Non-enzymatic browning in glucosamine and glucosamine-peptides reaction systems as a source of antioxidant and flavouring compounds

by

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Abstract

Non-enzymatic browning is a common and complex reaction that occurs in everyday cooking—indeed, it is a crucial step in food processing. A typical browning process includes both the Maillard reaction and caramelization, which normally occur only at extreme temperatures to produce both desired and distinctive flavours and an intense brown colour in foods. On one hand, advanced stage Maillard reaction compounds can be toxic, such as acrylamide and 5-hydroxymethylfurfural (5-HMF). And yet on the other hand, Maillard reaction compounds can possess bioactivities including taste enhancing, antioxidant and antimicrobial capacities; collectively these are known as Maillard reacted peptides (MRPs). This bizarre paradox among non-enzymatic browning reaction compounds has long deserved a more in depth investigation under controlled conditions.

To achieve these positive bioactivities yet reduce the accumulation of the harmful compounds associated with the Maillard reaction, a research strategy was proposed to produce and understand MRPs at lower temperatures. However, information on the antioxidant and sensorial properties MRPs at moderate temperatures is scarce. Glucosamine (GlcN) is an amino sugar recently revealed to be capable of triggering a fast Maillard reaction with protein at 25˚C. Additionally, due to the presence of both a carbonyl and amino group within the same molecule, GlcN can form substantial dicarbonyls at 37˚C—the precursors to desirable flavours. The main objective of this thesis was to study taste enhancement and antioxidant activity of GlcN-peptides in GlcN model systems. A total of 3 studies were designed representing the main building blocks of this project.

The first study focused on the potential of GlcN to modifying protein hydrolysates at 25 and 37˚C in a fish gelatin hydrolysates-GlcN model. Modification of the hydrolysates by GlcN was accomplished by two approaches: firstly, a Maillard-based glycation with GlcN, and secondly, an enzymatic glycosylation catalyzed by the transglutaminase (TGase), condensing the primary amine group of GlcN with the carboxamide group of a glutamine residue in a peptide. GlcN-induced modification was achieved at both 25 and 37˚C, and the antioxidant and antimicrobial activities were improved compared to native hydrolysates.

The second study focused on the taste enhancing property of GlcN-modified hydrolysed meat proteins produced at 37 and 50˚C in the presence or absence of TGase. Samples were formulated into seasoning compositions and evaluated by untrained consumers. The meat protein hydrolysate was perceived as the saltiest ($p<0.05$) whereas the glycated hydrolysate produced at 50˚C tended ($p= 0.0593$) to be the most savoury seasoning composition. This further confirmed the role of GlcN as an important component in modified hydrolysate by eliciting an umami taste, despite its inability to strike a balance between eliciting saltiness and savouriness.

The non-enzymatic browning of GlcN was further investigated in the third study. Chemical-physico changes and antioxidant activity were monitored in 3-level factorial models: in phosphate buffer versus ammonium hydroxide solution, at 40 versus 60˚C, and incubated up to 48 h. Incubation at 40˚C for 6 h produced a yellow-coloured caramel with the greatest levels of anti-radical activity and diacetyl—a volatile flavour compound of dicarbonyls.

Overall, this thesis showed GlcN to be a reactive amino sugar capable of key rapid peptide reactions and self-modifications at moderate temperatures. Compounds from these GlcN-mediated non-enzymatic browning reactions not only represent important flavour and colour agents, but also showed promise as antimicrobials and antioxidants. A future paradigm shift is anticipated for GlcN, evolving from its current status as a health supplement to become a multi-functional food ingredient.

Preface

This thesis is an original work by Pui Khoon Hong. The thesis is presented in a manuscript format that comprise of a total of six chapters.

Chapter 1 highlights the research problem, general hypothesis, main and specific objectives of three experimental studies.

Chapter 2 sums up the literature review of the synthesis and production of glucosamine, as well as the non-enzymatic browning reactions. The core of the thesis is described in Chapter 3, 4, and 5.

Chapter 3 (Study 1) is the modified version of the collaborative work published as: Hong, P.K., Gottardi, D., Ndagijimana, M., and Betti, M. 2014. Glycation and transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine enhance bioactivity. Food Chem 142: 285-293. Gottardi D. conducted the antimicrobial and cell antioxidant analyses.

Chapter 4 (Study 2) was published as Hong, P.K., Ndagijimana, M., and Betti, M. 2016. Glucosamine-induced glycation of hydrolysed meat proteins in the presence or absence of transglutaminase: Chemical modifications and taste-enhancing activity. Food Chem 97: 1143-1152.

The untrained consumer sensory evaluation described in the methodology, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Consumer sensory evaluation of taste enhancing food protein hydrolysates”, Project ID Pro00039803, Date: Feb 7th 2014-Feb 5th 2016.

Chapter 5 (Study 3) described the non-enzymatic browning reaction of glucosamine at mild conditions, focused on the colour, antioxidant activity and short chain α-dicarbonyls production. This chapter in part was published as Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243.

Chapter 6 summarizes the outcomes of this research. Short discussions in regards to the implications of the study and suggestions for future work are also included in this chapter.

I was responsible for the experimental design, data collection, analyses as well as manuscript preparation in the published articles and experimental chapters written in this thesis. Dr. M. Ndagijimana assisted with the technical support for experiment and the proof reading for the published manuscripts. Dr. M. Betti was the supervisory author and was involved in providing advisory inputs on the studies' concepts and work progress review, manuscript composition, editing as well as proof reading.

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I would like to thank Dr. Davide Gottardi who was a visiting graduate student from the University of Bologna (Italy) and collaborated in the first study (2012). He contributed his effort on the antimicrobial activity and cell antioxidant analyses.

Thank you to Dr. Alma Fernanda Sanchez Maldonado who was kind enough to perform some preliminary assessments on the antimicrobial activity in study 1 (2012).

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I am grateful for all the help and support from all my fellow lab mates and technicians in the Department of Agricultural, Food Science and Nutrition especially Gary Sedgwick, Joan (Nancy) Turchinsky and Susan Gibson.

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Last but not least, I cannot thank you enough to all my friends and family members who are always patient, caring, giving constant support in reaching my goals.

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and 60˚C) for for 0, 3, 6, 12, 24 and 48 h tested at different concentrations. Data points were fitted (R^2>0.98) and described with the equation:

$$y = B + \frac{(A - B)}{1 + 10^{(Log\ EC50 - x)\ H}}$$

where A and B= the top and bottom of plateaus in the units of the y-axis; EC50 = the effective concentration to reach to 50% ABTS anti-radical activity (EC_{50}, mg/mL) from samples (final concentration 0.1 mg/mL); x = concentration in log unit; H = hillslope. N=3. Figure adapted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd. *128*

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List of abbreviation

4-MEI, 4-methyl imidazole

5-HMF, 5-hydroxymethylfufural

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid

AGEs, advanced glycation end products

Alc, Alcalase

ANOVA, one way analysis of variance

ARP, Amadori rearrangement product

Asp, aspartic acid

B. subtilis, Bacillus subtilis

CAA, cellular antioxidant activity

CML, Nε-(carboxymethyl)lysine

DAD, Diode array detectors

DH, degree of hydrolysis

DOF, deoxyfructosazine

DPPH, 2,2-diphenyl-1-picrylhydrazyl

E. coli, Escherichia coli

EC_{50}, half maximal (median) effective concentration

F-6-P , fructose-6-phosphate

FAO, Food and Agriculture Organization

Flv, Flavourzyme

FRAP, ferric reducing power

FZ, fructosazine

G-, gram negative

G+, gram positive

G-6-P, glucose-6-phosphate

GFAT, glutamine:fructose-6-phosphate amidotransferase

GlcN, glucosamine

GlcN-6-P ,glucosamine-6-phosphate

GlcNAc, *N*-acetyl-glucosamine

Glu, glutamic acid

GLUT, glucose transporter

GSH, reduced glutathione

HAT, hydrogen atom transfer

HBP, hexosamine biosynthesis pathway

HepG2, human hepatocarcinoma cells

HPLC, High performance liquid chromatography

HRP, Heyns rearrangement product

IC_{50}, half maximal inhibitory concentration

ISP, isoelectric solubilization and precipitation process

JECFA, Joint Evaluation Committee for Food Additives

MALDI-TOF-MS, Matrix-assisted laser desorption ionization-time of flight-mass spectrometry

MBC, minimal bactericidal concentration

MIC, minimal inhibitory concentration

MRPs, Maillard reacted peptides

MSTM, Mechanically separated turkey meat

MW, molecular weight

MWCO, molecular weight cut off

PBS, Phosphate-bufferred saline

PPI, Poultry protein isolate

SEC, size exclusion chromatography

TE, Trolox equivalent

TGase, transglutaminase

UDP-GalNAc, uridine diphosphate-N-acetylgalactosamine

UDP-GlcNAc, uridine diphosphate-N-acetylglucosamine

UHPLC, Ultra High Performance Liquid Chromatography

Chapter 1: General introduction and objectives

Non-enzymatic browning reactions include caramelization and the Maillard reaction (also known as glycation), and are very important processes in determining the quality of food. Caramelization is defined as the dehydration of sugar, such as sucrose or glucose, followed by isomerization and polymerisation steps; while the Maillard reaction involves a reaction between amino acids and reducing sugars. Despite these simple definitions, both caramelization and the Maillard reaction involve a complex network of reactions which are even today still not well understood. A simplified schematic representation of the Maillard reaction is reported in Figure 2.4 (Chapter 2), where the reaction can be simplified to three major stages. The initial stage involves the condensation of the carbonyl group of a reducing sugar with an amino containing compound, leading to the formation of an Amadori rearrangement product. Subsequently, in the intermediate stage, the degradation of the sugar condensation products gives rise to alpha (α)-dicarbonyl compounds. These reactive α-dicarbonyl compounds combine with other compounds such as amines, amino acids, aldehydes, hydrogen sulphide and ammonia, leading to many important classes of compounds, including furans, pyrazines, pyrroles, oxazoles, thiophenes, thiazoles and heterocyclic compounds. In the final stage of the Maillard reaction, not only heterocyclic compounds are formed, but also polymeric molecules like melanoidins and modified peptides. These compounds generated from the final stage of the Maillard reaction are classified as advanced glycation end products (AGEs).

Non-enzymatic browning reactions normally occur at relatively high temperatures (e.g.: above 50˚C) and they influence the physical, chemical, sensory and nutritional properties of thermally-treated food products. For instance, the Maillard reaction

contributes to the formation of the golden-brown pigments which consist mainly of polymeric melanoidins, and are responsible for the colour of a typical bread crust or roasted meat. Furthermore, it contributes to the flavour and taste generation producing both volatile and non-volatile compounds, including a vast variety of heterocyclic compounds. Melanoidins are known to possess antioxidant and metal-chelating properties (Morales, Fernandez-Fraguas, and Jimenez-Perez 2005; Xu, Tao, and Ao 2007). Caramel, on the other hand, is a known food additive used in many food formulations, such as in soft drinks, soy sauce, and balsamic vinegar. Despite conferring positive attributes to foods, these reactions may also be responsible for the generation of toxicants like acrylamide (Stadler et al. 2002; Mottram, Wedzicha, and Dodson 2002), 5-hydroxymethylfurfural (5-HMF) (Abraham et al. 2011) and 4-methyl imidazole (4-MEI) (Hengel and Shibamoto 2013; Jang et al. 2013), particularly at very high temperatures in the presence of certain amino acids like asparagine (Stadler et al. 2002). Furthermore, it is known that the AGEs are glycotoxins due to their pro-oxidant and pro-inflammatory effects that can induce chronic diseases (Peppa et al. 2002). Studies have shown that dietary AGEs are the main contributor to the total body AGEs pool, and restrictions of AGEs can diminish these adverse effects (Uribarri et al. 2003; Vlassara et al. 2002). However, the AGE argpyrimidine has antioxidant properties and may not be toxic (Sreejayan et al. 2006, 2008).

A lot of simple model systems have been designed to better understand the Maillard reaction mechanism and identify its major products. For instance, some studies have focused on the Maillard reaction between a single amino acid or simple peptide and a reducing sugar in order to identify flavour compounds generated in these simple models

(Beksan et al. 2003; Kraehenbuehl et al. 2008; Ottinger, Soldo, and Hofmann 2003). Along with the heterocyclic compounds and polymeric substance produced in model systems, food scientists have also identified the so-called Maillard reacted peptides (MRPs) (Katsumata et al. 2008); these compounds still contain a peptide backbone but some of the amino residues in the sequence have been chemically modified forming a vast class of compounds. Sometimes a MRP can be a "simple" Amadori-glycopeptide or a more complex AGE-peptide where a cyclic structure can be present, such as with argpyrimidine (Nakadate et al. 2009). Interestingly, some of the MRPs have proven to possess a taste-enhancing activity. A taste enhancer is a compound capable of amplifying the taste of food including, saltiness, umami or sweetness. Several MRPs possessing taste enhancing activities have been identified and are associated to a new sensation coined as kokumi (Katsumata et al. 2008). The Japanese term 'kokumi' describes the nature of certain compounds that not only enhance sweet, salty and umami taste and flavour, but also increase the intensity of the mouthfulness, continuity and thickness sensations of food (Maruyama et al. 2012; Ueda et al. 1997). Moreover, some MRPs have exhibited antioxidant activities thus deemed as health-promoting and functional food ingredients (Silvan et al. 2006).

These interesting discoveries of the Maillard reaction prompted me to study on their antioxidant and taste enhancing properties. I also looked for possible applications for these MRPs in food formulations and new food product developments. For instance, in the last decade, the global food industry has seen a revolutionary change in the regulation of sodium in food following UK's government initiative in reducing sodium to promote health. Bread and processed meat products are the major items contributing to high sodium

in food (CTAC 2009). Since some MRPs have been identified to possess salt-enhancing properties, incorporating MRPs in food formulations could facillitate sodium reduction. However, as previously mentioned, the pivotal role of temperature in generating MRPs makes this a challenging task, since toxic compounds may be generated as a consequence of the high temperature used during the Maillard reaction (Mottram, Wedzicha, and Dodson 2002; van Boekel and Brands 1998). Hence, from a food toxicology perspective, generating MRPs while minimizing the dangerous AGEs is an important balance that must be achieved in developing low- or reduced-sodium food products.

In a preliminary study conducted in our research group, it was noted that when the amino sugar glucosamine (GlcN) was incubated with actomyosin, the solution produced a yellow colour within 24 h of incubation, and turned dark-brown after 1 week of incubation at ambient temperature (25˚C) (Hrynets, Ndagijimana, and Betti 2013). When GlcN was incubated at 37-50˚C, the formation of brown pigments could be noted already after 48 h—this phenomenon was not noted in any of the other reducing sugars (i.e. glucose) incubated in the same condition (Hincapie Martinez 2015; Hrynets, Ndagijimana, and Betti 2015a). I hypothesized that GlcN could undergo a non-enzymatic modification when incubated at a moderate temperature. Despite very few scientific reports available regarding GlcN's reactivity, one study ranked GlcN as the most reactive sugar compared to other reducing sugars like fructose, galactose, glucose and xylose (Kraehenbuehl et al. 2008). However, the temperature used in the study was at 125˚C. In another study, it was reported that GlcN could produce self-condensation (hydroxyalkyl pyrazines) products at 25-37˚C (Horowitz 1991; Hrynets, Ndagijimana, and Betti 2015a), as opposed to at higher temperatures when heterocyclic compounds are normally produced (Jia et al. 2014, 2015). However, the

authors of the studies were only able to identify these compounds and no quantitative evaluation was provided. Taken together these data, I hypothesized that GlcN could be a fast glycation agent indicating an opportunity to produce MRPs at lower temperature (i.e. 25-50˚C), thus minimizing the production of dangerous AGEs.

A unique property of GlcN is that it contains a free amino group in the C2 position next to a carbonyl. Chemically, GlcN can be considered as a primary amine, an important feature since GlcN may be potentially used by the enzyme transglutaminase (TGase) as a substrate for creating a stable iso-peptide bond with the amide group of a glutamine in a peptide or protein sequence. In this case, the formation of a glycopeptide would be possible, and this new enzyme-mediated process could be classified as an enzymatic glycation, also known as a glycosylation process. When I started my PhD program, I came across a report about the formation of a chitosan-gelatin conjugate with the help of TGase (Chen et al. 2003). Therefore, I hypothesized that GlcN can form glycosylated peptides when reacted in a mixture containing peptides, and TGase on top of the Amadori-peptide (glycopeptide) produced through the Maillard reaction. Since Amadori-glycopeptide from Maillard reaction has been linked to taste-enhancing properties, I expected that TGase glycosylated peptides may have a similar effect. Furthermore, another advantage of using a TGase-mediated glycosylation would be the use of a moderate reaction temperature, due to the high stability and reactivity of TGase between 20-40˚C (Ho et al. 2000).

Based on these considerations, the main scope of my research was to study the effect of different GlcN reaction systems in generating MRPs with both taste-enhancing activity and antioxidant capacity. To meet this objective three main studies were conducted.

The first study was the "Glycation and transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine enhance bioactivity" (Chapter 3), and was aimed to understand the potential of GlcN to modify hydrolysates at 25 and 37˚C, thus capitalizing on the fast glycation reactivity of GlcN and on its ability to participate a TGase-mediated reaction. The objectives of the study were:

a) To produce glycopeptides by glycosylating and glycating fish gelatin hydrolysates with GlcN at moderate temperatures (25 and 37˚C); and,

b) To characterise the bioactivity of the glycosylated and glycated hydrolysates.

Peptide (hydrolysate) modification by GlcN was monitored by using both spectroscopic and mass spectrometric techniques; in addition to that, the effect of these modifications on antioxidant activity was studied using *in vitro* studies and cell culture. For this study, fish gelatin produced from cold water fish was used to obtain a hydrolysate suitable for GlcN modification.

Prior to the beginning of the second study, a team member from our research group was able to quantify the major compounds produced from the non-enzymatic browning of GlcN at 37˚C (Hrynets, Ndagijimana, and Betti 2015a). Here GlcN could produce a significant amount of α-dicarbonyl compounds, the important intermediate precursors of flavour compounds (Hrynets, Ndagijimana, and Betti 2015a). At the early stage of my PhD program, such information was not available, as it was thought that GlcN would form Amadori glycopeptide from the "simple" reaction involving the carbonyl group of the GlcN with a free amino group of a lysine, arginine, histidine or a free amino acid. The discovery that GlcN can degrade to α-dicarbonyl compounds, has fully demonstrated the

potential of using GlcN as a source of carbonyl and dicarbonyl groups to modify peptides and thus produce MRPs, but at the same time, made the chemistry of GlcN glycation more complicated than expected.

The second study was the "Glucosamine-induced glycation of hydrolysed meat proteins in the presence or absence of transglutaminase: Chemical modifications and taste-enhancing activity" (Chapter 4), and was focused on the study of the taste enhancing activity in the GlcN modified poultry protein isolate (PPI) hydrolysates at moderate temperatures (37 and 50˚C). This study carried 3 objectives:

a) To assess the GlcN induced glycation and glycosylation of PPI hydrolysate at moderate temperatures;

b) To evaluate the saltiness and savouriness perception of the modified PPI hydrolysates when incorporated in a seasoning mixture; and,

c) To determine the interaction on the extent of chemical changes in the modified hydrolysate with the sensory attributes evaluated.

The chemical characteristics of the modified hydrolysates and GlcN were studied under spectroscopic and liquid chromatographic techniques. The α-dicarbonyl compounds were identified and quantified. Through consumer-based sensory evaluation sessions, the potential of glycated and glycosylated hydrolysates, as well as GlcN alone as a taste enhancer, was assessed in the form of seasoning composition.

The last study of this PhD work was focused on the non-enzymatic browning of GlcN alone, without the addition of any protein hydrolysate or peptide. As previously

reported, GlcN caramelization in phosphate buffer at 37˚C produced a variety of chemical compounds (Hrynets, Ndagijimana, and Betti 2015a). For instance, short chain α-dicarbonyls like diacetyl, methylglyoxal and glyoxal are known to possess an important antimicrobial activity (Daglia et al. 2007; Jay, Rivers, and Boisvert 1983), while diacetyl is a known primary flavouring compound in dairy products and buttered popcorn (Clarke and Winter 2015). Furthermore, the GlcN caramelization produces other important flavouring compounds like fructosazines (FZ) and deoxyfructosazines (DOF); and have been recognized to possess an anti-diabetic property (Bashiardes et al. 2002), the latter inhibits inflammation (Zhu et al. 2007). Hence, the major goal was devoted to determining the optimum conditions to maximize the production of flavouring and antioxidant compounds, while minimizing the formation of the dark pigments, melanoidins and methylglyoxal—the precursor of the toxic compound 4-MEI. Specific objectives were:

a) To study the kinetics of GlcN non-enzymatic browning using a 3-level factorial model (in different solution system consisting either PBS buffer or ammonium hydroxide, temperature [40 and 60˚C] and incubation time [0, 3, 6, 12, 24, 48 h]);

b) To quantify the flavouring compound diacetyl;

c) To quantify the short reactive α-dicarbonyls especially methylglyoxal which is associated with the production of 4-MEI;

d) To monitor the chemical-physico changes of GlcN during incubation; and,

e) To measure the antioxidant activity of GlcN in different incubation models.

Kinetics of caramelization was monitored at three different wavelengths (280, 320 and 420 nm) using spectroscopic techniques. Short chain α-dicarbonyls were quantified using liquid chromatographic techniques (HPLC–DAD system), while antioxidant capacity was measured with the DPPH, ABTS and FRAP assays.

Chapter 2: Literature review

2.1 Amino sugars in nature: occurrence, structure & biosynthesis

2.1.1 Amino sugar background

Amino sugars are monosaccharides which have one or more of their hydroxyl group replaced by an amine group. These sugars are found abundantly in nature in the form of monosaccharide and polymers. Among the common monosaccharide amino sugars are GlcN, muramic acid, galactosamine and mannosamine, and *N*-acetyl-glucosamine (Figure 2.1A). The amino sugar derivatives like *N*-glycolylmannosamine and *N*-glycolylglucosamine are precursors for biosynthesis of *N*-glycolylneuraminic acid, a mammalian sialic acid constituent (Bergfeld et al. 2012). The *N*-acetyl-glucosamine (GlcNAc) and GlcN are important components in the peptidoglycan and lipopolysaccharide cell wall components of Gram-negative (G-) bacteria (Plumbridge 1995). They are also found in soil fungi and some bacteria including Streptococcus, Salmonella species and *Escherichia coli* (Zhang and Amelung 1996). Furthermore, GlcN plays a vital role in the synthesis of proteoglycans and glycoaminoglycans in the articular cartilage and synovial fluid of animals. The GlcN derivatives include hyaluronic acid which serves as the backbone for linking aggrecans consisting of keratan sulfate and chondroitin sulfate (Jerosch 2011). GlcN is also involved in the production of heparin and heparan sulfate, a naturally occurring compound medically used as an anticoagulant (Figure 2.1B).

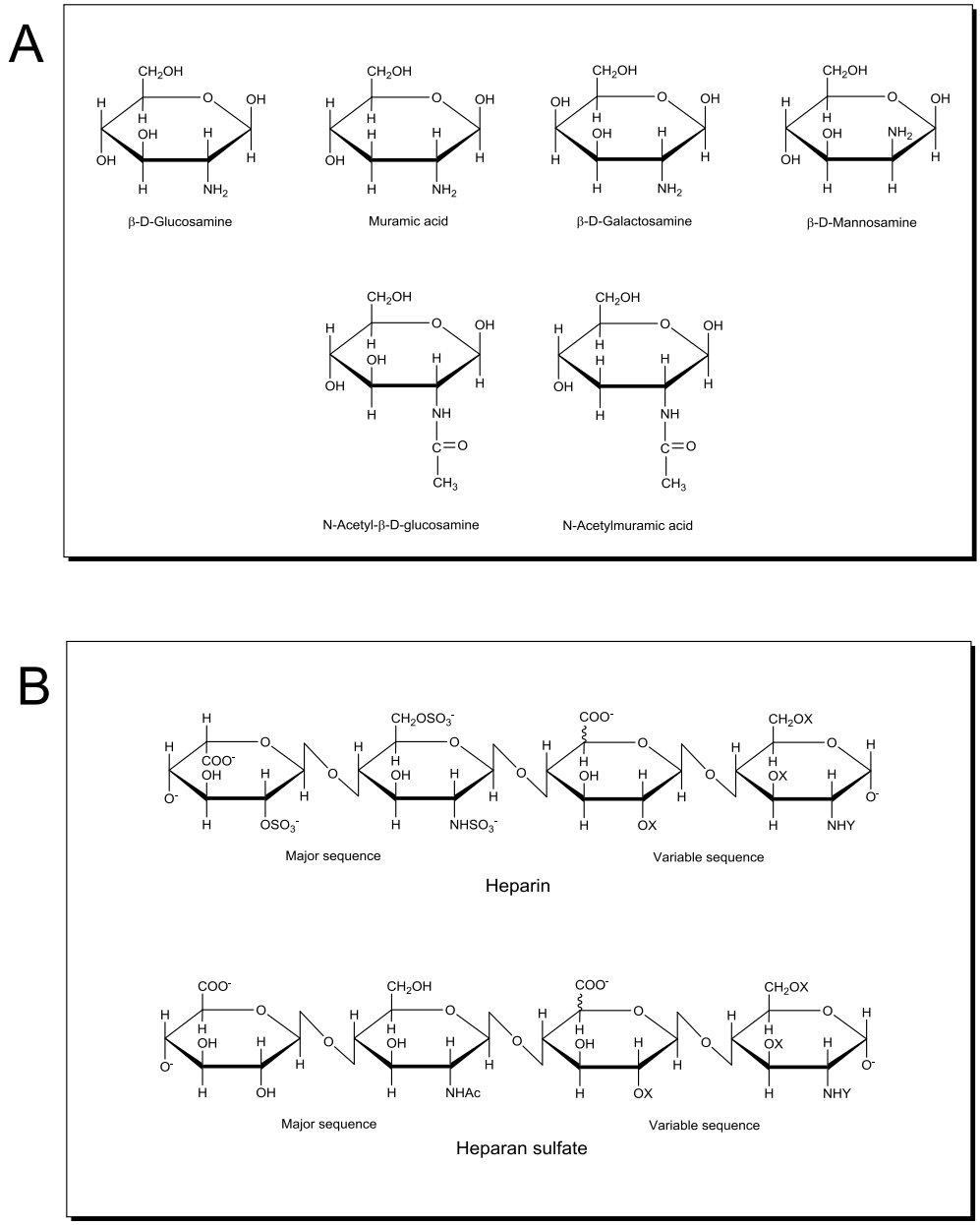


Figure 2.1: The structure of simple amino sugars (monosaccharides, A) and two examples of amino sugar in disaccharide forms: heparin and heparan sulfate (B). Major sequence for heparin consists of L-iduronic acid and sulfated GlcN; whereas major sequence for heparan sulfate is D-glucuronic acid and GlcNAc. The minor variable repeating units are shown on the right of each major sequences where X = SO^{3-} or H; whereas Y = SO^{3-} or $COCH_3$. Figure adapted with permission from Capila, I., and Lindhardt, R.J. 2002. Heparin - protein interactions. Angew Chem Int Ed 41:390-412. Copyright (2002) John Wiley and Sons.

The chemical composition of the amino sugar derived polymers is made up of a key pattern of repeating disaccharide units. For instance, heparan sulfate are sulfated linear polysaccharides with repeating disaccharide units consisting of uronic acid and GlcNAc (Capila and Lindhardt 2002). The amino group of the GlcN residue in heparin and heparan sulfate can be either a GlcNAc, N-sulfated GlcN, or an unsubstituted GlcN (Capila and Lindhardt 2002; Esko and Selleck 2002). For the uronic acid portion, it can be either a D-glucuronic acid, or D-glucuronic acid sulfated at C2, or L-iduronic acid or its sulfated acid at C-2 (see Figure 2.1B). Heparin is more sulfated than heparan sulfate and has more structure variety (Capila and Lindhardt 2002).

2.1.2 Biosynthesis of glucosamine

GlcN (2-amino-2-deoxy-D-glucopyranose, also known as 2-amino-2-deoxy-D-glucose) is ubiquitous in most living organisms. GlcN is found in biological fluids such as plasma and sinovial fluid, in combination with other monosaccharides such as glucose, galactose, other sugars and amino sugars (Roda et al. 2006). GlcN is synthesized *in vivo* via the hexosamine biosynthesis pathway (HBP), a minor branch of glycolysis. The process can be divided into two routes. Firstly, glucose enters the pathway and is transported from tissues to adipocytes or skeletal muscles by a glucose transport system known as glutamine:fructose-6-phosphate (F-6-P) amidotransferase (GFAT). Normally, as glucose enters the cell, it is rapidly phosphorylated to glucose-6-phosphate (G-6-P) and then converted to fructose-6-phospahte (F-6-P) by G-6-P isomerase. This HBP process typically involves a small percentage of glucose (about 3% of total glucose) (Marshall, Bacote, and Traxinger 1991). Subsequently, the GFAT enzyme converts F-6-P and glutamine to glucosamine-6-phosphate (GlcN-6-P) and glutamate. Alternatively, exogenous GlcN molecule can enter into the cell's HBP at a

concentration lower than that of glucose bypassing the GFAT route (Buse 2006). Here, the glucose transporter GLUT4 has an affinity for GlcN than its counterparts GLUT1 and GLUT2, since the amino group at carbon 2 renders the molecule more hydrophobic compared to glucose (Uldry et al. 2002). GlcN directly undergoes phosphorylation by hexokinase to form GlcN-6-P. Then, GlcN-6-P undergoes a series of rapid conversions to end products like uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), uridine diphosphate-N-acetylgalactosamine (UDP-GalNAc) and cytidine monophosphate-sialic acid. These components are important components of glycoproteins, glycolipids, proteoglycans and gangliosides (Buse 2006; Traxinger and Marshall 1991).

The UDP-GlcNAc in particular, serves as an indicator to the GFAT enzyme for its glucose intake activity—a HBP rate limiting step. The presence of insulin induces glucose uptake into cells (Cushman and Wardzala 1980; Suzuki and Kono 1980). The UDP-GlcNAc also mediates the *O*-linked glycosylation of various regulatory pathways (Marshall 2006). In turn, this can activate the expression of a series of genes encoding proteins that involves the insulin-responsive glucose transport system and triggers the onset of cellular insulin resistance, eventually desensitizing the insulin-responsive glucose transport system (Hawkins et al. 1997; Marshall, Bacote, and Traxinger 1991; Marshall 2006). This condition encourages an influx of glucose into the cell under hyperglycemic conditions (Marshall, Bacote, and Traxinger 1991).

2.1.3 GlcN for joint health

Aside from work focused on the relationship between HBP and insulin resistance, there are also some studies focused on the efficacy of GlcN for joint health. Oral administration of a

GlcN compound (GlcN sulfate, GlcN sulfate or GlcNAc), sometimes in combination with chondroitin sulfate, has been demonstrated to reduce osteoarthritis symptoms (Kelly 1998). The incorporation of chondroitin sulfate and manganese ascorbate in GlcN hydrochloride supplements in rabbits showed a synergistic effect in stimulating glycosaminoglycan synthesis (96.6%) compared to either agent alone (GlcN, 32%; chondroitin sulfate, 32%) (Lippiello et al. 2000). Most of the research evaluating the efficacy of GlcN in comparison to a placebo on humans was done by measuring the narrowing knee joint space. Two independent groups of researchers used a randomized, double-blind placebo controlled trials. Knee osteoarthritis patients were randomnly assigned 1500 mg of oral GlcN sulfate or placebo daily for 3 years in Belgium (placebo: n= 71; vs GlcN sulfate: n= 68) (Regnister et al. 2001) and Czech Republic (placebo: n= 55; vs GlcN sulfate: n= 66) (Pavelka et al. 2002). Both studies observed improvement of osteoarthritis symptoms (20-25%) and reduction of severe joint space narrowing based on GlcN sulfate supplemented orally at 1500 mg per day. However, another trial conducted in the United States (Glucosamine/chondroitin Arthritis Intervention Trial, GAIT, n= 1583) concluded daily supplementation of GlcN at 1500 mg per day, for 24 weeks in combination with chondroitin sulfate progressively reduced knee pain by 20% (Clegg et al. 2006). This shows that glucosamine is effective in retarding the development of osteoarthritis if used for long term treatment. According to a detailed clinical review by Hatchcock and Shao (2007), the observed safe level for GlcN is 2000 mg/day with complete absence of adverse effects. GlcN may serve as an alternative to relieve osteoarthritis symptoms compared to conventional analgesic drugs such as acetaminophen, and non-steroidal anti-inflammatory drugs, which have analgesic and anti-inflammatory properties, like ibuprofen,

indomethacin and diclofenac. Non-steroidal anti-inflammatory drugs are often associated with significant side effects such as gastrointestinal and cardiovascular toxicities and more (Koutroumpas, Simopoulou, and Sakkas 2012). The side effects observed with GlcN were relatively mild in comparison to non-steroidal anti-inflammatory drugs or other drugs and placebos (Zeng et al. 2015; Regnister et al. 2001; Setnikar et al. 1991).

2.1.4 Side effects of GlcN

At the same time, the oral administration safety of GlcN remains another topic of interest especially on the possibility of a diabetogenic effect. Anderson et al. (2005) compiled a comprehensive review on the assessing GlcN in both animals and humans. Of the 33 clinical studies representing 3063 participants, whom were supplemented with GlcN in the form of GlcN sulfate and GlcN hydrochloride salts (via oral and/or intravenous at the dose of 1000-3200 mg per day, majority of the studies used 1500 mg per day), Anderson and colleagues (2005) found no significant increase of the sugar levels in either healthy or diabetic patients. Recent studies from other groups confirmed no adverse effects on the insulin and fasting glucose levels in healthy and diabetic patients (Simon et al. 2010; Tannis, Barban, and Conquer 2004).

2.1.5 Antioxidant activity of GlcN

GlcN exhibits anti-inflammatory activity which was demonstrated in several animal studies (Kim et al. 2010; Setnikar et al. 1991; Wu et al. 2014). Most of these studies highlighted the ability of GlcN to inhibit the production of reactive oxygen species (Mendis et al. 2008; Setnikar et al. 1991; Wu et al. 2014), or nitric oxide (Meininger et al. 2000). Some studies attributed the protective effect of GlcN to its anti-radical capacity (Jamialahmadi et al.

2014; Mendis et al. 2008; Xing et al. 2006; Yang et al. 2007). Furthermore, GlcN demonstrated reducing power and ferrous ion-chelating activity (Xing et al. 2006; Yang et al. 2007).

2.2 Commercial production of GlcN

GlcN is derived from chitin, the second most abundantly available polysaccharide after cellulose. The chitin and chitosan polymers consist of varying amount of the linear β-1,4-linked N-acetyl-GlcN and GlcN monomers (Figure 2.2). Chitin is poorly water soluble due to a low amount of GlcN in its polymer. On the other hand, the solubility of chitosan depends on the degree of acetylation, which is usually less than 0.35 (Pillai, Paul, and Sharma 2009). Chitosan is insoluble in water but becomes more soluble in acidic aqueous conditions because the amine groups become more protonated (Singla and Chawla 2001).

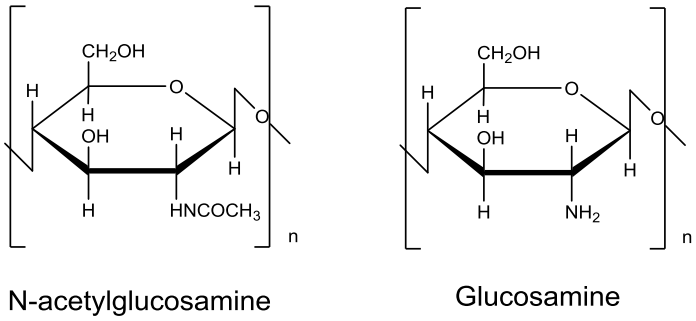


Figure 2.2: The monomer of chitin: N-acetylglucosamine and glucosamine.

