	10
THI 	231.1	153.1 195.5 213.1	40	10	15	10

DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.

An increase in temperature of caramelization from 50 to 70°C resulted in an increased generation of HMF in both vacuum and non-vacuum treatments. As mentioned before, HMF is formed from its precursor, 3-DG, upon dehydration, therefore most likely that higher temperatures of incubation favored 3-DG's dehydration. Grainger et al (2017). reported a weak negative correlation between pH and HMF and no correlation between MGO and HMF in Manuka honey. In this study a negative correlation ($r = -0.78$, $p < 0.01$) was observed between pH and HMF and a positive correlation ($r = 0.77$, $p < 0.01$) between 3-DG and HMF formation (refer to Appendix I). Shallenberger and Mattick (1983) showed that at pH 3, the rate of HMF formation from fructose was approximately double from that at pH 4-6; while at pH 2 and 1 it was about ten and nearly forty times as rapid, respectively. To the best of our knowledge, no HMF limits in caramels has been set. The levels of HMF found in commercial caramels vary considerably and are dependent on caramel type; where for instance Caramel type I range is 700-2700 mg/kg HMF (Aguilar et al., 2011). The results from this study showed that the HMF concentrations in GlcN caramels produced under vacuum or non-vacuum at 50-70°C were well less than the concentrations found in commercial caramels.

3.3.5. Identification of THI and 4-MEI

THI is an alkylimidazole formed as a by-product during thermal processing resulted from addition of ammonia caramel colourants, Class III and IV caramel colours (Aguilar et al., 2011; Mottier et al., 2017). THI was also identified in GlcN caramel solution produced in this study under both vacuum and non-vacuum conditions. The representative HPLC-UV chromatograms showed the peak of THI eluted before the HMF (Figure 3.1 C, D). For unambiguous THI identification its MS/MS spectra was acquired and compared to the standard THI solution, where the same mass fragments were found, confirming peak identification. Table 3.3 shows that significantly more THI was formed under vacuum conditions. On average 3.3 mg/L of THI was formed in vacuum treatments, while 1.3 mg/L was found in non-vacuum treatments. Higher temperatures also favored the formation of THI under both vacuum and non-vacuum conditions. The greatest concentration of THI was formed in GlcN under vacuum caramels produced at 70°C and was 4.7 mg/L; this concentration was still below the recommended level (Aguilar et al., 2011). It has been proposed (Kröplien et al., 1985) that the formation of THI during the caramelization of glucose in the presence of ammonia involves the condensation of fructosamine and MGO, two products formed via the Amadori rearrangement and alkaline degradation of glucose, respectively.

To verify this mechanism, the concentration of ammonia was also determined and showed a significantly less generation of ammonia in vacuum treated GlcN caramels at each incubation temperature (refer to Appendix G).

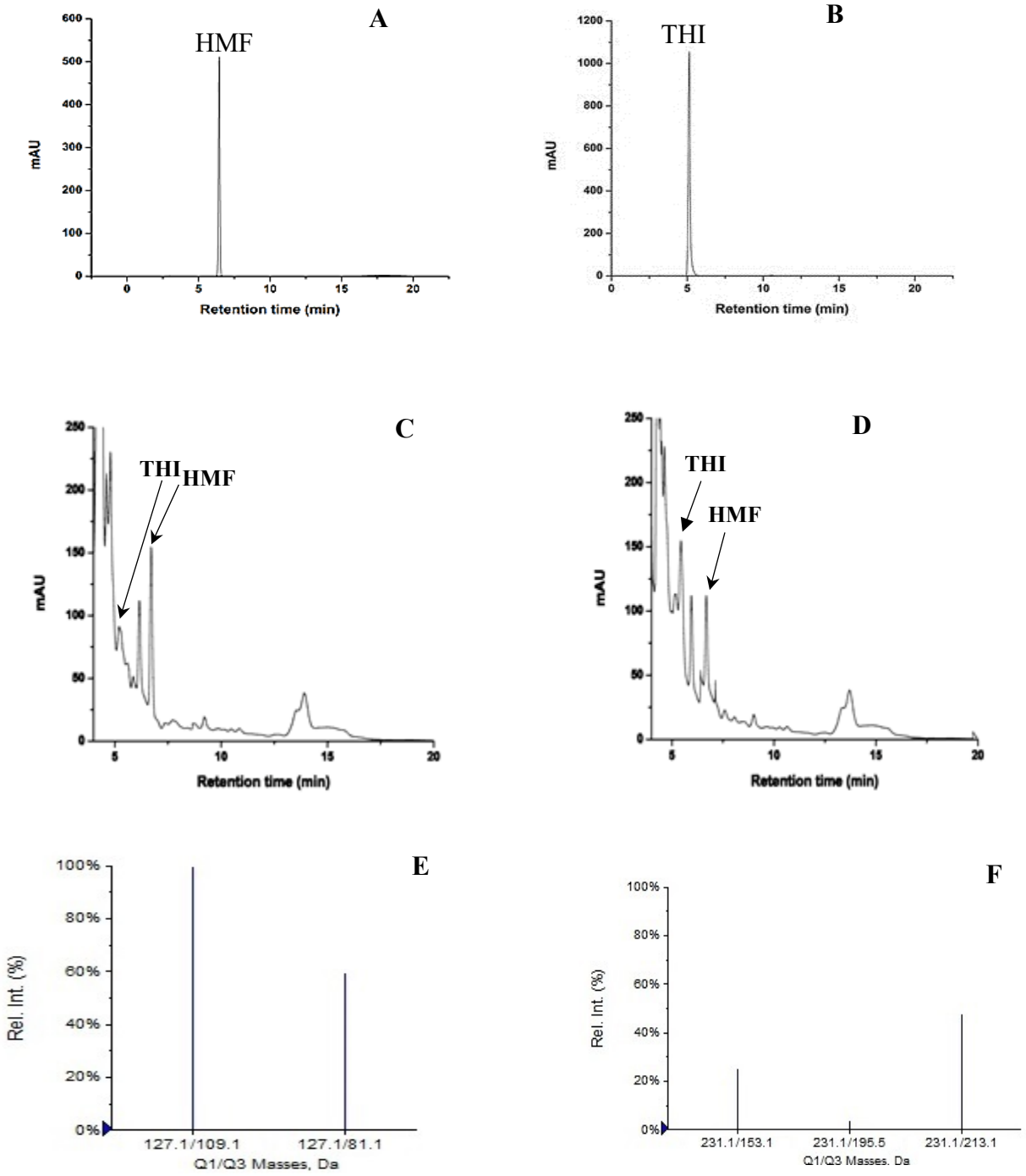


Figure 3. 1. HPLC and MS analyses of 5-hydroxymethyl-2-furfural (HMF) and 2-acetyl-(4)5 tetrahydroxylbutyl imidazole (THI). (I) Chromatograms of (A) HMF and (B) THI standards. (II) Representative chromatograms of HMF and THI in GlcN solution incubated at 50 °C under nonvacuum (C) and vacuum (D) for 12 h; ESI-MS/MS spectra of HMF (E) and THI (F) identified in GlcN (under vacuum) caramel solutions using SRM mode.

Since significantly more THI was formed in samples produced under vacuum, lower ammonia concentration detected in the same treatments suggest its greater involvement in THI formation. Significantly less MGO was also found in vacuum treated samples (Table 3.2), which may also imply its involvement in THI formation. On the basis of our experimental evidence, we propose the mechanism of THI formation during GlcN browning (Figure 3.2), where in the first step GlcN molecule condensation with ammonia results in formation of imine form of GlcN existing in equilibrium with its eneamine form. The reaction of enediamine with MGO and further rearrangements will form THI. Depending on which among C1-NH_2 or C2-NH_2 reacts with MGO, 5-THI or 4-THI, is formed, respectively. No 4-MEI was identified in GlcN caramels produced under the conditions of this study (refer to Appendix H). This is very important result as 4-MEI is a major concern in caramel-containing foods and beverages. A previous study (Yu et al., 2015) indicated that GlcN caramel produced at 120°C for 2 h generate 4-MEI; the moderate temperature used in this study allowed to produce 4-MEI-free caramel with a greater content of the aromatic diacetyl.

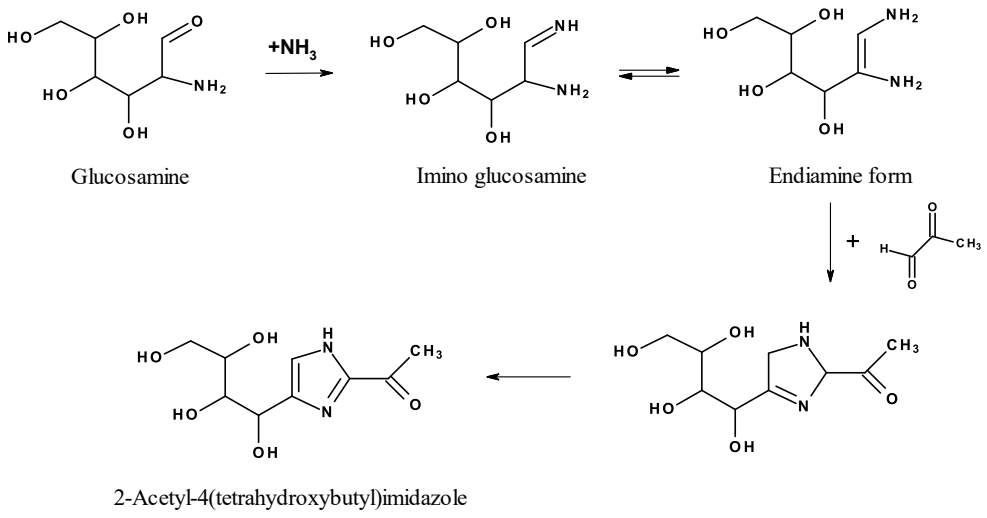


Figure 3. 2. Proposed formation mechanism of 2-acetyl-4(5)-tetrahydroxylbutylimidazole (THI) from GlcN.

3.3.6. Multivariate analyses: Principal components

Pooling the full set of analytical analyses enabled performing a principal component analyses, which showed that data could be distinguished into six separate groups (Figure 3.3), indicating that each caramel solution was of unique composition. Two principal components were extracted from the data showing 58.68% (PC1) and 29.52% (PC2) of the variation (Figure 3.3, Appendix J); implying that 88.2% of the total variance in the thirteen dependent variables determined could be condensed into two PCs (Appendix J).

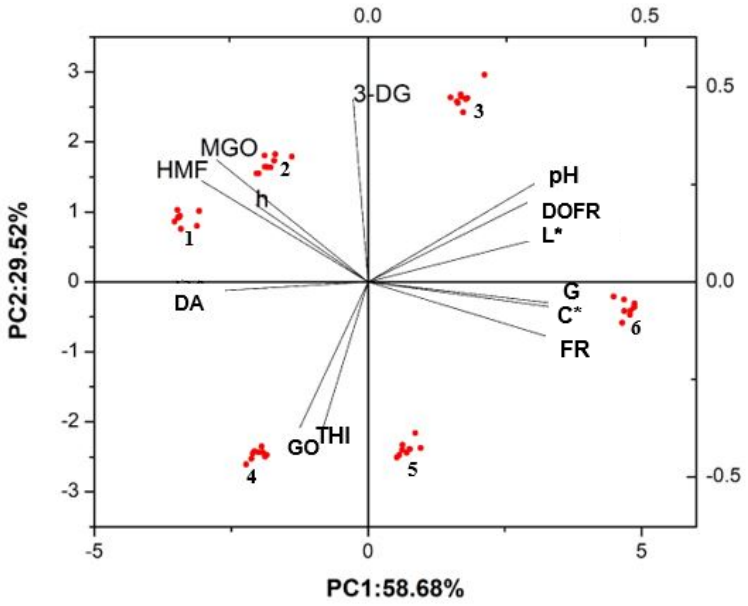


Figure 3. 3. PCA biplot for two principal components computed from the analytical analyses of GlcN caramel solutions composition produced under different conditions. The first principal component (PC1, incubation temperature) explains 58.68% of the variation, and the second principal component (PC2, vacuum/non-vacuum) explains 29.52% of the variation. Six groups are represented by the following treatments: 1, 50 °C non-vacuum; 2, 60 °C non-vacuum; 3, 70 °C non-vacuum; 4, 50 °C vacuum; 5, 60 °C vacuum; and 6, 70 °C vacuum.

PC1 had relatively large loadings of FR, G, DA, *L** and *C**, while 3-DG, THI, GO and MGO had large loadings on the PC2 (Table 3.5). Overall, PC2 was able to discriminate the caramel solutions produced under vacuum (4, 5, and 6) from caramel solutions produced in more oxidative condition (non-vacuum treatments), as the firsts are located in higher quadrants and the latter in

the lows (Figure 3.3). On the other hand, PC1 is separating the caramel solutions based on the non-enzymatic browning temperature (Figure 3.3). Therefore, FR, G, DA and colour characteristics (higher loading coefficients in PC1) can be used as possible markers to discriminate non-enzymatic browning temperature, while 3-DG, THI, GO and MGO (higher loading coefficients in PC2) can be used as markers to discriminate between vacuum and non-vacuum treatments.

Table 3. 5. Coefficients of the loading (eigen vectors) for the first two principal components (PC).

Dependent variables	PC1	PC2
Deoxyfructosazine (DOFR)	0.31	-0.21
Fructosazine (FR)	0.33	0.20
3-Deoxyglucosone (3-DG)	-0.03	0.47
Glucosone (G)	0.34	-0.06
Methylglyoxal (MGO)	-0.27	0.31
Glyoxal (GO)	-0.15	-0.45
Diacetyl (DA)	-0.35	-0.03
Hydroxymethylfurfural (HMF)	-0.30	0.26
2-Acetyl-5-tetrahydroxylbutyl imidazole (THI)	-0.10	-0.46
pH	0.30	0.26
*L**	0.33	0.12
*C**	0.35	-0.07
H°	-0.20	0.20

3.4. Conclusion

In conclusion, this study showed that the level of oxygen and temperature of incubation both play significant roles in determining physico-chemical properties and composition of GlcN caramel solutions. Combinations of different temperatures and vacuum/non-vacuum conditions result in GlcN caramel solutions that possess different acidity, browning level, and concentration of flavouring (i.e. pyrazines and diacetyl) and undesirable (HMF, THI) compounds. The treatments generated six unique caramels. In general, GlcN caramels produced under vacuum were slightly more acidic, lighter (less absorbance at 420 nm) and of a more intense colour compared to those generated under non-vacuum. In terms of butterscotch aromatic compound, GlcN caramels under

vacuum contained 1.3-times less DA, but almost 3-times more FR as compared to non-vacuum caramels. The treatments of 70ºC non-vacuum and vacuum conditions were the most favourable conditions to yield higher concentration of DA, 19 and 16.4 mg/L, respectively in this study. Vacuum caramels also contained less HMF, but more THI, which in either treatment were significantly less from those required by regulations. It was found that FR tend to be heat and oxygen unstable and decreased proportionally with an increased temperature of incubation. The opposite was found for DA, where its concentration increased with increasing temperatures. As for undesired HMF and THI, an increase in the temperature of incubation resulted in an increased concentration of both compounds. No 4-MEI was found in any of the GlcN caramels tested in this study. In accordance with the results, it is suggested to use a lower incubation temperature to minimize the formation of undesired HMF and THI, while still retaining high amount of flavouring agents DA and FR. The caramel solutions obtained in this study, particularly the ones with greater amount of FR, have the potential to be used in beer production against UV light-induced off-flavour generation as FR posses a strong light absorption in the UV-B range. Furthermore, the low pH (due to acetic and formic acids production) (Hrynets et al., 2016) and dark colour pave the possibility to produce a “chemical balsamic vinegar” from GlcN.

CHAPTER 4. The effect of amino acids on non-enzymatic browning of glucosamine: generation of butterscotch aromatic and bioactive health compounds.