Chitin is commonly found in the exoskeletons of insects, shell fish, and in certain strains of bacteria and fungi. The commercially available GlcN is mainly extracted from the shells of marine crustaceans (crab, shrimp and lobster) mostly because of its abundance as a waste product from the seafood processing industry. Approximately 6-8 million tonnes

of crustacean shells are produced globally every year (Yan and Chen 2015). In some regions of the world, the availability of these raw materials is seasonally dependent, and its composition differs according to the season (Aranaz et al. 2009). The issue of securing a consistent supply has created an interest in producing chitin from alternative sources such as cultured bacteria and fungi. Among the common filamentous fungi used for the GlcN production are Zygomycota, Ascomycota, Basidiomycota and Deuteromycota (Sitanggang, Sophia, and Wu 2012). Within the last decade, two companies - Cargill and Cyanotech - have launched non-shellfish based GlcN health-promoting products. Cargill's GlcN from *Aspergillus niger* is marketed under the trade name Regenasure®, whereas Cyanotech's JointAstin™ is adversited as 100% vegetable source GlcN. Genetically modified *Escherichia coli* is used for the microbial production of GlcN (Deng et al. 2005). *Bacillus subtilis* is another potential candidate for this application (Liu et al. 2013). Research on the microbial cultivation and optimization of biomass transformation to GlcN are recently of particular interest since the processing technology of these alternative sources is relatively new compared to the traditional shellfish-based systems.

There are many variations of GlcN production either from crustacean shells or microbial fermentation. A typical production flow of GlcN from shellfish and microbial cultivation is basically comprised of the following steps: deproteinization, demineralization, chemical hydrolysis of chitin, precipitation of GlcN, and then the crystallization of GlcN (Figure 2.3) (Benavente et al. 2015). The waste crustacean shells require additional steps such as deproteinization, demineralization, bleaching and grinding, this is because they contain 30-40% proteins, 30-50% calcium carbonate and lipid soluble pigments (e.g.: astaxanthin, astathin, lutein and β-carotene) (Aranaz et al. 2009). As for

fungal GlcN, the GlcN is presented in the form of chitin or chitosan found in cell walls. Recovery of biomass from the fermentation medium can be carried out by filtration (Sitanggang, Sophia, and Wu 2012). After the necessary pre-treatment steps, crude chitin from crustacean shell or fungal biomass is hydrolyzed with concentrated hydrochloric acid for several hours at 90-100˚C to depolymerise and deacetylate chitin to GlcN.HCl (European Food Safety Authority 2011; Sitanggang, Sophia, and Wu 2012). Recombinant *E. coli* can synthesize GlcNAc directly into the fermentation broth, so only a mild acid treatment of acetic acid or hydrochloric acid is required to obtain the GlcN (Sitanggang, Sophia, and Wu 2012). Following acid hydrolysis, the mixture is filtered to separate the GlcN.HCl-containing filtrate from solid particles. Then, the filtrate is crystalized at room temperature and ethyl alcohol is added to increase the crystallization rate at 5˚C (Benavente et al. 2015). Subsequently, the mixture is filtered to recover the crystals, which are then washed with ethyl alcohol and dried at 50˚C.

The commercial shell fish-based GlcN extraction procedure was regarded as not environmentally friendly because it involves both the use and disposal of a large amount of chemicals and demands the significant use of energy. Also from an operational perspective, the use of concentrated acid creates a challenge in the recovery of valuable shellfish by-products such as protein and pigments (Healy, Romo, and Bustos 1994). Therefore, Healy and colleagues (1994) proposed a new extraction method by combining both chemical and bacterial fermentation approaches during deproteinization and demineralization. This proved successful with several studies fermenting *Lactobacillus paracasei* and *Serratia marcescens* with crab shell wastes (Jung et al. 2007) and *Bacillus subtilis*-treated shrimp

shells (Sini, Santhosh, and Mathew 2007). However, chemical extraction is still preferred since it costs much less than the bacterial fermentation process.

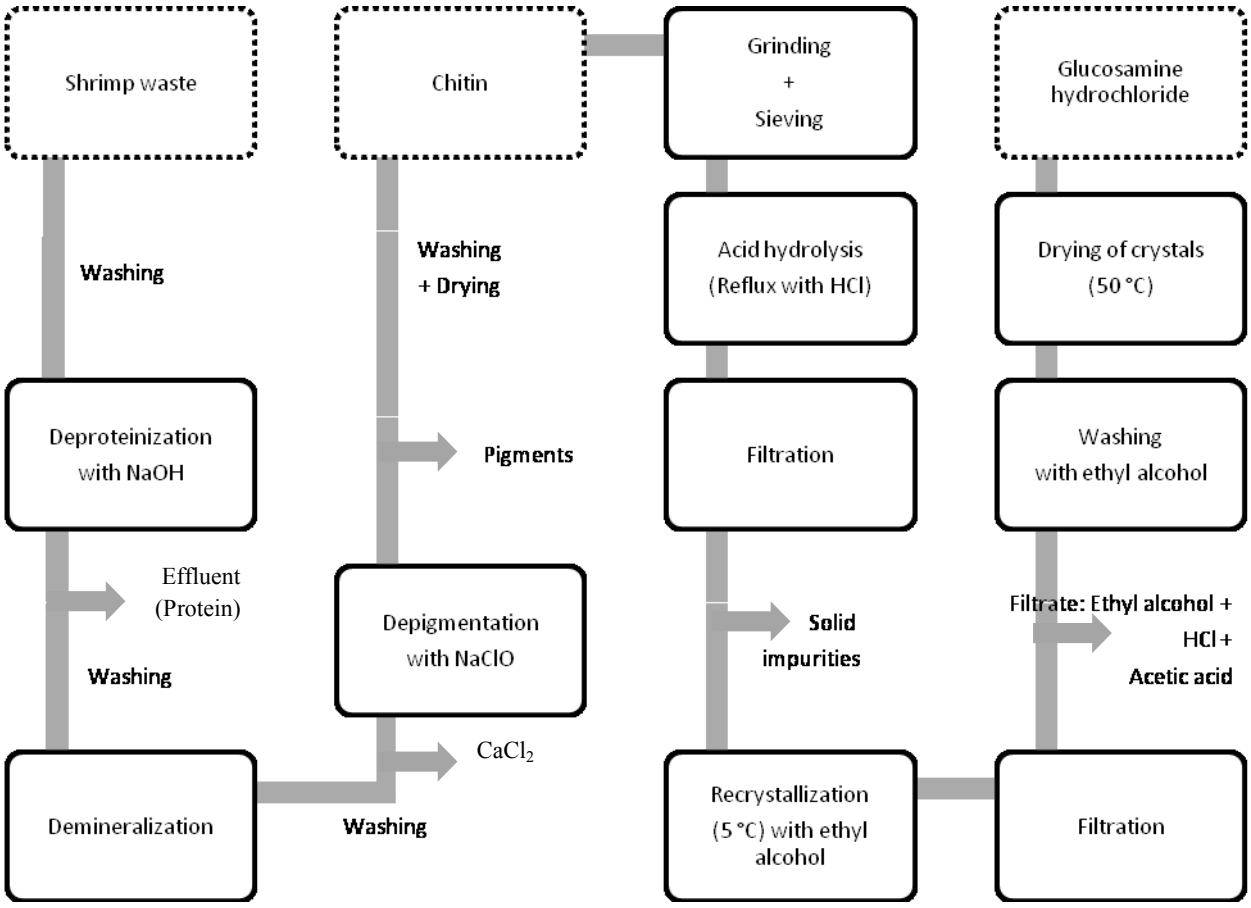


Figure 2.3: Process flow of glucosamine production from chitin. First, shrimp waste is pre-treated to obtain chitin, followed by acid hydrolysis, crystallization, and precipitation with ethyl alcohol. Finally, glucosamine crystals are recovered. Figure adapted with modifications from Benavente, M., Arias, S., Moreno, L., and Martinez, J. 2015. Production of glucosamine hydrochloride from crustacean shell. J Pharm Pharmacol 3:20-26. http://www.davidpublisher.org/Public/uploads/Contribute/55385fbd8a72d.pdf.

Fungal fermentation, on the other hand, yields less GlcN and GlcNAc in comparison to the recombinant *E. coli* technology due to the low GlcN content in fungal biomass. Its dry cell weight was less than 0.4 g/g (Liu et al. 2013). The recombinant *E. coli* approach can yield 73-110 g/L of GlcNAc, whereas the greatest yield ever achieved in

fungal culture was 14 g/L (Zhang and Amelung 1996). More detailed studies are required to expand the application of microbial fermentation in mass production of GlcN.

2.3 Overview on the non-enzymatic browning reactions

2.3.1 Sugar caramelization

Caramerlization is a sugar degradation process in the absence of amino group. This process is desirable in some processed foods such as in the making of caramel candy, bread and wine. On the contrary, it is undesirable when it comes to the crystallization of sucrose (Kroh 1994), one of the most important steps in table sugar production.

The process is favoured at temperatures higher than 120˚C and a pH range between 3-9 (Kroh, Fiedler, and Wagner 2008). When sucrose is heated at high temperatures, colourless sugar crystals progressively develop into a dark brown colour (Luna and Aguilera 2014). The process is not limited to sucrose only, there are a number of commercially available sources of carbohydrates for caramelization, which include glucose, fructose, invert sugar, dextrose and so forth (European Food Safety Authority 2011). Imposing quality control in caramel is very challenging, since the composition of caramel varies according to the types of sugar used and the various processing conditions. Therefore, the World Health Organization and the United Nations Joint Evaluation Committee for Food Additives (JECFA) collaborated with the European Union and the United States in establishing classes of caramels. Generally, the caramel colour can be divided into four main classes according to the materials used in the preparations (Table 2.1) (JECFA 1992, FAO 2000).

Table 2.1: The official caramel colour classes established by the World Health Organization, Joint Evaluation Committee for Food Additives (JEFCA), with the European Union and United States. The information is adapted from (FAO 2000, JECFA 1992).

Caramel Colour Class	Preparation	Common name
I	Heating carbohydrates with or without acids or alkalis, no ammonium or sulfite compounds are used.	Plain or spirit caramel
II	Heating carbohydrates with or without acids or alkalis in the presence of sulfide compounds; no ammonium compounds are used.	Caustic sulfite caramel
III	Heating carbohydrates with or without acids or alkalis in the presence of ammonium compounds; no sulfide compounds are used.	Ammonia or beer caramel, baker's and confectioner's caramel
IV	Heating carbohydrates with or without acids or alkalis in the presence of both sulfite and ammonium compounds.	Sulfite-ammonia, soft drink caramel, or acid-proof caramel

As reported in Table 2.1, the type of caramel produced can be distinguished based on the reactants used (European Food Safety Authority 2011). The presence of an ammonium compound in the production of caramel colour class III and IV produces caramel with higher colour intensity compared to class I and II, because it initiates the dehydration of sugar via β-elimination process. This process is a pivotal step for colour formation and permits class III and IV to caramelize at a relatively lower temperature than class I and II (Myers and Howell 1992; Kroh 1994). During sugar caramelization, a series of chemical reactions occur. These include enolization of the sugar molecule, then dehydration and various reaction pathways take place such as dicarbonyl cleavage and retro aldolization. These are subject to the sugar used and pH of the reaction environment (Luna and Aguilera 2014; Tsai et al. 2009). Flavour compounds are produced in the initial stage of caramelization; as the reaction progresses bitter flavour compounds start to accumulate in parallel with formation of high molecular compounds via radical polymerization (Luna and Aguilera 2014). As a result, caramel with various shades can be produced, and so to the

flavour yield which ranges from mild, caramel-like and sweet to bitter (Kroh 1994). The caramelized compounds are grouped as high and low molecular weight compounds. The high molecular weight compounds, which are non-volatile coloured constituents, contribute to 90-95% of the total mass, whereas the remaining 5-10% are low molecular weight compounds (Paravisini et al. 2012). Low molecular weight compounds, such as 5-HMF, furfural and hydroxyacetylfuran, are considered precursors of brown pigmented polymers (Kroh 1994). In addition, N-heterocyclic compounds and sulphonic acids are formed when ammonium or sulphites are present in the reaction (Myers and Howell 1992). On the contrary, information about the structure of the high molecular weight fraction is still unclear; more studies are required to clarify this matter.

2.3.2 Maillard reaction

The Maillard reaction is a non-enzymatic browning process that involves condensation of a carbonyl group of a reducing sugar with an amino group from a peptide or protein. The reaction is favoured above 50˚C between pH 4-7, which is a typical physico-chemical condition in food processing (Kroh 1994).

Hodge (1953) simplified the Maillard reaction into three stages: the initial, intermediate and final stages. In general, the initial stage of Maillard reaction involves the condensation between an amine group from a protein or peptide and a reducing carbohydrate or sugar (Figure 2.4, Reaction A). The resulting Schiff base is an unstable and reversible compound. If a ketose is involved in the reaction, it forms 2-amino-2-deoxyaldose, which is known as a Heyns rearrangement product (HRP); whereas an aldose gives rise to 1-amino-1-deoxy-2-ketose, and is referred to as an Amadori rearrangement

product (ARP, Figure 2.4, Reaction B). Products formed in the initial stage are colourless and often undetected in the ultraviolet range. In the intermediate stage, the sugar dehydrates as a result of Amadori product degradation (Reaction C), and fragmentation of the sugar produces reactive carbonyl compounds (Reaction D). At the same time, amino acid (Strecker) degradation (Reaction E) takes place. Since there is a cascade of reactions taking place at this stage, colourless to yellow compounds which have high intensity in the ultraviolet range can be detected. The production of low molecular weight carbonyl compounds in this stage plays a pivotal role as precursors for colour, aroma, flavour compounds and AGEs. In the final stage of the Maillard reaction, more spontaneous transformations occur due to divergent pathways in the intermediate stage; these include the products of aldol condensation (Reaction F) without the intervention of amino compounds; and aldehyde-amine condensation that leads to the formation of nitrogen-containing heterocyclic compounds, such as melanoidin (Reaction G). The mixture of these products usually appears brown to dark. The Namiki pathway (Namiki and Hayashi 1983) (Reaction H) is the latest inclusion in the Hodge's diagram, and involves the fission of a Maillard intermediate, and the production of a pyrazinium radical cation before the Amadori rearrangement.

Although the Hodge's classification of the Maillard reaction is widely used to describe the progress of the reaction, some researchers have commented that Hodge's approach in classification is not convenient (van Boekel 2001), being too simplistic and not sufficient to describe the complexity (Yaylayan 1997). Yaylayan (1997) proposed a different approach by describing the Maillard reaction pathways derived from three primary fragmentation pools consisting the sugar, amino acid and Amadori and Heyns

products. This classification was intended to simplify the prediction system of Maillard reaction in models.

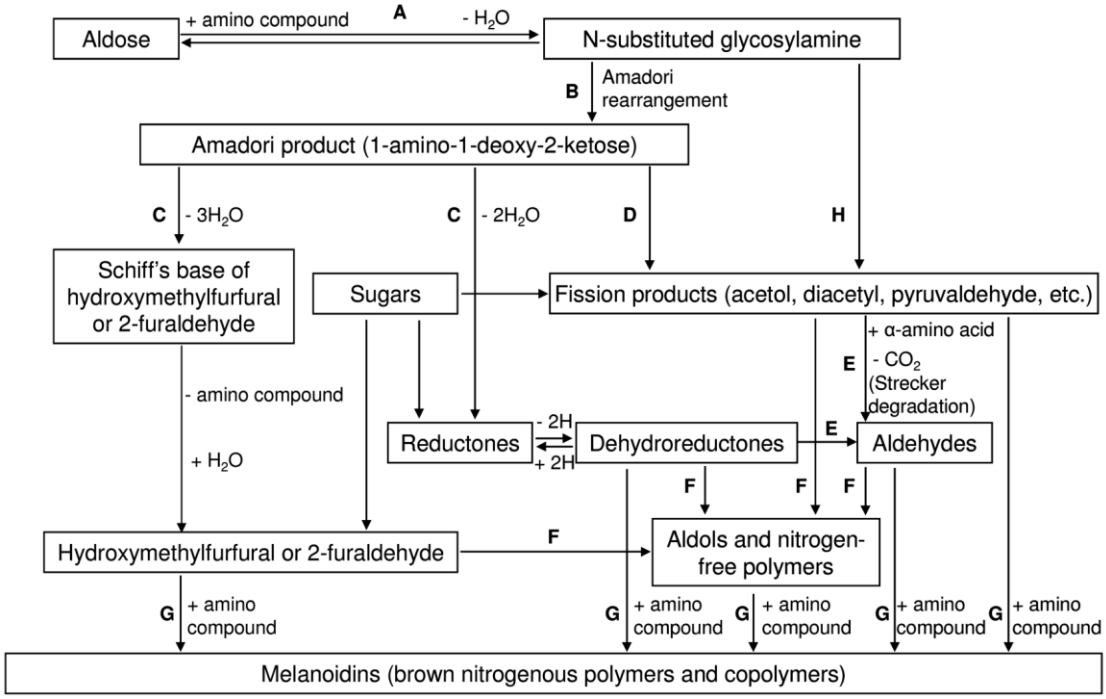


Figure 2.4: Hodge diagram, an extensive summary of the pathways of the Maillard reaction. Hodge summarized the reaction into 3 main stages that comprised of the initial stage (Reaction A & B), intermediate stage (Reaction C, D & E) and final stage (Reaction F & G). Free radical mediated degradation of the Maillard intermediates (Reaction H) was proposed by Namiki and Hayashi (1983). Figure reprinted with permission from Zhang, Q., Ames, J.M., Smith, R.D., Baynes, J.W., and Metz, T. 2009. A perspective on the Maillard reaction and the analysis of protein glycation by mass spectrometry: probing the pathogenesis of chronic disease. J Proteome Res 8 (2):754-769. Copyright (2009) American Chemical Society.

In order to demonstrate the diversity of the Maillard reaction, the role of its products will be discussed from the perspectives of flavour, aroma and antioxidation. In addition to that, its potential flavour and aroma precursors, as well as related potent antioxidant agents, will be highlighted.

2.3.3 The non-enzymatic browning of GlcN

2.3.3.1 α-dicarbonyl compounds

Hrynets, Ndagijimana, and Betti (2015a) demonstrated that GlcN is a Heyns compounds capable of producing α-dicarbonyls even at 37°C; approximately 1 - 6 mg of α-dicarbonyl compound was detected in every gram of incubated GlcN depending on the incubation time. The major α-dicarbonyls found during the non-enzymatic degradation were glucosone and 3-deoxyglucosone, but also found at lower levels were the short chain α-dicarbonyls like methylglyoxal, glyoxal and diacetyl. Glucosone is postulated to be formed through an oxidative pathway involving the Schiff base form of GlcN, while 3-deoxyglucosone is formed from the traditional 1,2-enolization pathway. A detailed scheme on the non-enzymatic degradation of GlcN to α-dicarbonyls compound is reported in Figure 2.5. Dicarbonyl compounds are generally regarded as one of the precursor groups for the generation of flavour and colour in food. Additionally, Hrynets and colleagues (2015a) proposed that the condensation between two dicarbonyl compounds, or alternatively, a dicarbonyl compound with GlcN—produced N-heterocyclic compounds such as FZ and DOF. A more in depth discussion on these autocondensation products is covered in section 2.3.3.2.

2.3.3.2 Autocondensation products from GlcN

GlcN can also undergo self-condensation forming a "symmetric" cyclic compound, yielding dihydrofructosazine [2,5-bis(arabino-tetrahydroxybutyl)dihydropyrazine)] with a $M + H^+$ m/z at 323.1454 (Hrynets, Ndagijimana, and Betti 2015a). Another group of GlcN autocondensation compounds are 2-(D-arabino-1',2',3',4'-tetrahydroxybutyl)-5-(D-erythro-

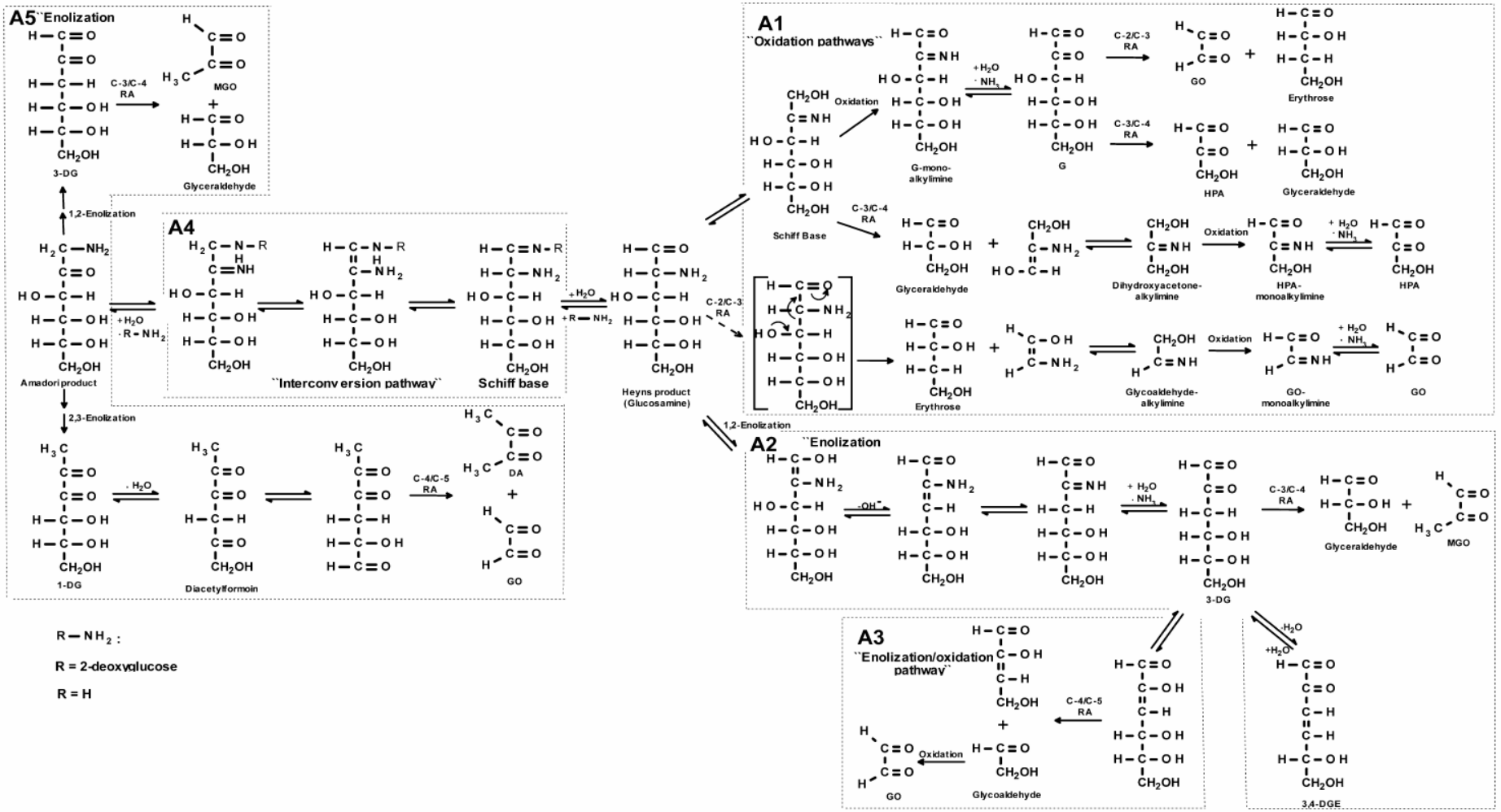


Figure 2.5: Proposed five pathways of α-dicaronyl formation from GlcN (a Heyns product) incubated at 37˚C: A1= oxidation, A2, A5= enolization, A3= enolization and oxidation, and A4= interconversion. G, glucosone; 3-DG, 3-deoxyglucosone; 1-DG, 1-deoxyglucosone; 3,4-DGE, 3,4-dideoxyglucosone-3-ene; GO, glyoxal; MGO, methylglyoxal; HPA, hydroxypyruvaldehyde; RA, retroaldolization. Figure reprinted with permission from Hrynets, Y., Ndagijimana, M., and Betti, M. 2015a. Studies on the formation of Maillard and caramelization products from glucosamine incubated at 37˚C. J Agric Food Chem. 63:6249-6261. Copyright (2015) American Chemical Society.

2",3",4"-trihydroxybutyl)pyrazinefructosazine (DOF) and 2,5-bis-(D-arabino-1,2,3,4-tetrahydroxybutyl)pyrazine (FZ) (Jia et al. 2014). Both DOF and FZ can be obtained from the same precursor—dihydrofructosazine. Dehydrogenation of dihydrofructosazine produces FZ ($M + H^+$ at m/z 321.15), further dehydration and isomerization yields DOF ($M + H^+$ at m/z 305.15) (Jia et al. 2015). DOF and FZ are pale yellow to yellow, are flavouring agents, and have been identified in soy sauce, caramel and tobacco (Henry et al. 2012). Interestingly, the DOF and FZ mixture, described as a light absorbing "colourless caramel", has been proposed as an additive for the prevention of light-induced off-flavour in light sensitive liquid food products such as beer (van der Ark et al. 2013). Due to the low toxicity of these compounds (LD_{50} > 2000 mg/kg, oral administration in mouse,) Aventis Pharma suggested a combination of FZ and DOF to prevent and treat type II diabetes (Bashiardes et al. 2002). In addition to that, when tested in an *in vitro* cell model in the form of FZ and DOF mixture, these GlcN derivatives exhibit superior anti-inflammatory activities compared to the native form—GlcN (Zhu et al. 2007).

Since DOF and FZ are versatile in the food flavour and pharmacological fields, researchers are trying to find ways to maximize the yield of the GlcN derivatives. Jang and colleagues used ionic liquid at 60 - 120˚C for 2 - 3 h to produce a large concentration of DOF and FZ, while minimizing the typical side products such as the condensation of GlcN that forms polymeric brown pigment compounds (Jia et al. 2014, 2015). Other possible pathways for DOF and FZ formed at 37˚C have also been proposed (Figure 2.6, Hrynets, Ndagijimana, and Betti 2015a). The condensation of a GlcN and 3-deoxyglucosone produces DOF, and interestingly, the cyclocondensation between two 3-deoxyglycosone yields a DOF isomer; whereas FZ forms when GlcN and glucosone condense.

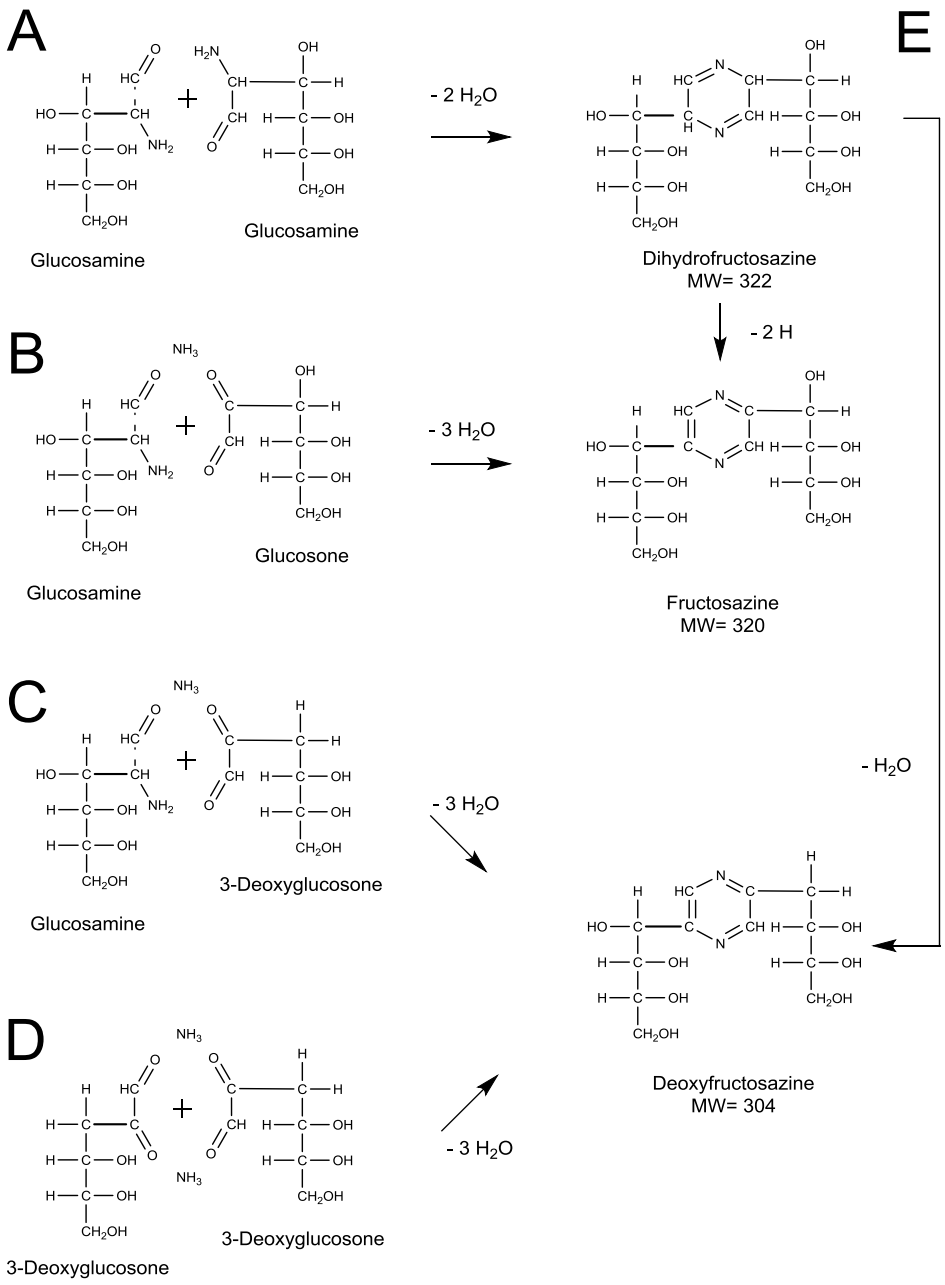


Figure 2.6: Proposed formation pathways of FZ (MW= 320) and DOF (MW= 304) from GlcN and α-dicarbonyls. In A, condensation between two molecules of GlcN gives dihydrofuctosazine as an intermediate, follow by dehydrogenation to yield FZ. Alternatively, it may be produced as illustrated in B, which is a condensation product of GlcN and glucosone. DOF can be obtained by reacting a GlcN with a 3-deoxyglucosone (C), or two molecules of 3-deoxyglucosone (D), or condensation of dihydrofructosazine (E). Figure adapted with permission from Hrynets, Y., Ndagijimana, M., and Betti, M. 2015a. Studies on the formation of Maillard and caramelization products from glucosamine incubated at 37˚C. J Agric Food Chem. 63:6249-6261. Copyright (2015) American Chemical Society.

2.3.4 The Maillard reaction products and their capacities as flavouring and antioxidant agents

2.3.4.1 α-dicarbonyl compounds

α-dicarbonyl compounds are the precursors of flavour, aroma and colour compounds and are mainly formed in the intermediate stage of the Maillard reaction. Henle's team (Degen, Hellwig, and Henle 2012) reported that the predominant dicarbonyls detected were 3-deoxyglucosone and 3-deoxygalactosone. On the other hand, methylglyoxal was identified as a minor component in a comprehensive study focusing on the concentration of dicarbonyl compounds in 173 commonly consumed food items (Degen, Hellwig, and Henle 2012). α-dicarbonyl compounds are usually obtained from the auto-oxidation of glucose (e.g.: glucosone), degradation of Amadori products (e.g.: 3-deoxyglucosone, 1-deoxyglucosone) and fragmentation of deoxyoxones (e.g.: C2, C3, C4 carbonyl compounds) (Gobert and Glomb 2009). Interestingly, deoxyoxones are able to form an imidazole and/or a pyrazine with ammonia (Belitz, Grosch, and Schieberle 2009), and thus are precursors for flavouring and browning.

Depending on the position of the carbonyls, some dicarbonyls such as 1-deoxyglucosone and glucosone can be called as reductones, due to the presence of enediol group next to a carbonyl (Kanzler, Haase, and Kroh 2014). These reductones can act as antioxidants by reducing the ferric ion or donating a hydrogen ion to quench a free radical molecule (Dittrich et al. 2003; Kanzler, Haase, and Kroh 2014; Kunz et al. 2013). As reductones, 1-deoxyglucosone and glucosone (Glomb and Tschirnich 2001) readily decompose through oxidative fragmentation. Glyoxal and methylglyoxal are the products

of the retro-aldolization of glucosone (Degen, Hellwig, and Henle 2012; Hofmann, Bors, and Stettmaier 1999) and 3-deoxyglucosone (Thornalley, Langborg, and Minhas 1999), respectively.

In another study, the concentration of GlcN derived dicarbonyls was investigated at 37˚C. Here the α-ketoamine structure resulted in a higher dicarbonyl contents in GlcN compared to glucose + ammonia reaction system (Hrynets, Ndagijimana, and Betti 2015a). These researchers also proposed several pathways that describe the formation of various α-dicarbonyl compounds from GlcN (Figure 2.5). The fundamental steps for short chain dicarbonyl formation focus on enolization, oxidation and fragmentation of GlcN (Figure 2.5, pathways A1- A3 and A5), with glyoxal and methylglyoxal as the common end products. Diacetyl which is an important flavour compound has been proposed as an 1-deoxyglucosone degradation product in pathway A5. Diacetyl is described as having a "buttery" or "butterscotch" aroma commonly found in dairy products, buttered popcorn and wine (Clarke and Winter 2015), and in some cases, it also described as a “caramel” odour (Chew and Smith 1992).

2.3.4.2 Maillard reacted peptides (MRPs) as taste flavour enhancers

The divergence of the Maillard reaction pathways gives rise to a variety of precursors for flavour and aroma. Many studies have been carried out to determine the direction of pathways that generate desired flavour and aroma compounds and also promote browning intensity. The Maillard reaction studies that have accumulated to date can be divided into two categories. Firstly, systematic approach models utilize a combination of simple amino acids and synthesized short chain peptides to react with a monosaccharide(s). Another

approach focuses on animal- or plant-derived proteins or peptides glycated with a monosaccharide(s). The latter approach often increases the yield of Maillard-based flavour enhancing glycated peptides, with the intention to assess its functionality and bioactivity. On the other hand, the former systematic approach enables researchers to manipulate the structural requirement of a taste enhancement property to steer a reaction towards an intended pathway. Regardless of the approaches, the Maillard reaction is capable of producing the so called “Maillard reacted peptides” (MRPs), a mixture of both Amadori-glycopeptide and AGE-peptides. Examples of AGE-peptides are reported in Figure 2.7.

A sodium reduction strategy was initiated by the United Kingdom Food Standards Agency (FSA) in 2003 (MacGregor, He, and Pombo-Rodrigues 2015); indeed, it caused a domino effect in the global food industry. This idea is adopted by Health Canada which targets to reduce sodium intake from 3400 to 2300 mg per day by 2016. The majority of Canadians' dietary sodium comes from processed foods. To aim for salt reduction, food manufacturers and the food service sectors are urged to reduce the sodium content to the lowest level possible without compromizing food safety and consumer acceptance (Health Canada 2012). Now flavour enhancers are used strategically to reduce sodium in food, and this has facilitated the creation of novel flavour enhancers over the past decade. Table 2.2 summarizes the recent development of Maillard-based flavour enhancers, including the MRP group. The major findings target the MRPs capacity to influence taste profiles of both umami and kokumi. Umami is related to the savouriness and meaty flavour and it can elevate the perception of saltiness. Kokumi is described as a sixth tastant and its first taste compound was isolated and described in garlic (Ueda et al. 1990). Its taste is bland but does enhance umami and indirectly enhances saltiness. In addition, kokumi can enhance

the perception of sweetness and increase mouthfulness, continuity and thickness intensity (Maruyama et al. 2012; Ueda et al. 1997). In 2006, Ogasawara et al. (2006) reported that the fractionated MRPs derived from soy peptide glycated with xylose (MW= 1-5 kDa) imparted both umami and kokumi when incorporated into consommé soup. Since then, soy peptides have been intensively studied. MRPs (MW= 1-5 kDa) produced with other reducing sugars such as galacturonic acid and GlcN, were also discovered as taste enhancers (Katsumata et al. 2008). Research is still in progress to define kokumi in a more

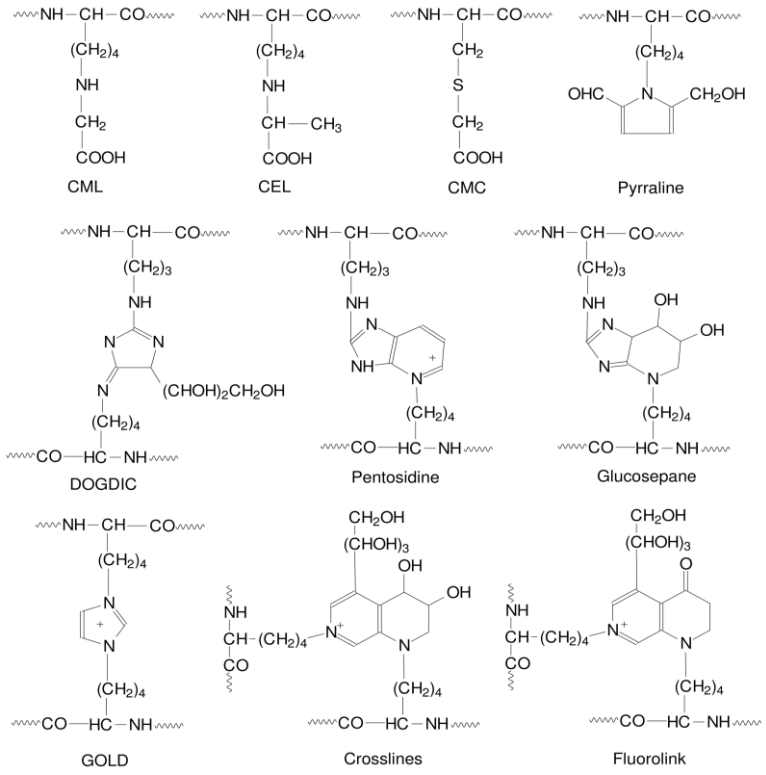


Figure 2.7: Some of the examples of AGE-peptides: Nε-(carboxymethyl)lysine (CML), Nε-(carboxylethyllysine) (CEL), S-(carboxymethyl)cysteine (CMC), pyrraline, 3-deoxyglucosone-derived imidazolium crosslink (DOGDIC), pentosidine, glucosepane, glyoxal lysine dimer (GOLD), crosslines, and fluorolink. Figure reprinted with permission from Zhang, Q., Ames, J.M., Smith, R.D., Baynes, J.W., and Metz, T. 2009. A perspective on the Maillard reaction and the analysis of protein glycation by mass spectrometry: probing the pathogenesis of chronic disease. J Proteome Res 8 (2):754-769. Copyright (2009) American Chemical Society.

precise manner. So far, calcium sensing receptors located in the tongue seem to be responsible for imparting kokumi the sensation triggered by the γ-glutamyl peptides. On the other hand, the MRPs are described as tasteless, odorless, non-pungent compounds, and they interact with TRPV1 taste receptor to impart salty taste in mice (Katsumata et al. 2008).

There is a growing market for clean ingredient labels. Health conscious consumers are avoiding autolysed yeast extract and hydrolysed vegetable protein ingredient declarations, since these are associated with monosodium glutamate (Watson 2011). Therefore, novel protein sources like sunflower hydrolysate, chicken peptide and shrimp hydrolysate are slowly gaining attention in the production of MRPs.

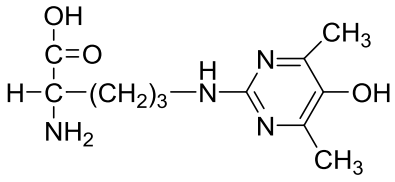
Most of the studies have not isolated nor identified the MRPs that impart umami or kokumi. The majority of these MRPs are produced under high temperatures combined with neutral to slightly basic conditions. On the contrary, Hofmann's group (Beksan et al. 2003; Ottinger, Soldo, and Hofmann 2003; Soldo, Blank, and Hofmann 2003) took a more systematic approach by synthesizing taste active MRP (i.e. alapyridaine) in both acidic and alkaline conditions, followed by identifying and characterizing the compounds that confer a given taste attribute.

Aside from the MRPs formed in the Maillard reaction models, enhanced meaty and seafood aroma can also be attributed by sulphur-containing compounds, nitrogen-containing compounds and aldehydes (Cai et al. 2016). However, these can impart a particular "burnt" taste at greater levels. Pyrazines are the most common volatiles generated

Table 2.2: Summary on the recent findings on the Maillard reaction mediated flavour imparting compounds.

Group	Substrates	Reaction condition	Findings
Ottinger et al.(2003)	Glucose + alanine	Reflux 30 min, pH 5.0	Alapyridaine intensified sweet, umami and salty taste (Soldo, Blank, and Hofmann 2003)
Beksan et al. (2003)	Monopotassium L-glutamate + Glucose/ β-D-fructose	75˚C, 1 h, non-aqueous, alkaline condition	Glycoconjugates impart umami taste
Ogasawara et al. (2006)	Isolated soy protein (1-5 kDa) + xylose	95˚C for 3.5 h	Enhanced umami, continuity and mouthfulness
Kraehenbuehl et al. (2008)	Monosaccharides : cysteine= 4:1 molar ratio	125˚C, 25 min, pH 6.5	Pyrazine and sulphur compounds formation favoured from amino sugar; high levels of formic/acid acids acidic sugars from acidic sugars
Katsumata et al. (2008)	Soy protein isolate (1-5 kDa) + Glucose / Xylose / Fructose / Glucosamine / Galacturonic acid	95˚C for 4.5 h	1-5 kDa fractions imparted salty taste
Lan et al. (2010)	Soy bean peptides + xylose	80-130˚C for 2 h	Crosslink of peptides (<1 kDa) resulted an increase in the 1 - 5 kDa fraction
Sun et al. (2010)	Chicken hydrolysate + glucose + xylose + vitamin B1 + cysteamine hydrochloride	90-120˚C, 1 h	Improved texture and sensory parameters
Liu et al. (2012)	Soy protein isolate + xylose	120˚C for 2 h	Enhanced effect on flavour: caramel-like, soy sauce-like odors, umami and mouthful tastes, reduced of bitterness
Huang et al. (2012)	Soy peptide + xylose + cysteine	80-120˚C for 1-2 h, cysteine was added at intervals	Cysteine inhibited color formation, produced meaty flavour
Song and Zhao (2013)	Soy protein hydrolysates (1 - 5 kDa) + xylose + cysteine + mTGase	120˚C for 4 h	Increased mouthfulness, umami, meaty flavour
Karangwa et al. (2014)	Sunflower protein hydrolysate/ free amino acid +xylose +/- cysteine	120˚C for 2 h, pH 7.4	Meat-like, umami and mouthfulness when cysteine was present in model; Caramel-like, bitter taste in models wihtout cysteine
Liu et al. (2015)	Chicken peptide + xylose	80 - 140˚C, 0.5-2 h, pH 6.5	>100˚C generated pyrazines that produced meaty aroma, ; mild heating generated umami and kokumi Maillard reaction product
Cai et al. (2016)	Shrimp waste hydrolysate + xylose	115˚C for 0-3 h, pH 8.0	Strong meaty and seafood aroma, umami and mouthfulness

at high temperatures, and they exhibit a roasted or toasted aroma, especially in foods that are heated in moisture-reduced, extruded systems (Ames, Guy, and Kipping 2001). Some of the MRPs, for instance the AGE-peptide can also have antioxidant activity. For example, argpyrimidine, a stable adduct derived from methylglyoxal and arginine residues in peptides (Nakadate et al. 2009), possesses a radical scavenging activity. The chemical structure of argpyrimidine is reported in Figure 2.8.



Argpyrimidine
MW= 254

Figure 2.8: Structure of agrpyrimidine.

2.3.4.3 Cyclic, heterocyclic compounds and melanoidins

With regards to flavour production in the Maillard reaction, the process can be traced back to as early as in the initial stage. The thermally unstable Amadori and Heyns rearrangement products do not contribute to the flavour profile, but do serve a pivotal point for flavour compound production (Mottram 1994).

Based on the depletion rates of amino acids, which is a common kinetic observed in the Maillard reaction, Jousse and colleagues (2002) developed a simplified model to describe the pathways involved in flavour generation, especially for volatiles (Figure 2.9). Overall, flavour generation pathways can be divided into 5 stages. Firstly, at elevated

temperatures, the formation of Amadori and Heyns rearrangement products (ARP, Figure 2.9) can be accompanied by sugar degradation (SD, Figure 2.9)—a caramelization process.

In the second stage, thermally unstable ARP or HRP degrade and cyclize to produce N-heterocyclic compounds such as pyrroles and pyridines (PY, Figure 2.9). Pyrroles are common aromas found in most cooked food, some of them described as caramel-like, sweet and corn-like (Mottram 1994). Fragmentation of the sugar backbone gives rise to rearranged sugars (RS, Figure 2.9). The RS either further react with ketones or rearrange themselves via ketonenol tautomerization, which gives back the amine.

Thirdly, RS cyclize to form O-heterocyclic compounds, such as furans and furfurals (FU, Figure 2.9), which are generally characterized as caramel-like, sweet and fruity (Mottram 1994). Alternatively, fragmentation of RS may take place, producing reactive α-dicarbonyl compounds (C, Figure 2.9) (short chain) that form FU.

In the subsequent stage, α-dicarbonyl compound can react with amino acid via Strecker degradation thus forming a Strecker intermediate (I, Figure 2.9). The amino acid in I provides nitrogen for pyrazine (PZ, Figure 2.9) formation. Pyrazine is an important class of flavour compound in the Maillard reaction. Some pyrazines can significantly contribute to some of the pleasant and desirable flavours at a sufficiently low threshold levels (at ppb range), including the roasted aroma in cooked foods, and also nutty and popcorn-like aromas (Ames, Guy, and Kipping 2001; Mottram 1994). Intact α-dicarbonyl favours the formation of Strecker aldehyes (SA, Figure 2.9), whereas other fragmented sugars generate odour-active acids (Hofmann, Munch, and Schieberle 1999). Although proline and hydroxyproline do not form amino-ketones and SA when reacting with

dicarbonyls due to their pyrrolidine ring (Mottram 1994), Jousse and colleagues (Jousse et al. 2002) grouped them under N-heterocylic compounds under the umbrella of SA. They are produced by reacting α-dicarbonyl with proline and hydroxyproline, respectively (Mottram 1994).

As the Maillard reaction proceeds, flavour compounds may form polymeric compounds known as melanoidins. Dicarbonyl compounds are the foundation of carbohydrate-based melanoidin formation generated at the final stage of the Maillard reaction. The production of melanoidin by methylglyoxal relies on the formation of a carbanion and then proceeds to aldol condensation under alkaline conditions. However, this has not been observed for glyoxal because glyoxal cannot form carbanion (Kroh, Fiedler, and Wagner 2008). The structure of melanoidin is still not well defined. It is generally grouped into two categories: high and low molecular weight melanoidins. High molecular weight melanoidin domains consists of several reacted dicarbonyls, pyrroles, pyrazines and pyridines, whereas low molecular domains are accompanied by furan, pyrrole and pyrrolinone structures and derivatives (Frank and Horfmann 2000; Kroh, Fiedler, and Wagner 2008; Wang, Qian, and Yao 2011). The high molecular size domains give a more intense colour than the low molecular size ones (Kroh, Fiedler, and Wagner 2008). Despite the lack of information on melanoidin structures, their antioxidant activity has been extensively studied. Some studies have shown an increased in the antioxidant activity correlated with browning intensity upon heating, whereas others did not observe this trend (Wang, Qian, and Yao 2011). Melanoidins also exhibit an *in vitro* iron chelating ability due to its anionic nature, however, no correlation has been established between browning and iron binding ability. The melanoidin chromophoric residues are not involved in the co-

ordination for iron complex formation (Morales, Fernandez-Fraguas, and Jimenez-Perez 2005), however, their anti-radical activity has been highly correlated with ferric reducing power (Delgado-Andrade, Rufian-Henares, and Morales 2005; Morales and Babbel 2002).

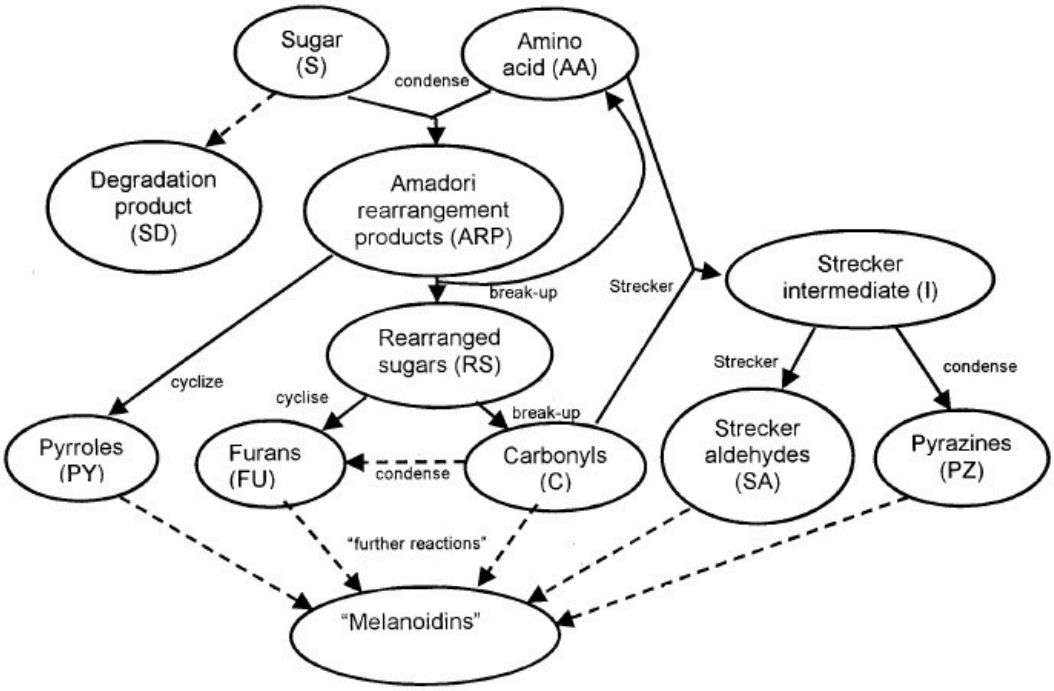


Figure 2.9: Possible volatile flavour synthesis pathways in the Maillard reaction that leads to formation of melanoidin. S= sugar; AA= amino acid; SD= sugar degradation product; ARP= Amadori rearrangement products; RS= Rearranged sugar; C= carbonyls; FU =furans (e.g.: furfurals, furanones); PY= pyrroles and pyridines; I= Streacker intermediate; SA= Strecker aldehyes; PZ= pyrazines. Figure adapted with permission from Jousse, E., Jongen, T., Agterof, W.,Russell, S., and Braat, P. 2002. Simplified kinetic scheme of flavor formaion by the Maillard reaction. J Food Sci 67 (7):2534-2542. Copyright (2006) John Wiley and Sons.

2.3.5 Toxic compounds from non-enzymatic browning reactions

There are multiple simultaneous reactions taking place in non-enzymatic browning reactions. This makes it very challenging to control the reaction, and the production of undesired side products is inevitable.

As discussed in section 2.3.4.1, α-dicarbonyl compounds are produced at the intermediate stage of the Maillard reaction because of glucose auto-oxidation, degradation of Amadori and Heyns products, and fragmentation of deoxyoxones (Gobert and Glomb 2009). Dicarbonyls are involved in flavour and colour generation in heated foods (exogenous Maillard reaction). On the other hand, endogenous formation of methyglyoxal and glyoxal is related to chronic diseases such as diabetes mellitus, central nervous system disorder and cardiovascular diseases (Maessen, Stehouwer, and Schalkwijk 2015; Papetti, Mascherpa, and Gazzani 2014). A study was conducted to compare dietary dicarbonyl intake levels with the corresponding levels *in vivo* (Degen, Hellwig, and Henle 2012). They found that elevated dietary dicarbonyl intake does not reflect a higher dicarbonyl concentration in the blood, suggesting that these compounds are not absorbed into the body.

The concentration of 5-HMF, 4-MEI and 2-acetyl-4-tetrahydroxybutylimidazole are undesirable components in caramel colours. The first two compounds are regarded as possible human carcinogens whereas the latter has immunosuppressive activity (European Food Safety Authority 2011; Goscinny et al. 2014). 5-HMF is formed under extreme heating and is the result of fructose or glucose degradation through the formation of 3-deoxyglucosone and its further dehydration (Capuano and Fogliano 2011; Nguyen et al. 2016). 4-MEI is formed under the reaction of methyglyoxal and ammonia at elevated temperatures above 120˚C (Karim and Smith 2016). First, ammonolysis of methylglyoxal leads to the formation of formamide, which later reacts with 2-aminopropanal to produce 4- or 5-MEI (Moon and Shibamoto 2011). 4-MEI is detected in class III and IV caramel

colours, whereas 2-acetyl-4-tetrahydroxybutylimidazole is exclusively found in class III only (European Food Safety Authority 2011).

AGEs are often described as glycotoxins and their prolonged presence in the body may pose a threat to health (Van Nguyen 2006). AGEs react with their receptors in plasma (RAGE) and disrupt intracelluar signalling, gene expression, and cause the release of pro-inflammatory substances and free radicals (Singh et al. 2014). Some researchers commented that among the AGEs identified, CML, pyrroles, imidazoles and pyridines are relatively non-reactive, and therefore non-toxic, whereas AGEs derived from 1-deoxyglucosone, 3-deoxyglucosone and methylglyoxal are toxic since they can further react in serum and tissue (Sato et al. 2006).

Acrylamide is another major concern in Maillard reaction products. The formation of major acrylamides is described with two main pathways (Nguyen et al. 2016). The first is a generic pathway which involving the interaction between a dicarbonyl compound from a reducing sugar and an acrylic amino acid, such as aspartic acid (Asp), carnosine, cysteine and serine via the Strecker degradation. In the second pathway, asparagine reacts with a reducing sugar forming a Schiff base, and directly undergoes decarboxylation to form acrylamide. This compound exhibits genotoxicity, neurotoxicity, and carcinogenicity in various organs (Capuano and Fogliano 2011).

2.4 Amino sugar as possible substrate for transglutaminase-mediated reaction

TGase (EC 2.3.2.13) belongs to a class of γ-glutamyltrasferases that facilitate the transamidation of the γ-carboxamide group of a glutamine residue (an acyl donor) to an

amine (an acyl acceptor). However, glutamine is deamidated when a suitable primary amine is not available. Commercialization of food grade microbial TGase in the 1990s had successfully expanded its application into the food industry. Now it is widely used to modify an array of proteins by the ε-(γ-glutamyl)-lysyl cross-linkage reaction in milk caseins, soy globulin, myosins and more (Motoki and Seguro 1998; Yokoyama, Nio, and Kikuchi 2004).

The TGase enzyme has poor recognition of the primary amine as a substrate. Therefore, almost any primary amine can become an acyl acceptor (Chica et al. 2004). GlcN which possess a primary amine in its structure is a potential acyl acceptor candidate for this transamidation. Chicken actomyosin glycosylated with intact GlcN at 37˚C showed an improvement on its emulsifying activity and stability (Hrynets, Ndagijimana, and Betti 2014). Studies have also demonstrated improvement in functional, textural and rheological properties of caseinate upon glycosylation with GlcN (Song and Zhao 2013; Zhu, Wang, and Zhao 2015).

Aside from the amino sugar GlcN, a pre-modified sugar moiety can be conjugated with the desired peptide in a block-wise manner (Ramos, Rollin, and Klaffke 2001). According to the Ramos et al. (2001), a pre-modified sugar portion can be protected by a glycoside when linked to an amino terminated spacer cross-linked with a peptide bound-glutamine residue in presence of TGase. This approach enables functionalized sugars to be added to a desired peptide.

2.5 Potential proteinaceous material suitable for the GlcN-induced glycation

2.5.1 Acid and alkaline recovered poultry proteins

There has been considerable attention focused on the isolation of valuable proteins from seafood and meat industry by-products since the 1970s. As a result, the fish surimi technology was invented and was eventually adopted into poultry protein isolate (PPI) processing techniques.

The basic steps in PPI production include (Appendix 1): meat grinding, homogenization, fat removal, protein solubilization (either at acidic [pH 2.5] or alkaline [pH 10.5] conditions), centrifugation and muscle protein isoelectic precipitation at pH 5.2, followed by the addition of cryoprotectants and then frozen storage (Khiari et al. 2014). A typical turkey derived PPI contains about 12-19% protein per wet weight (Hrynets et al. 2011; Khiari et al. 2014), and consists mainly of myofibrillar and sarcoplasmic proteins (Hrynets et al. 2011). Both acid and alkali extracted PPI do not differ in terms of protein functionality, however, extreme alkali conditions can cause deamidation of glutamine, meaning less substrate with which TGase to react. The acid-aided extracted protein in particular, is lighter appearance due to pigment removal and exhibits gel-like rheological characteristics. These improvements have enabled PPI to be successfully incorporated into marinated chicken breasts (Khiari et al. 2013) and processed poultry products, such as bologna and patties (Khiari et al. 2014; Omana, Pietrasik, and Betti 2012). Moreover, PPI can overcome the problem of "cereal-like flavour" reported for soy protein isolates—a popular ingredient used in meat processing (Williams and Zabik 1975). On the other hand, higher glutamic acid (Glu) in PPI compared to the raw meat (Hrynets et al. 2011) suggests it is a suitable ingredient for a savoury seasoning base. Therefore PPI may be a valuable proteinaceous material that can be subjected to enzymatic or chemical proteolysis to

produce peptides suitable for modification by GlcN non-enzymatic browning at moderate temperatures. This means the generation of MRPs with flavour and antioxidant compound components is possible.

2.5.2 Fish gelatin

Fish gelatin is hydrolysed collagen derived mainly from fish skins, common by-products in the fish processing industry. Commercial interest in fish gelatin is less encouraging than the bovine and porcine gelatin, mostly because of its suboptimal gelling point and lower gel strength (Eysturskarð et al. 2009). The hydroxyproline and proline contents in fish gelatin are lower than that of mammalian gelatines, meaning that fish gelatin is liquid at room temperature, putting significant limits on its application (Avena-Bustillos et al. 2006). On-going research has been conducted to improve the quality of fish gelatin extraction by optimizing the extraction method, or in finding ways to volarize the extracted gelatin. Some studies showed fish gelatin hydrolysates exhibit antioxidant activities (Mendis, Rajapakse, and Kim 2005; You, Regenstein, and Liu 2010; Zhang, Duan, and Zhuang 2012). Also, under-utilized fish protein hydrolysates have been demonstrated to have a better antioxidant activity when glycated with glucose (You et al. 2011). Taken together, these data suggest modification of fish gelatin hydrolysate via Maillard reaction may enhance its bioactivity and usefulness in the food industry.

Chapter 3 (Study 1): Glycation and transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine enhance bioactivity

This chapter is the modified version of the collaborative work published as "Hong, P.K., Gottardi, D,. Ndagijimana, M., and Betti, M. 2014. Glycation and transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine enhance bioactivity. Food Chem 142: 285-293."

3.1 Introduction

Glycation is a non-enzymatic reaction between amino acids and reducing sugars. It can occur in the body (endogenous) and in food systems (exogenous). Exogenous glycation, otherwise known as the Maillard reaction, has been studied extensively in food as it produces browning effects and flavour compounds. The accumulation of AGEs from Maillard reaction in human cells is regarded as detrimental to health. Often, it is associated with diseases such as diabetes, aging, renal failure and inflammation. In contrast to glycation, protein glycosylation is regarded as one of the major protein post-translational modifications in cells. It is an enzyme driven process that facilitates the production of biopolymers such as the production ofdeoxyribonucleic acid, ribonucleic acid, proteins and glycoproteins. Interest in the study of glycoproteins and glycopeptides has grown due to their potential biological applications in areas such as inflammatory response, reproductive system, immune system and neuronal development (Gamblin, Scanlan, and Davis 2009).

The potential of glycosylated and glycated protein and peptides is vast and can be further exploited in food systems. Incorporation of carbohydrate into protein has shown promising improvements in several aspects of functionality in glycosylated vegetable protein as well as in animal protein compared to its native protein form. There have been a handful of studies on determining the bioactivities of peptides but these have not fully examined the potential of glycopeptides in food systems. These bioactive compounds were successfully isolated from insects and plants.

In order to produce glycosylated and glycated peptides, the occurrence of a browning process and sugar degradation as part of the Maillard reaction in the reaction system seem to be a requirement. On top of that, the kinetics of the reaction depended on the type of sugars used (van Boekel 2001). As the sugar plays a pivotal role in the Maillard process, selection of a suitable candidate has to be carefully considered. A recent study conducted by Hrynets, Ndagijimana, and Betti (2013) demonstrated that the amino sugar GlcN was able to accelerate protein glycation at moderate temperatures (25 and 37˚C) as compared to glucose; therefore it could be a potential candidate also for peptide glycation.

TGase (EC 2.3.2.13) is a group of calcium-dependent enzymes that is involved in the post-translational modification of proteins. The use of TGase in food processing is popular in the dairy and meat processing sectors as it modifies the functional properties in proteins and peptides by introducing cross-links. It is more commonly referred to as an enzyme that catalyses acyl-transfer between the γ-carboxyamide group of peptide-bound glutamine residues that act as acyl donors with primary amines which are the acyl acceptors. In the absence of the amine substrate, TGase will undergo glutamine deamidation where water is used as an acyl acceptor. Furthermore, amino sugar such as

GlcN could be used as a substrate for creating a link between sugar and peptide or protein, which is referred as glycosylation. Since GlcN possess a free amino group, it could serve as an amine donor that will allow TGase to bind its α-amine group to a glutamine containing peptide. Thus, GlcN is an ideal candidate for such an interaction to modify the functionality and bioactivity of some common food protein hydrolysates. However, there is no literature reported thus far on producing glycosylated fish gelatin peptide via TGase using GlcN.

This study aimed to demonstrate a new approach for the production of glycosylated and glycated hydrolysates (Amadori-glycopeptides) derived from cold water fish skin gelatin and GlcN at moderate temperatures via TGase. The variations in bioactivity due to selected conditions of glycosylated and glycated hydrolysates were characterized.

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 Chemicals

Gelatin (from cold water fish skin), Alcalase (Alc, EC 3.4.21.14, from *Bacillus licheniformis*) and Flavourzyme (Flv, from *Aspergillus oryzae*), GlcN hydrochloride (GlcN), TGase from guinea pig liver, Folin-Ciocalteu reagent, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and Hanks' Balanced Salt Solution (HBSS) were purchased from Sigma-Aldrich (St. Louise, MO). All chemicals used in Size Exclusion Chromatography (SEC) and MALDI-TOF were of HPLC grade supplied by Sigma-Aldrich (St. Louise, MO), whereas other chemicals were of analytical grade. 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP) was obtained from Wako Chemicals USA, Inc. (Richmond, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA).

3.2.1.2 Bacterial strains and human HepG2 cells

Strains used for antimicrobial analyses were *Escherichia coli* AW1.7 (University of Alberta) and *Bacillus subtilis* FAD 110 (University of Alberta). HepG2 cells used in this study were purchased from ATCC (Manassas, VA).

3.2.2 Production of hydrolysates

Exactly 5% w/v of gelatin was dissolved in 0.05 M $(NH_4)HCO_3/NH_4OH$ buffer, the final pH of the mixture was pH 7.5 - 8.0. It was heated at 80˚C for 10 min. The gelatin mixture was cooled to 50˚C before adding Alc and Flv respectively at 1:10 enzyme:gelatin ratio followed by incubation in a shaker (50˚C, 3.5 h, 200 rpm). Incubation was terminated at 80˚C for 10 min, the hydrolysates were centrifuged at 10,000 × g (10˚C) for 15 min and filtered by using Whatman No. 1 filter paper. The filtrates from Alc hydrolysis (FA) and Flv hydrolysis (FF) were collected, lyophilized and stored at -18˚C for further use.

3.2.3 Glycation and TGase-meadiated glycosylation treaments

Samples obtained from the lyophilized hydrolysate powders (from FA and FF respectively) were weighed at 1.5 g each and added to GlcN at the weight ratio of 1:1. Each of the weighed powders was dissolved in 30 mL of 0.05 M $(NH_4)HCO_3/NH_4OH$ buffer (pH 7.0 ± 0.5). The experimental conditions were selected based on a preliminary study. In order to induce the production of TGase-mediated glycosylated peptides, FA and FF were mixed with GlcN respectively, and incubated at pH 7.5 with TGase (2 unit/g gelatin) at 25 and 37˚C for 3.5 h, producing FAT25, FAT37, FFT25, and FFT37 treatments (Figure 3.1). TGase was activated with 5 mM calcium chloride prior to use. On the other hand, the glycated peptides were prepared in the same manner, without the presence of TGase, thus

forming FAC25, FAC37, FFC25, and FFC37 treatments (Figure 3.1). Controls that consisted of the lyophilized hydrolysate were incubated at the same temperature without GlcN (Figure 3.1, FAH and FFH). At the end of incubation, all the mixtures were passed through a 0.2 µm nylon syringe filter (13 mm, Mandel, Ontario). TGase was removed by ultra-filtration with a molecular weight cut-off (MWCO) membrane of 10 kDa (3,900 × g, 20 min, 10˚C, Amicon Ultra Centrifugal filters (Millipore, Cork, Ireland)). Excess of GlcN was removed by dialysis membrane with a MWCO of 100-500 Da (Spectrum Laboratories, TX). Each of the retentates was collected and lyophilized, samples were then stored at -18˚C for further analysis.

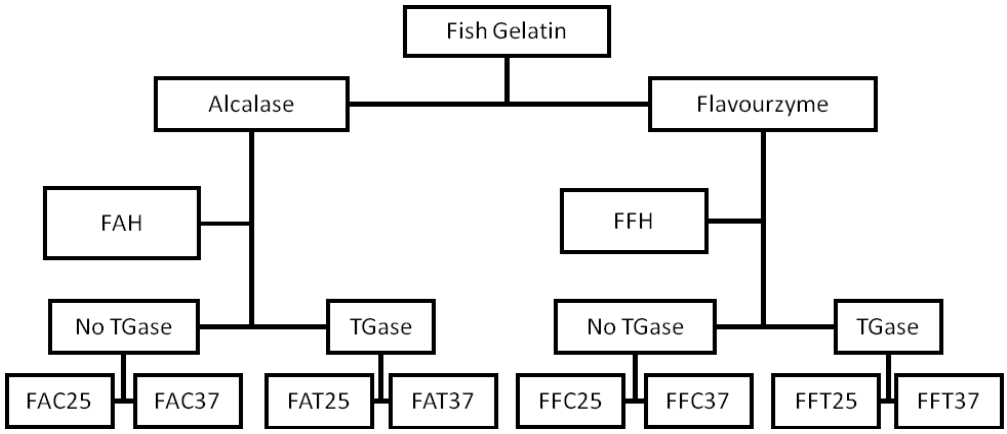


Figure 3.1: Production of glycated and TGase-mediated glycosylated fish gelatin hydrolysates. Cold water fish skin gelatin solutions were hydrolysed with Alc and Flv respectively, producing FA and FF. FA and FF were then incubated with GlcN in presence of TGase at 25 and 37˚C in order to obtain the TGase glycosylated treaments FAT25, FAT37, FFT25, and FFT37. Glycation treaments were prepared in the same manner without TGase producing FAC25, FAC37, FFC25 and FFC37. Controls were comprised of the respective hydrolysates (FAH and FFH). Figure reprinted with permission from Hong, P.K., Gottardi, D., Ndagijimana, M., and Betti, M. 2014. Glycation and transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine enhance bioactivity. Food Chem 142 (1):258-293. Copyright (2013) Elsevier Ltd.

3.2.4 Degree of hydrolysis (DH)

The measurement of DH was carried out according to O-phthaldialdehyde (OPA) method as stated by Nielsen, Petersen, and Dambmann (2001) by using serine as a standard for hydrolysis determination. Protein contents of gelatin samples were assessed. The percent of DH was calculated according to Alder-Nissen (1986).

3.2.5 Size exclusion chromatography (SEC)

Gelatin and hydrolysate samples were subjected to SEC using a HiLoad 16/60 Superdex 200 pg column (GE Healthcare Amersham Biosciences, Canada) connected to a fast protein liquid chromatography (GE Healthcare Amersham Biosciences, Canada). A sample volume of 500 µL (1 mg/mL) was injected and eluted isocratically at a flow rate of 1.2 mL/min with 50 mM phosphate buffer containing 0.15 M NaCl. Eluted peptides were detected at 215 nm. The mass calibration was performed using a protein mixture (MW 1332 Da-669 kDa).

As for glycosylated and glycated hydrolysates, each of the 100 µL (1 mg/mL) samples were eluted with a Superdex Peptide 10/300 GL column (GE Healthcare Amersham Biosciences, Canada) at a flow rate of 0.5 mL/min with the same mobile phase. Eluted peptides were detected at 215 and 280 nm. The mass calibration was performed using a protein mixture (MW 75 Da-12 kDa).

3.2.6 Determination of glycated and glycosylated peptides by Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS)

For analysis by MALDI-TOF-MS, the samples were diluted tenfold in 50% acetonitrile/water + 0.1% trifluoroacetic acid. One microliter of each sample was mixed with 1 μL of a-cyano-4-hydroxycinnamic acid (4-HCCA, 10 mg/ml in 50% acetonitrile/water + 0.1% trifluoroacetic acid). One microliter of the sample/matrix solution was then spotted onto a stainless steel target plate and allowed to air dry. All mass spectra were obtained using a Bruker Ultraflex MALDI-TOF/TOF (Bruker Daltonic, GmbH). Ions were analyzed in positive mode after acceleration from the ion source by 25 kV. External calibration was performed by use of a standard peptide mixture.

3.2.7 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging capacity was assessed by using Yen and Wu's method (1999) and modified by Hsu (2010). Exactly 200 μL of lyophilized sample was mixed into a test tube containing 400 μL of 0.5 mM DPPH and 1.4 mL of 99.5% methanol to achieve a final concentration of 2-10 mg/mL. L-ascorbic acid (0.1 mg/mL) was used to replace sample and referred to as positive control. The whole mixture was mixed thoroughly and incubated at room temperature in the dark for 60 min followed by reading its absorbance at 517 nm. The percentage of DPPH radical scavenging capacity was calculated as according to Hsu (2010). The EC_{50} value (concentration required for 50% reduction of activity) was determined.

3.2.8 Antioxidant activity in a linoleic acid peroxidation model system

Linoleic acid was oxidized in a model system according to Mendis, Rajapakse, and Kim (2005) with modifications from Li, Chen, Wang, Ji, and Wu (2007). Reagent mixture consisting of 1.5 mL of 0.1 M sodium phosphate buffer (pH 7.0), 1.5 mL of ethanol

containing 50 mM linoleic acid and 2 mL of 1 mg/mL of lyophilized sample dissolved in water were prepared. For the control, sample was replaced with 2 mM α-tocopherol and 2 mM BHT. All the mixtures were kept sealed and incubated in the dark at 40˚C. The oxidation level was measured every 24 h by using a ferric thiocyanate method as described by Osawa and Namiki (1981). Exactly 50 µL of aliquot from the test tube was mixed with 100 µL of 1 M HCl, 50 µL of 30% w/v ammonium thiocyanate, 50 µL of 20 mM ferrous chloride in 3.5% HCl and 2.25 mL of 75% ethanol. The mixture was homogenized and incubated at room temperature for 5 min before reading at 500 nm.

3.2.9 Preparation of human HepG2 cells

HepG2 cells were grown in growth medium (EMEM supplemented with 10% Fetal Bovine Serum (FBS), 50 units/mL penicillin and 50 µg/mL streptomycin) and were maintained at 37°C and 5% CO_2 as described previously by Wolfe and Liu (2007). Cells used in this study were between passages 5 and 10.

3.2.9.1 Cytotoxicity

HepG2 cells were prepared from the methods proposed from Wolfe and Liu (2007) and Kurosaki et al. (2008) with slight modifications. Cytotoxicity was determined using the protocols of the Water Soluble Tetrazolium Salts (WST1) assay. The inhibition of cell growth by the samples tested was referred as the percentage cell viability by referring to control. Concentrations of samples that decreased the absorbance by >10% when compared to the control were considered to be cytotoxic.