4.1. Introduction

Glucosamine (GlcN), also known as 2-amino-2-deoxy-D-glucose, is an amino monosaccharide receiving a recent research attention. In North America, it is used as a dietary supplement to treat osteoarthiritis (Salazar et al., 2014) and some studies have also shown it has efficacy as an anti-tumor agent as well (Chesnokov et al., 2014). Since GlcN has an amino group at the C-2 position next to the carbonyl group, it resembles a Heyns rearrangement product (HRP); a compound that is normally produced during the reaction between fructose and ammonia at elevated temperatures (Heyns & Koch, 1952). Although the Maillard reaction can be used to produce GlcN, most industrially produced GlcN is through the hydrolysis and deacetylation of chitin, the second most abundant natural polymer found on earth. Chitin is normally found in the exoskeleton of insects and also in shell fish which are used to produce chitosan and GlcN (Mojarrad et al., 2007). Our research group has conducted several studies about the non-enzymatic browning of GlcN at relatively moderate temperatures (37 to 70°C) (Hrynets et al., 2015a, 2016; Hong & Betti, 2016; Dhungel et al., 2018). As previously described, this is due to the unique structure of GlcN. Heyns or Amadori compounds are early Maillard reaction products capable of eliciting non-enzymatic browning reactions even at 37°C (Hrynets et al., 2015). Therefore, GlcN can be used to produce caramel solutions at moderate temperatures in more or less oxidative conditions to produce interesting compounds that possess aromatic, antioxidant (Hong & Betti, 2016), antimicrobial (Hrynets et al., 2016) and anti-inflammatory properties (Zhu et al., 2007). However, the concurrent production of endogenous dicarbonyls is associated with diabetic complications (Vlassara & Bucala, 1996). The use of moderate temperatures is a safer option compared to some toxicants found in industrially produced caramel like 4-MEI, THI and HMF

(Dhungel et al., 2018). In terms of aromatic compounds, GlcN can produce up to 16 mg/L of diacetyl (Dhungel et al., 2018), the butter-like aroma appreciated in many foods, particularly in dairy and baked products (Gemelas et al., 2016). To a certain concentration, it is also appreciated in darker beers (Coghe et al., 2004). GlcN non-enzymatic browning can also produce high amounts of the antimicrobial compound 3-deoxyglucosone through a 1,2-enolization reaction (MIC_{50} against Aw 1.7 *E. coli* ~ 1 g/L) (Hrynets et al., 2016). Glucosone, is another deoxysone produced during non-enzymatic browning, which can act as a reductone and radical scavenger (Kanzler, Haase, & Kroh, 2014). A study by Dhungel et al. (2018) demonstrated the generation of 854 mg/L of glucosone as a degradation product of GlcN under vacuum conditions. Betti et al. 2018 (under review), has demonstrated that glucosone can reduce Fe (III) to Fe (II) in the myoglobin molecule. However, the major compounds found in the GlcN caramel solutions prepared at these moderate temperatures are polyhydroxyalkyl pyrazines, fructosazine (FR) and deoxyfructosazine (DOFR). FR is produced through a self-condensation reaction of GlcN followed by dehydration and dehydrogenation, whereas self-condensation of GlcN followed by dehydration yields DOFR (Hrynets et al., 2016). GlcN non-enzymatic browning can produce up to 60.1 and 31.7 g/L FR and DOFR, respectively, in non-oxidative condition at 50°C (Dhungel et al., 2018). These molecules are gaining increasing interest due to their anti-inflammatory properties against type II diabetes (Zhu et al., 2007) and cartilage degradation (Giordani et al., 2006). Furthermore, the multinational company Heineken has produced a colourless, caramel-rich beer, where both FR and DOFR are present and can protect the beer against the generation of UV-induced off-flavours (Van Der Ark et al., 2013). FR and DOFR have also been found in roasted peanut (Magaletta & Ho, 1996), soy sauce (Tsuchida et al., 1990), caramel (Tsuchida et al., 1986), and tobacco smoke (Moldoveanu et

al., 2011). Hence, it could be of interest and benefit to the food industry to be able to better understand and control the production of these bioactive molecules.

Diacetyl is naturally occurring in many fermented foods including yogurt, butter, balsamic vinegar, wine, brandy, roasted coffee and honey whereas is added in foods like microwave popcorn (Clark & Winter, 2015). In yoghurt it can reach a concentration of 16 ppm, and in certain dark malted beer diacetyl can reach a desirable concentration between 5 and 10 ppm (Güler & Gürsoy-Balcı, 2011). Along with its distinctive buttery flavour, diacetyl has also been recognized to be one of the major contributors to the caramel-like odour (Chew & Smith, 1992). Therefore, from a practical point of view, producing a caramel solution rich in diacetyl could be used as the "mother" for subsequent dilutions in order to target specific food applications (i.e., cultured milks, beer, etc.). The advantage of using these types of GlcN caramel solutions in such food products could include both standardizing butter-scotch aroma and protecting against UV-light induced generation of off-flavour.

In light of these considerations, this study was aimed to react GlcN with different types of amino acids in order to select the best combination for generating the greatest amount of diacetyl and polyhydroxyalkyl pyrazines while minimizing the production of undesirable compounds like 4-MEI, THI and HMF. This represents the first milestone of a research project that, in the long run, aims not only to produce caramel solutions potentially rich in butter and caramel-like aroma with minimal toxic compounds, but also to generate different aroma profiles (i.e., fruity, roasted, among others) while maximizing antimicrobial and antioxidant activities.

4.2. Materials and Methods

4.2.1. Chemicals

D-glucosamine hydrochloride (GlcN, ≥99%), HPLC grade solvents (methanol, formic acid), *o*-phenylenediamine (OPD; 99.5%), lysine (≥98%), histidine (≥98%), serine (>99%), methionine (≥98%), valine (≥98%), glycine (≥98%), cysteine (97%), leucine (≥98%), arginine (≥98%), alanine (≥99.5%), threonine (≥98%), glucosone (G, ≥98%), glyoxal (GO, 40% in H_2O), methylglyoxal (MGO, 40% in H_2O), diethylene triamine pentaacetic acid (DTPA), 4(5)-MEI (98%), HMF (≥99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 3-deoxyglucosone (3-DG, ≥95%) and THI (≥95%) were from Cayman Chemical (Ann Arbor, MI, USA). Proline (99%) and diacetyl (DA, 99%) were from Acros Organics (NJ, USA). FR and 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 were from Fisher Scientific (NJ, USA). SPE tC-18 Sep-Pak Vac 6 cc columns were from Waters (Milford, MA, USA). 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. Preparation of reaction mixtures

Thirteen GlcN-amino acid combinations were prepared to study the formation of α-DCs and heterocyclic compounds in GlcN-amino acids caramels. GlcN solution (150 g/L) was filtered through 0.2 μm sterile filter and mixed in sterile tubes with different types of free amino acids (arginine, lysine, glycine, cysteine, proline, serine, methionine, histidine, threonine, alanine, valine and leucine) in 1:1 molar ratio. Glucosamine solutions incubated under identical conditions without amino acids are termed as control. The pH of original solution was adjusted to 7.40 with

1 M NaOH at time zero and left unadjusted during the incubation. Three independent batches of samples were prepared on different days resulting in a total number of 39 tubes (36 treatments + 3 control). The tubes were randomly assembled in the incubator and heated at 70˚C for 12 h.

4.2.3. Measurement of pH

After retrieval from incubator, pH measurements were performed using a pH meter (Orion 2-star, Thermo Scientific, Beverly, MA, USA). Each sample was measured twice and the average pH values were calculated.

4.2.4. Analysis of free α-dicarbonyl compounds

4.2.4.1. Pre-column derivatization

The extraction of α-DCs was performed as described by Hrynets et al. (2015) where 2 mL sample was spiked with OPD for derivatization followed by the adjustment of pH to 3.00 ± 0.02 with 4 N HCl. Obtained aliquots were incubated at 37 °C for 1 h. After retrieval from incubation, aliquots of the analytes were filtered with a PVDF syringe filter and subjected to HPLC and MS analyses.

4.2.4.2. HPLC and MS analyses

For HPLC analysis of α-DCs the method reported in Dhungel et al. (2018) was used. Chromatographic separations were performed on an HPLC Agilent 1100 system (Agilent Technologies, Inc., Santa Clara, CA, USA) consisting of a G-1312 binary pump, a G-1328A injector, a G-1322A degasser, and a G-1315A photodiode array detector. A C_{18} reversed-phase column (Ascentis Express Peptide; 15 cm × 4.6 mm × 2.7 µm; Supelco, PA, USA) at a flow rate of 0.3 mL/min was used. The injection volume was 10 µL and detection wavelength 314 nm. The α-DCs were identified by comparing the retention times to the standards of the quinoxaline derivatives of each α-DCs. The identification was also performed using an HPLC in tandem with mass spectrometry (MS) as described in Hrynets et al. (2016). MS conditions in positive mode

were spray voltage of 4.0 kV and source temperature 450°C. MS/MS product ions were produced by collision-induced dissociation (CID) of selected precursor ions using nitrogen as a collision gas under collision energy of 20-30 eV. The curtain gas pressure was set to 20 psi, and the decluttering and entrance potentials were set at 40 and 20 V, respectively.

The five-point calibration curves were constructed to quantitate α-DCs. The correlation coefficients for all calibration curves were $R^2 \geq 0.99$. The average limits of detection (LODs) were calculated as 3.6 ± 0.4 (G), 1.8 ± 0.3 (3-DG), 1.3 ± 0.08 (GO), 0.5 ± 0.0 (MGO) and 0.6 ± 0.0 μg/mL (DA) and the average limits of quantitation (LOQs) were 10.9 ± 1.3 (G), 5.5 ± 1.3 (3-DG), 4.2 ± 0.7 (GO), 1.6 ± 0.1 (MGO) and 1.8 ± 0.1 μg/mL (DA). The signal-to-noise ratios (S/N) were 3.3:1 and 10:1 for LOD and LOQ, respectively.

4.2.5. Analysis of fructosazine (FR) and deoxyfructosazine (DOFR)

Identification and quantitation of FR and DOFR was performed as described in detail by Hrynets et al. (2016). The HPLC apparatus and column were the same as described above for α-DCs. The detection wavelength was 275 nm, injection volume 10 μL and a flow rate 0.4 mL/min. The binary mobile phase containing 0.1% aqueous formic acid (A) and 100% methanol (B) was used and the gradient elution was programmed for 30 min. For identification, the retention times of the standard FR and DOFR were compared to the sample peaks followed by MS analyses. The MS identification of FR and DOFR was performed as described in Hrynets et al. (2016). The five-point calibration curves were constructed for the quantitation of FR and DOFR with $R^2 \geq 0.99$. For FR, the LODs and LOQs were 1.40 ± 0.00 and 4.24 ± 0.03 μg/mL, respectively and for DOFR were 0.05 ± 0.01 and 0.15 ± 0.08 μg/mL, respectively.

4.2.6. Analysis of heterocyclic compounds

HPLC analyses of 4-MEI, THI and HMF in GlcN-amino acid reaction mixtures were performed as described by Dhungel et al. (2018). The chromatographic separations were performed on the similar HPLC apparatus as described above for α-DCs. The mobile phase was a binary mixture of solvents (A) 0.05 M potassium dihydrogen phosphate and 0.005 M sodium octane sulfonate, adjusted to a pH of 3.0 ± 0.01 and (B) 100% methanol. To analyse 4-MEI, the mobile phase consisted of 85:15 buffer:methanol with detection at 215 nm, whereas the mobile phase used for the determination of THI and HMF was 92.5:7.5 buffer:methanol with a detection at 285 nm. The flow rate was maintained of 0.5 mL/min with an injection volume of 10 µL.

For the identification of 4-MEI, THI and HMF, MS analyses were conducted. The detection was performed on a QTRAP 4000 mass spectrometer (AB Sciex, ON, Canada) equipped with electrospray ionization (ESI) source. The LC procedures were performed exactly as described above. Data were collected in positive ionization mode with selected reaction monitoring (SRM) as described in Dhungel et al. (2018). The SRM settings were optimized using standard solutions. The acquisition was performed at spray voltage 3000 V, capillary temperature 350ºC, sheath and aux gas pressure 30 and 10, respectively.

HMF and THI were quantified by using the external five-point standard curves with a good linearity showing with regression coefficients of 0.998 and 0.997, respectively. The LODs were 1.4 ± 0.06 and 0.7 ± 0.03 µg/mL for THI and HMF, respectively. The LOQs were 4.2 ± 0.2 (THI) and 2.1 ± 0.17 µg/mL (HMF).

4.2.7. Statistical analyses

The data were analyzed statistically using one-way ANOVA with SPSS (IBM SPSS Statistics, Version 23, Armonk, NY, USA). The post hoc multiple-comparisons test between group means was performed using Tukey's HSD test ($p < 0.05$). All results were expressed as mean ± standard

error (SE) of the mean. A principal component analysis (PCA) was conducted using OriginPro 8.6 (OriginLab Corporation, MA, USA, 2012).

4.3. Results and Discussion

4.3.1. Effect of different amino acids on the formation of α-dicarbonyl compounds (α-DCs)

In this study, there are two main α-DCs sources: the 1,2-enolization of the Heyns compound GlcN (Figure 4.1), and the nucleophilic attack of the amino acids on the carbonyl of the GlcN molecule (Figure 4.2). The latter results in the production of the Amadori compound that, subsequently, through the 2,3- or 1,2-enolization rearrangements followed by a nucleophilic attack of water (Figure 4.2) yields 1-DG and 3-DG, respectively. These will then go trough retro-aldolization reaction forming the short chain α-DCs like GO, MGO and DA (Figure 4.1). The oxidation of GlcN to glucosone (Figure 4.1) is also another source of α-DCs. α-DCs are important precursors of aroma and browning compounds. For instance, 1- or 3-deoxyosones, or their fragmentation products like GO, DA (2,3-butanedione), or MGO (2-oxopropanal) provide the α-DC reactant for the Strecker degradation which is responsible for the formation of the Strecker aldehyde and the amino ketones. The latter are responsible for the production of volatile pyrazines associated with the production of aromatic compounds.

After derivatization of the major α-DCs to the respective quinoxalines its typical HPLC chromatogram is shown in Figure 4.3. To verify the identification of these α-DCs, the samples were also subjected to the LC-MS/MS analyses and the results are shown in Appendix K. Under the conditions of this study, the retention times were 21.9 min for G, 32.3 min for 3-DG, 63.1 min for GO, 76.3 min for MGO and 85.6 min for DA. Most likely the peak eluting at 33.2 min in GlcN-amino acids reaction system is 1-DG.