3.2.9.2 Cellular antioxidant activity (CAA) of hydrolyzed and conjugated samples

The CAA assay was carried out as described by Wolfe and Liu (2007). Each plate included triplicate samples, control and blank wells: control wells contained cells treated with DCFH-DA and oxidant; blank wells contained cells treated with dye and HBSS without oxidant. The CAA quantification was performed according to Wolfe and Liu (2007) and Xu and Chang (2010). The median effective concentration (EC_{50}) was defined as the dose required that result a 50% inhibition for sample calculated through software Prism 5.

3.2.10 Antimicrobial activity

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the mixture of glycosylated peptides were determined using *E.coli* AW1.7 and *B. subtilis* FAD 110. The strains were grown at 30˚C in Luria-Bertani Broth (LB) medium until their use. The bacterial culture was prepared at 104 cfu/mL in the 96-well microplates. Diluted sample solutions were dispensed into the wells providing final concentrations in the range of 0.21-40 mg/mL. The same tests were performed simultaneously for growth control (LB + bacteria) and sterility control (LB) using water instead of the mixture of glycopeptides. The final volume in each well was 200 µL, samples were incubated at 30˚C for 24 h.

The MIC of the samples was defined as the lowest concentrations preventing any discernible growth. From the same MIC well, 10 µL of suspension was plated in LB agar plate and incubated at 30˚C for 24 h. The absence of growth in the plate represented the MBC value, thus the capacity to completely kill all the bacteria. All the MIC and MBC were evaluated in triplicate.

3.2.11 Statistical analysis

Data were reported as means ± standard deviation of at least triplicates. Data from DPPH scavenging activity, CAA activity were reported as EC_{50} and IC_{50}, respectively, after analysed with Prism5 software (GraphPad Software Inc.). Means obtained from linoleic acid oxidation activity were analysed by one way analysis of variance (ANOVA), separated by Duncan test using PASW statistics software (Version 18). Means were considered significant when $p<0.05$.

3.3 Results and discussion

3.3.1 Characterization of fish gelatin, hydrolysates and glycopeptides by SEC

3.3.1.1 DH and characterization of fish gelatin and hydrolysates

Gelatin subjected to Alc digestion gave a higher DH of 4.39 ± 0.45% while Flv digestion showed better improvement in hydrolysis at 8.30 ± 0.19%. A closer look at the SEC result of the hydrolysates further confirmed that the Flv hydrolysate contained smaller peptide fractions than those derived from Alc (Figure 3.2A). The use of Alc and Flv in food protein hydrolysis has been extensively described. Alc is an endopeptidase and it has a range of specificity which commonly targets the cleavage site that contains hydrophobic residues in either the P′2 or P′3 positions especially when glutamine is located at the P1 position (Adamson and Reynolds 1996). Flv is a mixture of endoprotease and exopeptidase produced by *Aspergillus oryzae* giving it a broader range of action thus higher DH (Pedroche et al. 2002). Therefore, the higher DH contributed by Flv is in agreement with its broad spectrum for enzymatic activity.

3.3.1.2 Characterization of fish gelatin hydrolysates by SEC in response to GlcN treaments

The SEC chromatograms of the native hydrolysates and the glycoconjugted hydrolysates were presented in Figure 3.2 A-E. In general, the higher the level of modification, the lower the absorbance (at 215 nm) is. For instance, the FA hydrolysates glycosylated at 25˚C resulted in the most dramatic absorbance reduction at 215 nm, which concurred with the highest number of glycopeptides reported in the MALDI-TOF-MS spectrum (FAT25, Figure 3.3A). A possible explanation for this effect could be due to a shielding effect of the conjugated GlcN on the amide bonds. On the other hand, a different UV pattern was observed when the samples were analyzed at 280 nm; absorbance from all the GlcN treated samples was higher compared to the native hydrolysates as the temperature was increasing from 25 to 37˚C. Usually at 280 nm the relative amount of aromatic amino acids present in the sample is measured. Tyrosine and phenylalanine are the common aromatic amino acids found in cold water fish gelatin (Avena-Bustillos et al. 2006). It has been reported that non-enzymatic browning affects the amino acid composition and side chain groups of proteins and peptide; for instance the number of lysine and glutamine were reduced during the course of the reaction (Yoong et al. 1994). As a consequence, this might have increased the relative proportion of the aromatic amino acids resulting in a higher intensity at 280 nm; particularly at 37˚C where Maillard reaction was faster than at 25˚C.

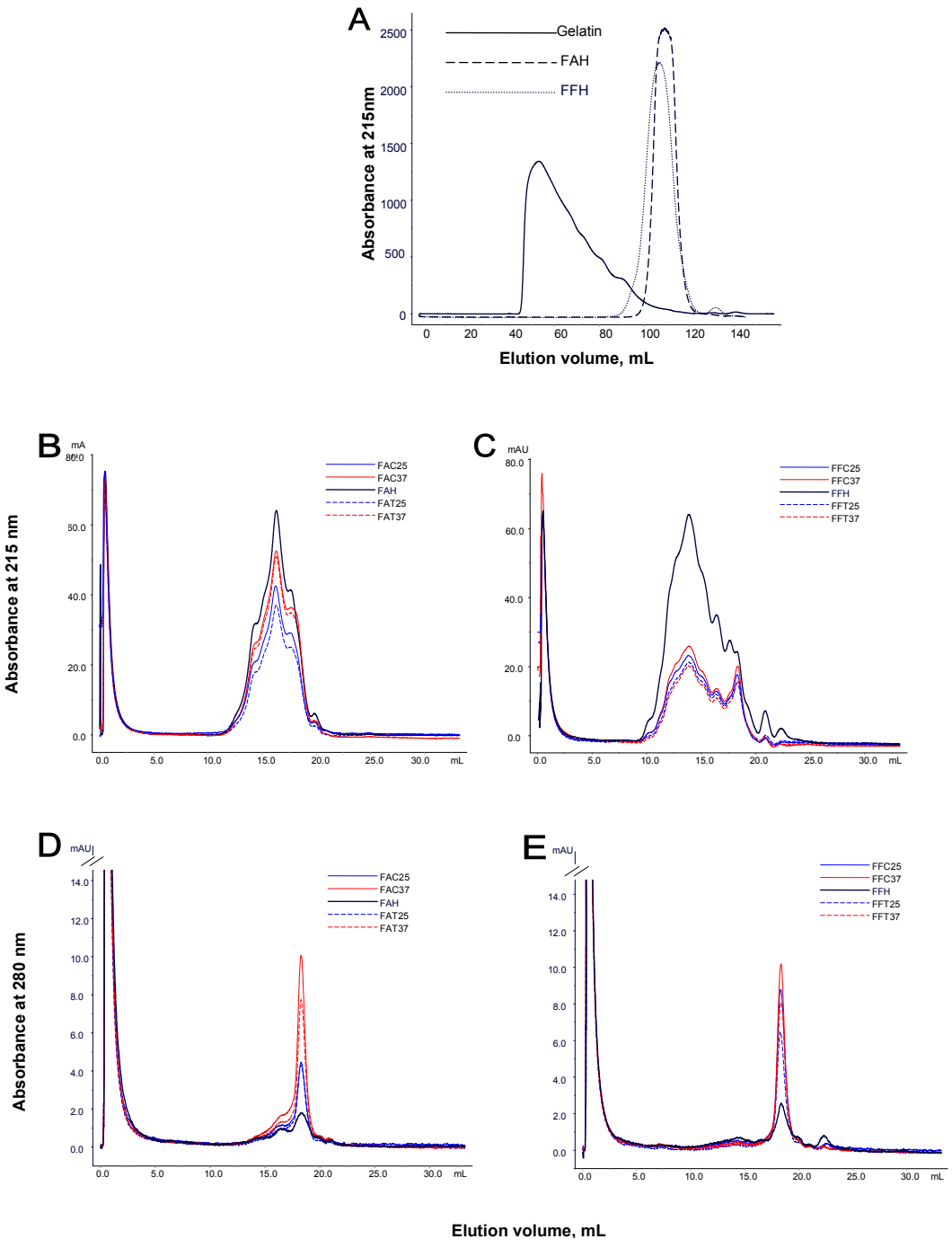


Figure 3.2. A: SEC profile of peptides obtained after fish skin gelatin was hydrolyzed with Alc (FA) and Flv (FF), respectively. B-E: SEC profiles of glycated (FAC25, FAC37, FFC25, FFC37) and TGase-mediated glycosylation treaments (FAT25, FAT37, FFT25, FFT37) of gelatin hydrolysates (FA, FF) with GlcN at 215 and 280 nm. Figure reprinted permission from Hong, P.K., Gottardi, D., Ndagijimana, M., and Betti, M. 2014. Glycation and transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine enhance bioactivity. Food Chem 142 (1):258-293. Copyright (2013) Elsevier Ltd.

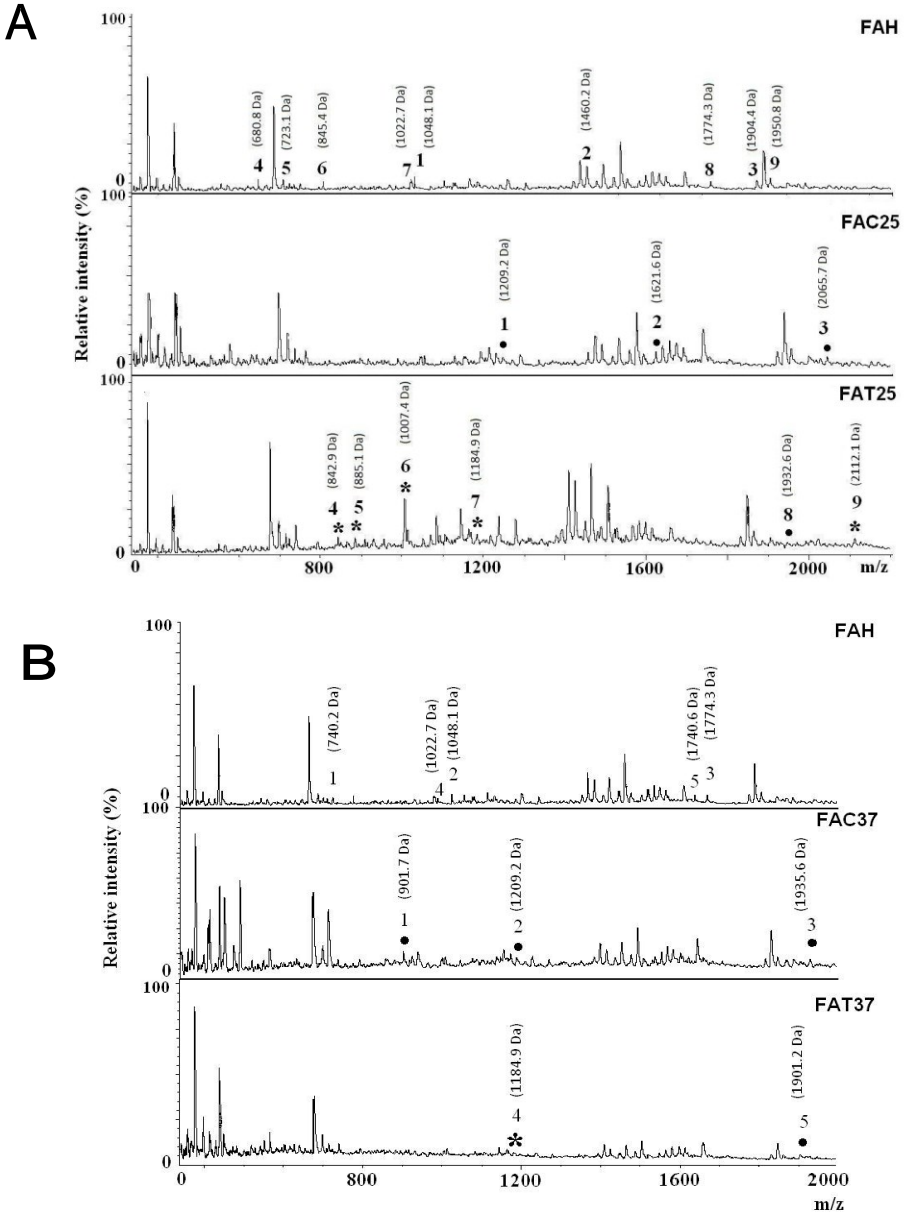


Figure 3.3: MALDI-TOF-MS spectra of hydrolyzed fish skin gelatin by Alc treaments (FAH, A and B), FA hydrolysate glycated with GlcN at 25˚C (FAC25, A) and 37˚C (FAC37, B); as well as FA hydrolysate glycosylated with GlcN at 25˚C (FAT25, A) and 37˚C (FAT37, B). Glycosylated peptides are marked with an asterisk (*) while Amadori-glycated peptides are marked with a filled circle (•). Glycated and glycosylated peptides (FAC25, FAC37, FAT25, and FAT37) and their native peptides (FAH) are indicated with the same number. Figure reprinted with permission from Hong, P.K., Gottardi, D., Ndagijimana, M., and Betti, M. 2014. Glycation and transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine enhance bioactivity. Food Chem 142 (1):258-293. Copyright (2013) Elsevier Ltd.

3.3.2 Proof of Amadori- and TGase-conjugated glycopeptides formation by MALDI-TOF-MS

The MW range of the peptides produced during hydrolysis of gelatin was determined using MALDI-TOF-MS. The MW of FAH was in the range of 500-2000 Da (Figure 3.3 A and B) while FFH showed a wider MW range (500-3600 Da, data not shown). The MW of Amadori-glycopeptides obtained after glycation at 25˚C (FAC25 in Figure 3.3A) and 37˚C (FAC37 in Figure 3.3B) respectively were reported. The result showed that the non-conjugated peptides and glycated peptides coexisted in the samples after GlcN treatments. The MW of glycated peptide was calculated by a mass shift of +161 Da between the native peptides and the glycated peptides, which indicates formation of an Amadori product. In fact, during glycation by GlcN, 1 mole of water was released (GlcN 179 Da – H_2O 18 Da). Three glycopeptides with the following protonated molecular masses were produced at both 25 and 37˚C: 1209.2, 1621.6, 2065.7; and, 901.7, 1209.2, and 1935.6 Da, respectively. Eventhough GlcN is one of the most reactive sugars, a low yield of glycation was evident at both temperatures due to the competition between GlcN self-condensation and glycation. For instance, GlcN can undergo a dimerization reaction (Zhu et al. 2007). When the Flv-derived hydrolysate (FF) was glycated with GlcN at 25˚C (FFC25) or 37˚C (FFC37), there was no difference in the amount of Amadori-glycopeptides produced compared to those obtained in glycated Alc-derived hydrolysate (FA) (data not shown).

The reaction between GlcN and fish hydrolysates was also performed in the presence of TGase from guinea pig liver. There are several types of TGase commercially available. In the food industry, microbial TGase is widely used in meat and fish to modify the texture by protein cross-linking. However, the commercial TGase contains sugars such

as maltodextrin or other carriers such as sodium caseinate. Therefore, pure TGase from guinea pig liver was used in this study in order to minimize any interference during glycosylation. This is because TGase recognizes the primary amino group of the glutamine bound peptide chain as an acyl donor, whereas GlcN could act as the acyl acceptor. Therefore this reaction could be regarded as glycosylation, as an acyl from the glutamine residue is attached to GlcN. The occurrence of glycosylation (glycosylated peptides) was determined by a mass shift of +162 Da (GlcN 179 Da – NH_3 17 Da). In addition, the possibility of GlcN which could react with peptides through the traditional mechanism of glycation was considered. Therefore, an additional mass shift of +161 Da related to glycation was taken into account when interpreting the result in FAT25 and FAT37, respectively.

As for FAT25 (Figure 3A) and FAT37 (Figure 3B), a mixture of peptides and glycopeptides (Amadori-glycopeptides + glycosylated peptides) was found; a total of six glycopeptides (5 glycosylated + 1 glycated) were produced at 25˚C; this is the highest number of total glycopeptides observed among all conditions tested. Their molecular masses were 842.9, 885.1, 1007.4, 1184.9, 1932.6, and 2112.1 Da. Regarding the treatment FAT37, only two glycopeptides (1 glycosylated + 1 glycated) with the molecular masses 1184.9 and 1901.2 Da were produced. Interestingly, temperature has a significant impact on the TGase mediated glycosylation, which was greater at 25˚C. However, differences in Amadori-glycopeptides production due to the temperature were not found. Similar trends were observed when FF was glycosylated at 25 or 37˚C where the total glycopeptides produced at 25˚C were greater than at 37˚C (data not shown).

To my best knowledge, this is the first report providing plausible evidence of glycosylation by GlcN of fish skin gelatin hydrolysates by means of TGase at moderate temperature. Jiang and Zhao (2011) attempted to incorporate GlcN into casein via microbial TGase at 37˚C. In order to deactivate the enzyme after the reaction, a heat treatment at 85˚C for five minutes was performed. The authors did not give any direct evidence of glycosylation. In addition, TGase deactivation at 85˚C was more likely to promote glycation and Maillard reactions involving unreacted GlcN.

3.3.3 DPPH radical-scavenging activity

The results obtained (Table 3.1) show that glycation and glycosylation treaments help in improving the overall DPPH radical scavenging activity in both hydrolysates. This is evident in FA as all the GlcN treaments only required 50% or less of the original hydrolysate (FAH) concentration to achieve EC_{50}. However, for FF samples, FFC25 did not show similar reduction in EC_{50} though the rest of the samples had a decrease in EC_{50} as compared to FFH. Oh and Lim (2008) suggested a dose dependent relationship of the *Dioscorea batatas* Decne glycoprotein's concentration with hydroxyl radical scavenging activity. The high polarity in the carbohydrate region enables oxygen scavenging activity, suggesting that glycoproteins could be both electron donors and electron acceptors in the biological system (van Boekel, 2001). In general, factors affecting the antioxidant capacity of glycopeptides are rather complex. For instance, the DPPH radical scavenging activity depends on the type of carbohydrate and protein content of a glycoprotein (Li et al. 2013).

Incubation temperature had an influence on the EC_{50}. FA and FF samples incubated at 37˚C for 3.5 h showed better DPPH radical scavenging activity compared to 25˚C.

FFT37 had the lowest EC_{50} (2.5 ± 0.2 mg/mL) followed by FAC37, FAT37 and FFC37 (2.6 ± 0.1, 2.6 ± 0.1, 2.9 ± 0.1 mg/mL, respectively). A possible explanation for this observation may be that incubation at 37˚C could induce higher formation of Maillard reaction products (i.e. AGEs) compared to the treatment at 25˚C. Another possible explanation for the higher scavenging activity observed at 37˚C may be due to the relative increase of aromatic amino acids as discussed in section 3.3.1.2. As a matter of fact, aromatic amino acids are regarded as effective radical scavengers (Rajapakse et al. 2005). On the other hand, radical scavenging activities of GlcN have been observed (Xing et al. 2006); therefore, using GlcN as a monosaccharide for conjugation may further improve the antioxidant capacity of the hydrolysates.

Table 3.1: DPPH scavenging activity and antimicrobial activity of Alc and Flv derived hydrolysates (FAH and FFH), and their glycated (FAC25, FAC37, FFC25, and FFC37) and glycosylated hydrolysates (FAT25, FAT37, FFT25, and FFT37). Table reprinted with permission from Hong Hong, P.K., Gottardi, D., Ndagijimana, M., and Betti, M. 2014. Glycation and transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine enhance bioactivity. Food Chem 142 (1):258-293. Copyright (2013) Elsevier Ltd.

Sample	DPPH scavenging activity		Antimicrobial activity, mg/mL			
			E. coli		*B. subtilis*	
	EC_{50} (mg/mL)	R^2	MIC	MBC	MIC	MBC
FAH	7.1±0.0	0.8099	-	-	-	-
FAC25	3.8±0.3	0.9837	-	-	-	-
FAC37	2.6±0.1	0.9517	-	-	-	-
FAT25	3.4±0.1	0.9797	40	40	-	-
FAT37	2.6±0.1	0.9928	40	40	-	-
FFH	4.8±0.3	0.9900	-	-	-	-
FFC25	5.2±0.1	0.7145	40	40	40	-
FFC37	2.9±0.1	0.8020	40	40	-	-
FFT25	3.4±0.1	0.9851	40	40	40	-
FFT37	2.5±0.2	0.9303	40	40	40	40

EC_{50} = Half maximal effective concentration
MIC = Minimal inhibitory concentration
MBC = Minimal bactericidal cocentration

3.3.4 Antioxidant activity in a linoleic acid oxidation model

Figure 3.4 A and B showed that absorbance gradually increased and reached a maximum level as the incubation time progressed, suggesting more lipid hydroperoxides were produced over time. FAH and FAT25 showed significantly higher antioxidant activity ($p<0.05$) than the other samples. In FF samples, FFC25 showed a positive response towards inhibiting lipid oxidation ($p<0.05$) while other samples in the group did not show any interesting antioxidant activity. The presence of hydrophobic peptides in sequences caused by Alc cleavage preferences during hydrolysis may contribute to the inhibition of lipid oxidation, since these peptides are more soluble in lipids (Adamson and Reynolds, 1996; Hsu, 2010). FAT25 showed a better overall stability than FAH ($p<0.05$) throughout the course of the test, and offered greater inhibition towards lipid oxidation. This suggests that the lipid oxidation inhibition in FAT25 is a concerted effect of a mixture of glycated and glycosylated peptides produced at 25˚C as well as the non-conjugated peptides.

Here it is postulated that FAH contained a certain level of hydrophobic peptides which were effective towards lipid oxidation. Furthermore, the reducing power and metal ion chelating activity of Alc mediated hydrolysates were documented (Kong and Xiong, 2006). In FAT25, some of the peptides were involved in glycosylation and glycation reactions, thus lowering its hydrophobicity. Despite the change in the hydrophilic-hydrophobic balance in FAT25 as compared to FAH, the combined effect of glycopeptides and hydrophobic peptides improved its oxidation inhibitory activity. In fact, this study had showed that glycoproteins possess metal chelating effect (Qian, Liu, and Eaton 1998). FAT37 had less glycosylated and glycated peptides in the mixture (Figure 3.3B), and showed less stability towards inhibition of lipid peroxidation. It appeared that a higher

amount of glycosylated peptides in FAT25 contributed to a reduction in lipid oxidation compared to the other treatments. Further study is necessary to investigate the mechanism for the mode of action of FAT25.

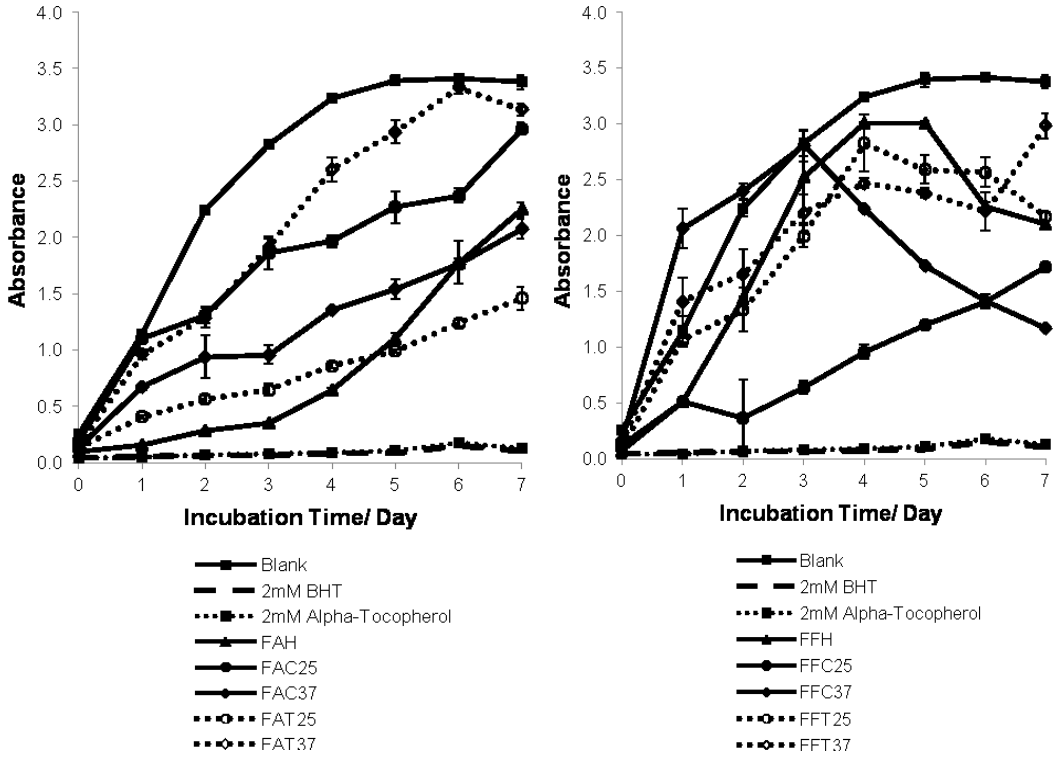


Figure 3.4: Antioxidant activity of Alc (A) Flv (B) derived fish hydrolysates (FAH, FFH) and their glycated (FAC25, FAC37, FFC25, and FFC37) and glycosylated hydrolysates (FAT25, FAT37, FFT25, and FFT37) in a linoleic acid oxidation model system. Figure reprinted with permission from Hong, P.K., Gottardi, D., Ndagijimana, M., and Betti, M. 2014. Glycation and transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine enhance bioactivity. Food Chem 142 (1):258-293. Copyright (2013) Elsevier Ltd.

3.3.5 CAA in HepG2 cells

Evaluation of antioxidant activity using cell cultures is a more biologically relevant method than chemical assays, because it provides information on the mode of action of antioxidants within cells (Carrosco-Castilla et al. 2012). Before measuring CAA, all the samples

(ranging from 0.1 to 1 mg/mL) were tested for their cytotoxicity level. All the cytotoxicity values for FA were higher than 0.5 mg/mL, while for FF the cytotoxicity values were above 0.1 mg/mL. These concentrations were viable for the cells tested with less than 10% of lethality, moreover, they were within the common range of concentrations used in other studies (Carrosco-Castilla et al. 2012). Subsequently, the samples were adjusted to concentrations lower than their respective cytotoxic levels in order to measure CAA. FAT25 showed a positive dose-dependent antioxidant activity with increasing sample concentration; the highest CAA was recorded at 29.6 ± 2.6% when tested at 500 µg/mL (Figure 3.5). The estimated IC_{50} for FAT25 was 95.49 µg/mL (R^2= 0.96). All other samples did not show any changes in this antioxidant assay.

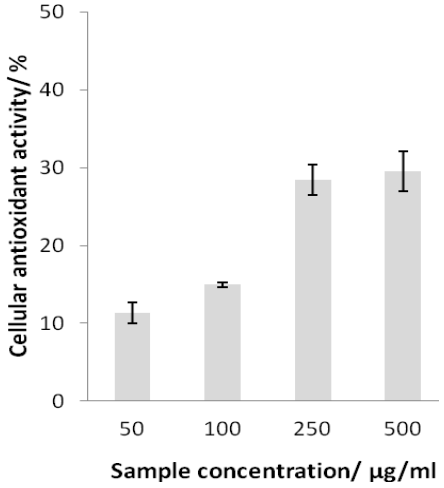


Figure 3.5: Cellular antioxidant activity of Alc-derived fish gelatin hydrolysate glycosylated at 25˚C (FAT25) analysed at different concentration (µg/mL). Figure reprinted with permission from Hong, P.K., Gottardi, D., Ndagijimana, M., and Betti, M. 2014. Glycation and transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine enhance bioactivity. Food Chem 142 (1):258-293. Copyright (2013) Elsevier Ltd.

The CAA assay was able to assess the ability of the compounds to prevent oxidation in two ways: firstly, at the cell membrane level by breaking up the peroxyl

radical chain reaction (which is found at the lipid bilayer); secondly, at the intracellular level by counteracting reactive oxygen species (ROS) (Wolfe and Liu 2007). Previously in the linoleic acid oxidation model, FAT25 showed capability in preventing lipid peroxidation as a function of time. A combined effect of glycopeptides plus hydrophobic peptides was hypothesized to explain the antioxidant capacity of FAT25 as compared to FAH. It is well known that hydrophobic amino acids enhance the antioxidant activity of a peptide by allowing the peptide to reach hydrophobic targets like cell membranes (Hsu 2010). Here this study has demonstrated that the formation of glycopeptides can increase the bioactivity of fish gelatin peptides.

3.3.6 Antimicrobial activity

E. coli (G-) and *B. subtilis* (G+) were tested for their minimal inhibitory concentration (MIC). When no microbial growth was observed, the relative minimum bactericidal concentration (MBC) was estimated. Results in Table 3.1 show that glycosylation and glycation improve the antimicrobial activity of the native hydrolysates. The antimicrobial activity of glycosylated and glycated treatments obtained from FF was independent from the use of TGase as a catalyst, particularly when tested with *E. coli*. All the treatments, except FFH, showed the same MIC and MBC values at 40 mg/mL. On the contrary, the same treatments were less effective against *B. subtilis*. Though FF treatments possessed microbial inhibiting properties, they were not found to be bactericidal at the same concentration, except for FFT37. This wider activity spectrum demonstrated in the FF treatments suggests that glycation could be responsible for the antimicrobial properties.

On the other hand, TGase had less impact on the antimicrobial activity of glycosylated samples obtained from FA treatments. The absence of driving factor (TGase) in the treatments FAH, FAC25 and FAC37 reaffirmed the notion that the activity is contributed by the glycosylated FA hydrolysates. In fact, both FAT25 and FAT37 showed antimicrobial effects that were specifically targeted against *E. coli* but not *B. subtilis*. This suggests that the antimicrobial effect may vary depending on the samples' initial peptide MWs and the position of glutamine in hydrolysates available for TGase reaction. Overall, all the treatments tested show more specificity towards *E. coli* compared to *B. subtilis*; glycated FF treatments seem to perform better in this case due to their wider antimicrobial spectrum. However, the high specificity of glycosylated hydrolysates (FAT25 and FAT37) toward the inhibition of *E. coli* is an aspect worthy of further exploration.

The antimicrobial activity of glycopeptides is widely documented in the literature. Some glycopeptides, such as Vancomycin (an amphoteric glycopeptide antibiotic) and its related class, are used against antibiotic-resistant pathogens. They work by interfering with the biosynthesis of the peptidoglycan layer of the bacterial membrane. They are effective against G+ bacteria but not G- bacteria, due to the outer membrane in G- bacteria that keeps glycopeptides from reaching their targets at the periplasmic face of the cytoplasmic membrane (Kahne, Leimkuhler, Lu, and Walsh 2005). Whereas there are glycopeptides that are effective against G- bacteria (Table 3.1). Drosocin, a glycopeptide isolated from insects, is a small proline rich peptide (MW: 2-4 kDa) with a disaccharide (galactose and N-acetylgalactosamine), which is mainly active towards G- bacteria (Bulet and Stöcklin 2005). Otvos and coworkers (2002) reported that the antibacterial activity of drosocin was enhanced approximately 100 times when glycosylated. Literature has also shown that

peptides rich in proline are potential antimicrobial peptides (Reddy, Yedery, and Aranha 2004), and proline is a common amino acid found in gelatin. The antibacterial mechanism depends on the interaction with the lipopolysaccharides of G- bacteria as well as the bacterial chaperone and heat shock proteins GroEL and DnaK. This interaction inhibits protein folding (Kragol et al. 2001). This mechanism could explain how our samples containing glycopeptides work as antimicrobial compounds.

3.4 Conclusions

In this study, it is verified that glycopeptides were produced by GlcN treaments at 25 and 37˚C in presence or absence of TGase. The results highlighted that the GlcN-induced glycation and glycosylation can enhance the antioxidant and antimicrobial properties which were not found in the untreated hydrolysates; particularly the treatment of FAT25 which contains a mixture of hydrolyzed, glycated and glycosylated peptides prepared at 25˚C for 3.5 h. These properties could be further improved by fractionation in order to purify the active compounds. The potential of glycopeptide application in foods is promising and it is expanding in the nutraceutical field. The strategy adopted in this study showed a promising future in producing new glycopeptides from animal by-products. Work on developing glycopeptides' sensory properties is presented in Chapter 4.

Chapter 4 (Study 2): Glucosamine induced glycation of hydrolyzed meat proteins in the presence or absence of transglutaminase: Chemical modifications and taste enhancing activity

This chapter in full has been published as "Hong, P.K., Ndagijimana, M. and Betti, M. 2016. Glucosamine-induced glycation of hydrolyzed meat proteins in the presence or absence of transglutaminase: Chemical modifications and taste-enhancing activity. Food Chem 197: 1143-1152."

4.1 Introduction

The global trend to reduce sodium in food is due to health concerns of hypertension and cardiovascular disease. One of the strategies to reduce salt intake is through the addition of flavour enhancers in processed foods. Unlike food flavours, flavour enhancers do not possess flavour or taste themselves, but rather intensify the flavours of other compounds. Common commercial flavour enhancers are inosinates, guanylates and glutamates. Since consumers are demanding "natural" food ingredients, several studies explored alternatives such as the deamidation of wheat gluten protein (Liao et al. 2010; Schlichtherle-Cerny and Amado 2002), enzymatic hydrolysis of shrimp protein (Cheung and Li-Chan 2014), and chicken muscle protein (Maehashi et al. 1999). These peptides and proteins were reported to enhance the umami (savoury) and salty taste. For instance, Maehashi et al. (1999) isolated an umami fraction from chicken proteins hydrolyzed with bromelain; within this fraction several di- and tri-peptides containing Glu (i.e. Glu-Glu and Ala-Glu-Asp,

respectively) were identified. These peptides demonstrated an increase to the umami taste when used in combination with 5'-inosine monophosphate, a commercial flavour enhancer. Also, meat proteins contain high levels of Glu and Asp that can be released with chemical or enzymatic hydrolysis to increase the umami taste. The recent interest in the valorization of meat and fish processing by-products has led to the production of muscle protein isolates recovered through the isoelectric solubilization and precipitation process (ISP). This can be a suitable and an economic protein-based biomass to hydrolyze and unleash the potential of Glu in amplifying the deliciousness of food. For instance, Hyrnets et al. (2011) found that Glu significantly increased after the ISP of mechanically separated poultry meat compared to the starting material. Even without hydrolysis, poultry protein isolate (PPI) was successfully incorporated in chicken patties with positive consumer sensory acceptability (Khiari et al. 2014).

The Maillard reaction, also known as glycation, is a common process to generate food flavours. This reaction involves the condensation of the carbonyl group of a reducing sugar with an amino compound, followed by the degradation of the condensation products to α-dicarbonyl compounds. Subsequently, α-dicarbonyl compounds react with other compounds such as amines, amino acids, aldehydes, hydrogen sulfide and ammonia, leading to many important classes of flavour compounds including furans, pyrazines, pyrroles, oxazoles, thiophenes, thiazoles and other heterocyclic compounds. Several studies have been conducted in a simple model system consisting of an amino acid or a peptide and a reducing sugar to generate specific flavour compounds (Lee, Jo, and Kim 2010; Xu et al. 2013). Recently, some compounds generated through the Maillard reaction, specifically the so called "MRPs" (Maillard reacted peptides), possess flavour-enhancing properties. For

instance, soy protein isolate glycated with various reducing sugars (Katsumata et al. 2008; Lan et al. 2010; Ogasawara, Katsumata, and Egi 2006; Song et al. 2013) as well as xylose glycated with sunflower protein hydrolysate (Eric et al. 2013) yield a mixture of MRPs (modified peptides + glycopeptides), which not only enhanced the savoury taste, but also increased the intensity of the mouthfulness and continuity sensations known as "kokumi". On the other hand, despite the usefulness of the Maillard reaction to generate both flavours and MRPs, one of the negative effects is the production of browning compounds. These compounds are usually associated with toxic substances, such as acrylamide and AGEs. This becomes particularly relevant when the Maillard reaction is conducted at elevated temperatures (i.e. >100˚C), especially during cooking (Mottram, Wedzicha, and Dodson 2002; Poulsen et al. 2013; Stadler et al. 2002). Consumers demand the production of "clean" and "natural" label ingredients. Therefore, alternative approaches which minimize the formation of browning compounds while increasing the production of the flavour enhancing MRP are of interest. In this aspect, Hong et al. (2014) and Hrynets et al. (2013) had successfully glycated fish gelatin hydrolysate and myofibrillar proteins at moderate temperature (37˚C) to generate modified peptides and proteins with enhanced bioactivity (i.e. antioxidant capacity) and functionality (i.e. solubility), respectively. This was likely due to the use of the highly reactive amino sugar GlcN in concert with the enzyme transglutaminase (TGase). Here, novel glycopeptides can be produced due the ability of TGase to catalyze the transfer of an acyl group from a glutamine amino acid in a protein or peptide sequence to the amino group of GlcN forming a stable isopeptide bond. The effectiveness of using GlcN as a way to modify peptides has been already exploited by Katsumata et al. (2008). In this study, a fraction of soy protein hydrolysate was glycated at

95˚C for 4.5 h, generating MRPs that modulate the salty taste. However, at 95˚C, browning and heterocyclic compounds may be generated. It is my intention in this current research to generate MRPs using GlcN at significantly lower temperatures that still amplify saltiness and increase savouriness.