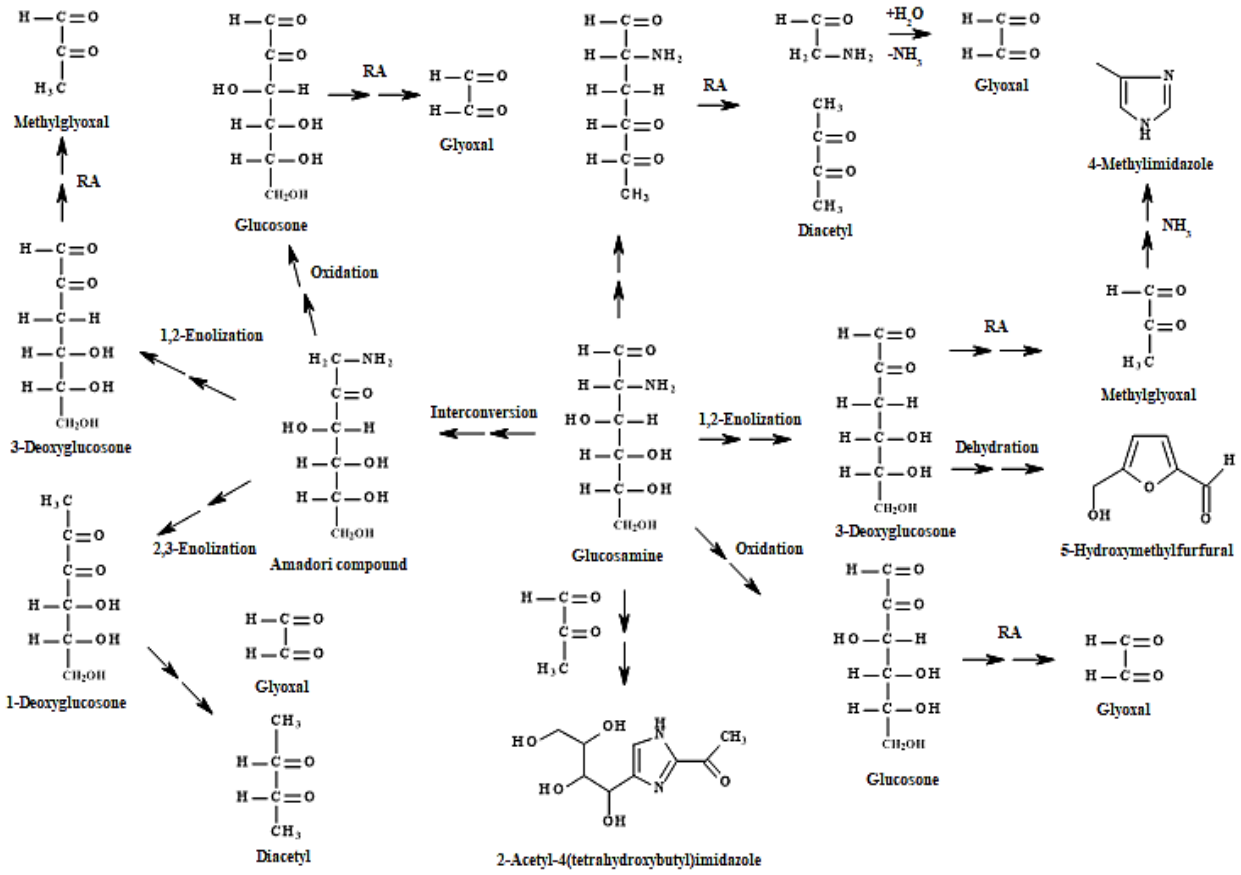


Figure 4. 1. General scheme of the pathways involved in glucosamine degradation. RA – Retro-aldolization.

Among the α-DC, G is known to be a reductone compound and a radical scavenging compound (Kanzler, Haase, & Kroh, 2014). Glucosone can go through a C-2/C-3 retro-aldolization reaction forming GO and erythrose, and as it has been reported (Dhungel et al. 2018) that the redox environment can affect it consumption to polymeric compounds like melanoidins. For instance, a more oxidative environment would consume G forming more browning compounds. The greatest ($p < 0.05$) amount of G was found in the GlcN-Met treatment at a level of 171.2 ± 5.7 mg/L, while the lowest concentration was 24.0 ± 1.1 mg/L in GlcN-Pro (Table 4.1). This indicates a protective effect of this non-polar and sulfur containing amino acids on glucosone degradation. Indeed, methionine residues are known for their greater susceptibility to oxidation

compared to the other amino acids (Kim et al., 2014). Therefore, the greatest amount of glucosone in GlcN-Met caramels might be associated with methionine's ability to be oxidized protecting G. Histidine is also an amino acid that is susceptible to oxidation and (Li et al., 1995), as a matter of fact, generated the second greatest level (145.8 mg/L) of G when was incubated with GlcN, supporting prevoius consideration. Cysteine is another amino acid that can be oxidized in proteins (Kim et al., 2014), however in this case, the amount of G was 48.1 ± 7.5 mg/L. The reason for this phenomenon is not immediately clear and requires more investigation.

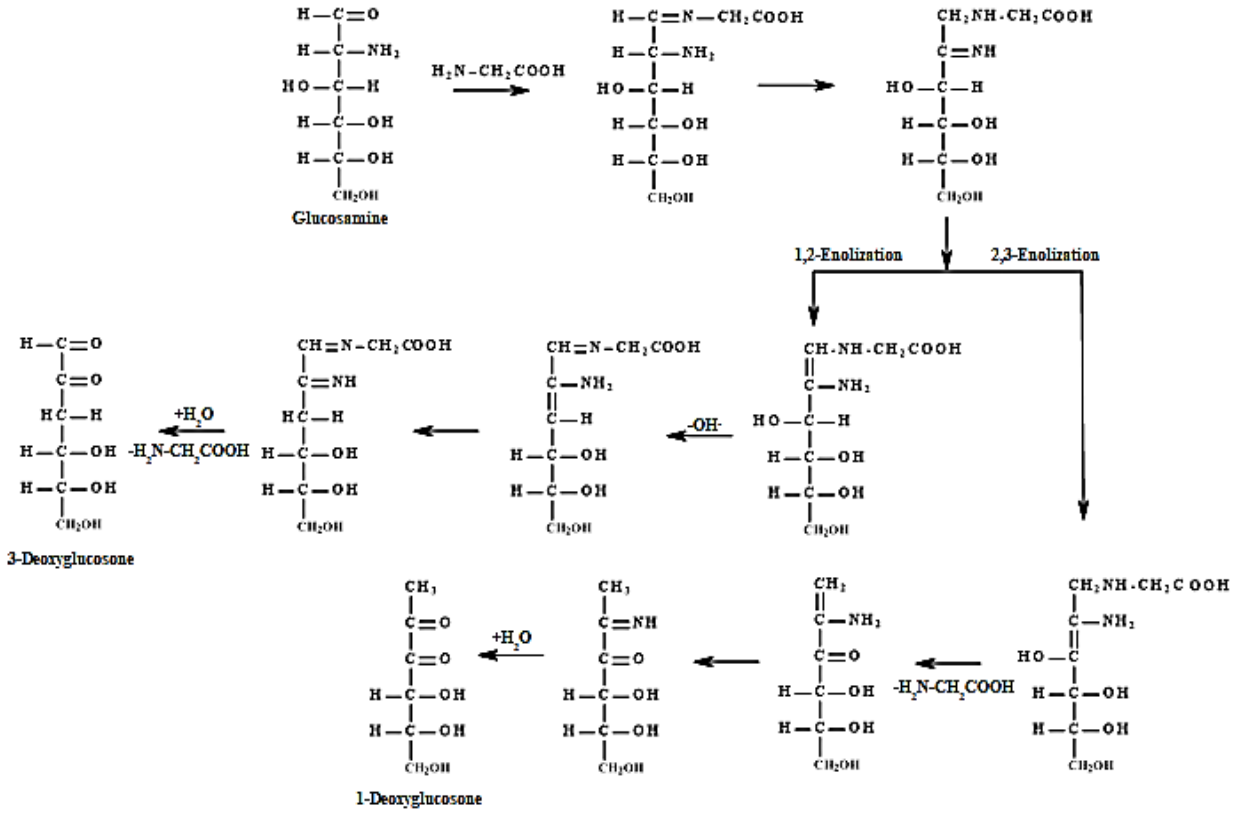


Figure 4. 2. Formation of 3- and 1-deoxyglucosone in glucosamine-glycine reaction mixtures.

3-DG is formed through the 1,2-enolization of GlcN or through the 2,3-enolization of Amadori compound (Figure 4.1, 4.2). The latter will occur if carbonyl group of GlcN reacts with

an amino acid. 3-DG can also go through a C-3/C-4 retroaldolization forming MGO and glyceraldehyde (Hrynets et al., 2015). 3-DG can also form HMF upon double dehydration and cyclization (Perez Locas & Yaylayan, 2008).

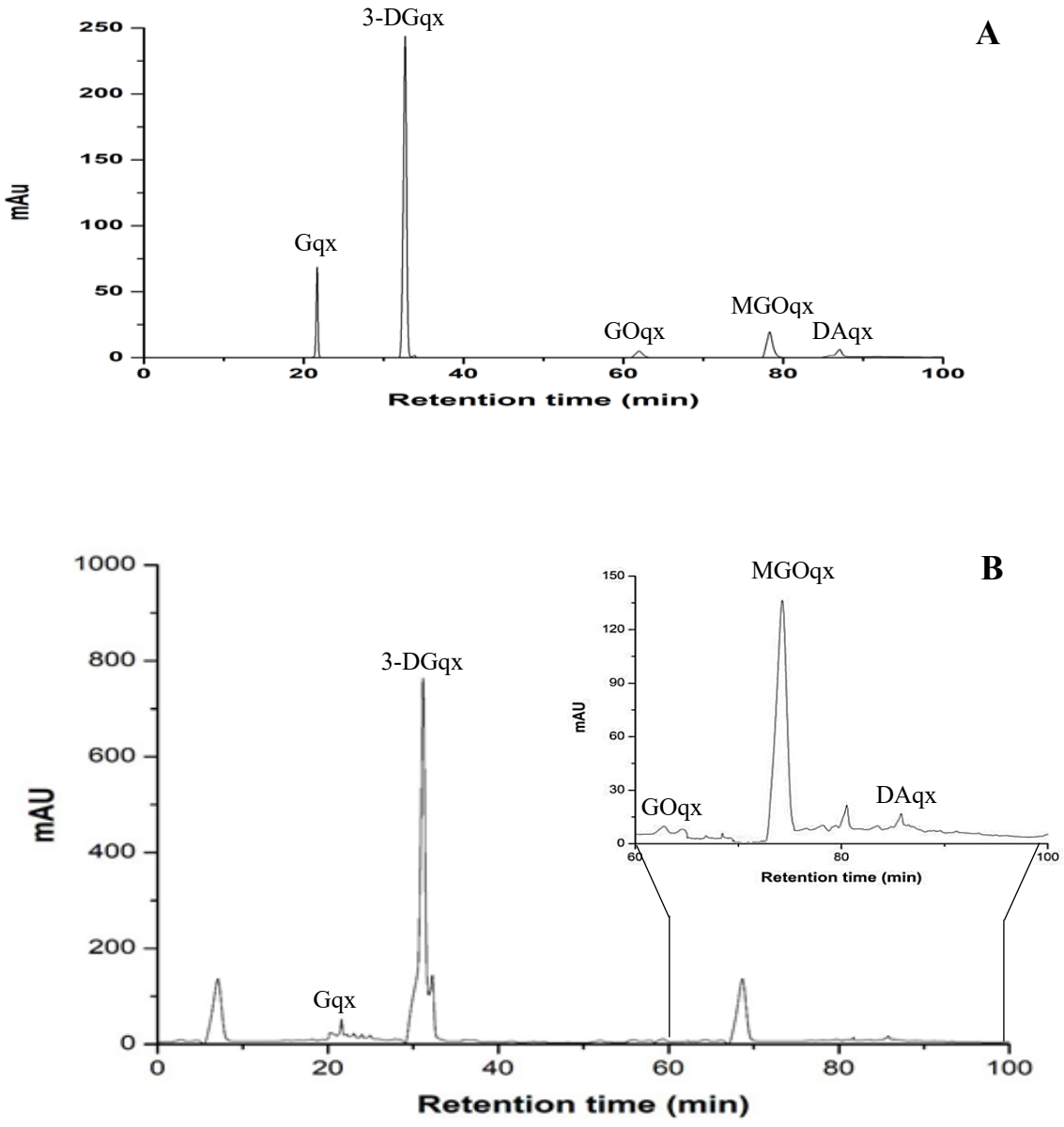


Figure 4. 3. HPLC analyses of α-DCs (in the form of quinoxaline derivatives). (A) Chromatogram of standard quinoxaline mixtures of glucosone (G), 3-deoxyglucosone (3-DG), glyoxal (GO), methylglyoxal (MGO) and diacetyl (DA) absorbed at 314 nm and (B) Representative HPLC chromatogram of α-DCs in GlcN-Gly mixtures incubated at 70°C for 12 h and derivatized with OPD, absorbed at 314 nm. Inset in (B) shows a zoomed-in view of the peaks eluted at 60-100 min.

Interestingly, Hrynets et al. (2016) has demostrated that this long chain α-DC possesses antimicrobial activity against heat resistant *E. coli* AW 1.7 at a concentration of ~1 g/L. Hence, treatment that produces more than 1 g/L of 3-DG could result in a caramel solution with antimicrobial activity. The greatest concentration of 3-DG of 1557 ± 14 mg/L was found in GlcN-Ser combination and was the least, 432 ± 24 mg/L, in GlcN-Thr (Table 4.1). Interestingly, the GlcN-Ser combination resulting in concentration well above 1 g/L, which could potentially give antimicrobial activity. However, further research is needed to prove these hypothesis.

The above-mentioned pattern seems to be mirrored also for GO and MGO except for serine which produced higher amount of GO. As mentioned before, G can undergo a retro-aldolization producing GO. The least amount of GO was found in the GlcN-His (4.5 ± 0.8 mg/L), GlcN-Cys (4.7 ± 0.7 mg/L) and GlcN-Val (4.7 ± 0.1 mg/L) treaments, while the greatest amount was found in the GlcN-Ser (14.1 ± 0.7 mg/L) and GlcN-Leu (13.2 ± 0.7 mg/L) (Table 4.1). MGO is the product of retro-aldolization of 3-DG (Hryntes et al., 2016). Table 4.1 shows that the greatest amount of MGO is formed in GlcN-Gly (57.7 ± 2.2 mg/L) and least in the GlcN-Lys (2.0 ± 0.1 mg/L). There was also a significant difference ($p < 0.05$) between polar and non-polar amino acids in the formation of MGO where the concentration of MGO generated by the GlcN-non-polar amino acids (20.2 ± 7.3 mg/L) was greater than that of the GlcN-Polar amino acid (4.3 ± 0.6 mg/L).

4.3.2. Effect of different amino acids on formation of diacetyl (butterscotch aromatic compound)

It has been suggested that DA forms by retroaldolization of 1,4-dideoxyglucosone during caramelization and the Maillard reaction. 1,4-Dideoxyglucosone is the dehydration product of 1-DG (Hollnagel & Kroh, 1998). However, Betti et al. (2018, under review) has recently proposed a new mechanism for the formation of DA involving a double dehydration of GlcN followed by retro-aldolization reaction (Figure 4.1). Table 4.1 shows that the GlcN control (GlcN incubated

alone) generated 18.5 mg/L of diacetyl that agrees to the findings of Dhungel et al. (2018). Among the different combinations, the treatment GlcN-Gly generated the greatest ($p < 0.05$) amount of DA of 32.0 ± 0.9 mg/L, while GlcN-Thr yielded the least ($p < 0.05$) of 4.8 ± 1.0 mg/L. Overall, only the GlcN-Gly combination resulted in an increased amount of the butterscotch aroma compound compared to the other treatments, and except for two combinations (GlcN-Ala and GlcN-His) they produced less ($p < 0.05$) DA compared to the GlcN control. Hence, the addition of glycine to a GlcN solution promotes the formation of this aromatic compound. The aroma threshold for DA depends on the type of food matrix and is reported in the range of 0.001 to 0.550 ppm with lowest threshold in cheese (Smit et al., 2005). For all the combination treatments tested in this study, the diacetyl levels were well above the threshold. As mentioned in the introduction, having a "mother" caramel solution rich in DA might be positive for a practical point of view, as appropriate dilution can be used for specific food application. The solid content of this caramel solution would be around 15%, and normally caramel in food applications like soft drink, yellow rice wine, soy sauce, seasonings and bakery foods are used in the range of 0.05-5%, and therefore this would bring DA concentration to a range of 0.1-10.7 mg/L.

Table 4. 1. Concentration (mg/L) of α-dicarbonyl compounds in glucosamine (GlcN) control and GlcN-amino acid reaction mixtures incubated at 70°C for 12 h.[a]

	G	3-DG	Total long chain α-DCs	GO	MGO	DA	Total short α-DCs
Treatment							
GlcN control	72.5 ± 2de	665 ± 23^{d}	737 ± 12^{d}	8.9 ± 0.4^{bc}	4.8 ± 0.4^{de}	18.5 ± 0.8^{b}	32.4 ± 0.3^{c}
GlcN-Polar amino acid							
GlcN-Lys	63.4 ± 4^{ef}	1051 ± 47^{b}	1114 ± 30^{b}	8.6 ± 0.1^{c}	2.0 ± 0.1^{f}	13.0 ± 0.6^{de}	23.7 ± 0.4^{ef}
GlcN-His	146 ± 3^{b}	466 ± 25^{f}	612 ± 13^{fg}	4.5 ± 0.8^{e}	5.0 ±0.1^{de}	16.2 ± 1.3^{bc}	25.8 ± 1.1^{de}
GlcN-Ser	49 ± 1^{g}	1557 ± 14^{a}	1606 ± 7^{a}	14.1 ± 0.7^{a}	2.4 ± 0.4^{f}	5.7 ± 0.7^{g}	22.2 ± 0.4^{efg}
GlcN-Thr	113 ± 1^{c}	432 ± 24^{f}	545 ± 15^{g}	6.3 ± 0.6^{d}	4.9 ± 0.3^{de}	4.8 ± 1.0^{g}	4.8 ± 0.6^{i}
GlcN- Arg	145 ± 6^{b}	578 ± 34^{e}	722 ± 17de	5.5 ± 0.1^{de}	2.4 ± 0.4^{f}	9.7 ± 0.6^{f}	9.7 ± 0.3^{h}
GlcN-Cys	48.1 ± 7^{g}	927 ± 2^{c}	975 ± 4^{c}	4.7 ± 0.3^{de}	9.3 ± 0.1^{b}	4.6 ± 0.5^{g}	18.6 ± 0.3^{g}
GlcN-Non-polar amino acid							
GlcN-Val	15.2 ± 1^{h}	736 ± 38^{d}	751 ± 22^{d}	4.7 ± 0.1^{de}	9.0 ± 0.3^{bc}	6.1 ± 0.4^{g}	19.8 ± 0.4^{fg}
GlcN-Leu	54.5 ± 1^{fg}	926 ± 22^{c}	980 ± 13^{c}	13.2 ± 0.7^{a}	8.8 ± 0.3^{bc}	14.9 ± 0.9^{cd}	36.9 ± 0.3^{b}
GlcN-Pro	24.0 ± 1^{h}	715 ± 20^{d}	739 ± 12^{d}	10.5 ± 0.9^{b}	3.1 ± 0.4^{ef}	10.6 ± 0.6^{ef}	10.6 ± 0.4^{h}
GlcN-Met	171 ± 5^{a}	475 ± 23^{f}	646 ± 10^{ef}	6.0 ± 0.2^{de}	6.9 ±0.6^{cd}	13.0 ± 0.8^{de}	25.9 ± 0.8^{de}
GlcN-Ala	142 ± 2^{b}	496 ± 41^{ef}	638 ± 23^{f}	6.1 ± 0.2^{de}	5.7 ± 0.4^{d}	17.2 ± 1.0^{bc}	29.0 ± 0.8^{cd}
GlcN-Gly	82.7 ± 5^{d}	855 ± 26^{c}	937 ± 14^{c}	5.4 ± 0.1^{de}	57.7 ± 2.2^{a}	32.0 ± 0.9^{a}	95.1 ± 2.1^{a}
Source of variation				***p-value***			
Treatment	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

The results are expressed as mean (n = 3) ± SE. [a]Means within the same column with no common superscript differ significantly ($p < 0.05$).

4.3.3. Effect of different amino acids on formation of FR and DOFR

2,5-Bis(D-*arabino*-tetrahydroxybutyl) pyrazine (FR) and 2-(D-*arabino*-tetrahydroxybutyl)-5-(D-*erythro*-2,3,4-trihydroxybutyl) pyrazine (DOFR) are the most abundant non-volatile pyrazines formed during GlcN non-enzymatic browning. Apart from their potential as pharmacological agents, they are also used as a flavouring agent in food industry (Tsuchida et al., 1990). In this study, however, several hydroxylalkyl pyrazine analogues may be produced from the following reactions: i) self-condensation of two molecules of GlcN primarily forming 2,5-FR and 2,5-DOFR; ii) condensation of the Heyns compound GlcN with the Amadori compound (fructosamine) yielding the 2,6-FR and 2,6-DOFR and iii) condensation reaction between GlcN, NH_3 and 3-DG or G forming 2,5- and 2,6-FR (Hrynets et al. 2015). Since 2,5- and 2,6-polyhydroxylalkyl pyrazines are isomers and possess the same polarity, they are very difficult to be separated under the chromatographic conditions used in our study. Therefore, in this study we indicate FR and DOFR as mixture of 2,5 and 2,6 positional isomers. The representative HPLC chromatogram and MS/MS spectra of identification of FR and DOFR in GlcN-Gly mixture are shown in Appendix L & M. Results reported in Table 4.2 showed the greatest concentration of polyhydroxylalkyl pyrazines (FR and DOFR) in GlcN-Gly model system. Glycine, a non-polar amino acid, when mixed with 15% GlcN generated higher amount of FR (19.0 ± 1.7 g/L) and DOFR (105.0 ± 3.3 g/L), while GlcN-Ala yielded the least amount of FR (1.0 ± 0.0 g/L) and DOFR (3.1 ± 0.0 g/L) among all combinations. The amount of FR and DOFR in GlcN-Gly was approximately 5 times greater than GlcN control caramels. However, not all the GlcN-amino model systems showed an increase in concentration of FR and DOFR as compared to GlcN control caramels. In summary, GlcN-Gly model system generated the greatest amount of FR along with the greatest concentration of diacetyl.

4.3.4. Effect of different amino acids on formation of HMF, THI and 4-MEI

Among the heterocyclic compounds formed during caramelization and Maillard reaction, HMF, THI and 4-MEI are the most widely studied due to their potential toxicity (Kim et al., 2013). Many studies indicated HMF possess mutagenic, cytotoxic and carcinogenic effects in human health (Janzowski et al., 2000). HMF is a classic indicator of browning and lengthy storage times in carbohydrate containing foods (Shinoda et al., 2005). HMF is a heterocyclic compound formed by the dehydration of GlcN via dehydration of 3-DG (Figure 4.1). The amount of HMF produced during non-enzymatic browning reaction is dependent upon temperature, water activity and pH (Ghaderi et al., 2015). Studies have shown the varying concentration of HMF in different foods. It is present in lower concentrations in meat products (below 0.9 mg/kg) and baby foods (i.e., less than 22 mg/kg) and in higher concentrations in caramel products (110 to 9500 mg/kg) (Murkovic and Pichler, 2006).

Appendix N & O show the HPLC and MS/MS identification of HMF in GlcN-Gly reaction models. Table 4.2 shows that in general, addition of amino acid to GlcN resulted in almost 9 times greater concentration of HMF compared to GlcN control. The greatest amount of HMF was found in GlcN-Arg models (58.7 ± 1.3 mg/L); while the least was in GlcN control (6.4 ± 0.1 mg/L). Göğüş et al. (1998) demonstrated that addition of amino acid/s in the glucose and fructose model system increased the rate of HMF accumulation, that agrees with our results. The same authors also studied the accumulation of HMF in a model system of glucose-fructose in combination with Arg, Gln and Pro; the results demonstrated a significantly greater ($p < 0.05$) accumulation of HMF in model systems containing Arg and Gln than in those containing Pro which is in agreement with our results, where GlcN-Arg models showed an increased accumulation (more than 2 times) of HMF than in GlcN-Pro models (23.7 ± 1.1 mg/L).

THI and 4-MEI are the molecules with a low molecular weight and are considered "caramel markers". These alkylimidazoles are prevalent in foods and beverages containing ammonia caramel colour (E150c) and ammonium sulphite caramel colour (E150d) (Guan et al., 2014). In previous studies the presence of THI and 4-MEI was detected not only in caramel colours but also in naturally brewed soy sauce, licorice, roasted coffee and coffee substitutes (Cunha et al., 2016). According to the European Commission, the legal limits of THI and 4-MEI is 10 and 200 mg/kg, respectively, for Class III caramel colour (Commission Regulation EU, 2012) whereas the limit of 4-MEI in Class III and IV caramel colour and THI in class III caramel colour in Canada is 200, 250 and 250 mg/kg respectively. THI is associated with the immunosuppressant activity in rodent models whereas 4-MEI has exhibited carcinogenic activity in animal experiments (Elsinghorst et al., 2013). Therefore, it is prudent to scrutinize and limit the amounts of HMF, THI and 4-MEI in caramel colourants. As pointed out in our previous study (Dhungel et al., 2018), THI is formed by the condensation of iminofructosamine, or iminoglucosamine with MGO in the presence of ammonia (Figure 4.1). 4-MEI is suggested to form by the ammonolysis of MGO (Moon & Shibamoto, 2010) (refer to Figure 4.1). In this study, THI was detected only in GlcN control samples whereas it was absent in GlcN-amino acids combinations (Table 4.2). 4-MEI was not detected in GlcN control samples as well as GlcN-amino acid samples. This suggests that the pathway for formation of these imidazoles may have taken a different route with possibility of formation of more melanoidins or other heterocyclic compounds in the final stage of the non-enzymatic browning reactions. For instance, MGO, one of the precursors of 4-MEI and THI, goes through a Strecker degradation yielding the Strecker aldehyde and the amino-ketones, in which latter can condense to form volatile pyrazines. The absence of these alkylimidazoles (i.e., THI and

4-MEI) is a desirable property of our caramels, since the GlcN-amino acid combinations used in our study enable us to produce the caramels free of these neo-formed contaminants.

Table 4. 2. Concentration of fructosazine (FR), deoxyfructosazine (DOFR), 5-hydroxymethyl-2-furfural (HMF) and 2-acetyl-(4)5-tetrahydroxylbutyl imidazole (THI) in glucosamine (GlcN) control and GlcN-amino acid reaction mixtures incubated at 70°C for 12 h.[a]

	FR (g/L)	DOFR (g/L)	Total FR and DOFR (g/L)	HMF (mg/L)	THI (mg/L)
Treatment					
GlcN control	4.1 ± 0.4^{d}	19.3 ± 0.5^{g}	23.5 ± 0.8^{f}	6.4 ± 0.1^{h}	2.7 ± 0.2
GlcN-Polar amino acid					
GlcN-Lys	10.2 ± 0.5^{c}	97.7 ± 1.9^{b}	107.9 ± 2.0^{b}	52.0 ± 1.5^{b}	
GlcN-His	2.3 ± 0.0^{efg}	6.8 ± 0.2^{hi}	9.1 ± 0.1^{ghi}	10.2 ± 0.2^{fg}	
GlcN-Ser	3.2 ± 0.2^{def}	25.2 ± 1.6^{f}	28.4 ± 1.5^{f}	12.7 ± 0.5^{ef}	ND
GlcN-Thr	1.4 ± 0.0^{fg}	4.4 ± 0.1^{hi}	5.8 ± 0.1^{hi}	9.8 ± 0.5^{fg}	
GlcN-Arg	10.1 ± 0.1^{c}	46.1 ± 1.1^{d}	56.2 ± 1.1^{d}	58.7 ± 1.3^{a}	
GlcN-Cys	13.8 ± 0.3^{b}	71.0 ± 0.4^{c}	84.8 ± 0.4^{c}	8.6 ± 0.1^{gh}	
GlcN-Non-polar amino acid					
GlcN-Val	1.5 ± 0.2^{fg}	9.7 ± 0.1^{h}	11.2 ± 0.9^{g}	13.2 ± 1.2^{e}	
GlcN-Leu	1.7 ± 0.1^{fg}	7.7 ± 0.2^{hi}	9.4 ± 0.3^{gh}	11.5 ± 0.5^{efg}	
GlcN-Pro	3.6 ± 0.4^{de}	34.9 ± 1.9^{e}	38.5 ± 1.5^{e}	23.7 ± 1.1^{d}	ND
GlcN-Met	1.1 ± 0.0^{g}	3.1 ± 0.2^{i}	4.2 ± 0.2^{i}	9.7 ± 0.1^{g}	
GlcN-Ala	1.0 ± 0.0^{g}	3.1 ± 0.0^{i}	4.2 ± 0.1^{i}	10.4 ± 0.1^{efg}	
GlcN-Gly	19.0 ± 1.7^{a}	105.0 ± 3.3^{a}	125.0 ± 0.9^{a}	30.5 ± 2.4^{c}	
Source of variation			***p-value***		
Treatment	<0.001	<0.001	<0.001	<0.001	-

The results are expressed as mean (n = 3) ± SE. [a]Means within the same column with no common superscript differ significantly ($p < 0.05$). ND = not detected.

4.3.5. Principal component analysis

The goal for the use of PCA was to better interpret the data set by visualizing the main sources of variability between the different GlcN-amino acid combinations. Figure 4.4 shows bi-dimensional representation of PC1 and PC2 scores for nine tested variables and GlcN caramels generated with different amino acids combinations. Appendix P & Q report principal component eigen values and coefficients of the loading (eigen vectors) for PC1 and PC2, respectively. The

PC1 and PC2 accounted for 37.37 and 25.07% of the total variance, respectively. As shown, GlcN-Lys and GlcN-Cys are placed on the positive side of PC1, whereas the GlcN-Arg and GlcN-Gly are located on the negative half of the PC2. The GlcN-Gly combination was very well discriminated by PC1 from the other combinations and from GlcN control. The combinations of GlcN-Lys, GlcN-Cys and GlcN-Arg were also distinguished from the other mixtures by PC1. The products of non-enzymatic browning that showed higher loadings on PC1 and thus enabling to differentiate the above-mentioned combinations were FR, DOFR, MGO, DA and HMF (Table S3). PC2 was able to distinguish GlcN-Ser combination as different from GlcN-Lec/Pro/Val/Thr/Ala/Met and GlcN control. Moving downward the PC2 axis there was a clear trend for the clusters between GlcN-Leu and GlcN-Pro on the positive half of the graph and the cluster GlcN-Ala, GlcN-His and GlcN-Met on the negative half of the PC2 axis. GlcN-amino acids mixtures found on PC2 were mainly distinguished from the GlcN-amino acids placed on PC1 by 3-DG and GO.

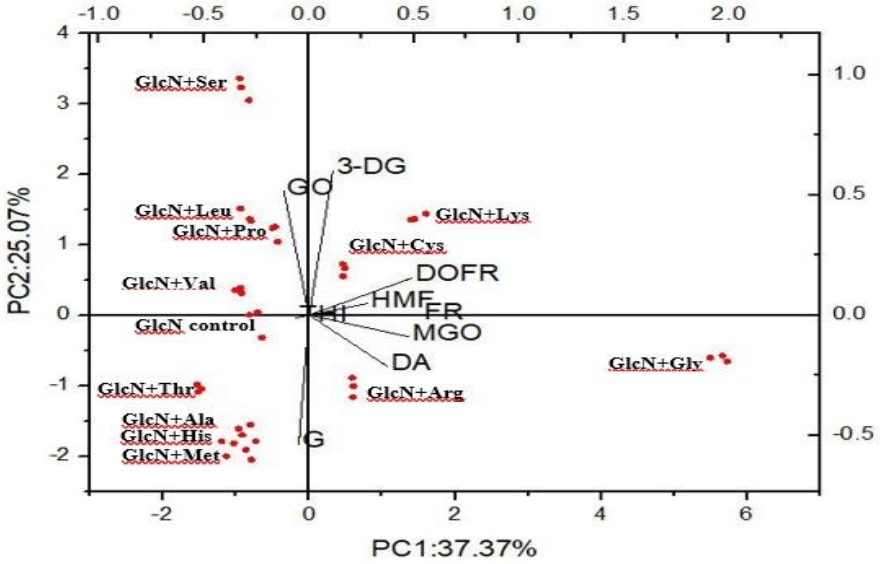


Figure 4. 4. Biplot showing two principal components (PC1 and PC2) in multivariate analysis.