The main objective was to evaluate the chemical and enzymatic modifications of hydrolyzed poultry meat proteins in response to GlcN glycation in the presence or absence of TGase at moderate temperatures (37 and 50˚C). I examined how this affected the saltiness and savoury perception of the modified hydrolysates in the seasoning composition. The PPI extracted at a pilot plant facility with the ISP process was hydrolyzed with a commercial protease Alc and subjected to GlcN glycation treatment in presence or absence of TGase. Then, it was used to formulate a liquid seasoning composition which was subsequently used for a consumer sensory evaluation. Chemical changes regarding composition and structure due to GlcN treatments were analyzed and correlated to the consumer panel results.

4.2 Materials and methods

4.2.1 Chemicals

Mechanically separated turkey meat (MSTM) was obtained from Lilydale Inc. (Edmonton, Canada). Alcalase was a gift from Novozymes (Franklinton, NC). GlcN hydrochloride and reduced glutathione (GSH) were purchased from PureBulk (Roseburg, Oregon). Food grade microbial TGase (ACTIVA® TI) was manufactured by Ajinomoto (France). Food grade hydrochloric acid, sodium hydroxide and acetic acid were purchased from Fisher Scientific (Fisher Scientific Company, Ottawa, ON). Monosodium glutamate was purchased from Ajinomoto (La Victoria, Peru). Chemicals used for poultry protein

isolation and sensory evaluation were of food grade. All other chemicals and solvents used in other analysis and liquid chromatography were of analytical grade or HPLC grade. They were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Lois, MO) and Fisher Scientific (Fisher Scientific Company, Ottawa, ON).

4.2.2 Experimental Design

Poultry protein was isolated from commercial MSTM using the acid-aided ISP process. The poultry protein was extracted from 80 kg of MSTM in four batches. Subsequently, they were pooled into one batch, stored at -20˚C and used within 3 months. Extracted protein (6.0-6.5% w/v) was hydrolyzed with Alcalase in 11 batches and pooled. Followed enzymatic hydrolysis, the glycation treatment was performed. The hydrolysate (5% protein) was incubated with GlcN either in the presence or absence of TGase at moderate temperatures (37 or 50˚C) and at pH 7.0 ± 0.5 resulting in the following treatments: native hydrolysate 37˚C, native hydrolysate 50˚C, glycated hydrolysate 37˚C in absence of TGase, glycated hydrolysate 37˚C in presence of TGase, and, glycated hydrolysate 50˚C in absence of TGase.

For chemical analysis purpose, all the treatments were acidified to pH 4.9 with food grade HCl at the end point of incubation, pasteurized at 80˚C for 10 min, lyophilized and kept at -18˚C until use. Chemical modification of the hydrolysate in response to GlcN glycation was assessed using spectroscopic techniques (see section 4.2.4) and α-dicarbonyl compounds formation (section 4.2.5).

For the sensory evaluation, the native and glycated hydrolysates were used to formulate a composition (a liquid seasoning) similar to the ones commercially available (i.e. Maggi seasoning). Therefore, acetic acid and HCl (food grade acids) were used to

bring the pH of the seasoning to 4.9 at the end point of incubation and before they were pasteurized (80˚C for 10 min). These liquid seasonings were kept frozen at -18˚C and used within two weeks. Protein and Na^+ content were standardized to 3% w/v and 0.3 M, respectively prior to sensory evaluation. The taste enhancing properties (saltiness and savouriness) were studied using ranking tests. Seasoning control samples containing glutamate ("savoury seasoning"), glutathione ("kokumi seasoning"), and, a mixture of the two ("kokumi + savoury seasoning") were also produced and standardized at the same level of Na^+ and pH. For the evaluation of saltiness and savouriness of the seasonings, a randomized complete design was used with sample treatment as experimental unit. Participants were asked to evaluate the intensity of saltiness and savoury taste perceived in two separate sessions.

4.2.2.1 Poultry protein isolate

Poultry protein was obtained from the MSTM (moisture 67.0%, protein 14.1%, fat 17.6%, ash 1.2%, carbohydrate 0.1%) using the ISP procedures. The PPI was prepared at a certified food-grade pilot plant at the food processing developing centre (FPDC) in Leduc, Alberta (Canada) as according to the optimized method of Khiari et al. (2014, Appendix 1A) with modifications. The extraction was carried out in 4 batches. For each batch, about 20 kg of minced MSTM was mixed with ice-water mixture at a 1:5 weight ratio. Citric acid (0.038% w/w) was added into the mixture prior to homogenization to help in reducing the total lipid and pigment in MSTM. Fat was scooped out from the mixture 30 min after resting from homogenization at 4˚C. The protein solution was adjusted to pH 2.5 with 2 M HCl. The soluble proteins were obtained by centrifugation (bowl speed 8,500 rpm; Westfalia Separator AG, Model NA 7-06-076, Oelde, Germany) to remove the insoluble

particles. Then, the soluble proteins were precipitated at pH 5.2 (isoelectric point) with 2 M NaOH, followed by centrifugation. The precipitate was collected and added with water (0-4˚C) at a ratio of 1:1 (w/w) pH 5.2. The mixture was centrifuged for the second time in order to remove soluble pigments and to collect the precipitated protein. Cryoprotectants (0.3% w/w sodium tripolyphosphate, 0.03% w/w sodium nitrite and 0.4% w/w sodium bicarbonate) were added to the PPI (w/w: moisture 91.4%, protein 7%), they were pooled and stored at -18˚C and used within 3 months.

4.2.2.2 Hydrolysis of PPI

The preparation of the PPI hydrolysates was carried out in 11 batches at a designated food preparation laboratory at the University of Alberta. The food grade PPI hydrolysate was prepared by hydrolysing with Alcalase (EC 3.4.21.62, 15 U/g PPI hydrolysate protein) at 50˚C for 3.5 h (pH 7.5-8.0) with constant stirring. In a preliminary study, the PPI had a poor solubility problem, probably due to freezing denaturation during storage without the addition of sucrose as a cryoprotectant component. Therefore, prior to hydrolysis, the PPI solution was adjusted to approximately to pH 10.5 for 30 min (Appendix 1B) to encourage protein unfolding to improve its solubility; then it was adjusted to pH 7.5-8.0 and 6.0-6.5% w/v protein for hydrolysis. The pH of the hydrolysate was maintained at 7.5-8.0 by adding 2 M sodium hydroxide. The hydrolysate was heated to 80˚C for 10 min and cooled down to 4˚C. Then, it was left at 4˚C overnight for phase separation. The clear supernatant was collected and filtered with filter paper to remove impurities. The filtrate (w/v: protein 6.3%, Na^+ 0.4 M) was pooled and stored at -18˚C until use. The pH stat method was used to assess the degree of hydrolysis (% DH) of three random batches of PPI hydrolysates. The DH was calculated as according to Adler-Nissen (1986).

4.2.2.3 Crude protein and Na^+ determination

Crude protein was measured using a nitrogen determinator (Leco TruSpec C/N Analyzer, Leco Corporation, St. Joseph, MI) and applying a conversion factor of 6.25 on the percentage of N. It is known that during the ISP process NaCl is produced. Hence, monitoring the Na^+ content is important in order to formulate a standardized seasoning composition for the sensory evaluation. Na^+ content was determined with Atomic Absorption Spectroscopy (Varian AA240FS Sequential Atomic Absorption Spectrometer, Agilent Technologies, Santa Clara, CA) at 589.6 nm using a fuel lean air/acetylene flame as according to manufacturer's manual.

4.2.2.4 Preparation of GlcN treated hydrolysates

The preparation of GlcN treated hydrolysates was carried out in 3 batches for each treatments at a designated food preparation laboratory at the University of Alberta. For each batch, about 0.5 L of the PPI hydrolysate solution containing 5% w/v protein was added to GlcN at a weight ratio of 4:1. Sodium hydroxide (2 M) was used to adjust the pH to 7.0 ± 0.5 (Appendix 1C). Hydrolysates were glycated with GlcN in the absence of TGase, at 37 and 50˚C respectively, whilst, another glycated hydrolysate treatment was incubated at 37˚C in the presence of TGase. In this case, food grade microbial TGase (26 ± 5 unit/g enzyme; at a ratio of 2 unit/g protein) was used. As for controls, the PPI hydrolysates were incubated at the same manner as indicated above without GlcN. GlcN solutions were incubated at 37 and 50˚C as controls for the GlcN treated hydrolysates. All treatments were incubated for 3.5 h, then, they were adjusted to pH 4.9 with 2 M HCl and pasteurized at 80˚C for 10 min. Subsequently, they were cooled to 4˚C and filtered with a

Minimate TFF Capsule fitted with an Omega™ MWCO 10k Da membrane (Pall Canada Ltd., Quebec, QC, Canada). All the treatments were pooled, lyophilized and stored at -18˚C until used for chemical analysis.

4.2.3 Molecular weight characterization of PPI hydrolysate

The molecular weight distribution of the samples was analyzed by matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF/TOF-MS) as described in Chapter 3 (Study 1).

4.2.4 Spectral characteristics of hydrolyzed poultry protein in relation to GlcN treatment

4.2.4.1 UV-vis profile

In order to monitor the progress of Maillard reaction in the GlcN treated hydrolysates, all the treatments were scanned at UV-visible wavelengths. Approximately 5 mg/mL of each glycated samples (1.25 mg/mL for GlcN samples) in a 1 cm quartz cuvette was scanned at λ= 200-500 nm using the Spectramax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA). The intensity of each of the samples was recorded and compared with other treatments.

4.2.4.2 AGE-fluorescence compounds

The GlcN treated hydrolysates were diluted to 5 mg/mL, whereas the GlcN samples were diluted to 1.25 mg/mL which correspond to the ratio of protein:GlcN= 4:1 used during glycation of hydrolysates. The fluorescence emission spectra (excitation= 347 nm; emission= 400-600 nm) were assessed using a 1 cm quartz cuvette and the Spectramax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA).

4.2.5 Quantification of α-dicarbonyl compounds

The extraction of the α-dicarbonyl compounds from glycated hydrolysates was based on the 3-step procedure method as described by da Silva Ferreira et al. (2007) and modified by Papetti et al. (2013). Exactly 6 mL of lyophilized GlcN modified hydrolysates (20 mg/mL, dissolved in DI water) from each treatments was passed through a pre-conditioned Sep-Pak C-18 cartridge (flow rate= 2 mL/min, 10 mL methanol and 20 mL DI water). Each of the polar fraction obtained was spiked with 6 mg of 1,2-diaminobenzene (*O*-phenylenediamine (OPD)) to derivatize the α-dicarbonyl compounds into their respective quinoxaline derivatives at 37˚C for 1 h (pH 3.00 ± 0.02). Later, the derivatized samples were passed through another C-18 cartridge with 4 mL of 90:10 v/v of pure methanol/water mixture (flow rate 2 mL/min). The first 1 mL was discarded and the subsequent 2 mL eluted from the C-18 cartridge was collected. Separation of the α-dicarbonyl compounds was carried out using an Ascentis Express Peptide ES-C18 column (Dimension: 150 mm x 4.6 mm ID, 2.7 µm; Sigma-Aldrich, St. Louis, MO) connected to an Ultra High Performance Liquid Chromatography (UHPLC) apparatus (Shimadzu, Columbia, MD). The UHPLC system comprised a binary pump, a degasser, an auto-sampler, an oven set at 25.0 ± 0.5˚C, and a diode array detector set at 314 nm (Shimadzu, Columbia, MD). The separation procedures were carried out as according to Papetti et al. (Papetti et al. 2013). All the samples were injected in triplicate (N=3). The identification of the α-dicarbonyl compounds was based on the retention times in comparison to the pure standards (quinoxaline derivatives). The quantification of the α-dicarbonyl compounds (as quinoxaline derivatives) was achieved by external calibration. Quinoxaline derivatives of glucosone (11.1-89.1 mg/L), 3-deoxyglucosone (20.3-648.6 mg/L), glyoxal (0.1-11.6 mg/L), methylglyoxal (0.1-3.6 mg/L), and diacetyl (0.4-1.7 mg/L) were prepared. The limit of detection (LOD)

determined from the triplicate blanks was as follow: 675 (glucosone), 375 (3-deoxyglucosone), 34 (glyoxal), 1 (methylglyoxal) and 6 ng/L (diacetyl); whereas their limit of quantification (LOQ) was 2046 (glucosone), 1137 (3-deoxyglucosone), 104 (glyoxal), 4 (methylglyoxal), and 19 ng/L (diacetyl).

4.2.6 Quantification of savoury free amino acids in relation to GlcN treatments

The free amino acid content of the GlcN treated hydrolysates, native hydrolysates and GlcN were performed as described by Sedgwick et al. (1991) with pre-column *o*-phthaldialdehyde (OPA) derivatization. Calibration was done by using 0.1 μM β-amino-n-butyric acid (BABA) as internal standard, amino acid standard mixture (Sigma-Aldrich, St. Louis, MO) supplemented with glutamine, asparagine, and tryptophan. Data was acquired and processed using the Galaxie Chromatography Software (Agilent Technologies, Santa Clara, CA). The total umami amino acids was a sum of Glu and Asp.

4.2.7 Seasoning preparation and sensory evaluation on the saltiness and savouriness intensity

In order to assess the potential of the hydrolysed PPI as a taste enhancer, the native hydrolysates and the GlcN treated hydrolysated were formulated in a way which was similar to the seasoning liquids available in the market. The glycated hydrolysates were prepared as mentioned at section 4.2.2.4 with slight modification prior to pasteurization; as for GlcN, only the GlcN solution incubated at 50˚C was prepared for sensory evaluation. Acetic acid (2 M) was added (1% v/v) and 2 M HCl were added to adjust to pH 4.9 towards the end of incubation. Acetic acid was added in order to benchmark a commercial liquid seasoning (Maggi Seasoning) in which the acetic acid is one of the key main ingredients.

The hydrolysates and controls were pasteurized at 80˚C for 10 min, cooled down to 4˚C and stored at -18˚C. Samples were consumed within two weeks.

The hydrolysate and GlcN treated hydrolysates were diluted to a final protein content of approximately 3.0% w/v prior to sensory evaluation. The sodium content in all the treatments was standardized to approximately 0.3 M Na^+. Four standard liquid seasonings were prepared as controls (Table 4.1). They consisted of 'sodium seasoning' (0.3 M Na^+), 'savoury seasoning' (Na^+ + Monosodium glutamate (MSG), 3 mM), 'kokumi seasoning' (0.3 M Na^+ + Reduced glutathione (GSH), 5 mM) and 'savoury + kokumi seasoning' (0.3 M Na^+ + 3 mM MSG + 5 mM GSH).

Table 4.1: Seasoning composition used for sensory evaluation. Table reprinted with permission from Hong, P.K., Ndagijimana, M., and Betti, M. 2016. Glucosamine-induced glycation of hydrolysed meat proteins in the presence or absence of transglutaminase: chemical modifications and taste-enhancing activity. Food Chem 197 (B):1143-1152. Copyright (2015) Elsevier Ltd.

Solution	Concentration, % w/v		
	Na^+*	Protein from hydrolysate	Taste enhancer
Native hydrolysate 37˚C	0.7	3	-
Native hydrolysate 50˚C	0.7	3	-
Glycated hydrolysate 37˚C in absence of TGase	0.7	3	-
Glycated hydrolysate 37˚C in presence of TGase	0.7	3	-
Glycated hydrolysate 50˚C in absence of TGase	0.7	3	-
Glucosamine 50˚C	0.7	-	-
Salty seasoning (Na^+)	0.7	-	-
Savoury seasoning (Na^+ + monosodium glutamate (MSG))	0.7	-	0.05 MSG**
Kokumi seasoning (Na^+ + Glutathione (GSH))	0.7	-	0.15 GSH***
Savoury + Kokumi seasoning (Na^+ + MSG + GSH)	0.7	-	0.05 MSG + 0.15 GSH

* 0.7% Na^+ w/v = 0.3 M Na^+
** 0.05% MSG w/v = 3 mM MSG
*** 0.15% GSH w/v = 5mM GSH

A total of 125 consumers (age 18-69, Appendix 2) consisting of students and staff from the University of Alberta were hired to participate in two sensory evaluation sessions. They met the following criteria: healthy, free from food allergy, sensitivity, intolerance or discomfort to the ingredients used in the sensory evaluation. The sensory evaluation study was approved by the University of Alberta's Human Ethics Research Office and written consent was obtained from participants during recruitment.

Samples were served warm (50-60˚C) in covered plastic cups (approximately 15 mL in each cup) labelled with randomized three digits. Participants were requested to evaluate the samples using the sit-and-spit method, they were told to hold each sample in their mouth for approximately 10 s before spitting it out. All sensory evaluation sessions were carried out in red light booths. In the first session, 64 consumers were asked to rank the saltiness intensity of the 10 samples served randomly; another 61 consumers were asked to rank the savoury intensity in the second session. Score 1 was given to sample with the highest intensity whereas score 10 was given to sample possessing the lowest intensity tested.

4.2.8 Data statistical analysis

The means of α-dicarbonyl compounds and free amino acid content were analyzed with One-way ANOVA and separated by Duncan test. The means were considered statistically significant at $p<0.05$. The data obtained from the sensory evaluation sessions were compiled in the form of box plot. Then, the mean rank scores obtained from sensory evaluation sessions were analyzed with Friedman's test and separated with Bonferroni adjustment. The average rank scores were considered statistically significant at $p<0.05$.

4.3 Results and discussions

4.3.1 Characterization of PPI hydrolysate

The PPI was hydrolyzed with the endopeptidase Alc, an alkaline bacterial protease produced from *Bacillus licheniformis*. This enzyme was selected based on its ability to release savoury amino acids; for instance, it has been successfully used in wheat gluten hydrolysis to release Glu, producing a potential taste enhancing hydrolysate (Koo et al. 2014). Flv, a fungal protease and peptidase complex produced from *Aspergillus oryzae*, was also used to hydrolyze the PPI (data not shown). In a preliminary sensory panel, this enzyme has an intrinsic savoury taste that could have invalidated our study (data not shown), therefore it was not used in the current study.

Based on the optimized hydrolysis duration (Figure 4.1A), the 3.5 h time point was chosen as it represented the hydrolysis plateau, with an average degree of hydrolysis (DH) of 6.9 ± 1.0%. The DH obtained in this study was not particular high likely due to the combined effect of ISP and freeze-denaturation occurred during freezing storage, resulting in protein aggregation with subsequent negative effects on the hydrolysis process. Several research teams have targeted peptides at the MW range of 1-5 kDa as a prerequisite in the production of kokumi taste enhancing peptides (Eric et al. 2013; Katsumata et al. 2008; Lan et al. 2010; Ogasawara et al. 2006; Song et al. 2013). The m/z of the PPI hydrolysate was 448.264-2905.925 (Figure 4.1B), which was confined to the range mentioned.

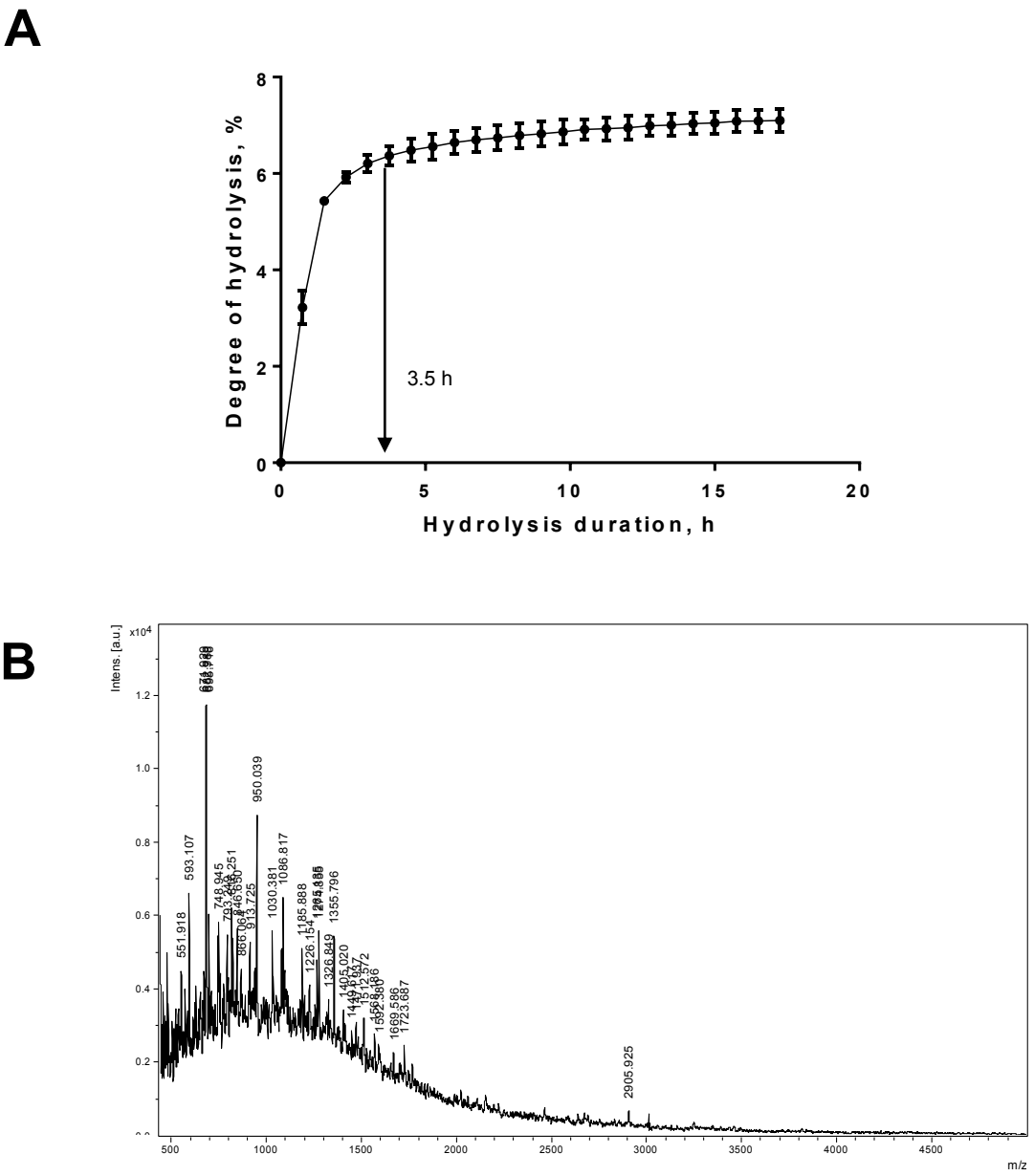


Figure 4.1: A: Hydrolysis curve of poultry protein isolate and Alc for 17 h (15 U/g protein, pH 7.5 - 8.0, 50˚C). Means ± standard deviation of three random batches of PPI hydrolyzed with Alc were plotted. B: MALDI-TOF-MS spectrum of poultry protein hydrolyzed with Alc. Figure reprinted with permission from Hong, P.K., Ndagijimana, M., and Betti, M. 2016. Glucosamine-induced glycation of hydrolysed meat proteins in the presence or absence of transglutaminase: chemical modifications and taste-enhancing activity. Food Chem 197 (B):1143-1152. Copyright (2015) Elsevier Ltd.

4.3.2 UV-VIS and fluorescence profiles of hydrolyzed poultry proteins in relation to GlcN treatments

UV-visible analysis is a simple yet rapid method to monitor and characterize the changes occurring during the Maillard reaction. Figure 4.2A is the UV spectra of native and GlcN-treated hydrolysates in the presence and absence of TGase, and also GlcN solutions incubated alone at the same concentration used in the glycation treatment. In all the treatments two peaks were present. The first sharp peak was in the 220-230 nm region, and the second one was approximately in the 270-280 nm region. The changes of absorbance in relation to GlcN-induced glycation were prominent in the second peak, and related to aromatic amino acids like tryptophan and tyrosine as well as FZ and other condensation products resulting from GlcN cyclocondensation (Hrynets et al. 2015a). GlcN incubated alone without hydrolysates at 37 and 50˚C showed lower absorbance at 280 nm, whereas native hydrolysates along with the glycated samples at 37˚C showed higher absorbance values. The highest absorbance was observed when the native hydrolysates were glycated with GlcN at 50˚C. Glycation performed at 37˚C in the presence or absence of TGase did not change the spectra profiles compared to the native hydrolysates. This was contrary to when glycation was carried at 50˚C, where the prominent increase in absorbance observed at 280 nm was likely due to the formation cyclocondensation products (pyrazines) from GlcN and GlcN self-reaction, GlcN and α-dicarbonyl reaction (Hrynets et al. 2015a) as well as Strecker degradation, a reaction which occurs between α-dicarbonyl compounds and free amino acids and peptides.

Fluorescence emission is a good indicator of formation of AGEs (Matiacevich and Buera 2006) as products of the Maillard reaction. AGEs are normally produced by α-

dicarbonyl compounds, like 3-deoxyglucosone, by attacking the free amino groups of peptides and proteins. Samples were excited at 347 nm and emitted at 400-600 nm, these are the typical wavelengths used to assess the AGEs of the Maillard reaction (Ferrer et al. 2005; Morales and Jimenez-Perez, 2001). All the GlcN treated hydrolysates and the GlcN solutions showed a broad emission peak at the 400-450 nm region, with an maximum emission at 410-430 nm, whereas the native hydrolysates did not show any emission (Figure 4.2B). As expected, hydrolysate glycated at 50˚C resulted in the highest peak emission, indicating a higher production of AGEs compared to the other treatments. Several fluorescent AGEs could be responsible for the increased fluorescence intensity observed in response to GlcN glycation, possibly including pentosidine, argpyrimidine, pirropyridine, pentodilysine, and also the cyclocondensation products from the GlcN auto reaction as described previously for UV-VIS analysis (Ferrer et al. 2005; Wilker et al. 2001). When comparing GlcN incubation alone with GlcN treated poultry meat protein peptides, the latter produced more AGEs. The reason for this phenomenon is likely due to a higher amount of accessible $-NH_2$ groups in the system containing both peptides and GlcN.

In summary, the spectroscopic analyses indicate that hydrolyzed poultry proteins are chemically modified in response to GlcN treatments and the extent of modification is greater at 50˚C. Production of AGEs was lower at 37˚C compared to at 50˚C, indicating that a lower temperature would be more favourable to produce MRPs from a food safety point of view.

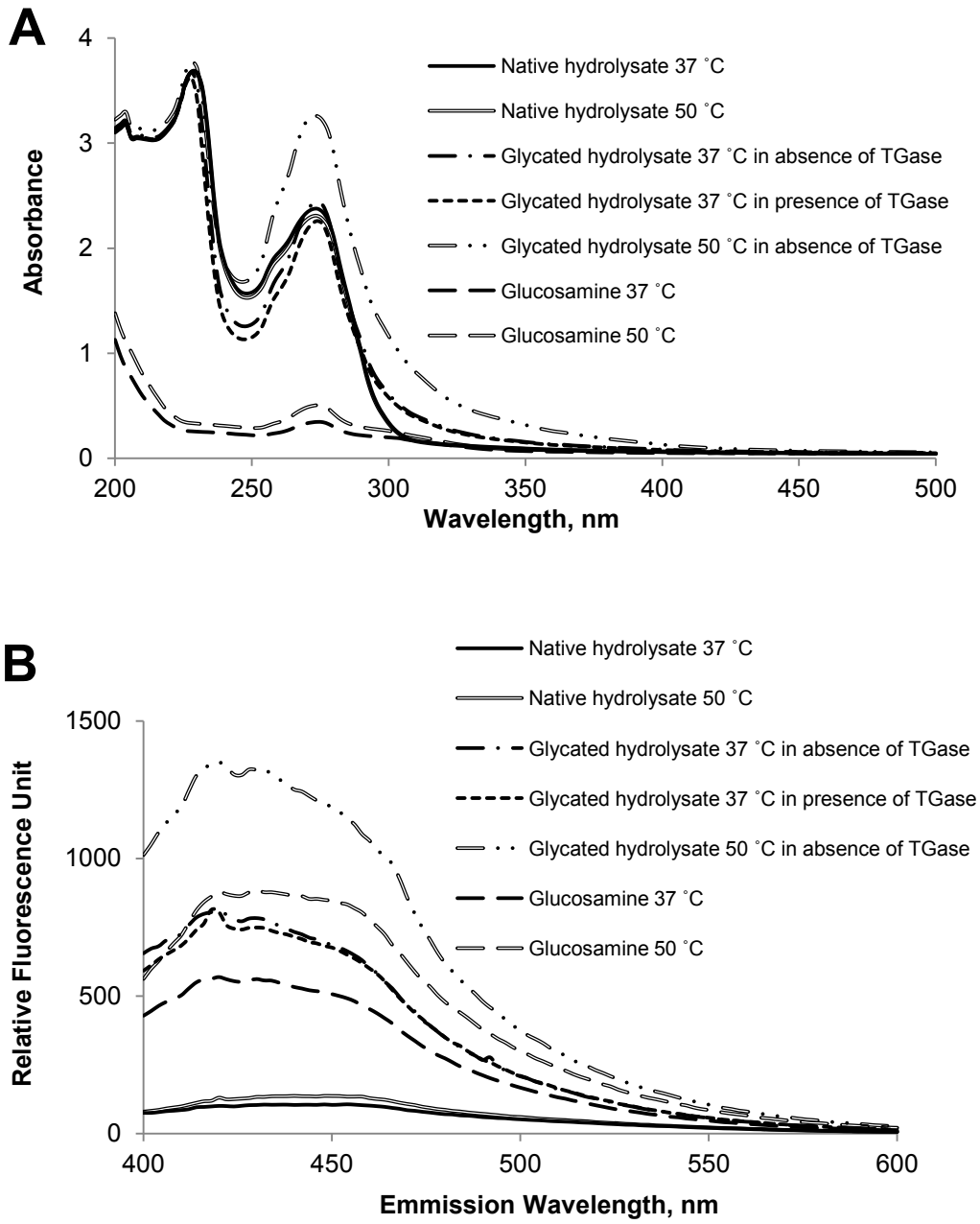


Figure 4.2: Spectral characteristics of hydrolysates, GlcN treated hydrolysates and glucosamine solutions. Ultra-violet spectra (200 - 500 nm, A); AGE-Fluorescence spectra (Excitation= 347 nm, Emission= 400 - 600 nm, B). N=3. Figure reprinted with permission from Hong, P.K., Ndagijimana, M., and Betti, M. 2016. Glucosamine-induced glycation of hydrolysed meat proteins in the presence or absence of transglutaminase: chemical modifications and taste-enhancing activity. Food Chem 197 (B):1143-1152. Copyright (2015) Elsevier Ltd.

4.3.3 Identification and quantitation of the major α-dicarbonyl compounds

As previously discussed, α-dicarbonyl compounds are very important intermediate products from the Maillard reaction that have the ability to condense with free amino groups in proteins and peptides inducing important structural modifications. They are the precursors of browning and flavouring compounds during the Maillard reaction. As reported by Hrynets et al. (2013, 2014), since GlcN is a Heyns compound, it has the ability to produce significant α-dicarbonyl compounds at 37˚C that can then condense with free amino groups. These modifications may be crucial to produce MRPs that possess flavour-enhancing properties.

Figure 4.3 illustrates the representative UHPLC chromatograms of collected α-dicarbonyl compounds produced from all the treatments. Glucosone, 3-deoxyglucosone, glyoxal, methylglyoxal and diacetyl were identified and quantified in all the glycated treatments (Table 4.2), whereas no α-dicarbonyl compounds were detected in native hyrolysates. The amount of glucosone detected was significantly different among the treatments. GlcN treated hydrolysates produced significantly greater glucosone content than the GlcN solution incubated at 37˚C ($p<0.05$) but was not statistically different from the GlcN solution incubated at 50˚C. Glycation in absence of TGase conducted at 50˚C produced almost three times greater 3-deoxyglucosone (188.2 ± 36.5 mg/L) compared to other GlcN treatments at 37˚C. Furthermore, the concentration of 3-deoxyglucosone was greater in the treatments containing the hydrolyzed meat proteins than the GlcN solution incubated alone, likely due to the higher free amino groups available for the Maillard reaction. A similar result was obtained by Hrynets et al. (2015b). 3-deoxyglucosone is a predominant α-dicarbonyl compound in food such as fruit juice (trace-410 mg/L), balsamic

vinegar (median 361 mg/L), and bread (13-619 mg/kg)., It was also detected at 212 mg/L in liquid condiments and seasonings (Degen, Hellwig, and Henle 2012). In the current study, the amount of 3-deoxyglucosone found was less compared to these commercial food products mentioned.

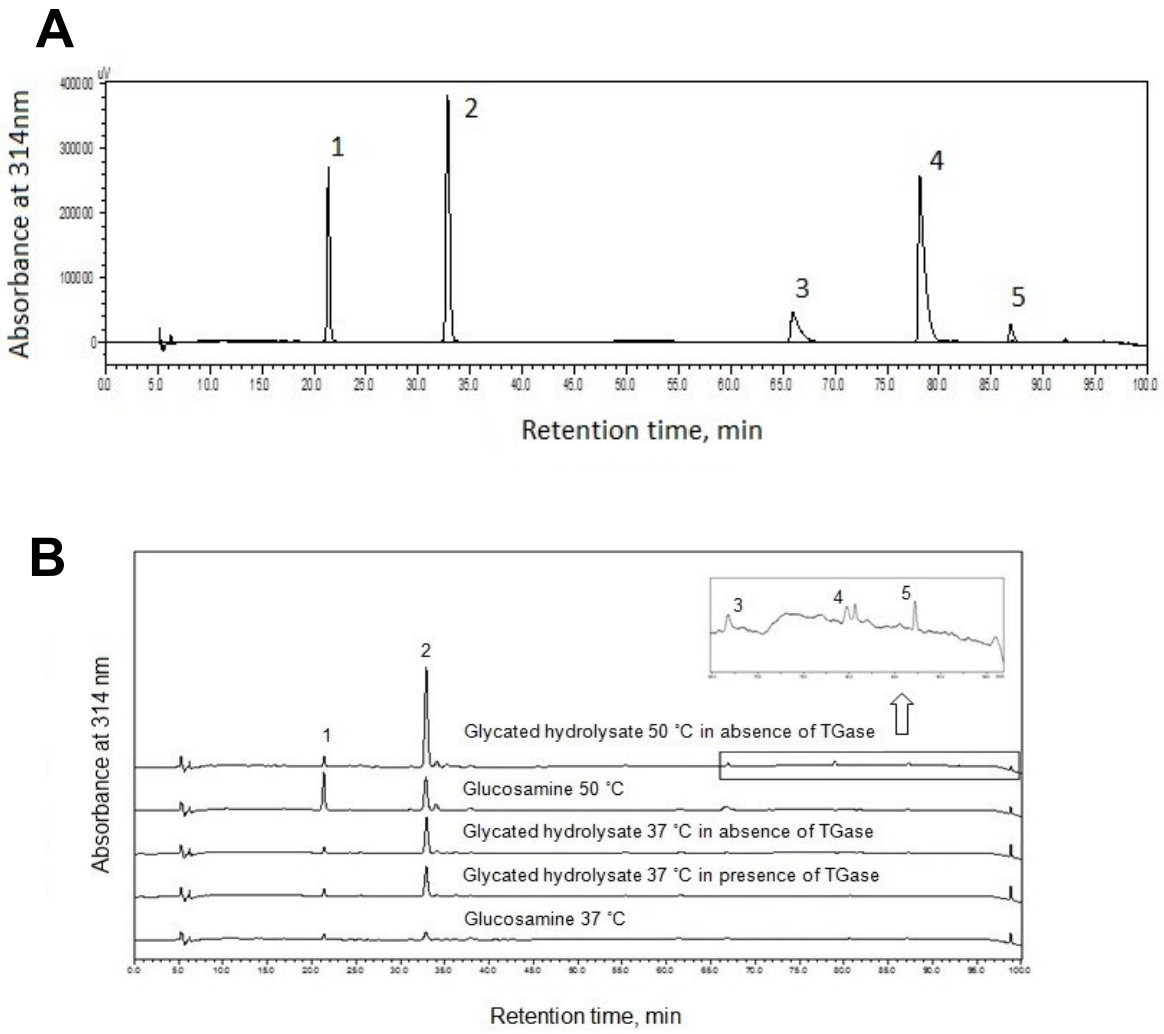


Figure 4.3: A: UHPLC chromatogram of α-dicarbonyl compound standards: 1= glucosone (21 min), 2= 3-deoxyglucosone (33 min), 3= glyoxal (66 min), 4= methylglyoxal (79 min), and, 5= diacetyl (89 min). B: UHPLC chromatogram of α-dicarbonyl compounds in glycated hydrolysates and GlcN solutions (N=3): 1= glucosone, 2= 3-deoxyglucosone, 3= glyoxal, 4= methylglyoxal, and, 5= diacetyl. Figure reprinted with permission from Hong, P.K., Ndagijimana, M., and Betti, M. 2016. Glucosamine-induced glycation of hydrolysed meat proteins in the presence or absence of transglutaminase: chemical modifications and taste-enhancing activity. Food Chem 197 (B):1143-1152. Copyright (2015) Elsevier Ltd.