4.4. Conclusion

In summary, this study showed that the addition of different amino acids can influence the chemical degradation of GlcN, yielding a "caramel" product with various chemical compositions. The pattern of pooling of the GlcN-amino acid caramels in the biplot indicates that GlcN-Ala, GlcN-His and GlcN-Met caramels can have similar compositions, whereas the alignment of GlcN-Gly caramel pool implies that it is unique among the other GlcN-amino acid caramels. This study demonstrated that the GlcN-Gly model system has the capacity to produce "caramel" solutions rich in diacetyl (butterscotch aroma) and FR+DOFR. Furthermore, the caramel components with toxicity are not produced when amino acids are added to GlcN. Gly is a relatively inexpensive amino acid and may be a viable option to produce economically a "caramel solution" with a butter scotch aroma. The influence of amino acid addition on other volatile aromatic compounds needs further investigation.

CHAPTER 5. Conclusions, implications and future research

This thesis provides the overview on the non-enzymatic browning of GlcN under vacuum and GlcN-amino acid model reaction mixtures. Up to now, the research on investigating the chemistry of GlcN caramel under sous-vide conditions was not performed. Although several studies have been conducted on the Maillard reaction between the sugar-amino acid model system (prevalently in glucose/sucrose-amino acid model), no research has been performed in glucosamine-amino acid mixtures. Overall, this research contributed knowledge to the following areas:

a) Chemistry of non-enzymatic browning under vacuum: This thesis provides a deeper understanding on the chemistry of GlcN caramel prepared under sous-vide conditions as compared to oxidative conditions. Diacetyl was found in greater concentrations in GlcN caramels generated under non-vacuum conditions, while caramels produced under vacuum contained more of bioactive fructosazine. The toxic 4-MEI was not detected in any of the six different caramels, while the THI amounts were well below toxicity levels. A new mechanism of THI production from GlcN was proposed.

b) Chemistry of GlcN-amino acid caramels: This study provides knowledge about the chemistry of GlcN-amino acid caramels compared to GlcN caramels;

c) Quantification of flavour compounds and bioactive compounds: Sous-vide is a popular approach to cook foods at a precise temperature in vacuum pouches which retains flavours. Therefore, this study was conducted under sous-vide condition to quantify the compounds responsible for generating flavour and aroma and their precursors. Diacetyl, a compound responsible for buttery notes and compounds with bioactivity (i.e. fructosazine

and deoxyfructosazine) was found in the greatest amount in GlcN-glycine. Potentially toxic alkylimidazoles were not present in any of the GlcN-amino acid caramels;

d) Quantification of potentially toxic compounds: Previous research have shown that industrially produced caramels colours contain potentially toxic compounds such as 4-MeI, THI and HMF. Therefore, quantification of aforementioned compounds was performed revealing that GlcN caramels (with or without amino acids) produced under moderate temperatures can be considered safe since they contain very low or none of these undesirable compounds.

Since the caramel produced in this study is rich in diacetyl, it can be diluted as needed and incorporated into bakery products, breakfast sausages, balsamic vinegar, dark ale, buttered and breaded meat products etc. Therefore, in future the GlcN-based caramels can be added to certain range of food products where buttery flavour and/or bioactivity is crucial.

Overall, this research demonstrated that glucosamine caramels show promise to fill a niche in the food and beverage industries. The caramelization conditions, either being under vacuum or in an oxidative environment, can greatly influence the characteristics of the final caramel. This means that different caramels can be generated to be used in specific food formulations. GlcN caramels are relatively inexpensive and uncomplicated to produce, rich in both flavourant diacetyl and bioactive fructosazine and deoxyfructosazine compounds. In addition, GlcN caramels have none or very low levels of undesired alkylimidazoles. GlcN in combination with amino acids gives the possibility to diversify the aroma of the caramels without producing a large concentration of undesired alkylimidazoles. In keeping with this, GlcN caramels can not only be used to impart colour to food product, but also can be incorporated to impart certain bioactivities.

From the studies completed in this thesis, several future projects of interest were identified:

a) Development of flavour and aroma lexicon of different GlcN caramels. Flavour and aroma profiling with trained panelists could be conducted for GlcN and GlcN-amino acid caramels;

b) Previous studies indicated FR and DOFR as bioactive compounds. Since, these pyrazines are produced in significant amounts (Chapter 4), it would be of great interest to verify the benefits of FR and DOFR in the studies involving animal models;

As mentioned before, a multinational beer company Heineken patented "colourless caramel" which stabilizes beer's shelf life. A recent study performed in our research group (Bhattacherjee et al., 2016) showed that fructosazine possess an antimicrobial activity against heat-resistant *Escherichia coli* AW 1.7. Therefore, antimicrobial and antioxidant activities of GlcN and GlcN-amino acids caramels could be tested alone or incorporated in a food matrix, like for instance, meat bars or dark ale beverages.

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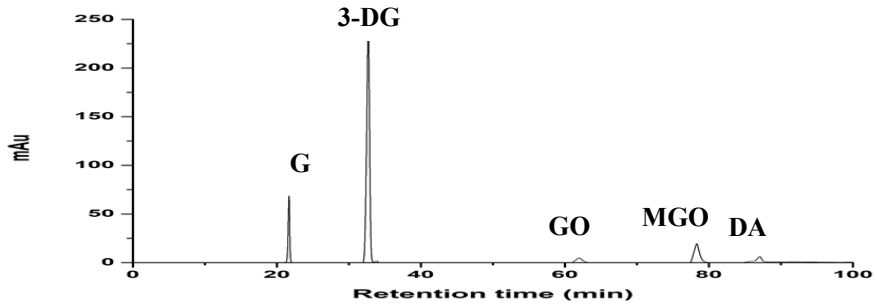
Appendix A. Changes in redness (*a**) and yellowness (*b**) during incubation of GlcN solutions at 50, 60 and 70°C under non-vacuum and vacuum conditions for 12 h.

		a*	***b****
Level of oxygen			
Non-vacuum		2.3^{b}	0.9^{b}
Vacuum		7.4^{a}	2.5^{a}
SEM		0.7	0.3
Temperature			
50°C		9.9^{a}	3.3^{a}
60°C		3.7^{b}	1.3^{b}
70°C		1.0^{c}	0.5^{c}
SEM		0.5	0.2
Interaction (Level of oxygen*Temperature)			
Non-vacuum	50°C	5.8^{c}	1.4^{c}
	60°C	0.6^{e}	0.9^{d}
	70°C	0.5^{f}	0.3^{f}
Vacuum	50°C	14.0^{a}	5.2^{a}
	60°C	6.7^{b}	1.7^{b}
	70°C	1.5^{d}	0.6^{e}
SEM		0.005	0.01
Sources of variation		***p-values***	
Treatment		<0.001	<0.001
Temperature		<0.001	<0.001
Interaction		<0.001	<0.001

[a–f]Means within the same column with no common superscript differ significantly ($p<0.05$).
SEM-pooled standard error of the means.

Appendix B. HPLC analyses of α-dicarbonyl compounds (in the form of quinoxaline derivatives). (I) Chromatograms of standard quinoxaline mixtures of glucosone (G), 3-deoxyglucosone (3-DG), glyoxal (GO), methylglyoxal (MGO) and diacetyl (DA) absorbed at 314 nm. (II) Representative HPLC chromatograms of α-dicarbonyl compounds in GlcN solutions incubated at 50, 60 and 70°C under non-vacuum and vacuum conditions for 12 h absorbed at 314 nm. Inset shows a zoomed-in view of the peaks eluted at 60-90 min. GlcN – glucosamine.

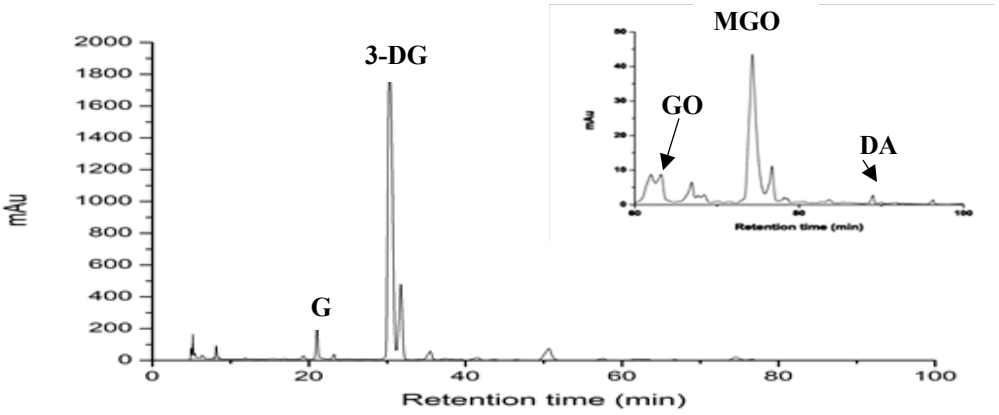
I. Standards



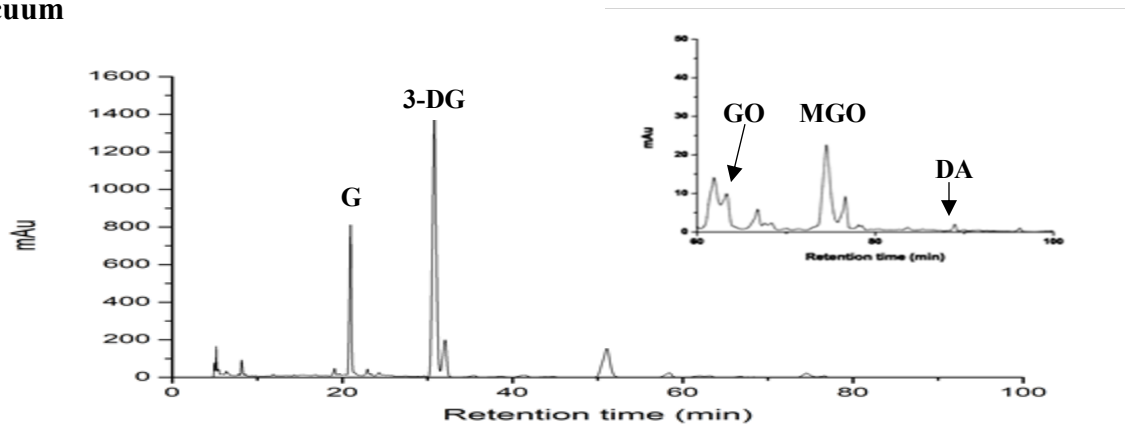
II. Treatments

50°C

Non-vacuum

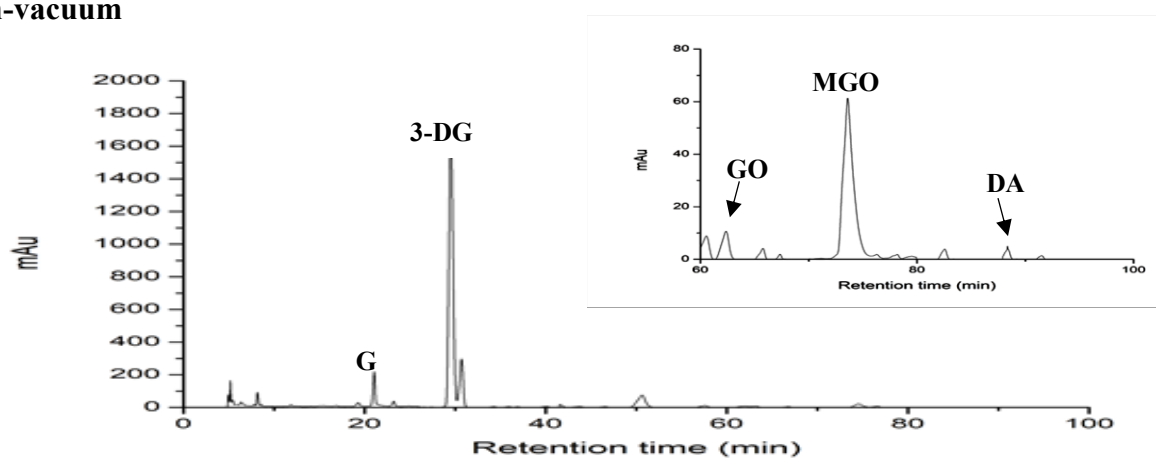


Vacuum

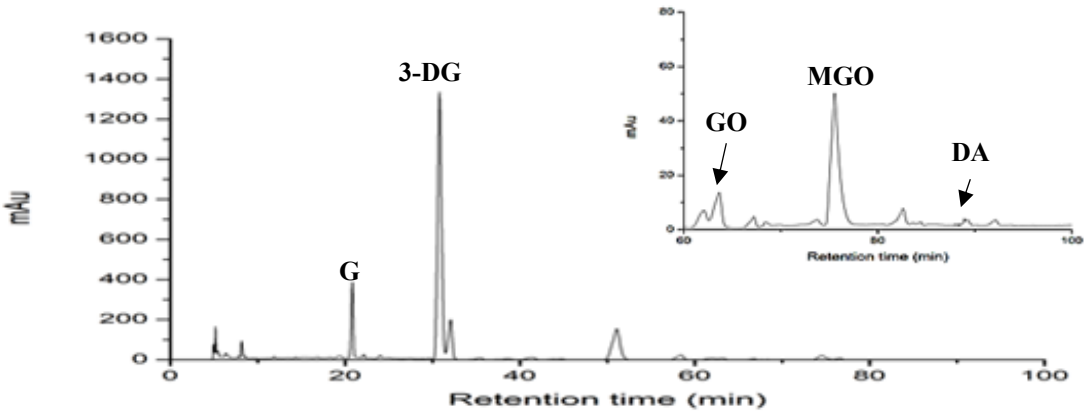


60°C

Non-vacuum

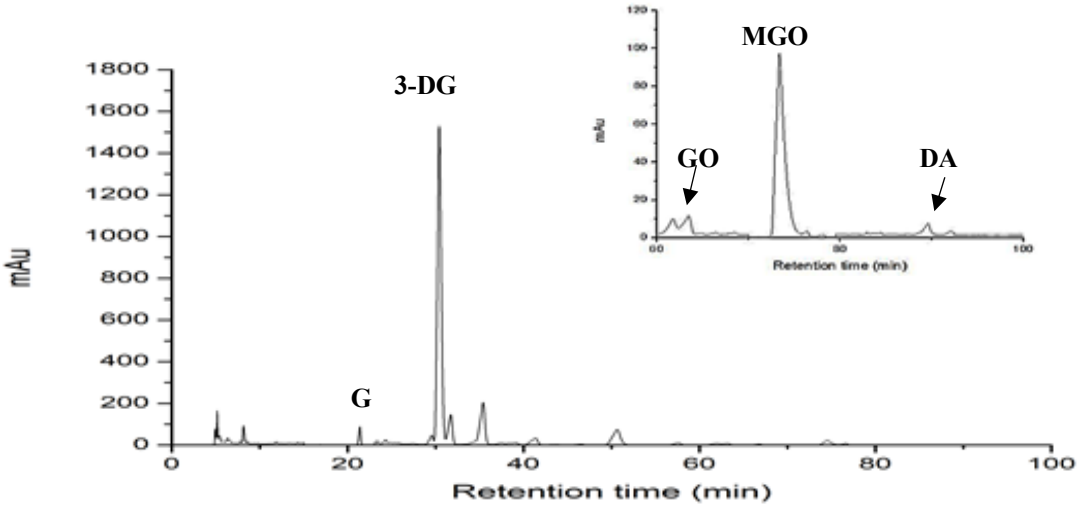


Vacuum

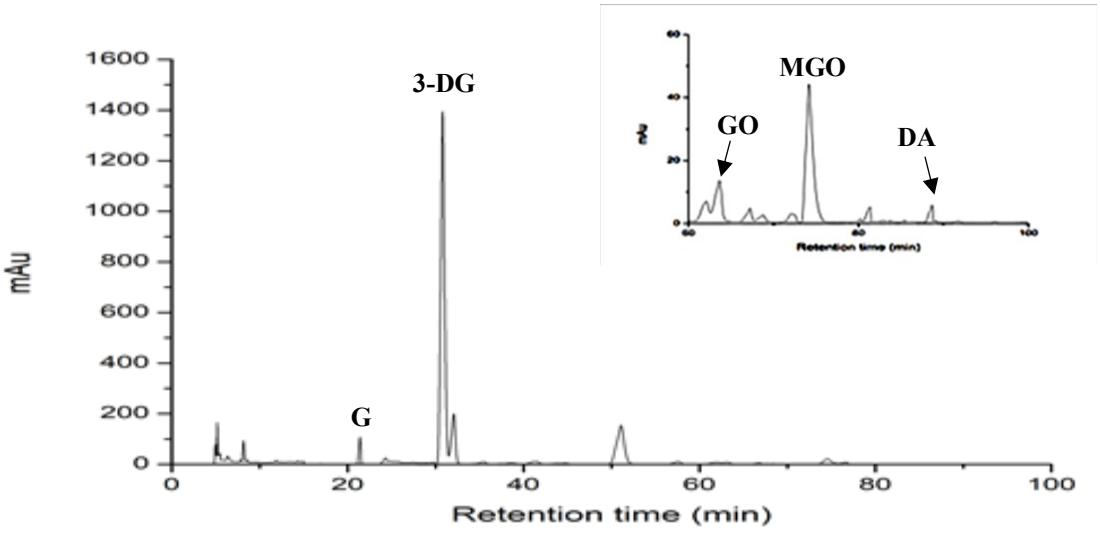


70°C

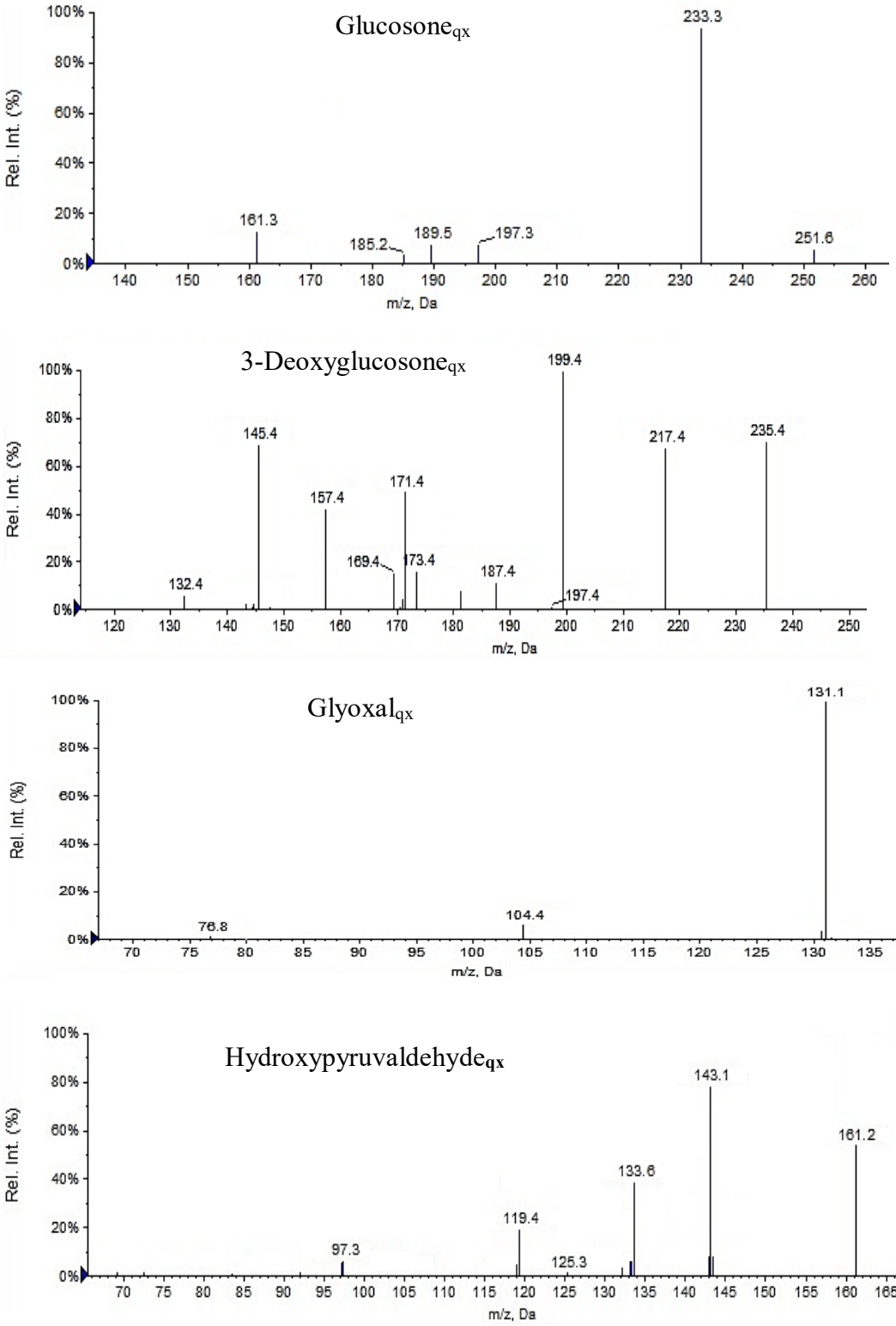
Non-vacuum



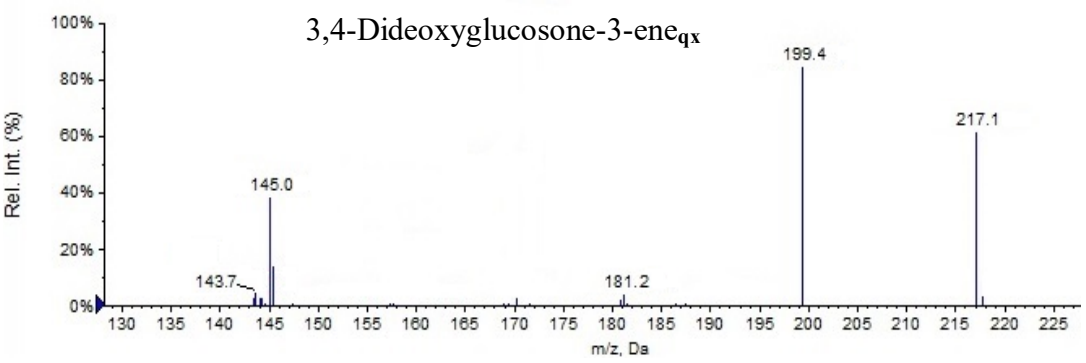
Vacuum



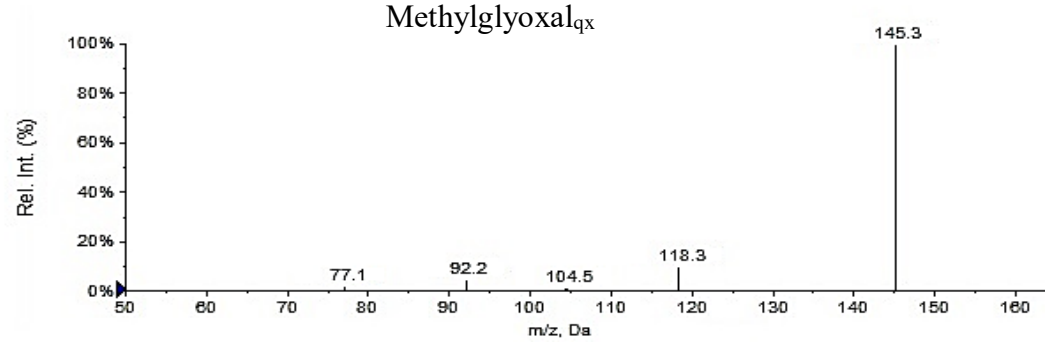
Appendix C. MS/MS spectra of quinoxaline α-dicarbonyl compounds produced during glucosamine caramelization: glucosone$_{qx}$; 3-deoxyglucosone$_{qx}$; glyoxal$_{qx}$; hydroxypyruvaldehyde$_{qx}$; 3,4-dideoxyglucosone-3-ene$_{qx}$; methylglyoxal$_{qx}$ and diacetyl$_{qx}$.



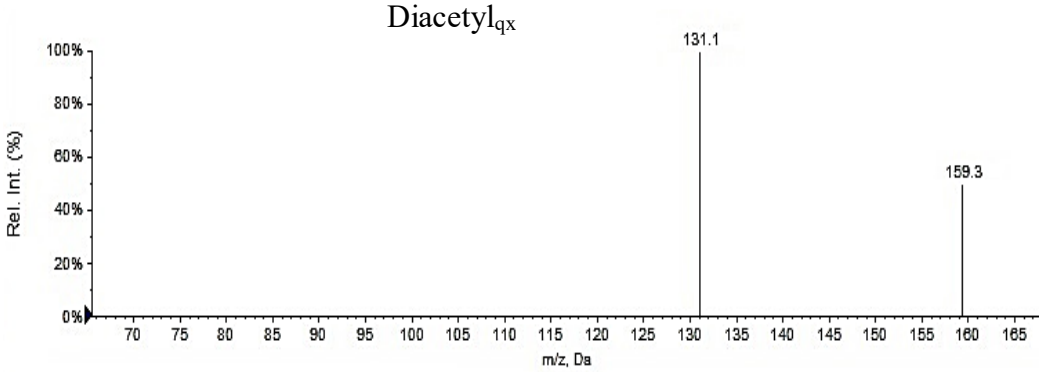
3,4-Dideoxyglucosone-3-ene$_{qx}$



Methylglyoxal$_{qx}$



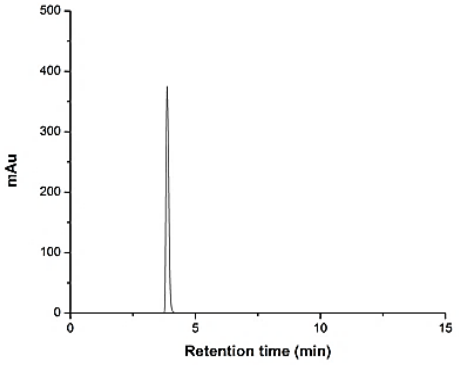
Diacetyl$_{qx}$



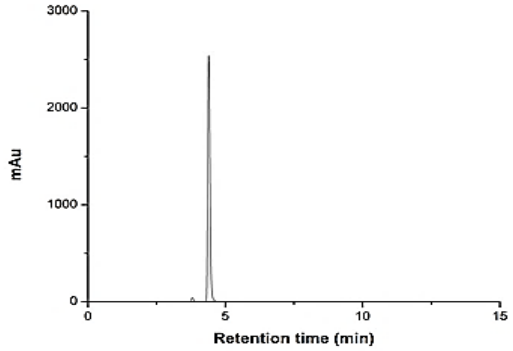
Appendix D. HPLC analyses of fructosazine (FR) and deoxyfructosazine (DOFR). (A) Chromatograms of FR and DOFR commercial standards absorbed at 275 nm. (B) Representative HPLC chromatograms of FR and DOFR in GlcN solutions incubated at 50, 60 and 70°C under non-vacuum and vacuum conditions for 12 h absorbed at 275 nm. GlcN – glucosamine.

A. Standards

Fructosazine (FR) standard

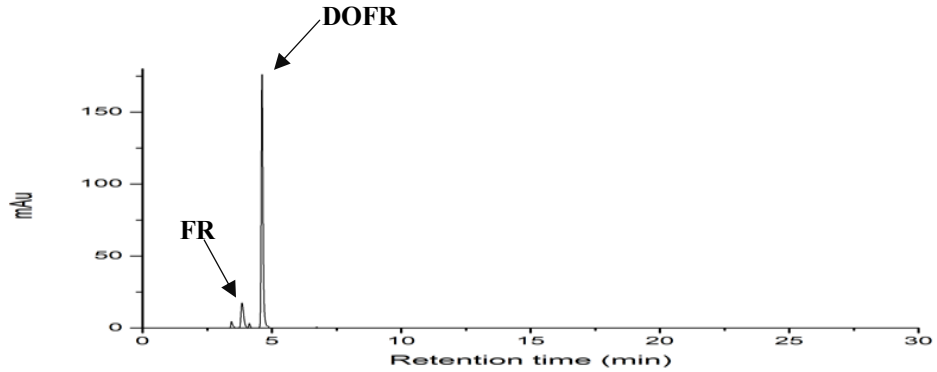


Deoxyfructosazine (DOFR) standard

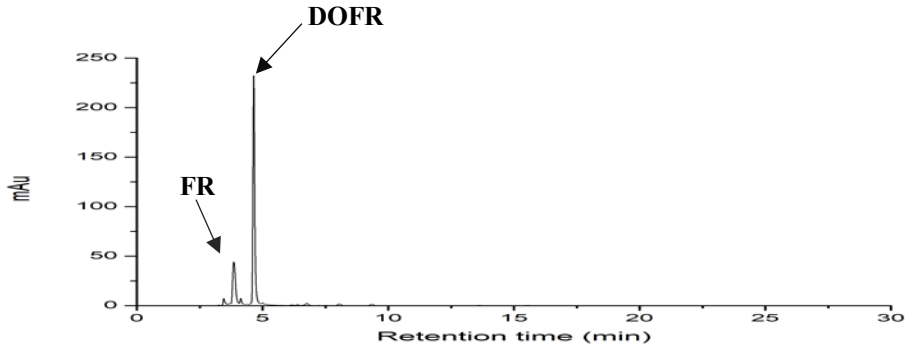


B. Treatments

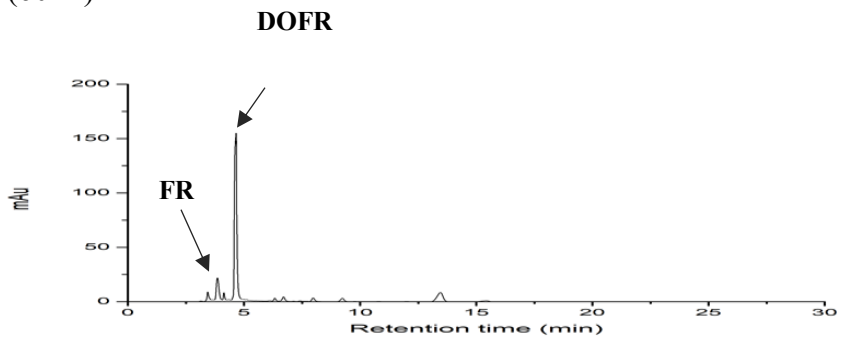
Non-vacuum (50°C)



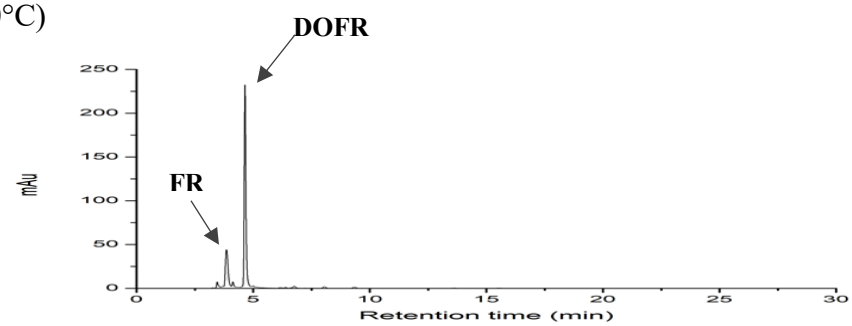
Vacuum (50°C)