Table 4.2: Major α-dicarbonyl compounds identified in the GlcN solutions and the GlcN treated hydrolysates produced at moderate temperatures (37 and 50˚C). Table reprinted with permission from Hong, P.K., Ndagijimana, M., and Betti, M. 2016. Glucosamine-induced glycation of hydrolysed meat proteins in the presence or absence of transglutaminase: chemical modifications and taste-enhancing activity. Food Chem 197 (B):1143-1152. Copyright (2015) Elsevier Ltd.

Treatments	α-dicarbonyl compounds, mg/L				
	Glucosone	3-Deoxyglucosone	Glyoxal	Methylglyoxal	Diacetyl
Glycated hydrolysate 37˚C in absence of TGase	57±5 ^{b}	67±5 ^{b}	0.3±0.1 ^{c}	0.3±0.0 ^{bc}	0.6±0.1 ^{b}
Glycated hydrolysate 37˚C in presence of TGase	66±9 ^{ab}	71±10 ^{b}	0.2±0.1 ^{c}	0.2±0.0 ^{c}	0.6±0.1 ^{b}
Glycated hydrolysate 50˚C in absence of TGase	78±10 ^{a}	188±37 ^{a}	0.5±0.1 ^{b}	0.6±0.1 ^{a}	1.1±0.1 ^{a}
Glucosamine 37˚C	29±2 ^{c}	9±2 ^{c}	1.1±0.1 ^{a}	0.3±0.0 ^{b}	1.1±0.3 ^{a}
Glucosamine 50˚C	70±7 ^{ab}	17±3 ^{c}	1.2±0.2 ^{a}	0.3±0.0 ^{bc}	0.4±0.1 ^{b}
p-value	<0.001	<0.001	<0.001	<0.001	0.001

Means ± standard deviation; N=3.
Means with different superscripted letters in the same column are significantly different ($p<0.05$).

The glyoxal and methylglyoxal and diacetyl content in the treatments are considered as low, ranging between 0.2-1.2 mg per L. The level of these short chain α-dicarbonyl compounds found in the current study are lower than the commercial balsamic vinegars (1.3-27.9 mg/L) (Papetti et al. 2013). However, they are conforming to the range reported for coffee brew, barley coffee brew and soy sauce (0.1-2.7 mg/L) (Papetti, Mascherpa, and Gazzani 2014). Glyoxal and methylglyoxal are described as mild sour, pungent and slightly nutty aromas (Kokkinidou, 2013). The formation of glyoxal likely originated from the cleavage at the C_2-C_3 bond of glucosone (Degen et al. 2012; Hofmann, Bors, and Stettmaier 1999). A significantly higher content of glyoxal was observed in both GlcN treatments incubated at 37 and 50˚C (Table 4.2, $p<0.05$). While the highest 3-deoxyglucosone was found in the glycation treatment at 50˚C resulting in the greatest

methylglyoxal level. This makes sense since 3-deoxyglucosone serves as a precursor for the production of methylglyoxal (pyruvaldehyde) (Weenen 1998; Yaylayan and Keyhani 2000) through the fragmentation of 3-deoxyglucosone by retroaldol condensation (Thornalley, Langborg, and Minhas 1999).

In summary, these α-dicarbonyl compounds results agree with the spectroscopic analysis reported in the previous section. It highlighted that the major modifications observed at 50˚C were at least in part due to a higher production of α-dicarbonyl compounds. GlcN treated hydrolysates at 37˚C also produced α-dicarbonyl compounds with the potential of inducing structural modifications to meat protein peptides. In section 4.3.4, the discussion focuses on whether these modifications were sufficient to impart an appreciable effect on the perceived saltiness and savouriness or not.

4.3.4 Sensory evaluation and quantification of savoury free amino acids in relation to GlcN treatments

In order to understand whether treating of hydrolyzed poultry protein with GlcN had an effect on its sensory attributes, ranking tests were conducted. Specifically, the saltiness and the savouriness in seasoning compositions, acidified with acetic acid to a final pH of 4.9, were evaluated. It was hypothesized that production of MRPs through glycation with GlcN would enhance these two attributes in comparison to the untreated hydrolysate. Commercial taste enhancer formulations, reported in Table 4.1, were also used as a comparison to the treatments.

Figure 4.4 presents the results from the first sensory panel, in which the saltiness of the seasoning samples was assessed. Despite several panellists' comment that the seasonings were too salty, the GlcN treated hydrolysates at 37 and 50˚C together with the

native hydrolysate incubated at 37˚C was ranked the highest intensity ($p<0.05$) as compared to the negative control containing only NaCl, implying a superior salt perception of the treatments that contain hydrolyzed PPI. Moreover, the meat protein hydrolysate glycated at 37˚C without TGase was perceived as more salty when compared to the seasonings formulated with the commercial taste enhancers. At the same time, GlcN treated hydrolysates were not different ($p>0.05$) in terms of salty perception as compared to the native hydrolysate, indicating that the chemical modifications induced by the Maillard reaction at 37 and 50˚C are not robust enough to trigger the salt enhancement effects.

In general, the majority of the samples which contained the hydrolyzed meat proteins (except for the hydrolysate incubated at 50˚C) possessed salt-enhancing properties as compared to the seasoning formulated with addition of NaCl alone. This is likely due to the presence of free Glu and Asp along with other specific peptides which have the ability to increase the salty sensation. The effectiveness of Glu and Asp to amplify saltiness is well-documented in the literature (Fuke and Ueda 1996). The amount of savoury amino acids (Glu + Asp) found in this study ranged from 93.2 to 139.2 mg/L (Figure 4.5) and was not statistically different among the GlcN treatments and the native hydrolysates ($p>0.05$). Nevertheless, the concentrations found in this work were lower as compared to the ones reported in the previous studies (Lioe et al. 2006) in terms of triggering umami and salty sensation. However, the specific matrix composition formulated in this study which includes acetic acid at 13 mM may have played a role in amplifying the salty sensation. In addition, Maehashi et al. (1999) reported that chicken meat protein hydrolysate contains several acidic peptides with capacity to increase both the umami and salty sensation. In summary, the amounts of Glu and Asp in combination with acetic acid were able to

generate a seasoning composition that increased the perceived saltiness compared to the seasoning containing only NaCl and acetic acid. GlcN treated hydrolysates, whether in presence or absence of TGase did not increase the salty perception of the poultry meat protein hydrolysate.

In the second sensory evaluation, the savoury taste perceived in all of the seasoning compositions was not statistically different ($p= 0.0593$). These data were quite surprising as one would expect to find convergent results between salt intensity and savouriness, since the positive association between savouriness and saltiness has been proven before by Fuke and Ueda (1996). For instance, high savouriness is usually associated with high salt perception, a "formula" that has been applied to cut down the sodium level in the processed food by subsequently increasing the amount of savoury compounds like, for instance, Glu or 5'-inosine monophosphate. In the current study, it appears that saltiness and savouriness are two independent attributes with minimal association. Panellists seemed to define saltiness differently from savouriness, except for one treatment—the hydrolysate glycated at 50˚C, where saltiness and savouriness were scored almost identically. This phenomenon could be ascribed to the fact that the untrained panelists rather than trained ones were recruited to evaluate the seasoning samples. Another possible reason could be due to the relative high level of sodium (Na^+) used in the seasoning formulation (0.7% w/v). As a consequence, the addition of hydrolyzed meat proteins to an already salty seasoning may have further increased the salty perception but not necessarily resulting in a balanced, savoury seasoning. For instance, the native hydrolysates which had high mean ranking scores for saltiness, ranked at the bottom in terms of savouriness. In general, in spite of the analysis of variance for savoury perception which was not technically significant, the p-

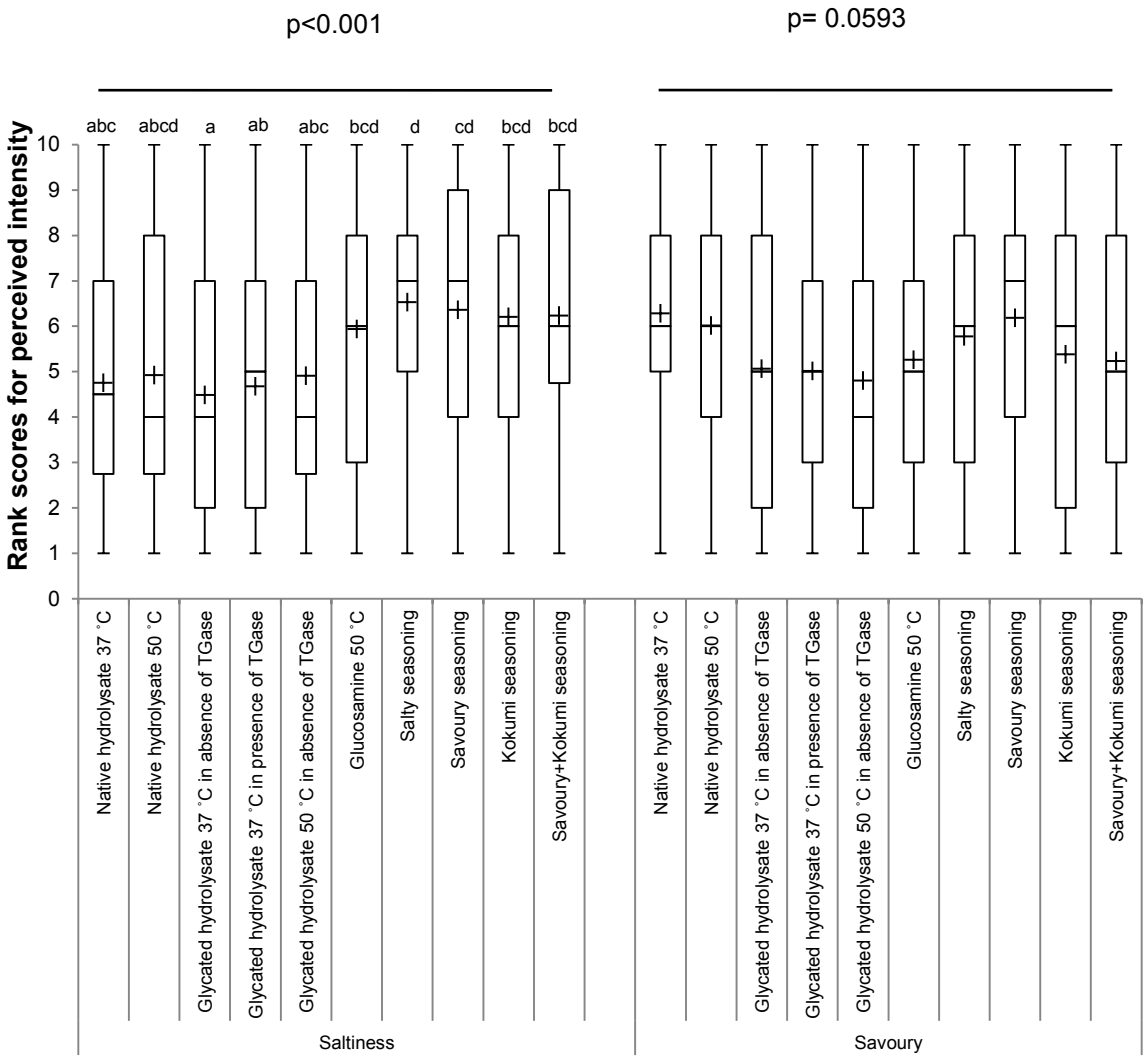


Figure 4.4: Comparison of the perceived saltiness (N=64) and savouriness (N=61) intensity of poultry protein hydrolysate in relation to the GlcN treatments. The cross (+) represents the mean rank score in each box plot. Lower mean rank scores indicate higher perceived intensity and higher mean rank scores indicate lower perceived intensity. Means with different letters in the same sensory attribute are significantly different ($p<0.05$). Figure reprinted with permission from Hong, P.K., Ndagijimana, M., and Betti, M. 2016. Glucosamine-induced glycation of hydrolysed meat proteins in the presence or absence of transglutaminase: chemical modifications and taste-enhancing activity. Food Chem 197 (B):1143-1152. Copyright (2015) Elsevier Ltd.

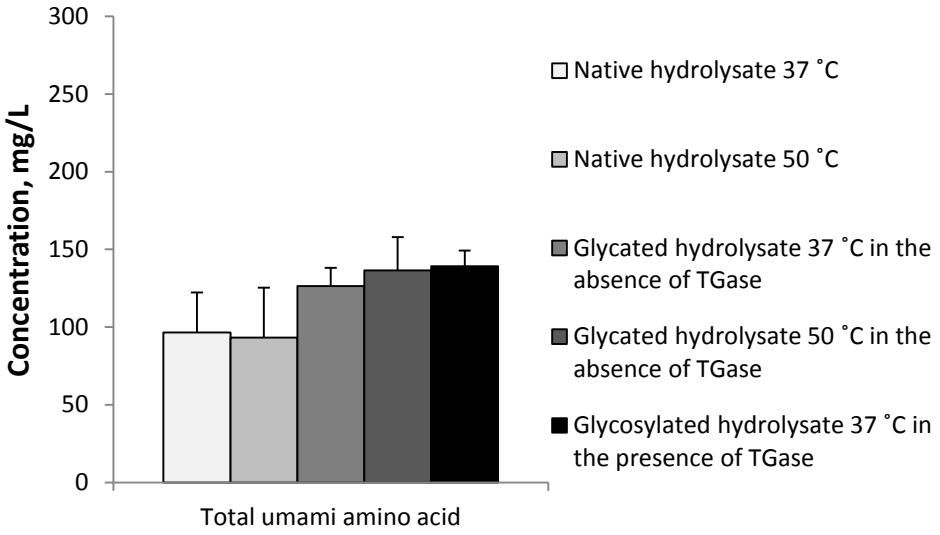


Figure 4.5: Umami profile of poultry protein hydrolysate in relation to glucosamine treatment (N=3). Total umami amino acids= Glu + Asp. Figure reprinted with permission from Hong, P.K., Ndagijimana, M., and Betti, M. 2016. Glucosamine-induced glycation of hydrolysed meat proteins in the presence or absence of transglutaminase: chemical modifications and taste-enhancing activity. Food Chem 197 (B):1143-1152. Copyright (2015) Elsevier Ltd.

value was in a range that could be considered a tendency ($p= 0.0593$). Among all of the treatments, glycation of meat hydrolysate conducted at 50˚C resulted in the greatest mean rank score, likely due to the greater production of α-dicarbonyl compounds contributing to more MRPs and thus the precursors of important flavour compounds (see section 4.3.3). On the other hand, the native hydrolysates scored the least. In summary, despite the GlcN treatments were not as effective as expected in term of increasing saltiness and savouriness, the glycation conditions used in this study were rather mild in order to minimize production of AGEs, but also limiting GlcN's potential to modify the meat protein hydrolysate.

4.4 Conclusions

This study is able to demonstrate that poultry meat proteins hydrolyzed with Alc have the capacity to enhance saltiness in seasoning composition due a synergistic effect between the free Glu and Asp. Despite the GlcN-induced chemical modification of the hydrolysates, the enhancement on the perceived saltiness and savouriness were minimal. A possible future improvement would be optimizing the glycation conditions, such as increasing the concentration of GlcN and duration of the reaction.

Chapter 5 (Study 3): Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl production

This chapter in parts has been published as "Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212: 234-243."

5.1 Introduction

Non-enzymatic browning reactions represent a very important and complex phenomenon occurring in food during processing and storage. They are favoured by heat treatments and include reactions such as the Maillard reaction, caramelization, and oxidation of ascorbic acid. Caramelization is the degradation of sugar in the absence of an amino group where the initial sugar enolization proceeds with a series of steps: dehydration, fragmentation, oxidation and so forth. This process normally occurs at higher temperatures (from 120˚C to >200˚C) (Coca et al. 2004; Kroh 1994) where reactive intermediate compounds such as reductones (Rhee and Kim 1975) and reactive carbonyl compounds are produced, which give rise to heterocyclic compounds such as furfurals and brown polymeric molecules.

The Maillard reaction involves the condensation of an amino group from a protein or peptide to a carbonyl group of a reducing sugar at high temperatures. The reaction leads to the formation of a Schiff base product that rapidly transformes to an Amadori rearrangement product (ARP) or Heyns rearrangement product (HRP) depending on whether the reaction involves an aldose or ketose, respectively. Both Maillard reaction and

caramelization yield some similar products such as reductones, flavour compounds, brown pigments, organic acids and a variety of heterocyclic compounds. Furthermore, in Maillard reaction, degraded ARP or HRP will undergo further condensation by incorporating nitrogen which produces dark pigment melanoidins. Since these two reactions share similar products, there is not a definitive way to directly trace back the origin of a compound to either a Maillard reaction or caramelization.

GlcN is known as a natural health product for the relief of osteoarthritis symptoms. It is derived from the hydrolysis and deacetylation of chitin. The chemical structure of GlcN is rather unique; it contains an amino group at the C2 position next to the carbonyl group forming an aldose-amine structure that resembles a HRP. This peculiar structure makes GlcN an unstable molecule capable of self-condensation forming the hydroxyalkyl pyrazines (i.e. FZ) and degradative reactions forming the reactive dicarbonyls even at 37˚C (Hrynets, Ndagijimana, and Betti 2015a). Hence, GlcN non-enzymatic browning is a mixture of caramelization and Maillard reaction products. Due to its reactivity in mild conditions, various studies have been conducted in both protein and peptide reaction systems in order to generate novel food ingredients. For instance, Hrynets and colleagues were able to produce novel glycoproteins and amyloid-like structures by glycating myofibrillar proteins (Hrynets, Ndagijimana, and Betti 2014) and myoglobin at 37˚C (Hrynets, Ndagijimana, and Betti 2015b), respectively. The possibilities of GlcN in being an antimicrobial (Hrynets et al. 2016), producing antioxidant compounds (Oyaizu 1986; Gottardi et al. 2014; see Chapter 3) or a taste enhancer (see Chapter 4) were also demonstrated when incubated at moderate temperatures between 25-50˚C. The advantages of working at lower temperatures are that the rate of reaction is relatively more

manageable, and, this approach can minimize the production of highly polymerized compounds (i.e. the dark coloured melanoidins) and toxic compounds (AGEs and 4-MEI).

Besides the research conducted at moderate temperatures, new studies focusing on how to produce and isolate important molecules (i.e. FZ) from the GlcN self-reaction system are emerging. FZ and DOF seem to have interesting applications to prevent and treat diabetes (Bashiardes et al. 2002), as well as flavouring agents for food and tobacco (Henry et al. 2012). Recent works from Jia and colleagues (2014; 2015) have demonstrated that high yields in FZ and DOF can be achieved using a specific basic ionic liquid as a catalyst. Hrynets and colleagues (2016) were able to convert around 50% of GlcN to FZ in a GlcN-iron catalyzed reaction system incubated at 50˚C.

In light of these considerations, GlcN is a versatile amino sugar that can be used to generate bioactive molecules. The objective of the present work is to study the effect of two different type of solutions (PBS and ammonium hydroxide), temperature (40, 60˚C) and incubation time (0, 3, 6, 12, 24 and 48 h) on the kinetics of GlcN non-enzymatic browning. The study is also aimed to evaluate the impact of different stages of the reaction models on the colour, antioxidant properties and short chain α-dicarbonyl production with flavour activity. This research may represent the foundation for developing GlcN-derived caramel solutions produced at mild temperatures with specific properties as controlled by the conditions of the reactions.

5.2 Materials and methods

GlcN.HCl was purchased from MP Biochemicals Canada. Quinoxaline (99%), methylquinoxaline (98%) and the derivatization agent 1,2-diaminobenzene (*o*-phenylenediamine (OPD)), and 2,4,6-tris-2,4,6-tripyridyl-2-triazine (TPTZ) were obtained

from Sigma-Aldrich (St. Louis, MO). Diacetyl, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Fisher Scientific (Ottawa, ON, Canada). All other chemicals and solvents used in assays and liquid chromatography were of analytical grade or HPLC grade.

5.2.1 Experimental design

A preliminary study was (a 3×3×3×5 factorial design) conducted with the aim to screen the most important factors that influence the kinetic of GlcN non-enzymatic browning. Factors included were glucosamine concentration (1.0, 2.5, 5.0 % w/v), solution (PBS, ammonium hydroxide and water), temperature (40, 50 and 60˚C) and time (3, 6, 12, 24 and 48 h).

Following the screening study, glucosamine 5% was chosen as the sole concentration used in this study. PBS and ammonium hydroxide were selected in the solution factor. In addition to that, two levels of temperature (40 and 60˚C) and 6 time points were chosen (0, 3, 6, 12, 24 and 48 h). Thus, this experiment represents a 2×2×6 factorial design where the effects of type of solution, temperature and time were tested on the physico-chemical and antioxidant properties of GlcN caramels. Specifically, 5% GlcN was dissolved in two different solutions: 50 mM PBS or 50 mM ammonium hydroxide. The solutions were then incubated at either 40 or 60˚C for 0, 3, 6, 12, 24 and 48 h resulting in a total of 24 treatments. Three independent trials (24 tubes × 3 trials) were conducted resulting is a total number of 72 observations. After incubation, tubes were cooled in ice-water bath and part of the GlcN caramels in the tubes were immediately tested for physical-chemical properties such as pH, colour measurement and spectroscopic analyses. Tubes were then frozen and kept at -18˚C for HPLC analysis of α-dicarbonyls, SEC and

antioxidant assays (DPPH, ABTS and FRAP). For both α-dicarbonyls and SEC analyses only two tubes per treatment were used.

5.2.2 Sample preparation

Exactly 5% w/v of GlcN was prepared in solutions containing either 50 mM PBS or 50 mM ammonium hydroxide. The solutions were adjusted WITH 2M NaOH to pH 7.4 ± 0.1 prior to incubation, sodium azide was added to a final concentration of 0.02% v/v to prevent microbial growth. Exactly 10 mL of the solutions were transferred into screwed cap glass tubes. The tubes were randomly placed in the 40 and 60˚C incubators and left incubated for 3, 6, 12, 24, and 48 h. Tubes containing PBS incubated at 40 and 60˚C were labelled as P40 and P60 respectively, whereas A40 and A60 were abbreviations for samples in ammonium hydroxide solutions incubated at 40 and 60˚C. Reaction mixtures prepared at 0 h were treated as controls. The pH was not regulated during the course of the incubation duration. Tubes were retrieved at the end of each incubation endpoints and cooled immediately in an ice-water bath.

5.2.3 Chemical-physico properties

5.2.3.1 UV & UV-vis spectroscopic analysis

The aliquots of incubated GlcN reaction mixtures were diluted 40 times (1.25 mg/mL) with deionized water. The samples were measured at 280, 320, and 420 nm against water as blank in a Spectramax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA) with a 1-cm quartz cuvette. The absorbance intensities obtained were compared against their respective controls at T= 0 h.

5.2.3.2 pH kinetics on the GlcN non-enzymatic browning reaction

The pH values of the incubated GlcN reaction mixtures were monitored over time. The solutions were measured directly with a pH probe (Denver Instrument Ultra Basic pH meter) after equilibrating the samples to room temperature.

5.2.3.3 Colour characteristics of GlcN non-enzymatic browning

The sample aliquots were pipetted into a 1-cm plastic cuvette. The colour of the aliquots was measured with a tristimulus colorimeter (Minolta CR-400, Konica Minolta Sensing Americas, Inc., Ramsey, NJ) by obtaining the CIE L*a*b* and L*, chroma (c*), hue (h*) colour space values. Choma is represented by the equation:

$$Chroma = \sqrt{a^{*2} + b^{*2}}$$

Whereas hue is expressed as :

$$Hue = \arctan\frac{b^*}{a^*}$$

Each of the colour space value reported was the mean of three measurements.

5.2.4 Intermediate reactive products from GlcN caramelization - α-dicarbonyl compounds

Two millilitres from each of the collected aliquotes was diluted to 6 mL with deionized water. The extraction of the α-dicarbonyl compounds from the incubated GlcN solution was carried out as described in Chapter 4 (Study 2).

The derivatization and separation of α-dicarbonyl compounds was carried out as according Papetti et al. (2013) and Hong et al. (2016). Standard curve for quinoxaline and methylquinoxaline are expressed as y= 8.8323x (R^2= 0.9974) and, y= 7.5834x (R^2= 0.9995) respectively, where y= peak area, and x= concentration in μM. An Ascentis Express Peptide ES-C18 column (150 mm × 4.6 mm ID, 2.7 μm; Sigma-Aldrich, St. Louis,

MO) was connected to an Agilent 1100 series HPLC system (Agilent Technologies, Foster City, CA), running at a flow rate of 0.3 mL/min at 25.0 ± 0.5˚C and a diode array detector (DAD) was set at 314 nm. The mobile phases consisted of A: water acidified with 0.1% formic acid, and B: 100% methanol. The analyte solution was filtered with PVDF syringe filter (Millipore (Canada) Ltd., Billerica, MA). Exactly 10 μL of the analyte was injected and eluted under a 120 min gradient: 0-5 min, 90-85% A; 5-13 min, 85-80% A; 13-40 min, 80% A; 40-65 min, 80-70%; 65-90 min, 70-50% A; 90-100 min, 50-0% A; 100-105 min, 0% A; and 105-110 min, 0-90% A; the column was re-equilibrated at 90% A for 10 min.

The post-run data were analysed with the Agilent ChemStation software. The major α-dicarbonyl compounds were identified by comparing the retention times with their respective quinoxaline derivatives as external standards. Calibration curves were used to quantify the quinoxaline derivatives of the α-dicarbonyl compounds (glyoxal, methylglyoxal and diacetyl). The values expressed was the mean of two experimental repetitions.

5.2.5 Condensation and polymeric products from the GlcN non-enzymatic browning

The analyte solutions were filtered with a 0.22 μm syringe filter (Millipore (Canada) Ltd., Etobicoke, Ontario/ Lab Water Business Unit, Billerica, MA). A Superdex Peptide 10/300 GL column (GE Healthcare Life Sciences, Baied'Urfe, QC, Canada) was used to analyse the molecular weight distribution of the analytes. The column was connected to an Agilent 1100 series HPLC system (Agilent Technologies, Foster City, CA), 10 μL of sample was injected and eluted isocratically with 50 mM PBS buffer (pH 7.4, supplemented with 0.15 M NaCl) at a flow rate of 0.45 mL/min. A diode array detector (DAD) was set at 280 nm to

estimate the molecular weight range in the incubated GlcN solutions. The molecular weight calibration was carried out with a standard mixture (MW 380-12000 Da, Appendix 3). The post-run data were analysed with the Agilent ChemStation software.

5.2.6 Antioxidant activity

5.2.6.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity in the models was evaluated using the method as described in Chapter 4 (Study 2). Samples from the models were diluted to achieve a final concentration of 0.05-2.00 mg/mL in the assay. Trolox was used as a standard for the assay (final concentration 0.3-12.5 μg/mL). In order to eliminate the interference of the samples, the absorbance values were corrected using 'sample blank' and 'solution blank'. Sample blank consisted of sample (200 μL) and methanol (1800 μL), whereas the solution blank contained either diluted PBS or ammonium hydroxide solution (200 μL, without GlcN) and methanol (1800 μL). The percentage of DPPH radical scavenged was compared against a control (containing only DPPH) and calculated as below:

$$\%DPPH\ scavenging\ activity = \frac{Control - (Sample - Sample\ blank - Solution\ blank)}{Control} \times 100\%$$

The percentage of DPPH scavenging activity was then used to estimate the amount of sample necessary to decrease the DPPH radicals by 50% (EC_{50}). Aside from that, the anti-radical activity (at a final concentration of 0.1 mg/mL) was expressed in μmol Trolox equivalent/g sample (μmol TE/ g) in order to compare with other antioxidant assays in this study.

5.2.6.2 2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging activity

The ABTS radical scavenging activity was conducted according to Ozgen et al. (2006). A 7 mM $ABTS^{\bullet+}$ stock solution was prepared in 20 mM sodium acetate buffer (pH 4.5) supplemented with 2.45 mM potassium persulfate. The stock solution was kept in the dark at 4˚C for 12-16 h until a stable oxidative state was achieved. Later, the stock solution was diluted with 20 mM acetic buffer to get an absorbance of 0.70 ± 0.01 at 734 nm. Samples were diluted to various concentrations prior to test in order to achieve 0.02-0.50 mg/mL in the $ABTS^{\bullet+}$ assay. Exactly 30 µL of the sample aliquot was mixed with 3000 µL of diluted $ABTS^{\bullet+}$ solution and left incubated in the dark for 30 min. The anti-radical activity was monitored at 734 nm with a spectrophotometer. The absorbance values were compared with 'control' which has $ABTS^{\bullet+}$ only and corrected with 'sample blank' which contained sample aliquot and 20 mM sodium acetate buffer. The percentage of $ABTS^{\bullet+}$ scavenging activity was calculated as below:

$$\%ABTS\ radical\ scavenging\ activity = \frac{Control - (Sample - Sample\ blank)}{Control} \times 100\%$$

Similar to DPPH assay, the percentage of ABTS scavenging activity was used for EC_{50} calculation. Trolox solution was used as a positive control for the antioxidant assay. The anti-radical activity of the samples (tested at 0.1 mg/mL) was expressed in terms of µmol trolox equivalent (TE)/g sample.

5.2.6.3 Ferric reducing antioxidant power (FRAP) assay activity

The FRAP assay activity was carried out as stated by Benzie and Strain (1996). Briefly, 10 mM TPTZ solubilised in 40 mM HCl, 20 mM $FeCl_3$ in water and 300 mM sodium acetate buffer (pH 3.6) were mixed at a volume ratio of 1:1:10. Sample aliquots were diluted to various concentrations in order to accomplish a final concentration of 0.05-2.00 mg/mL in the FRAP assay. The diluted sample aliquot (10 µL) was added to 200 µL of the FRAP

assay and incubated in the dark for 30 min. Then, the samples were measured at 593 nm. The absorbance values obtained from the samples were corrected with 'sample blank' that basically had the same content as the test assay except TPTZ and $FeCl_3$ solution. Ascorbic acid and trolox were used as positive controls for the assay. The FRAP activity was expressed as μmol ascorbic acid equivalent/g sample. The result was also expressed in μmol TE/g sample in order to compare with DPPH and ABTS radical scavenging activity.

5.2.7 Statistical analysis

A factorial analysis of variance (ANOVA) was performed using the general linear model (GLM) procedures in order to investigate the effects of various factors and their interactions. The model tested the main effects of solution, temperature and time and their interactions as following:

$$Y= \mu + [S] + [T] + [t] + [S*T] + [S*t] + [T*t] + [S*T*t] + e$$

where, Y= dependent variable; μ= grand mean; S= solution; T= temperature; t= time; e= error.

The percentage of contribution from the main factors and their interactions to each parameters were calculated based on the sum of squared deviation compared to the respective total corrected sums (Chen and Kitts 2011). Tukey's test was applied to separate the means whenever a significant difference was detected at $p<0.05$.

Kinetics models about spectroscopic analyses, pH decline, dicarbonyl production, DPPH and ABTS radical scavenging activity were fitted with a non-linear regression analysis (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA). Specifically, the fitted curves for spectroscopic changes and methylglyoxal production over time were expressed with the following exponential growth equation:

$$y = y_0 + (P - y_0) \times (1 - e^{-Kt})$$

where y_0= the intensity or concentration at T= 0 h; P= intensity or concentration at plateau; K= rate constant, t= time.

The fitted curve about pH decline over time was described with an exponential decay equation:

$$y = (y_0 - P) \times (e^{-Kt}) + P$$

where y_0= the intensity or concentration at T= 0 h; P= intensity or concentration at plateau; K= rate constant, t= time.

Finally, DPPH and ABTS radical scavenging activities were described using sigmoid curves:

$$\mathrm{y} = B + \frac{(A - B)}{1 + 10^{(Log\ EC50 - x)\ H}}$$

where A and B= the top and bottom of plateaus in the units of the y-axis; EC_{50}= the effective concentration to reach to 50% of the antioxidant activity (EC_{50}, mg/mL) of DPPH and ABTS from samples (final concentration 0.1 mg/mL); x= concentration in log unit; H= hillslope.

Bivariate Pearson correlation analysis among the parameters tested was also conducted using SPSS statistics (version 17).

5.3 Results and discussion

5.3.1 UV-VIS absorbance intensity

UV-visible analysis is a simple approach to monitor the progress of GlcN caramelization (Hrynets, Ndagijimana, and Betti 2015a). For instance, absorbance at 280 nm gives an indication about the production of condensation products (i.e. FZ and DOF) while pre-melanoidins and melanoidins compounds (dark-brown pigments), normally formed at the last stage of Maillard reaction, can be better estimated at 320 and 420 nm, respectively.

Table 5.1 summarizes the results obtained from the factorial ANOVA test. The contribution percentage of the main factors (solution [*S*], temperature [*T*] and time [*t*]) and their interactions ([$S \times T$], [$S \times t$], [$T \times t$]) as well as the level of significance (p-values) on the spectroscopic changes were reported. Overall, it is apparent that the main effects of *T* and *t* alone accounted for most of the variability (>80%) of the UV-vis absorbance measured at the three different wavelengths. On the contrary, despite the main effect of *S* was significant ($p<0.001$), it only explained 3-5% of the total variability for the absorbance measured at 280 and 320 nm, respectively, while its contribution at 420 nm was negligible. Among the interaction terms, $T \times t$ contributes to 11.6, 3.2, and 6.9% at 280, 320 and 420 nm ($p<0.001$), respectively, while $T \times t \times S$ seems to give a certain robust contribution (7.1%) for the intensity of 320 nm.

To better interpret the effect of the treatments on the non-enzymatic browning of GlcN at the aforementioned wavelengths, kinetic models were fitted (Figure 5.1). Figure 5.1A depicts the evolution of the GlcN condensation products at 40 and 60˚C. When GlcN was incubated at 40˚C, the PBS model (P40) resulted a higher intensity at 280 nm during the course of incubation as compared to the ammonium hydroxide model (A40) in which the absorbance intensity increased steadily. However at 60˚C, the absorbance intensity was equally high regardless of the solution used. At 60˚C, P60 reached to a plateau earlier (7 h, $R^2= 0.9995$) than in A60 (16 h, $R^2= 0.9991$). This indicates a faster GlcN condensation rate in the PBS solution as compared to ammonium hydroxide solution.