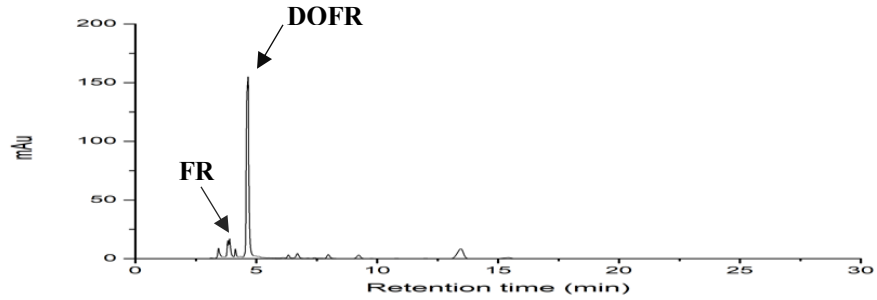
Non-vacuum (60°C)



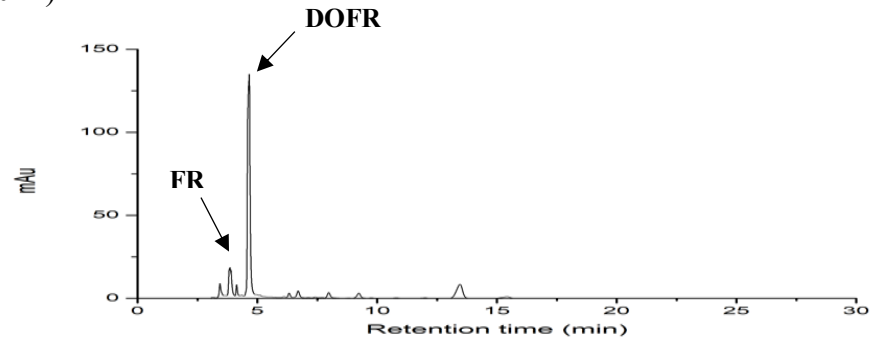
Vacuum (60°C)



Non-vacuum (70°C)

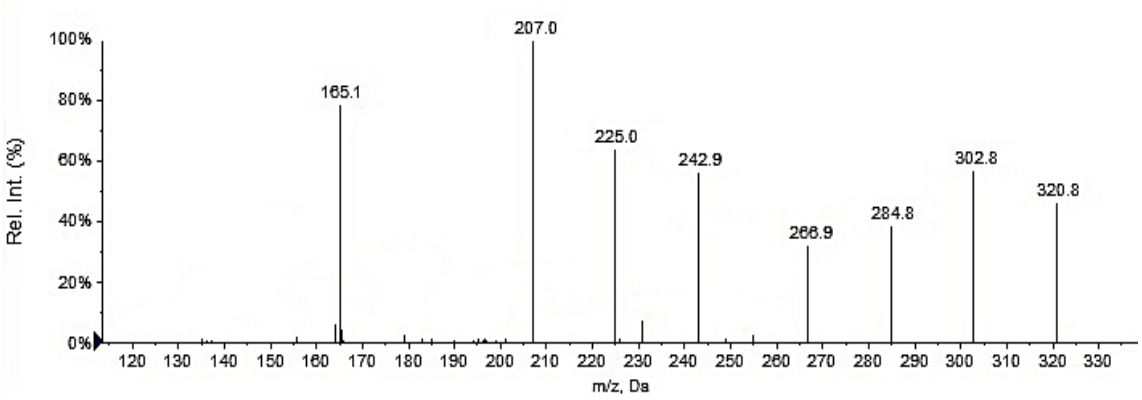


Vacuum (70°C)

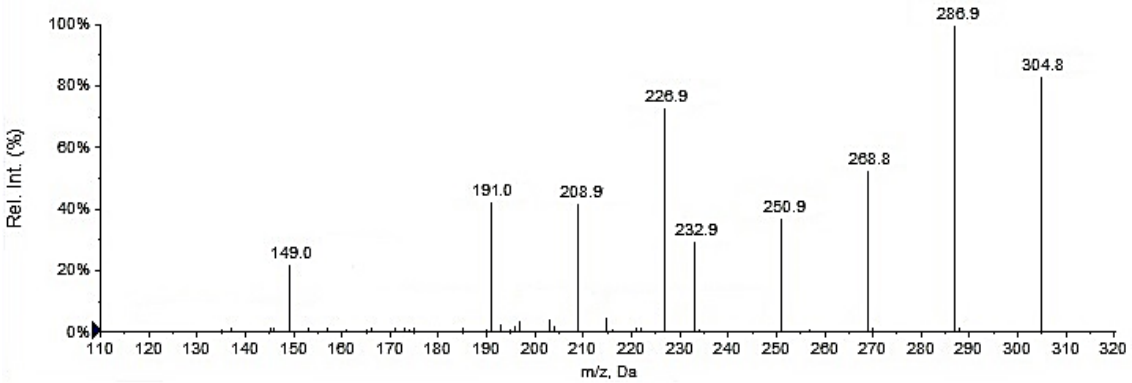


Appendix E. Representative MS/MS spectra of (A) fructosazine and (B) deoxyfructosazine identified in GlcN caramel solutions generated under vacuum. GlcN – glucosamine.

A.



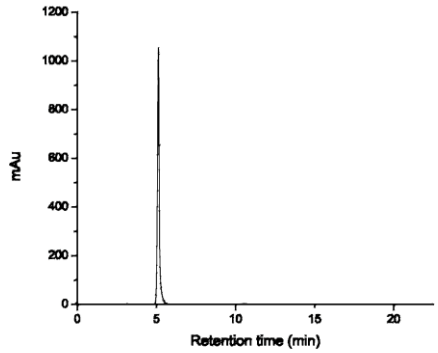
B.



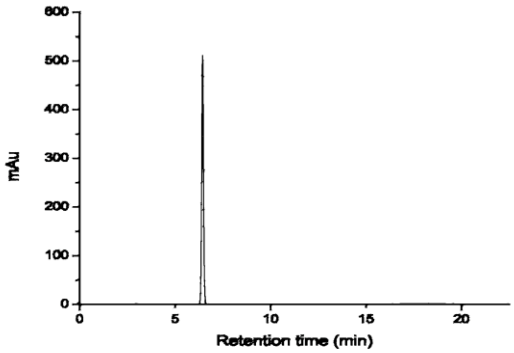
Appendix F. HPLC analyses of 2-acetyl-5-tetrahydroxylbutyl imidazole (THI) and 5-hydroxymethyl-2-furfural (HMF). (A) Chromatograms of THI and HMF standard solutions absorbed at 285 nm. (B) Representative HPLC chromatograms of HMF and THI in GlcN solutions incubated at 50, 60 and 70°C under non-vacuum and vacuum conditions for 12 h absorbed at 285 nm. GlcN – glucosamine.

A. Standards

THI standard

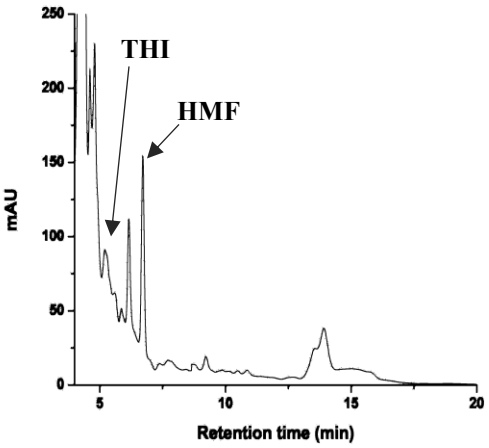


HMF standard

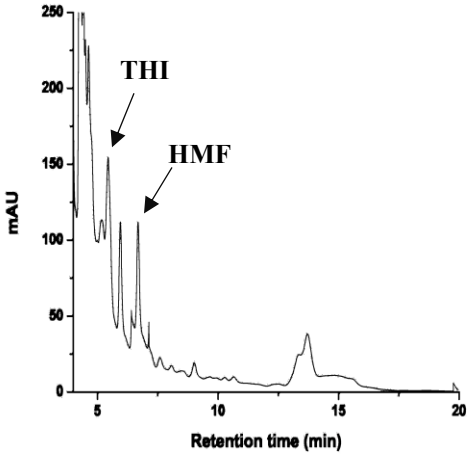


B. Treatments

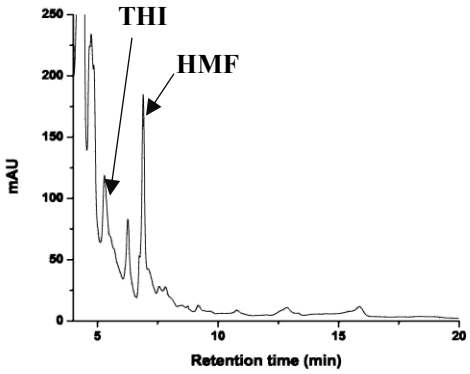
Non-vacuum (50°C)



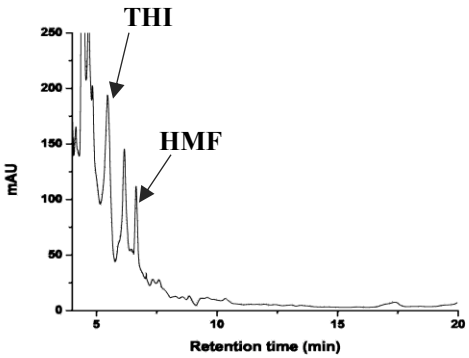
Vacuum (50°C)



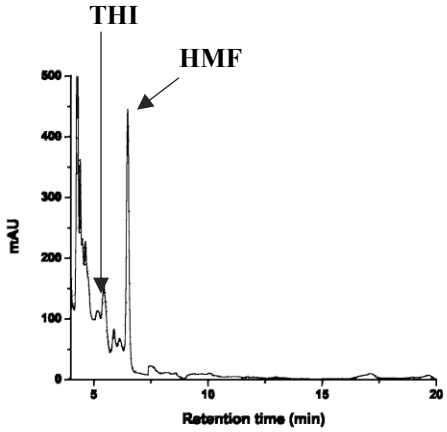
Non-vacuum (60°C)



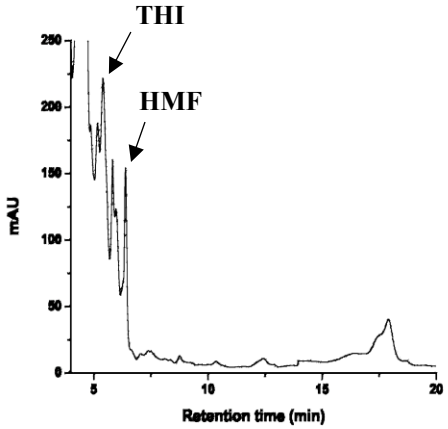
Vacuum (60°C)



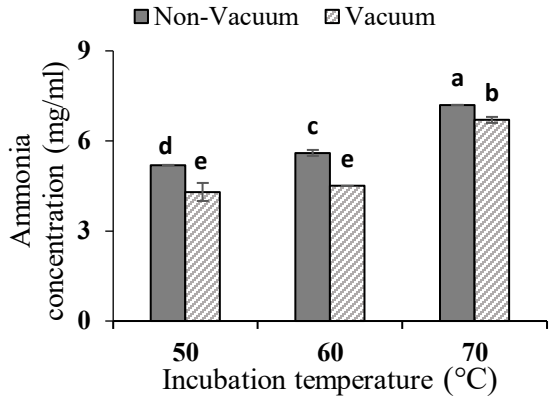
Non-vacuum (70°C)



Vacuum (70°C)



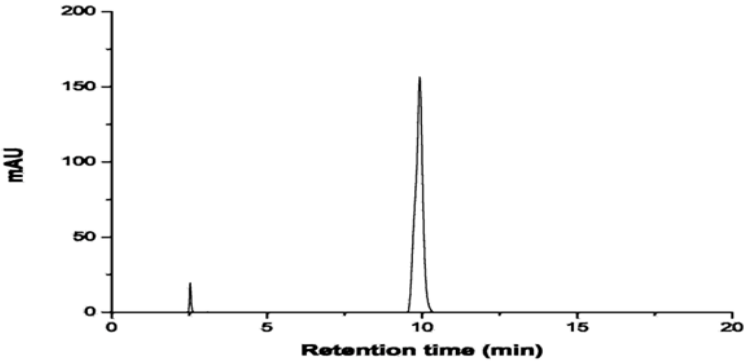
Appendix G. Concentration of ammonia in GlcN solutions generated under non-vacuum and vacuum conditions incubated at 50, 60 and 70°C for 12 h. The results are expressed as mean (n = 9) ± standard deviation. Different letters (a-f) represent statistical differences ($p < 0.05$). GlcN – glucosamine.



Appendix H. HPLC analyses of 4-methylimidazole (4-MEI). (A) Chromatogram of 4-MEI standard absorbed at 215 nm. (B) Representative HPLC chromatogram of 4-MEI in GlcN solutions incubated at 70°C under non-vacuum and vacuum conditions for 12 h absorbed at 215 nm. GlcN – glucosamine.

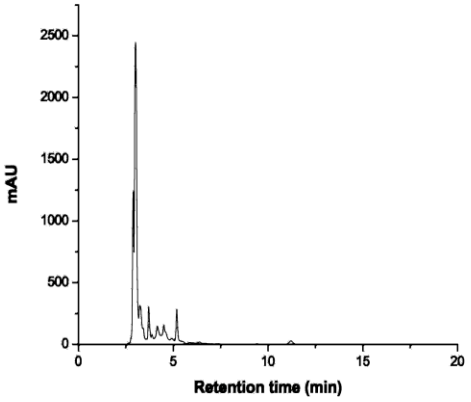
A.

4-Methylimidazole (4-MEI) standard

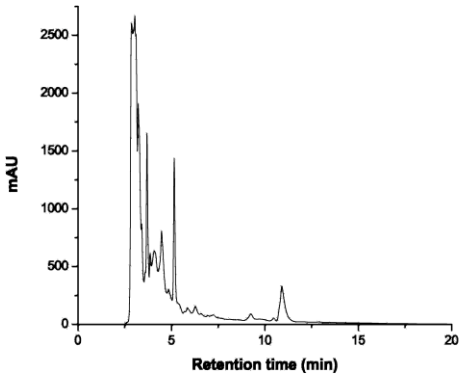


B.