Table 5.1: Contribution of factors (%) and their interactions on the chemical-physico and antioxidant properties in the GlcN models. Table reprinted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd.

	Response									
Factors	280 nm	320 nm	420 nm	pH	L*	Chroma	Hue	DPPH	ABTS	FRAP
Solution (*S*)	3.2***	5.0***	0.0	0.5***	1.2***	0.8***	3.3***	13.2***	18.1***	35.5***
Temperature (*T*)	49.0***	47.5***	43.4***	43.0***	46.5***	22.3***	5.1***	30.1***	31.4***	1.8
Time (*t*)	32.6***	35.7***	41.0***	47.0***	31.6***	11.7***	6.6***	29.5***	20.4***	9.8**
$S \times T$	0.6***	0.5***	3.1***	0.0	1.4***	0.1	0.0	0.2	0.0	9.4***
$S \times t$	0.2***	0.1	0.8***	0.0	0.7***	9.4***	7.1***	1.7**	10.0***	6.7*
$T \times t$	11.6***	3.2***	6.9***	8.9***	16.1***	48.9***	70.7***	17.5***	4.1***	1.9
$S \times T \times t$	2.7***	7.1***	4.2***	0.4***	1.8***	5.0***	1.5	4.4***	12.0***	16.9***
Error	0.1	0.8	0.6	0.2	0.6	1.9	5.8	3.5	3.9	18.0
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

% Contribution = Sum of square from each factors/ total corrected sum of square

* $p<0.05$

** $p<0.01$

*** $p<0.001$

N=3

In Figure 5.1B, the progress on the formation of pre-melanoidins at 320 nm is shown. At 40˚C, P40 produced higher absorbance intensity than A40. However in P60, pre-melanoidin formations were rapidly developed in the first 12 h, where they reached to a reaction plateau around 8.5 h (R^2= 0.9607). Prolonged incubation times (more than 24 h) resulted in a higher intensity in A60, in which a reaction plateau was recorded at 31.5 h (R^2= 0.9923).

Figure 5.1C describes the formation of melanoidins in incubated GlcN solutions. At 40˚C, the intensity of P40 dominated over the one incubated in A40. On the other hand, higher temperature gave an opposite effect on the formation of melanoidins. Both models (P60 and A60) showed increasing intensity in the first 12 h, then, A60 produced relatively higher amount of melanoidins after the 12 h time point.

Based on the results above, the role of PBS and ammonium hydroxide solutions was different in the non-enzymatic browning reaction of GlcN. In PBS models, GlcN showed a slow onset of 420 nm-browning in the entire incubation course, whilst the ammonium hydroxide solution increased the formation of dark-brown pigments towards the later stage of the incubation. The formation of melanoidin requires a reducing carbohydrate and an amine group, and suggests the presence of ammonium (NH_4^+) ions to provide additional amine group to the formation of melanoidins, and a higher incubation temperature accelerates the melanoidin conversion rate.

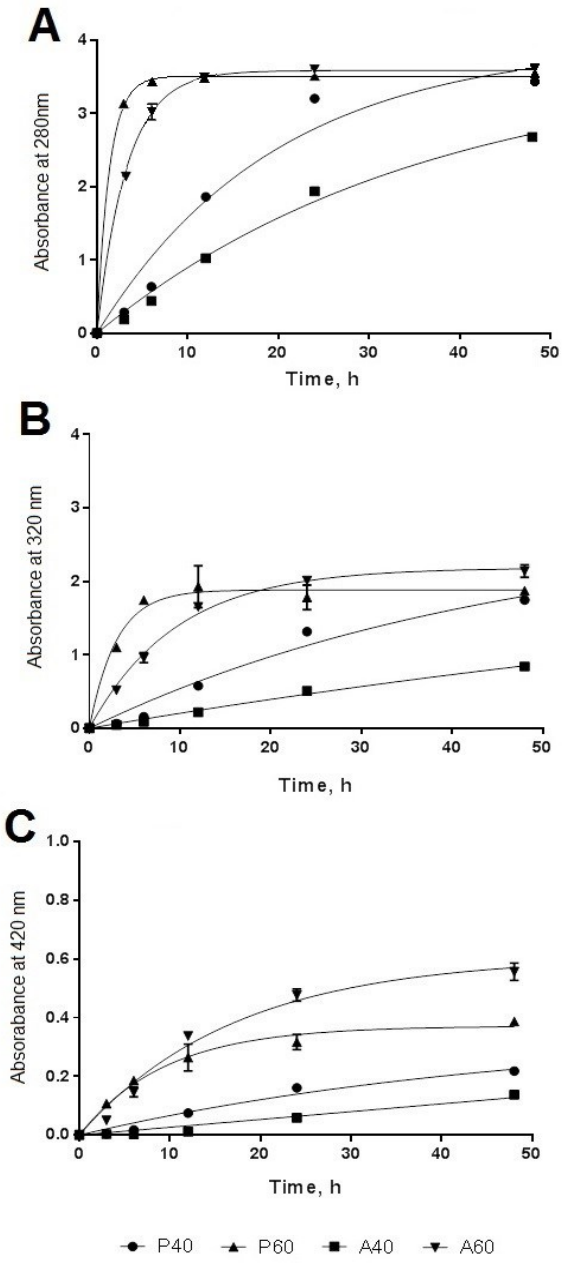


Figure 5.1: Non-enzymatic browning kinetics at 280 (R^2>0.95, A), 320 (R^2>0.96, B) and 420 nm (R^2>0.94, C) of GlcN reaction mixtures incubated in PBS [P] at 40 (P40) and 60˚C (P60), respectively, and in ammonium hydroxide [A] at the same temperatures (A40 and A60). All samples were analysed at 0, 3, 6, 12, 24, 48 h and data were fitted with the following non-linear equation: $y= y_0 + (P-y_0) \times (1-e^{-Kt})$, where y_0= the absorbance intensity at T= 0 h, P= plateau, K= rate constant, t= time. N=3. Figure adapted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd.

5.3.2 pH kinetics on the GlcN non-enzymatic browning reaction

Both caramelization and the Maillard reaction are known to produce organic acids (i.e: formic and acetic acid) and cause pH decrease (Martins and van Boekel 2005). Being a Heyns compound, GlcN is expected to go through a rapid degradation, thus producing reactive dicarbonyl compounds and, subsequently, organic acids.

Figure 5.2 describes the variations of pH in the models of non-enzymatic browning of GlcN. Overall, the pH decline rate in 40˚C were relatively small compared to at 60˚C. P40 and A40 remained above pH 6 after incubated for 48 h, whereas P60 and A60 hovered around at pH 3.5. Results indicated that reaction temperature exerted a greater influence on the pH kinetics rather than the type of solutions used in the models. This is in line with the results presented in Table 5.1 which the two main factors contributing to the pH variability were temperature and time. Both factors contributed to a total of 90% of the variability, whilst the solution factor only contributed 0.5%.

In sugar caramelization, thermal and acid-driven dehydration and cyclisation reaction dominats, resulting an acidification of the caramel (Kroh 1994). The significant decline in pH values is likely due to the accumulation of organic acids, like acetic acid and formic acid (van Boekel and Brands 1998). It has been reported previously (Davidek et al. 2006) that hydrolytic cleavage of α-dicarbonyl compound, 1-deoxy-2,3-hexodiulose, produces acetic acid and erythrose, while cleavage of 3-deoxyhexos-2-ulose leads to a formation of formic acid and 2-deoxy-arabinose. Recent study from Hrynets et al. 2016 confirmed this hypothesis. The same authors reported generation of acetic and formic acids during GlcN browning at 50˚C.

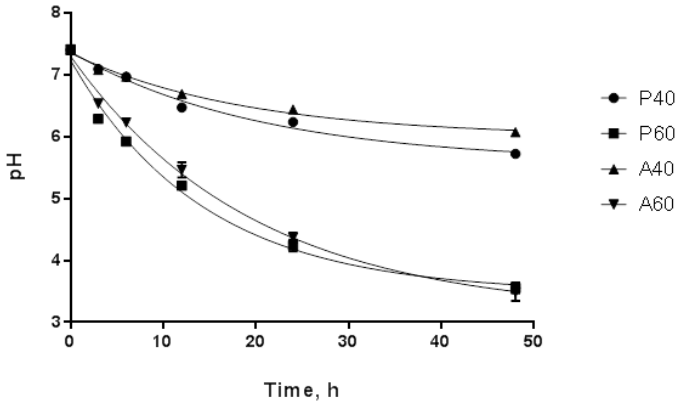


Figure 5.2: The pH kinetic of GlcN reaction mixtures incubated in PBS [P] at 40 (P40) and 60˚C (P60), respectively, and in ammonium hydroxide [A] at the same temperatures (A40 and A60). All samples were analysed at 0, 3, 6, 12, 24, 48 h and data were fitted (R^2>0.98) with the following non-linear equation: $y= (y_0-P) \times e^{(-Kt)} + P$, where y_0= pH value at T= 0 h, P= plateau, K= rate constant, t= time. N=3. Figure adapted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd.

5.3.3 Intermediate reactive products from GlcN caramelization - α-dicarbonyl compounds

Approximately 1 to 6 mg of α-dicarbonyl compounds are produced in every gram of GlcN when when incubated at 37˚C for up to 12 days (Hrynets, Ndagijimana, and Betti 2015a) in which up to 80% of the total α-dicarbonyl content is contributed by 3-deoxyglucosone. From a food science perspective, α-dicarbonyls are important precursors of flavour and colouring compounds. Moreover, among the known α-dicarbonyl compounds, diacetyl has been recognized to possess a pleasant buttery flavour (Clarke and Winter 2015). Short chain α-dicarbonyls (glyoxal, methylglyoxal and diacetyl) also possess antimicrobial activity (Jay, Rivers, and Boisvert 1983; Maletta and Were 2012), a characteristic that can be eventually exploited in the production of GlcN caramel solutions. However, from the health and safety perspective, the production of α-dicarbonyl compounds has to be

monitored since they are capable of reacting with metabolic proteins. Furthermore, methylgloxal is the precursor of 4-methyl-imidazole (4-MEI), a toxic compound that has been associated with caramel production (Yu, Xu, and Yu 2015). Therefore, it is important to beware of and monitor on the formation of these short chain reactive compounds in this study.

Figure 5.3 depicts the formation of various short-chain α-dicarbonyl compounds as a function of time from different GlcN reaction model systems. For glyoxal (Figure 5.3A), all the systems showed an upward trend at the first 3-6 h of incubation, and a steady decrease thereafter, reaching to a final concentration of 0.30-0.46 mg/L (8-11 mg/g GlcN) at 48 h. Hrynets and colleagues (2015a) had proposed GlcN can be converted to glyoxal via several pathways, including the oxidation pathway, the enolization pathway, or a combination of the both, or, interconversion reaction of HRP into ARP followed by 2,3-enolization (Chapter 2, Figure 2.5).

The methylglyoxal (Figure 5.3B) levels in all the treatments were primarily influenced by the main effects of temperature and incubation time ($p<0.001$, Table 5.1). Methylglyoxal showed an upward trend followed by a concentration plateau at different time points throughout the incubation duration: P60= 0.93 mg/L GlcN (5.3 h); A60= 1.17 mg/L (10.2 h), A40= 0.58 mg/L (15.1 h) and P40= 0.99 mg/L (22.9 h). Although P60 was the earliest to reach to a plateau, the methylglyoxal level in the A60 model was greater, suggesting that higher incubation temperature and presence of NH_4^+ ions increased the formation of methylglyoxal. The accumulation of carcinogenic 4-MEI in caramels depends on the level of methylglyoxal (Moon and Shibamoto 2011; Yu, Xu, and Yu 2015). Methylglyoxal is a product from the retroaldolization of 3-deoxyglucosone (Thornalley,

Langborg, and Minhas 1999). During caramelization and Maillard reactions, methylglyoxal and ammonia are capable of producing 2-aminopropanal, which further reacts with formaldehyde to form 4-MEI (Yu, Xu, and Yu 2015). The models clearly demonstrated that a higher temperature (60˚C) and the presence of $NH4^{+}$ ions from the ammonium hydroxide solution promote the formation of methylglyoxal, an important precursor of 4-MEI. However, the methylglyoxal concentrations were relatively lower compared to balsamic vinegar (4.4-4.6 mg/L) (Papetti et al. 2013) or GlcN caramel mixtures incubated at 120˚C for 2 h (26 mg/L) (Yu, Xu, and Yu 2015). In addition, the amount of methylglyoxal can be reduced to half of A60 when the temperature is lowered (40˚C).

Diacetyl is known for its sensory property in butter, dairy products, microwave popcorn and beer (Clarke and Winter 2015). The diacetyl levels are reported in Figure 5.3C. All treatments showed a trend similar to glyoxal. Incubation temperature played a major role in the accumulation of diacetyl over time. A milder temperature (40˚C) yielded more diacetyl as compared to higher temperature (60˚C), this is in agreement with the previous study where GlcN was incubated at 37 and 50˚C (Chapter 4). At 40˚C, the diacetyl levels in P40 and A40 reached to their maximum levels at 6 h (2.2 mg/L) and 12 h (1.8 mg/L) respectively. This suggests that a combination of mild temperature and shorter heating time is more appropriate in order to maximize the flavouring compound content in the GlcN mixture. The amount of diacetyl found in the current study similar to that found in yogurt, wine and butter, foods known typically to be rich in these short chain α-dicarbonyls (Clark and Winter, 2015).

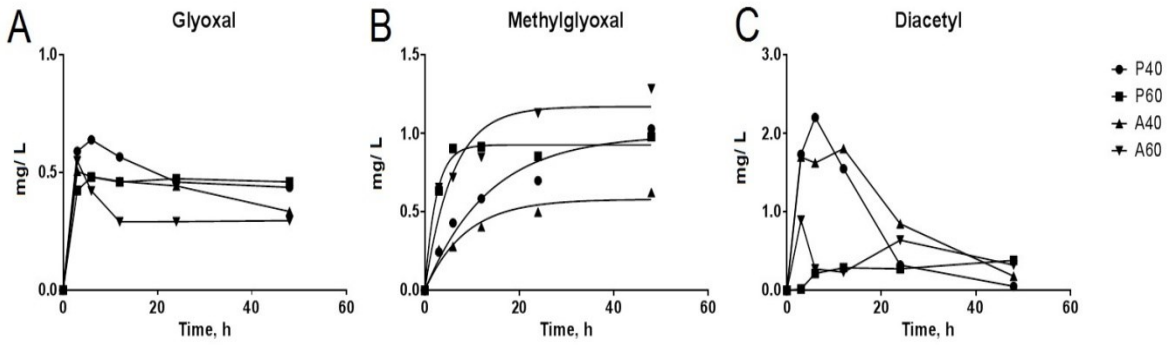


Figure 5.3: Short chain α-dicarbonyls production of GlcN solutions incubated in PBS [P] at 40 (P40) and 60˚C (P60), respectively, and in ammonium hydroxide [A] at the same temperatures (A40 and A60). All samples were analysed at 0, 3, 6, 12, 24, 48 h. Methylglyoxal's data were analysed with a non-linear fitting (R^2>0.96): $y= y_0 + (P-y_0) \times (1-e^{(-Kt)})$, where y_0= the corrected concentration at T= 0 h; P= concentration at plateau; K= rate constant, t= time. N=2. Figure adapted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd.

5.3.4 Condensation and polymeric products from GlcN non-enzymatic browning reaction

The formation of higher molecular weight compound is attributed to the accumulation of GlcN self condensation products such as dihydrofructosazine (MW= 322), FZ (MW= 320) and DOF (MW= 304) which can be detected at 275 nm (Hrynets, Ndagijimana, and Betti 2015a; Tsuchida et al. 1973). FZ and DOF are flavouring agents, that also have anti-diabetic (Bashiardes et al. 2002) and anti-inflammatory (Zhu et al. 2007) properties. However, in the final stage of non-enzymatic browning reactions, coloured and high MW (> 300 Da) compounds are normally generated. Typical examples are pre-melanoidins and melanoidins, which possess interesting properties such as antimicrobial, antioxidant, anti-hypertensive and prebiotic (Borrelli and Fogliano 2005; Jimenez-Zamora, Pastoriza, and Rufian-Henares 2015). The mechanism of the antioxidant effect of melanoidins is based on

their ability to trap positively charged electrophilic species, to scavenge oxygen radicals, or to carry out metal chelation to form inactive complexes (Delgado-Andrade, Rufián-Henares and Morales, 2005). Their chelating properties towards metal ions also contribute to their antioxidant and antimicrobial properties in foods (Rufian-Henares and Morales 2007; Tagliazucchi, Verzelloni, and Conte 2010a, 2010b). Therefore, in order measure the abundance of the high molecular weight compounds produced during GlcN caramelization, SEC was performed and monitored at two different wavelenghts: one at 280 nm for FZ and DOF estimation, and, the other one at 320 nm for pre-melanoidins estimation.

Figure 5.4 depicts the changes of absorbance intensity at 280 nm (top) and 320 nm (bottom) as a function of incubation time. The kinetics of the peak formation was in line with the result of UV-vis spectroscopy reported in section 5.3.1. In all the models tested, the peak area of the treatments increased with incubation duration. The area and absorbance intensity of the peaks were at their least at the begining of incubation while they reached to their maxiumum at T= 48 h, except for P60 in which the largest peak was recorded at T= 12 h. At 40˚C, the models incubated in PBS and ammonium hydroxide solutions showed similar rates in developing higher molecular weight compounds at both 280 and 320 nm. The PBS model showed at least 3 times increment of the P40's absorption intensity when the temperature increased to 60˚C; however, this was not observed in the ammonium hydroxide model, where the kinetics of peaks formation in A60 were similar to P40 and A40 when measured at 280 and 320 nm. According to the molecular weight calibration curve consisted of peptides monitored under 280 nm (Appendix 3), the peaks observed at 3 h and later incubation times were in a range between 200 to 1,400 Da, while no peaks were detected in fresh GlcN (0 h). A similar trend was observed at 320nm. This

suggests that the GlcN molecules (MW= 179) condense to form higher molecular weight compounds and progress to form pre-melanoidin compounds within the first 3 h of incubation.

5.3.5 Colour characteristics of GlcN caramel in response to treatments

The tristimulus colorimetry is a traditional approach that is widely used to rapidly assess the progress of Maillard reaction (Morales and van Boekel 1999) by referring to the L* a* b* values. However, the extent of browning can be better understood when described in the psychochromatic terms which employs the use of L*, c* and h. The L* value is a rapid indicator on the browning intensity in caramel processing. L* describes the darkness (-) and lightness (+) of the solutions; while a* expresses the greenness (-) and the redness (+) of samples; and b* indicates blue on its negative value and yellow on its positive values. The c* and h values are the elaborated a* and b* values. The c* value expresses the degree of departure from gray (when a* and b*= 0) to pure chromatic colour (+) (McGuire 1992; Morales and Jimenez-Perez 2001); whereas the h value interprets colours perceived in the spectrum in terms of degree, whether it is red, yellow, green or blue, or a combination of the two shades.

As described in section 5.3.1, the rate of browning (measured at 420 nm) was more influenced by incubation time and temperature, rather than types of solutions used in the models (Table 5.1). The spectrum of brown colour developed in the models was different. L* values declined significantly over time as the dark shade in the GlcN models developed (Table 2, $p<0.05$). Nonetheless, temperature had a larger influence on the L* values ($p<0.001$, Table 5.1). At 40˚C, the darkness of the incubated GlcN solutions could be separated to at least 4 shades according to the incubation time (Figure 5.5, $p<0.05$).

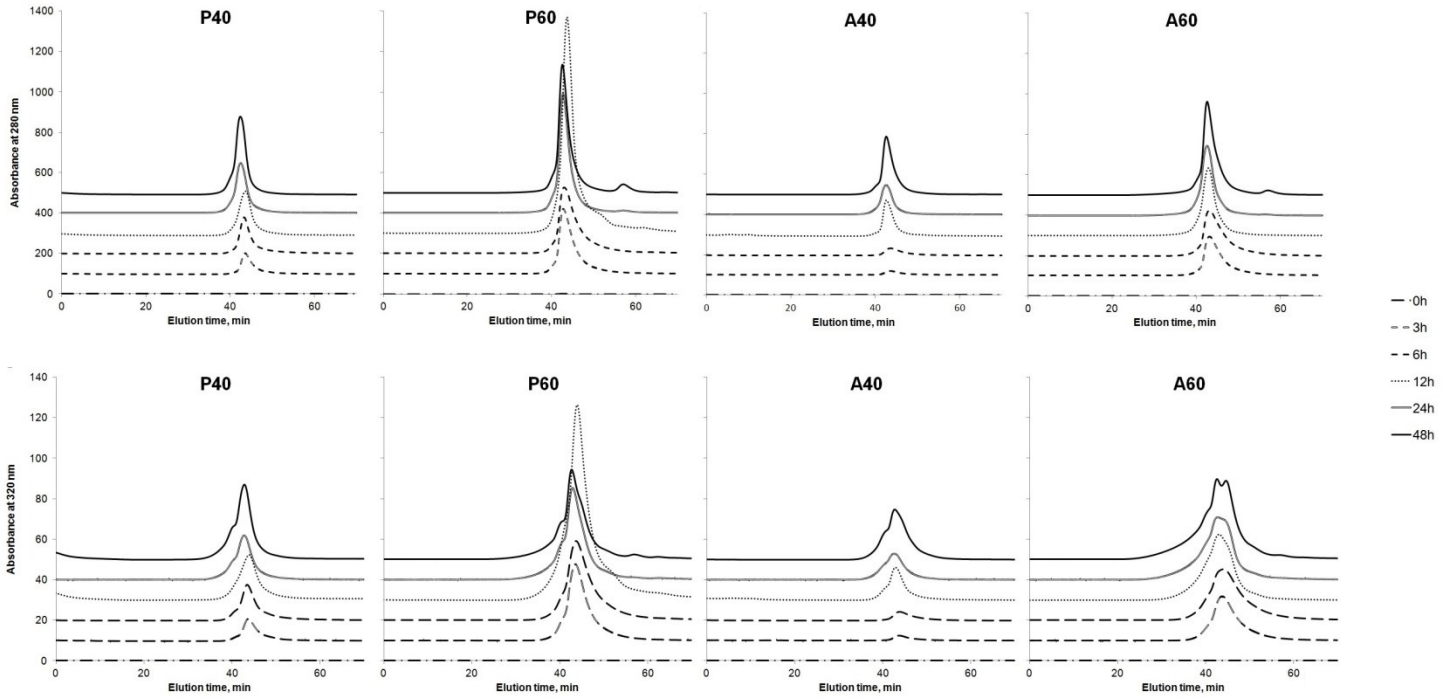


Figure 5.4: Kinetics on the formation of GlcN condensation products (top, 280 nm) and pre-melanoidin compounds (bottom, 320 nm) in GlcN models assessed by SEC. The models consisted of GlcN incubated in two different solutions (PBS [P] and ammonium hydroxide [A]), incubated at 40 (P40 and A40) and 60˚C (P60 and A60) for up to 48 h.

Contrarily, at 60˚C, the lightness and darkness of both the PBS and ammonium hydroxide models were reduced to 3 shades (Figure 5.5, $p<0.05$), samples incubated at 6 h were equally dark as compared to the ones at 48 h (Table 5.2, $p<0.05$). This suggests that a lower incubation temperature better produced the desired quality of colour, since a rapid colour development at 60˚C means less control and limited selection on the shades of colour yielded.

Based on a correlation analysis (Table 5.6), the L* values in the models were highly correlated to the pH values ($r= 0.72$, $p<0.01$). This means the darker the GlcN caramel gets, the lower the pH is. Low pH (around pH 4) will further limit the browning reaction and produce an acidic environment in favour of food preservation. The appearance of GlcN-derived browning products can be managed by regulating the pH of the reaction; therefore, giving better control in achieving desired characteristics in the reaction product.

The chroma values of the 40˚C models were similar, where they increased during incubation until reaching to their maximum values and gradually declined thereafter (Table 5.2). The chroma values in the 60˚C models decreased significantly, as is evident in the appearance of the samples, which were no longer bright and light. The hue value 0˚ indicates red-purple, 90˚ is perceived as yellow, 180˚ is depicted as bluish-green, and 270˚ is interpreted as blue (McGuire 1992). The hue values obtained in the models were between 35˚ to 104˚ (Table 5.2) which fall between the orange-red to yellow shades. The 40˚C models showed a gradual decrease in the hue value as the amino sugar was incubated ($p<0.05$). Both P40 and A40 appeared to be greenish-yellow at the beginning of incubation and transformed to orange-red at 48 h (Figure 5.6). Totally opposite trend was observed

when GlcN was incubated at 60˚C. Both P60 and A60 were orange-red at the initial stage of incubation and acquired yellow hue towards 48 h.

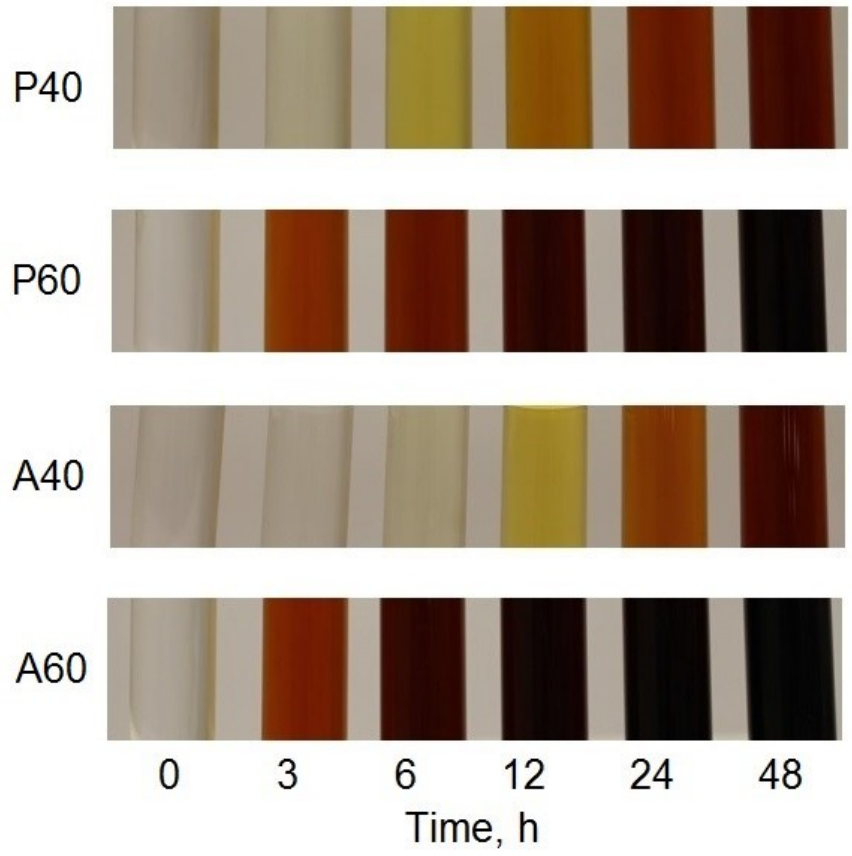


Figure 5.5: Change of colour shades in the GlcN models as a function of time. Samples were prepared by incubating GlcN in PBS (P) and ammonium hydroxide (A) solutions, at 40 and 60˚C for up to 48 h. Low incubation temperature (40˚C) resulted gradual change and more colour variety in brown colour shades (4 shades in P40 andA40) compared to at 60˚C (3 shades in P60 and A60). Figure adapted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd.

Table 5.2: Changes in colour characteristics: L*, chroma (C) and hue (h), in GlcN models incubated with PBS [P] and ammonium hydroxide [A] solutions at 40 and 60˚C for up to 48 h. Table adapted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd.

Model	Time, h	L*	C	h
P40	3	28.3±0.1 ^{a}	3.3±0.9 ^{c}	103.1±0.6 ^{a}
	6	26.7±0.1 ^{b}	9.9±0.6 ^{a}	95.5±0.8 ^{b}
	12	22.9±0.4 ^{c}	9.0±0.3 ^{a}	65.8±3.4 ^{c}
	24	20.5±0.0 ^{d}	6.4±0.1 ^{b}	41.1±0.9 ^{d}
	48	19.3±0.1 ^{e}	3.4±0.2 ^{c}	36.1±0.9 ^{e}
P60	3	20.6±0.8 ^{a}	6.7±0.6 ^{a}	45.3±3.7 ^{c}
	6	20.0±0.6 ^{ab}	4.4±0.7 ^{b}	43.5±2.5 ^{c}
	12	19.3±0.0 ^{b}	2.4±0.2 ^{c}	49.2±2.4 ^{c}
	24	18.8±0.1 ^{b}	1.6±0.1 ^{cd}	64.9±0.6 ^{b}
	48	18.7±0.2 ^{b}	1.2±0.1 ^{d}	86.2±2.4 ^{a}
A40	3	28.2±0.1 ^{b}	1.2±0.1 ^{d}	93.8±0.4 ^{b}
	6	28.8±0.2 ^{a}	3.6±0.3 ^{c}	98.6±0.5 ^{a}
	12	27.1±0.2 ^{c}	10.5±0.4 ^{b}	91.5±0.9 ^{b}
	24	22.3±0.3 ^{d}	8.8±0.2 ^{a}	55.0±3.3 ^{d}
	48	19.6±0.1 ^{e}	4.0±0.1 ^{c}	41.9±1.0 ^{e}
A60	3	21.1±0.3 ^{a}	7.2±0.5 ^{a}	45.7±1.6 ^{b}
	6	19.2±0.1 ^{b}	2.5±0.1 ^{b}	53.5±2.5 ^{ab}
	12	18.9±0.2 ^{b}	1.5±0.0 ^{b}	75.9±1.5 ^{ab}
	24	18.8±0.3 ^{b}	1.3±0.0 ^{b}	84.9±4.0 ^{a}
	48	19.2±0.7 ^{b}	2.0±1.6 ^{b}	71.4±28.3 ^{ab}

Data were expressed as mean (N=3) ± standard deviation.
Different superscripted letters in the columns within the same model (P40, P60, A40, A60) indicate significant difference at p=0.05.

The hue value and a* value (green-red index) were inversely correlated (Table 5.6, r= -0.85, p< 0.001), the redness intensity played a pivotal role that determined on how the solution colour is perceived. Whilst, chroma was positively influenced by b* (blue-yellow index) (Table 5.6, r= 0.97, p< 0.001). Figure 5.6 describes the progress of b* and a* during the course of incubation. The 40˚C models formed curves that circulated clockwise as the incubation progressed. The 40˚C curves were similar to what was reported by Morales et al. (2001) in which sugar-amino acid models were incubated at 100˚C for 24 h. When heated at higher temperature, 60˚C models showed a reduced magnitude of b* and a* when incubated.

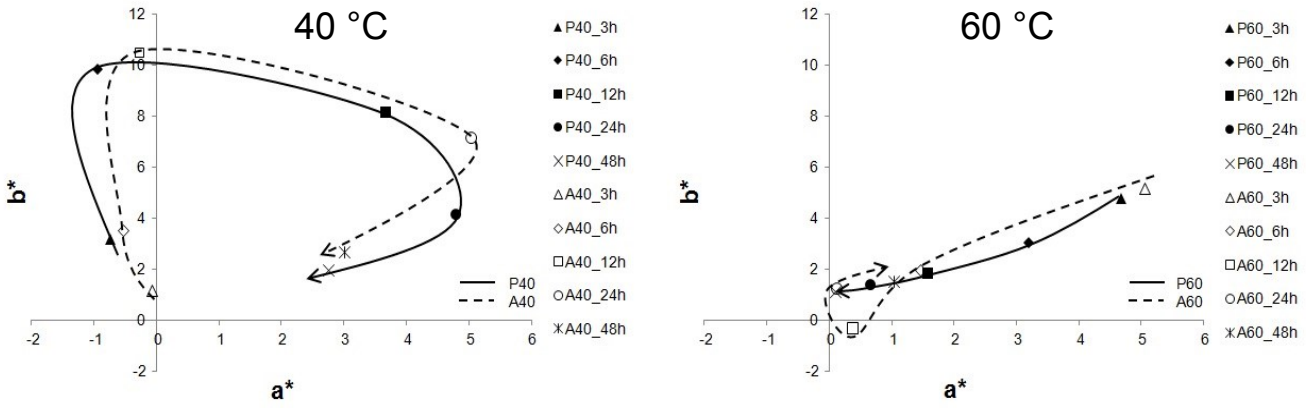


Figure 5.6: Changes in the planar coordinates of b* (blue[-] to yellow[+]) and a* (green[-] to red[+]) in GlcN models. GlcN were incubated in different two solutions (PBS [P] and ammonium hydroxide [A]), temperature (40 and 60˚C) and incubated up to 48 h. N=3.

5.3.6 Antioxidant activity

The pioneer work on GlcN browning conducted by Oyaizu (1986) showed that GlcN powder incubated at 37˚C for up to 30 d started to develop a brown coloud after 12 d. Browning continued to increase up to 30 d and its intensity was proportional to the antioxidant activity. In this study, we attempted to study the relationship between browning

and antioxidant activity (measure DPPH and ABTS assays) in a liquid system at 40 and 60˚C in two different types of solution with the aim to generate caramel solutions with radical scavenging activity in shorter times while maximizing the production of diacetyl.

5.3.6.1 DPPH radical scavenging activity

The DPPH radicals are widely used to estimate the antioxidant activity contributed by the electron and hydrogen atom transfer (HAT) mechanism (Xie and Schaich 2014). Production of browning compounds (i.e. melanoidins) is normally associated with an increase of antioxidant capacity (i.e. reduced radical formation) of caramels and Maillard reaction products (Bressa et al. 1996; Tsai et al. 2009). Since majority of the components in the brown pigment mixture are soluble in alcohol (Bekedam, Roos, and Schols 2008; Tsuchida et al. 1973), it is justifiable to use DPPH to measure the antioxidant capacity in the caramels as the assay is a methanol based-assay.

The efficacy of various GlcN caramels in scavenging free DPPH radicals tested at various concentrations is reported in Figure 5.7 A-E. It is expressed in term of % radicals scavenged as a function of concentration and incubation time. Generally, all the GlcN-derived caramels solutions obtained at different times exhibited DPPH radical scavenging activity in a dose-dependent manner. In addition to that, caramel solutions produced at 40˚C showed a progressive increase in antioxidant activity with prolonged incubation duration, but this trend was not observed at 60˚C.

Results reported in Table 5.1 indicates that the rate of GlcN in scavenging DPPH radicals is mainly influenced by the effects of temperature and incubation time (Table 5.1, $p<0.001$) and to a lesser extent by the types of solution. However, a significant interaction between temperature and incubation time was also observed. Prolonged incubation time

caused a widening of the fitted sigmoid curve and a shift to the left of the graph in the 40˚C models (Figure 5.7, P40 and A40). However, the effect of incubation time on the radical scavenging activity of P60 and A60 was not as evident as at 40˚C; in this case, sample concentration mainly influenced the DPPH activity.

Based on the fitted sigmoid curves, the EC_{50} of DPPH radical scavenging activity in the GlcN caramel solutions was determined (Table 5.3). The DPPH radical scavenging activity is expressed in Trolox equivalent (TE) unit as well. Generally, an increased in the TE values were observed throughout the incubation time ($p<0.05$). The greatest TE was observed in P40 at 48 h (651 µmol/g), then P60 at 12 h (568 µmol/g), A60 at 24 h (426 µmol/g) and A40 at 48 h (386 µmol/g). Therefore, the less the EC_{50} value the greater the antioxidant effect. Here, the lowest EC_{50} values were observed at 48 h, with P40 as the most effective anti-radical caramel solution (0.15 mg/mL), followed by P60 (0.16 mg/mL), A60 (0.21 mg/mL) and A40 (0.27 mg/mL).

By referring the Pearson's correlation coefficients (Table 5.6), DPPH scavenging activity showed strong interactions with absorbance at 280nm ($r= 0.92$), 320 nm ($r= 0.87$) and inversely correlated to L* ($r= -0.88$). A moderate correlation was observed between the dark-brown pigments (measured at 420 nm) and DPPH radical scavenging activity ($r= 0.64$, $p<0.001$). This suggests that the DPPH scavenging activity did not entirely come from the melanoidins; the formation of the UV-absorbing intermediates (280-320 nm) may also have contributed to this activity. As mentioned earlier, the DPPH radicals can be quenched by the electron and HAT (Xie and Schaich 2014). Another possible explanation of antioxidant behaviour in the GlcN-model could be the dehydrogenation of dihydrofructosazine to form a more stable FZ molecule (Jia et al. 2015). This is in

agreement with the accumulation of FZ which is detectable at 280 nm as the browning in GlcN progresses.

The role of coloured pigments in scavenging free radicals remains inconclusive when it is assessed with one antioxidant assay only, especially when the models contain both colourless and pigmented compounds. Therefore, ABTS assay was used to further investigate the antioxidant activity in the GlcN models.

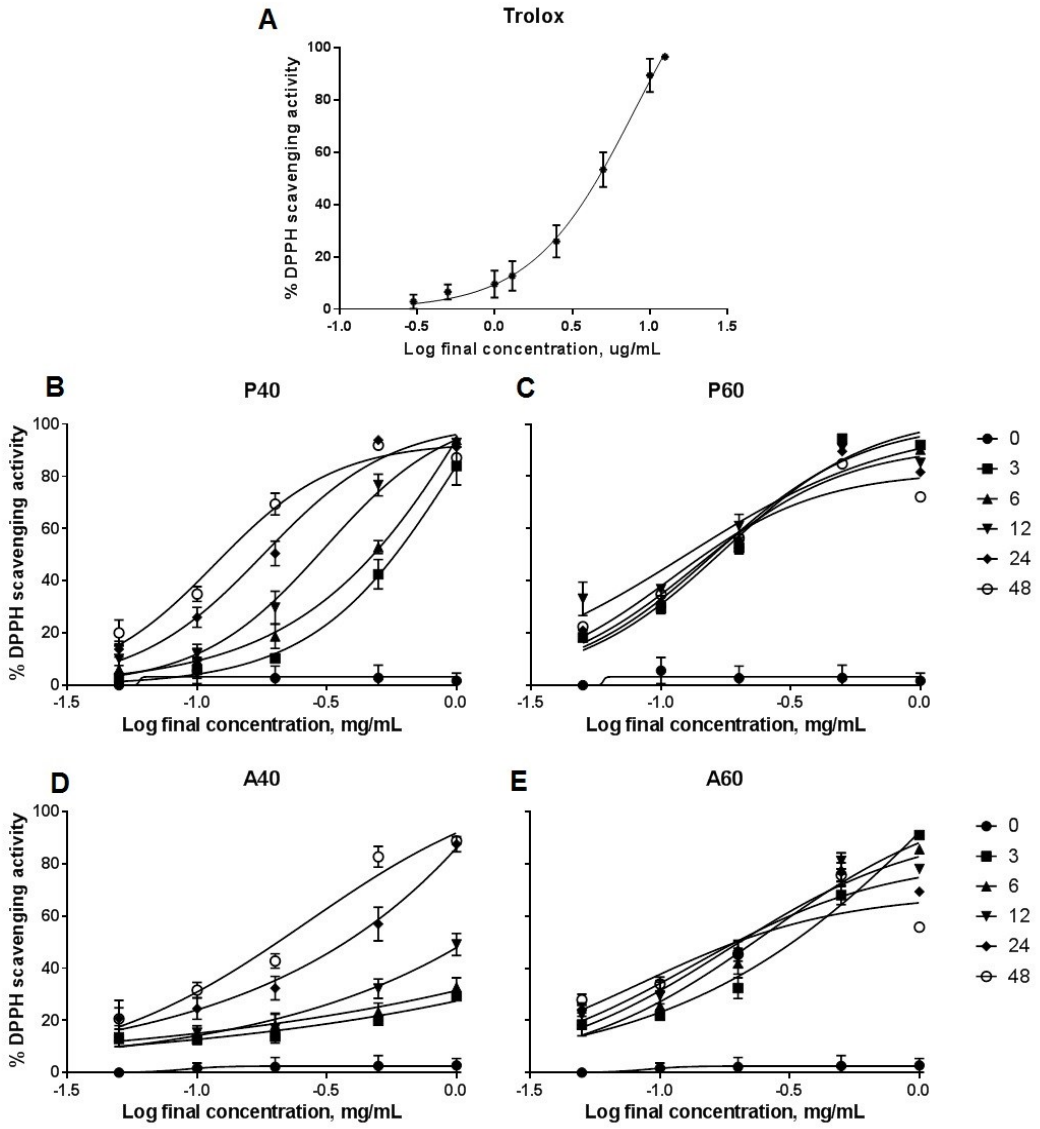


Figure 5.7 A-E: DPPH radical scavenging activity of Trolox (positive control) and GlcN incubated in two solutions (PBS [P] and ammonium hydroxide [A]) at two temperatures (40 and 60˚C) for 0, 3, 6, 12, 24 and 48 h tested at different concentrations. Data points were fitted (R^2> 0.82) and described with the equation:

$$y = B + \frac{(A - B)}{1 + 10^{(Log\ EC50 - x)\ H}}$$

where A and B= the top and bottom of plateaus in the units of the y-axis; EC50= the effective concentration to reach to 50% DPPH anti-radical activity (EC_{50}, mg/mL) from samples (final concentration 0.1 mg/mL); x= concentration in log unit; H= hillslope. N=3. Figure adapted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd.

Table 5.3: Trolox equivalent (μmol/g) and EC_{50} values (mg/mL) of DPPH scavenging activity in GlcN models. Table adapted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd.

	P40			P60			A40			A60		
Time, h	TE	EC_{50}	R^2	TE	EC_{50}	R^2	TE	EC_{50}	R^2	TE	EC_{50}	R^2
3	63±17 ^{d}	0.65	0.9840	485±23 ^{b}	0.21	0.9902	99±9 ^{b}	0.68	0.9867	283±40 ^{b}	0.42	0.9943
6	147±46 ^{cd}	0.76	0.9832	503±32 ^{ab}	0.20	0.9850	143±45 ^{b}	ND	0.8280	377±41 ^{ab}	0.26	0.9800
12	257±63 ^{cd}	0.31	0.9892	568±45 ^{a}	0.20	0.9781	129±55 ^{b}	0.59	0.9543	398±37 ^{a}	0.23	0.9758
24	463±47 ^{b}	0.21	0.9858	511±7 ^{ab}	0.19	0.9810	283±45 ^{a}	1.09	0.9783	426±40 ^{a}	0.21	0.9604
48	651±42 ^{a}	0.15	0.9872	521±2 ^{ab}	0.16	0.9555	386±27 ^{a}	0.27	0.9772	412±49 ^{a}	0.21	0.8315
p value	<0.001			0.032			<0.001			0.012		

TE (trolox equivalent) values are expressed as mean (N=3) ± standard deviation.
Different superscripted letters in the same column indicate significant difference in the mean values, $p<0.05$.
ND = not determined.
Trolox EC_{50}: 6.44 μg/mL, R^2=0.9865

5.3.6.2 ABTS radical scavenging activity

The ABTS assay is an aqueous assay that measures the anti-radical activity based on two mechanisms: electron transfer and HAT (Tian and Schaich 2013). In Maillard reaction models, most of the intermediate compounds including the cyclic and acyclic ones are soluble in water. For example, brewed coffee is well known for its antioxidant activity, the majority of low MW extracts (MW< 12 kDa) are highly polar and soluble in water (Bekedam, Roos, and Schols 2008). Therefore, the ABTS assay is a suitable approach to assess the antioxidant activity in the GlcN models.

The reaction rate of ABTS anti-radical activity in the GlcN models weas similar when compared with their corresponding DPPH radical scavenging activity. The antioxidant activity increased as the GlcN concentration increased. Figure 5.8 showed the sigmoid curves were consistent in most of the models, except for P40. The narrow sigmoid curves indicate that the ABTS radical scavenging activity in P60, A40 and A60 was not greatly affected by incubation time but rather was affected by the GlcN caramel concentration. In P40, the sigmoid curves shifted to the left of the graph indicating less sample required to achieve greater percentage in anti-radical activity.

The TE values in ABTS assay were consistently in agreement with the anti-radical activity in DPPH assay (Table 5.3 and 5.4). Both sets of data showed an increment in the TE values during prolonged incubation time. P40 and P60 gave equally greatest TE at 48 h (about 1859 μmol/g) followed by A60 (1741 μmol/g) and A40 (1467 μmol/g). The EC_{50} value of each treatment was estimated based on the curves' best fit. The values were at their least when incubated for 48 h, and they were similar to each other ranging from 0.08 to 0.13 mg/mL, accounting for about one-tenth of trolox's anti-radical capacity.

The ABTS radical scavenging activity was influenced by a combination of solution, temperature and time (Table 5.1, $p<0.001$); however, temperature played a critical role. The result was well correlated with DPPH anti-radical activity (Table 5.6, $r= 0.86$, $p<0.001$). Moreover, ABTS scavenging activity was positively interacted with absorbance at 320 (Table 5.6, $r= 0.86$, $p<0.001$), 280 ($r= 0.83$, $p<0.001$) and, and fairly correlated well with 420 nm ($r= 0.69$, $p<0.001$). This further suggests that, complementary to the DPPH assay, which is targeted to the melanoidins and other methanolic-soluble compounds, the hydrophilic cyclic and heterocyclic compounds could have contributed to the radical scavenging activity.

Aside from that, the role of certain reductones in anti-radical activity has also proposed. Reductones react by donating electron and react with free radicals to terminate the radical chain reaction (Jayaprakasha, Tamil Selvi, and Sakariah 2003). Furthermore, another possible mechanism may involve the dihydrofructosazines througha HAT mechanism which would promote the formation of fructosazine. When both the quantitative responses of DPPH and ABTS assays are compared in terms of trolox equivalent (TE) value, the ABTS assay gave a greater TE value in the GlcN models, though similar trends are observed in both assays. Similarly, the cane brown sugar extract gave a greater TE response in the ABTS than the DPPH assay (Payet, Sing, and Smadja, 2005), this is in line with the current study.

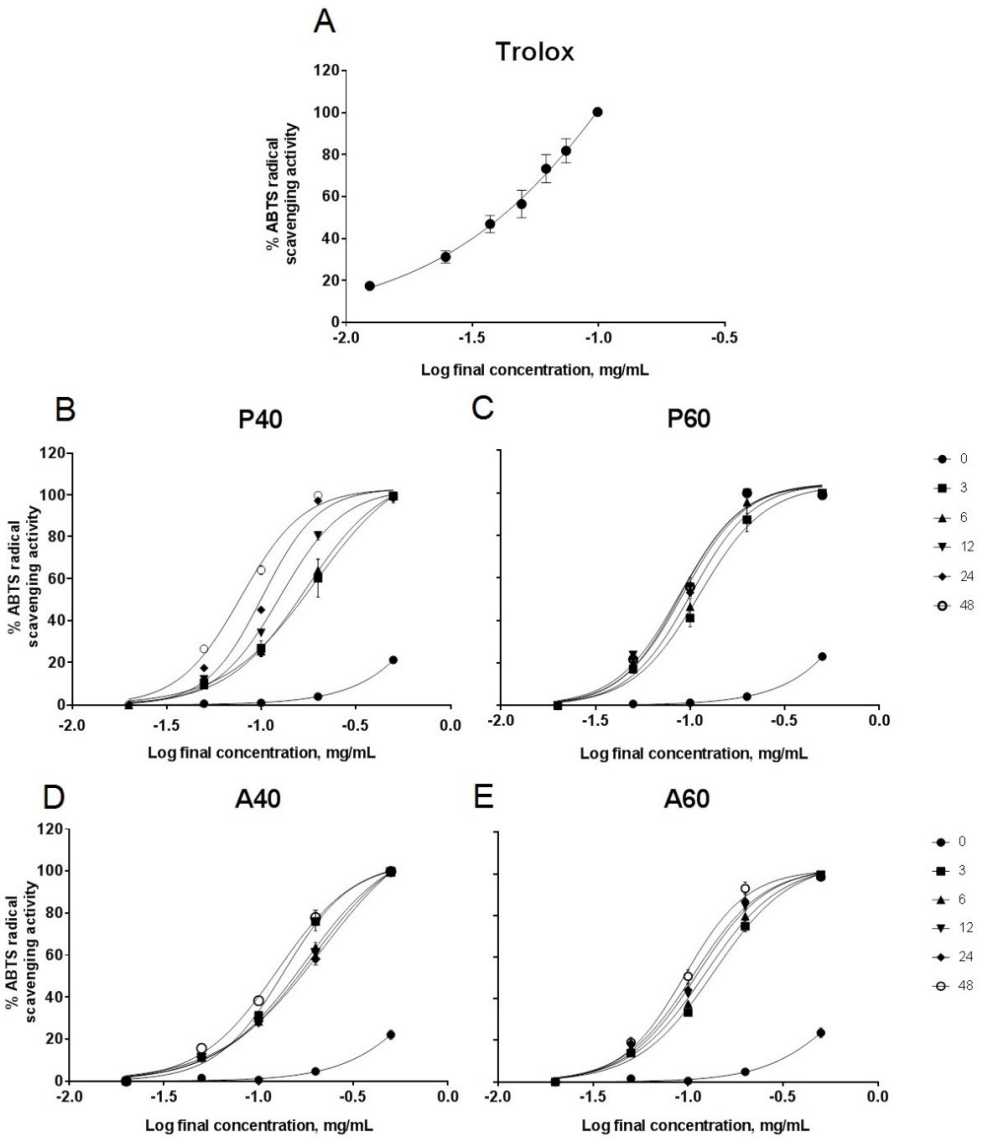


Figure 5.8 A-E: ABTS radical scavenging activity of trolox (positive control) and GlcN in two solutions (PBS [P] and ammonium hydroxide [A]), incubated at two temperatures (40 and 60˚C) for for 0, 3, 6, 12, 24 and 48 h tested at different concentrations. Data points were fitted (R^2>0.98) and described with the equation:

$$y = B + \frac{(A - B)}{1 + 10^{(Log\ EC50 - x)\ H}}$$

where A and B = the top and bottom of plateaus in the units of the y-axis; EC50 = the effective concentration to reach to 50% ABTS anti-radical activity (EC_{50}, mg/mL) from samples (final concentration 0.1 mg/mL); x = concentration in log unit; H = hillslope. N=3. Figure adapted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd.

Table 5.4: Trolox equivalent (μmol/g) and EC_{50} values (mg/mL) of ABTS scavenging activity in GlcN models. Table adapted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd.

	P40			P60			A40			A60		
Time, h	TE	EC_{50}	R^2	TE	EC_{50}	R^2	TE	EC_{50}	R^2	TE	EC_{50}	R^2
3	1131±165 ^{c}	0.20	0.9897	1635±101 ^{b}	0.11	0.9888	1433±79 ^{a}	0.14	0.9957	1410±48 ^{d}	0.14	0.9964
6	1198±102 ^{c}	0.17	0.9953	1783±89 ^{ab}	0.10	0.9827	1199±42 ^{b}	0.18	0.9984	1499±33 ^{cd}	0.12	0.9953
12	1510±37 ^{b}	0.13	0.9969	1861±11 ^{a}	0.09	0.9869	1148±14 ^{b}	0.20	0.9994	1590±42 ^{bc}	0.11	0.9957
24	1813±26 ^{a}	0.11	0.9862	1852±10 ^{a}	0.09	0.9860	1096±55 ^{b}	0.24	0.9989	1633±66 ^{ab}	0.11	0.9921
48	1859±3 ^{a}	0.08	0.9928	1860±2 ^{a}	0.09	0.9868	1467±62 ^{a}	0.13	0.9963	1741±54 ^{a}	0.10	0.9918
p value	<0.001			0.004			<0.001			<0.001		

TE (trolox equivalent) values are expressed as mean (N=3) ± standard deviation.
Different superscripted letters in the same column indicate significant difference in the mean values, $p<0.05$.
Trolox EC_{50}= 0.01 mg/mL, R^2= 0.9782.

5.3.6.3 Ferric reducing antioxidant power (FRAP) assay activity

Determining the capacity of GlcN caramels in reducing the non-soluble ferric ion to the soluble ferrous ion remains is important as the majority of iron in foods is in its least soluble form Fe^{3+} (Conrad and Umbreit 2000; Sharp 2010). The FRAP assay is the measurement of reductants to reduce the ferric ion (Fe^{3+}) to ferrous form (Fe^{2+}). The results of FRAP activity were expressed in μmol ascorbic acid/g GlcN and are reported in Table 5.5.

According to Table 5.5, P40, A40 and A60 show a sharp increase in ferric reducing activity, these models achieved their maximum levels at 3 h; then declined as it progressed to 48 h. This suggests that short incubation time gave greater FRAP activity. On the contrary, the P60 treatment had a reverse trend, the reducing activity slowly developed and reached to its maximum at the end of incubation (48 h).

By referring to Table 5.6, the FRAP activity was negatively and moderately correlated with 320 nm ($r= -0.46$, $p<0.001$), DPPH activity ($r= -0.46$, $p<0.001$), ABTS activity ($r= -0.45$, $p<0.001$), 280 nm ($r= -0.44$, $p<0.001$) and 420 nm ($r= -0.31$, $p<0.05$). In the previous discussion (section 5.3.6.1 and 5.3.6.2), the anti-radical activities were strongly correlated to the above parameters. However, it is not the case for the ferric reduction activity; it seems that a shorter incubation time produces GlcN caramels with greater ability to reduce Fe^{3+} than caramel produced at a longer incubation time.

An important compound produced in greater amount in GlcN caramels especially at shorter incubation time is the reductone D-arabino-hexo-2-ulose (D-glucosone) (Hrynets, Ndagijimana, and Betti 2015a). It was detected when GlcN was incubated at both 37 and 50˚C in the previous study (Chapter 4) as well as at 40 and 60˚C in this study (Appendix

4). Kanzler, Haase, and Kroh (2014) reported that the enediol structure of D-glucosone can act as antioxidant via 3 proposed pathways: a) oxidation of enediol to triketone; b) hydrogen abstraction and forming a stable radical; and, c) disproportion of the two stable radicals to form reductone and triketone. Hence, it seems plausible that glucosone may donate electrons to Fe^{3+} ions. Other volatile reductones like furoneol (MW= 128) and norfuraneol (MW= 114) which are formed during GlcN maillard reaction might also involved in ferric reduction (Kikugawa et al. 1998; Kraehenbuehl et al. 2008). Oxidation of dihydrofructosazine to FZ may also be involved in the reduction of ferric iron (Shimamura et al. 2003).

Table 5.5: Ferric reducing antioxidant power (FRAP) activity in GlcN models. The values were expressed in ascorbic acid equivalent unit (μmol ascorbic acid/g GlcN).

Time, h	P40	P60	A40	A60
3	117±20 [a]	87±21	145±9	148±5 [a]
6	94±7 [ab]	92±11	125±5	120±16 [b]
12	95±2 [ab]	109±4	143±2	104±2 [b]
24	78±8 [b]	107±2	132±5	100±7 [b]
48	94±6 [ab]	121±22	129±16	106±14 [b]
p value	0.017	0.108	0.067	0.001

Values are expressed as mean (N=3) ± standard deviation.
Different superscripted letters in the same column indicate significant difference in the mean values, $p<0.05$.

Table 5.6: Pearson's correlation coefficients of tested parameters in the GlcN models. Table reprinted with permission from Hong, P.K. and Betti, M. 2016. Non-enzymatic browning reaction of glucosamine at mild conditions: Relationship between colour formation, radical scavenging activity and α-dicarbonyl compounds production. Food Chem 212:234-243. Copyright (2016) Elsevier Ltd.

	L*	a*	b*	Chroma	Hue	pH	UV280nm	UV320nm	UV420nm	DPPH_TE	ABTS_TE	FRAP_TE
L*	1	-.41**	.50**	.37**	.65**	.72**	-.97**	-.87**	-.74**	-.88**	-.77**	.33**
a*	-.41**	1	.17	.39**	-.85**	.13	.35**	.10	-.12	.36**	.19	-.18
b*	.50**	.17	1	.97**	.11	.54**	-.51**	-.60**	-.61**	-.48**	-.59**	.07
Chroma	.37**	.39**	.97**	1	-.10	.56**	-.40**	-.54**	-.60**	-.37**	-.51**	.00
Hue	.65**	-.85**	.11	-.10	1	.09	-.62**	-.40**	-.11	-.67**	-.49**	.28*
pH	.72**	.13	.54**	.56**	.09	1	-.76**	-.87**	-.95**	-.65**	-.69**	.21
UV280nm	-.97**	.35**	-.51**	-.40**	-.62**	-.76**	1	.94**	.80**	.92**	.83**	-.44**
UV320nm	-.87**	.10	-.60**	-.54**	-.40**	-.87**	.94**	1	.91**	.87**	.86**	-.46**
UV420nm	-.74**	-.12	-.61**	-.60**	-.11	-.95**	.80**	.91**	1	.64**	.69**	-.31*
DPPH_TE	-.88**	.36**	-.48**	-.37**	-.67**	-.65**	.92**	.87**	.64**	1	.86**	-.46**
ABTS_TE	-.77**	.19	-.59**	-.51**	-.49**	-.69**	.83**	.86**	.69**	.86**	1	-.45**
FRAP_TE	.33**	-.18	.07	.00	.28*	.21	-.44**	-.46**	-.31*	-.46**	-.45**	1

* Correlation is significant at p=0.05 level (2-tailed).
** Correlation is significant at p=0.01 level (2-tailed).
N=3

5.4 Conclusions

In this study, PBS and ammonium hydroxide solutions, incubation time and temperature have all been demonstrated to play a role in the kinetics of GlcN non-enzymatic browning. Combinations of different factors result in GlcN caramel solutions that possess different levels of browning, antioxidant activity and flavouring compounds (i.e. diacetyl). In order to produce a caramel-like product with the greatest amount of diacetyl, the utilization of PBS at 40˚C for a shorter incubation time (6 h) would be preferable. At this incubation time, the radical scavenging activity would be relatively high and the production of melanoidins would be minimized resulting in a yellow-light brown caramel. In line with this, Heineken, the multinational beer manufacturer, has developed a new GlcN-derived ingredient called "colourless caramel" which absorbs the UV light to help stabilize the shelf life of beer. Overall, this study showed that GlcN is a reactive amino-sugar at relatively moderate temperatures of 40 and 60˚C. This offers an option to produce caramel-like products in a rapid and energy saving manner.

Chapter 6: Conclusions, implications and future research

Overall, this doctoral work advanced knowledge in the field of non-enzymatic browning of the amino sugar glucosamine. Non-enzymatic browning includes both Maillard (glycation) and caramelization reactions. To date, most of the studies about glucosamine have focused on its effect against osteoarthritis. Very little is known about the reactivity of GlcN and the possibility of using this amino sugar to generate flavouring, antioxidant and antimicrobial compounds for food formulations.

Based on the limited research literature that was available in 2011 when I started this doctoral project, our research team took the initiative to explore and find ways to gain control over the non-enzymatic browning reaction of GlcN at moderate temperatures (25-50˚C). My research was focused on studying the reactivity of GlcN at moderate temperatures in producing the Maillard reacted peptides (MRPs= Amadori-glycopeptides + AGE-peptides) that exhibit taste enhancing and antioxidant activities; volatile aroma compounds like diacetyl. Concurrently, I had a priority to minimize the formation of undesirable polymerized and toxic compounds in this reaction, which was the basis of the moderate temperature approach. I also explored the possibility of using TGase to produce glycosylated peptides using GlcN as amine donor to the amide group of glutamine-containing peptides such as the hydrolysates of fish gelatin and poultry meat.

Upon completion of this thesis, the majority of the objectives listed in Chapter 1 (Introduction) have been accomplished. The main findings are:

1. Evidence of GlcN-induced peptide modification at moderate temperatures of 25-50˚C

   This thesis demonstrated indirect evidence verifying the occurrence of MRPs and GlcN caramelization products at 25-50˚C using spectroscopic techniques and size exclusion chromatography (SEC).

2. Plausible evidence on the formation of Amadori-glycopeptides and TGase glycosylated peptides

   In conjunction with the above finding, "direct but partially inconclusive" proof regarding the formation of Amadori-glycopeptides and TGase-glycosylated peptides was established with MALDI-TOF/TOF-MS. This first identification was based on the MW shift occurring to a peptide as a consequence of glycation (+161 Da= GlcN 179 Da – H_2O 18 Da) and glycosylation (+162 Da= GlcN 179 Da – NH_3 17 Da).

3. GlcN-induced modification at moderate temperatures enhances antioxidant activity *in vitro* and in cell model

   By studying the antioxidant activity in GlcN-induced modified peptides and in all the model systems, it was confirmed that GlcN is the driving force that contributes to improvements in radical scavenging and reducing activities, both *in vitro* as well as in cell model, making the impact of this modification more biologically understood and relevant.

4. GlcN-induced peptide modification enhances antimicrobial activity

The GlcN-modified peptides and caramelization products can inhibit growth that targets both classes of bacteria: *Escherichia coli* AW1.7 (G-) and *Bacillus subtilis* FAD110 (G+).

5. GlcN-modified peptide and GlcN caramelization products as potential taste enhancers

   The highly reactive dicarbonyl compounds and their role in creating MRPs at moderate temperatures suggest possible applications as a salt-enhancing or kokumi products.

6. Model study gives an insight to better control the properties of GlcN caramels for food applications

   The main factors were identified in a model study that affect GlcN caramel colour and relate to its antioxidant activity. The interaction between a lower heating temperature (40˚C) and a short duration (<6 h) offers optimal conditions for developing GlcN caramel with significant antioxidant activity and light brown colour.

7. Diacetyl as a substantial volatile flavour component in GlcN caramel

   α-dicarbonyl compounds are significant products in GlcN caramel. Short chain α-dicarbonyl compounds (such as diacetyl) were identified and quantified because they represent an important portion of the volatiles in GlcN caramels.

Although the isolation and full mass spectrometry identification of Amadori–glycopeptides and AGE-peptides were not provided in this doctoral work, this thesis can be considered an important contribution for future research in pursuit of a better understanding on the effect of GlcN in modifying peptides. For example, my colleagues were able to

adapt the idea of producing antimicrobial Amadori-glycopeptides at moderate temperatures. This idea has been extended to be a MSc-level study by isolating the glycopeptides and more thoroughly screening antimicrobial activity with the same bacteria—*E.coli* AW 1.7.

Also, this thesis opens the door to a new application of GlcN caramels—as a functional food ingredient. Specifically, GlcN non-enzymatic browning at mild conditions may produce caramels which possess antioxidant and antimicrobial activities, as well as flavouring compounds and anti-diabetic activity (i.e. DOF). Furthermore, despite the inconclusive findings for the taste enhancing property in study 2 (Chapter 4), it was able to provide a direction for further improvement of the reaction conditions to achieving the intended objective. More attention should focus on the development of α-dicarbonyl compounds, especially diacetyl which is characterized as "buttery" and "butterscotch-like". These are the important future precursors for colour and flavour development. In line with my current research, Heineken—the multinational beer manufacturer—has incorporated a new GlcN-derived ingredient called "colourless caramel" which absorbs UV light to help stabilize beer's shelf life. I believe GlcN caramel has the potential to become a multi-functional food ingredient with adequate applied research in the future.

Despite the positive findings obtained in this thesis, there are certainly areas that could be improved to better substantiate the results obtained. For instance, in study 1, the identification of glycopeptides could have been be improved by isolating them through affinity chromatography followed by tandem mass spectrometry. In study 2, hiring the same batches of untrained consumers for both sensory evaluation sessions could have ensured the repeatability of the test, rather than using different consumers as test panels in

all the sessions. Additionally, the high sodium contents in all the samples used in the sensory evaluations could have been reduced to avoid confusion among panels. Ideally, the best and desired salt level for sensory evaluation would have required a series of pre-testing sessions before the actual sensory evaluations were to commence.

In terms of possible further research work, the isolation and quantification of dihydrofructosazine, FZ and DOF produced during GlcN non-enzymatic browning would be an interesting area to explore. These compounds are associated with flavour and are normally found in roasted foods and tobacco smoke. DOF also possess anti-diabetic and anti-inflammatory properties. Identification of these hydroxyalkyl pyrazines at 37˚C have already been proven by Horowitz (1991) and Hrynets et. al (2015a), but no quantification has been reported so far. If significant amounts of FZ and DOF are proven to be formed at the low temperature reported in this study, GlcN-derived caramel may indeed have the potential to become a health-enhancing nutraceutical syrup.

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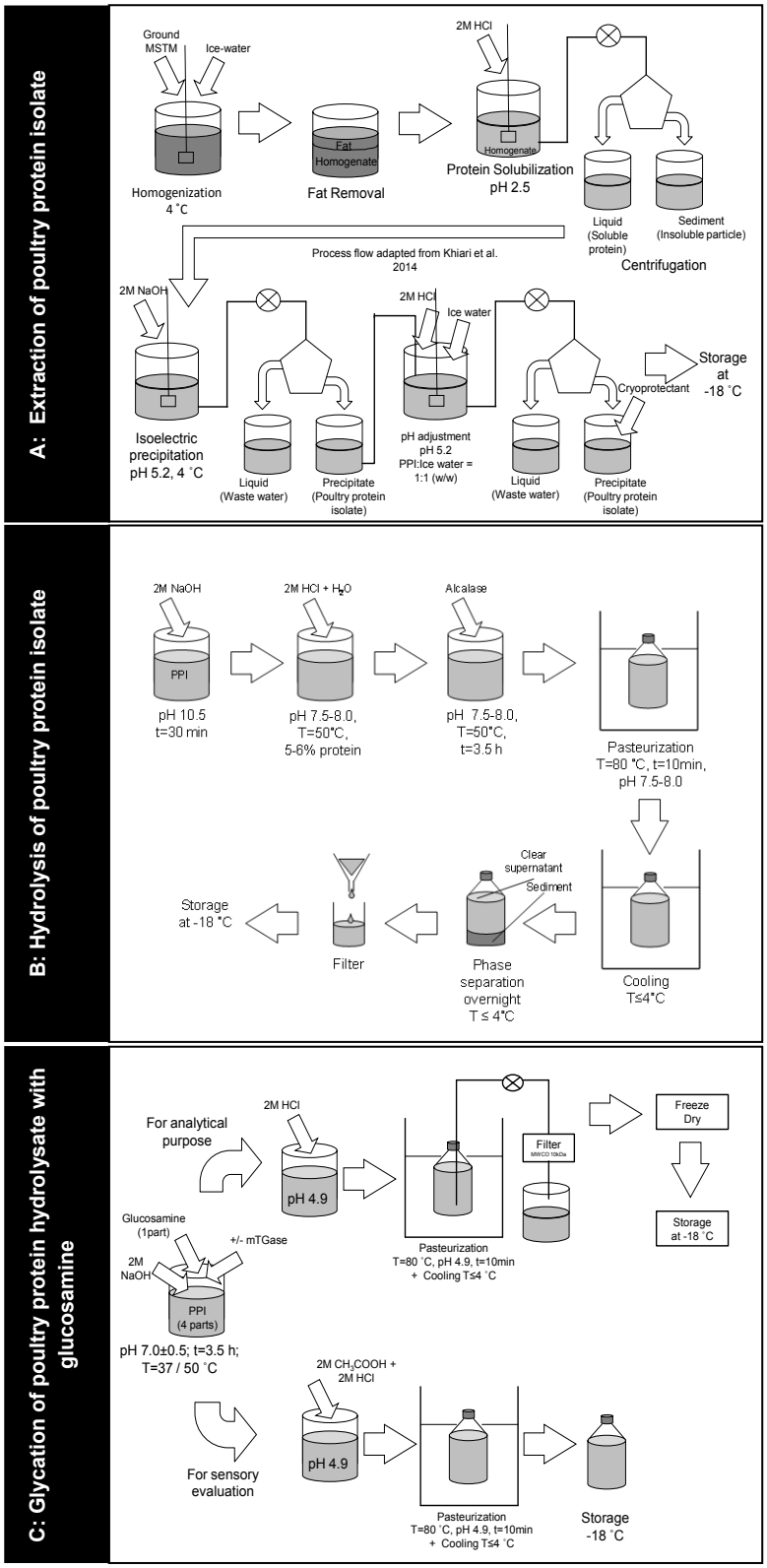
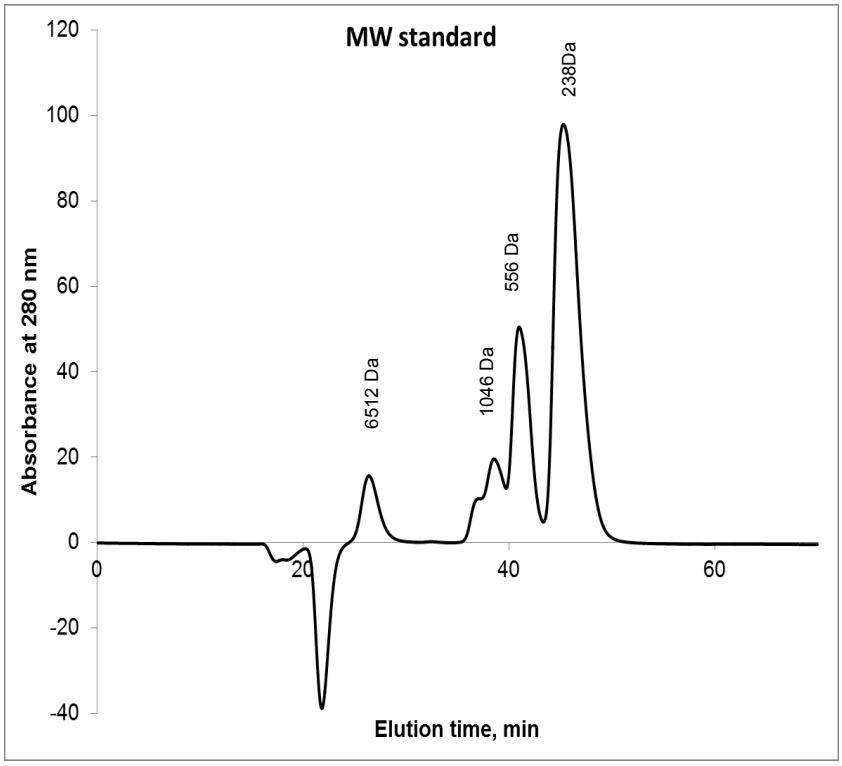
Appendix 1: Process flow of poultry protein processing from the protein extraction phase to the GlcN-induced glycation phase. (A) Isolation of poultry protein from mechanically separated turkey meat. (B) Hydrolysis of poultry protein isolate with Alcalase. (C) Glycation of poultry protein isolate hydrolysate to glucosamine. Figure reprinted with permission from Hong, P.K., Ndagijimana, M., and Betti, M. 2016. Glucosamine-induced glycation of hydrolysed meat proteins in the presence or absence of transglutaminase: chemical modifications and taste-enhancing activity. Food Chem 197 (B):1143-1152. Copyright (2015) Elsevier Ltd.



Appendix 2: Demographic distribution of the untrained consumers recruited for ranking test on the sensory evaluation of the perceived saltiness (N=64) and savouriness (N=61) intensity in poultry meat protein-based seasoning compositions. Table reprinted with permission from Hong, P.K., Ndagijimana, M., and Betti, M. 2016. Glucosamine-induced glycation of hydrolysed meat proteins in the presence or absence of transglutaminase: chemical modifications and taste-enhancing activity. Food Chem 197 (B):1143-1152. Copyright (2015) Elsevier Ltd.

Variable		Saltiness		Savouriness	
		Number of consumers	%	Number of consumers	%
Gender	Male	21	32.8	31	50.8
	Female	43	67.2	30	49.2
Age	18-29	53	82.8	50	82.0
	30-39	6	9.4	9	14.8
	40-49	1	1.6	2	3.3
	50-59	3	4.7	0	0
	60-69	1	1.6	0	0

Appendix 3: SEC profile of the standard molecular weight mixture (390 Da-12 kDa) eluted at 280 nm.



Appendix 4: Glucosone and 3-deoxyglucosone production of GlcN solutions (reported in term of chromatogram peak area) incubated in PBS [P] at 40 (P40) and 60˚C (P60), respectively, and in ammonium hydroxide [A] at the same temperatures (A40 and A60). All samples were analysed at 0, 3, 6, 12, 24, 48 h. N=2.