Non-vacuum (70°C)



Vacuum (70°C)



Appendix I. Pearson's correlation coefficients among some of tested parameters during GlcN incubation at different conditions.

	pH	**HMF content**
pH	1	-0.78**
HMF content	-0.78**	1

	3-DG content	**HMF content**
3-DG content	1	0.77**
HMF content	0.77**	1

	Ammonia concentration	**THI content**
Ammonia concentration	1	0.57**
THI content	0.57**	1

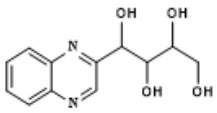
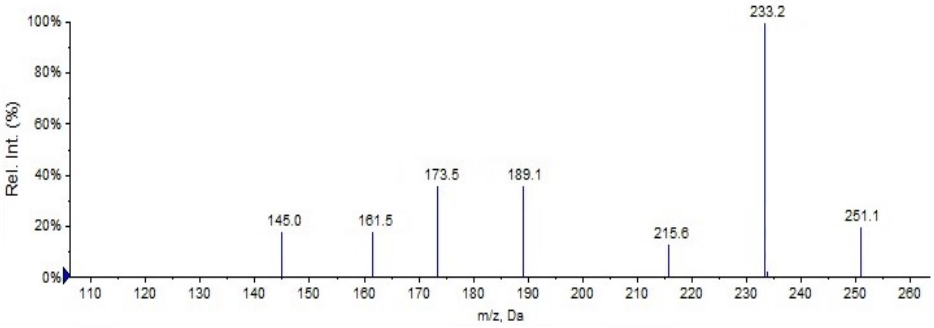
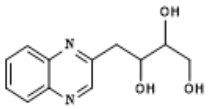
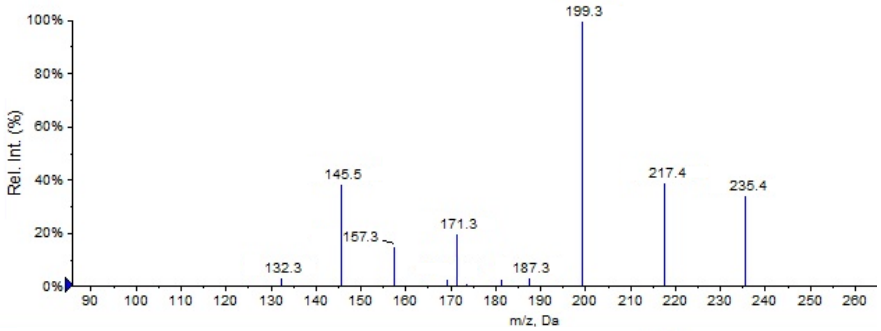
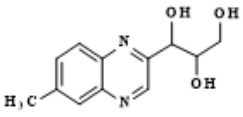
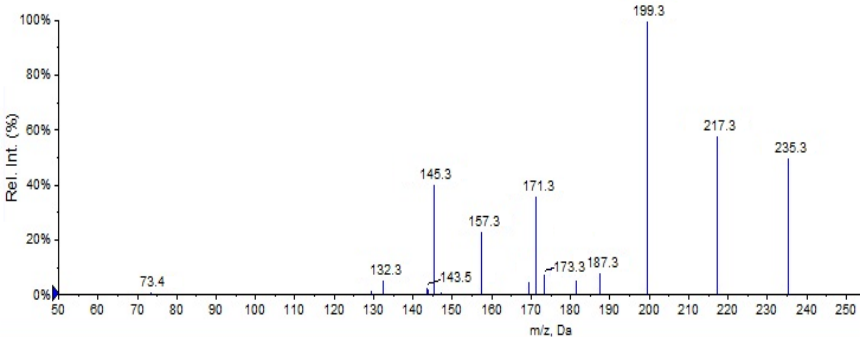
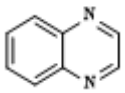
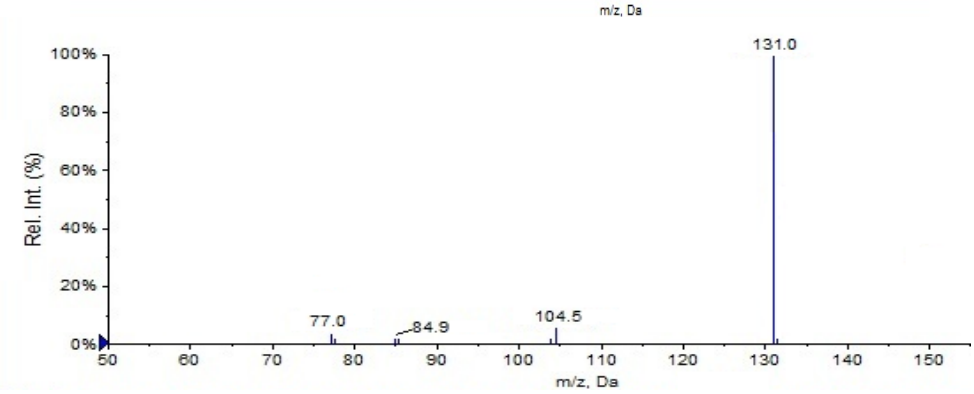
**Correlation is significant at $p < 0.01$ (2-tailed). n = 9.
HMF – hydroxymethylfurfural; 3-DG – 3-deoxyglucosone; 2-acetyl-4(5) (tetrahydroxybutyl)imidazole (THI).

Appendix J. Principal component eigen values for the principal component analysis.[a]

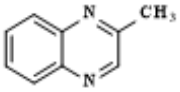
Principal Components	Eigen value	Proportion of total variance (%)	Cumulative variance proportion (%)
PC1	7.63	58.68	**58.68**
PC2	3.84	29.52	**88.20**
PC3	0.84	6.45	94.66
PC4	0.32	2.50	97.15
PC5	0.20	1.52	98.67
PC6	0.11	0.82	99.49
PC7	0.03	0.20	99.69
PC8	0.02	0.13	99.81
PC9	0.01	0.09	99.90
PC10	0.006	0.04	99.94
PC11	0.004	0.03	99.97
PC12	0.002	0.02	99.99
PC13	0.001	0.01	100.00

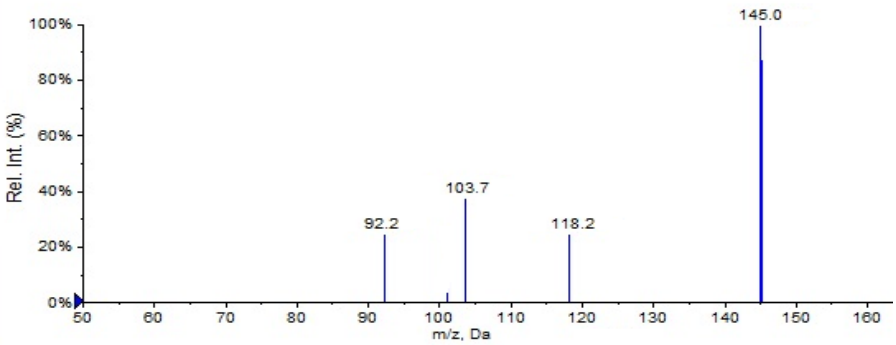
[a]PC= Principal component.

Appendix K. MS/MS spectra of quinoxaline derivatives of glucosone; 3-deoxyglucosone; 1-deoxyglucosone; glyoxal, methylglyoxal and diacetyl.

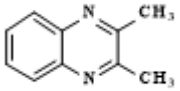
Quinoxaline derivatives	MS/MS fragmentation spectra
Glucosone$_{qx}$	 
3-Deoxyglucosone$_{qx}$	 
1-Deoxyglucosone$_{qx}$	 
Glyoxal$_{qx}$	 

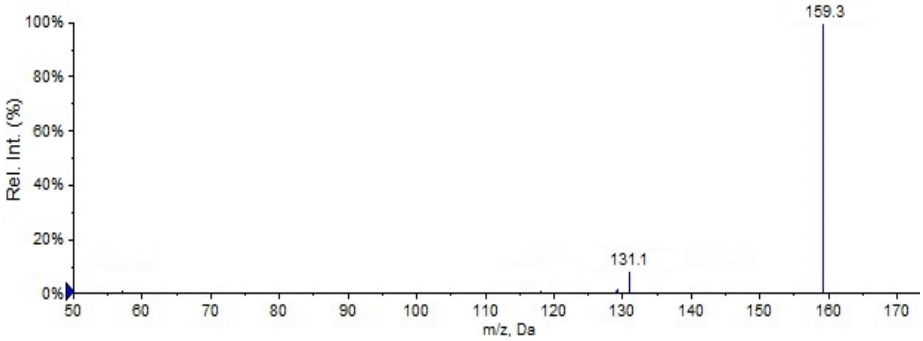
Methylglyoxal$_{qx}$





Diacetyl$_{qx}$

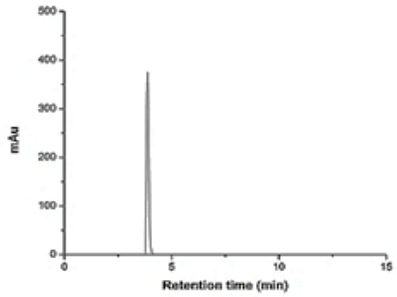




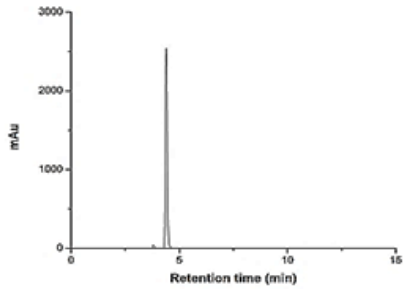
Appendix L. HPLC analyses of fructosazine (FR) and deoxyfructosazine (DOFR). (A) Chromatograms of FR and DOFR commercial standards and (B) Representative HPLC chromatogram of FR and DOFR in GlcN-Gly mixtures incubated at 70°C for 12 h absorbed at 275 nm.

A.

FR standard

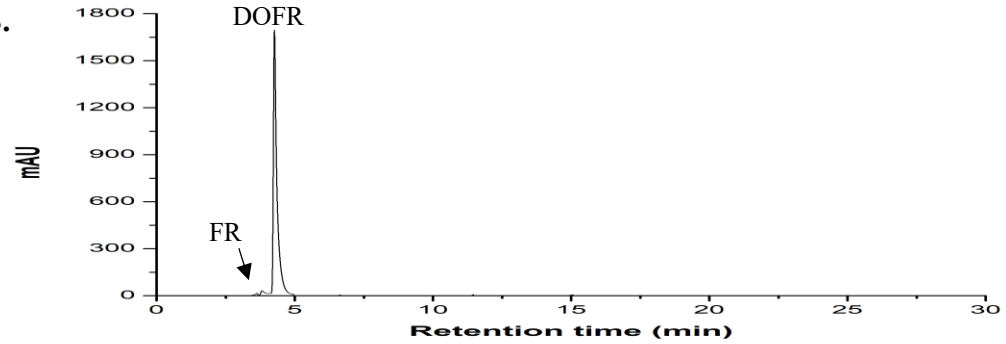


DOFR standard

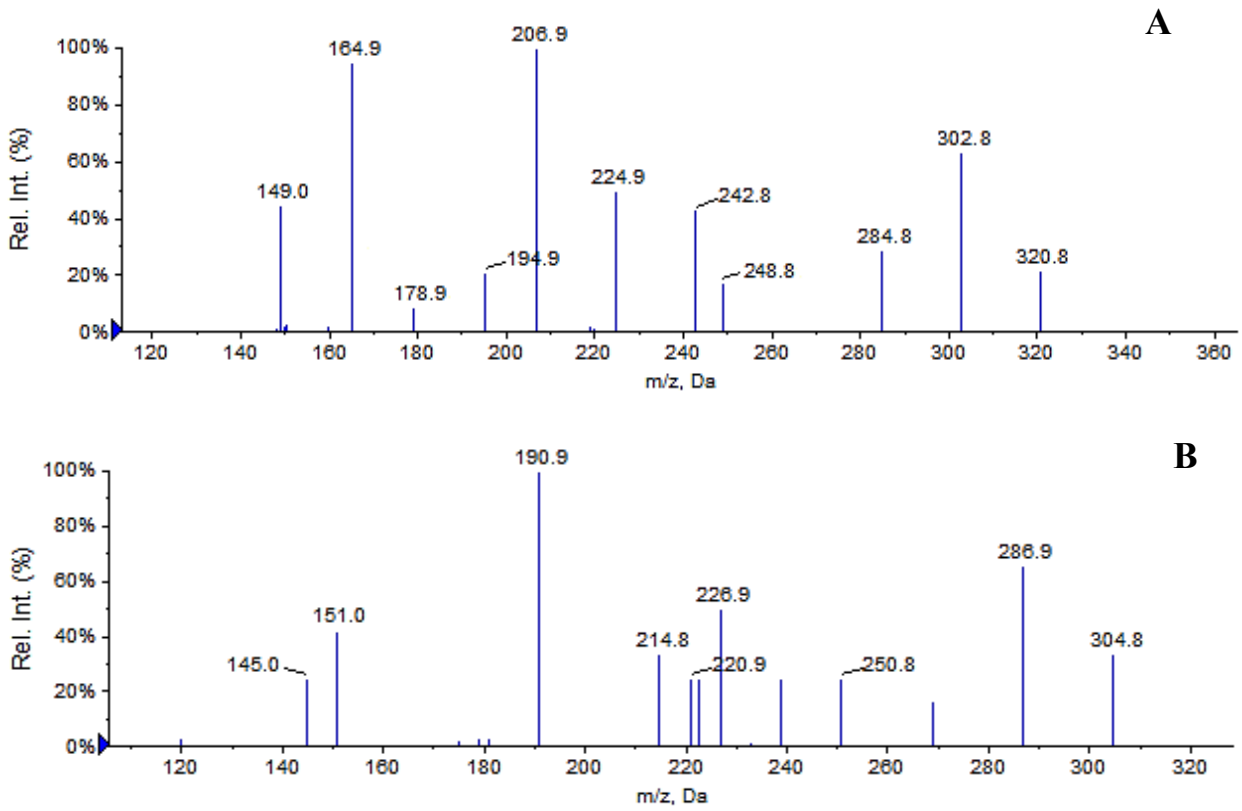


(Concentration of FR and DOFR was 1.25 mg/ml and 2 mg/ml respectively)

B.

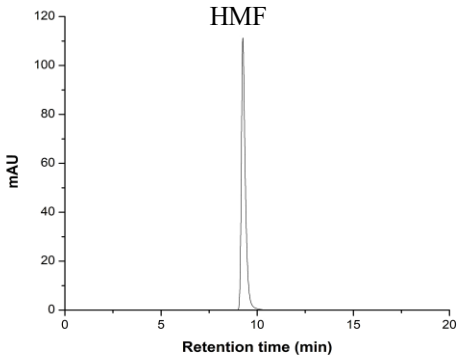


Appendix M. Representative MS/MS spectra of (A) fructosazine and (B) deoxyfructosazine identified in GlcN-Gly reaction mixtures.

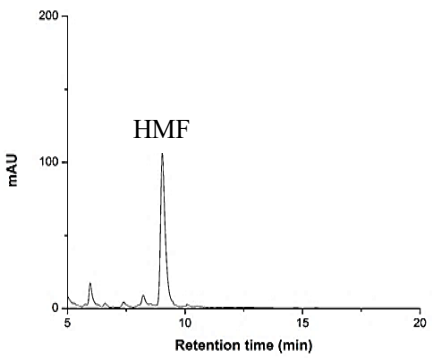


Appendix N. HPLC analyses of 5-hydroxymethyl-2-furfural (HMF). (A) Chromatogram of HMF standard solutions absorbed at 285 nm. (B) Representative HPLC chromatogram of HMF in GlcN-Gly mixtures incubated at 70°C for 12 h absorbed at 285 nm.

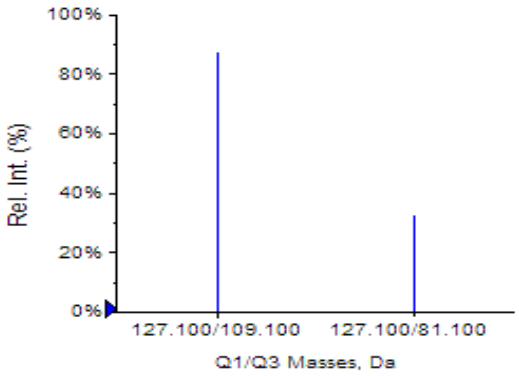
A.



B.



Appendix O. Representative MS/MS spectra of HMF identified in GlcN-Gly mixtures incubated at 70°C for 12 h.



Appendix P. Principal component eigen values for the principal component analysis.[a]

Principal Components	Eigen value	Proportion of total variance (%)	Cumulative variance proportion (%)
PC1	3.36	37.37	**37.37**
PC2	2.26	25.07	**62.44**
PC3	1.38	15.29	77.73
PC4	0.82	9.14	86.87
PC5	0.69	7.61	94.48
PC6	0.30	3.28	97.76
PC7	0.11	1.18	98.94
PC8	0.08	0.92	99.86
PC9	0.01	0.14	100.00

[a]PC= Principal component.

Appendix Q. Coefficients of the loading (eigen vectors) for the first two principal components (PC).

Dependent variables	PC1	PC2
Fructosazine (FR)	0.53	-0.001
Deoxyfructosazine (DOFR)	0.49	0.16
Glucosone (G)	-0.05	-0.53
3-Deoxyglucosone (3-DG)	0.11	0.60
Glyoxal (GO)	-0.12	0.52
Methylglyoxal (MGO)	0.48	-0.09
Diacetyl (DA)	0.37	-0.21
5-hydroxymethyl-2-furfural (HMF)	0.27	0.05
2-Acetyl-(4)5-tetrahydroxylbutyl imidazole (THI)	-0.06	-0.01