Glucosamine and Glucosamine-Peptides Antimicrobial Compounds

by

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ABSTRACT

Bacterial resistance to chemical and physical methods in food processing, and the consumers' demand for food free of chemical additives challenge the food industry to identify new approaches for food preservation. Affordable and novel antimicrobial compounds from food derived sources are an interesting field of research. This study investigated antimicrobial compounds derived from glucosamine or from glucosamine and fish gelatin peptides.

Reaction of glucosamine in aqueous solution at 50 °C in the presence and absence of iron produced the five α-dicarbonyl compounds glucosone, 3-deoxyglucosone, glyoxal, methylglyoxal and diacetyl. The reaction was followed up to 48 h of incubation by UV/Vis absorbance profiles and pH variations, in order to understand where the major changes occurred during the progress of the reaction. Major changes in UV/Vis profiles were found after 3 h and 48 h of incubation. The reaction mixture exhibited antimicrobial activity at 5% (w/v) against *Escherichia coli* AW 1.7 and this activity was partially attributable to the α-dicarbonyls.

Furthermore, fish gelatin peptides were modified by chemical glycation or enzymatic glycosylation with glucosamine at room temperature; the five α-dicarbonyls glucosone, 3-deoxyglucosone, glyoxal, methylglyoxal and diacetyl were also found in the conjugated mixtures. The conjugated samples of fish gelatin peptides with glucosamine were fractionated by lectin affinity and reverse phase liquid chromatography. The resultant fractions of glycopeptides reduced the

growth of *E.coli* AW 1.7 at lower concentration of 1% (w/v) compared with the original conjugates.

In this study, antimicrobial compounds such as α-dicarbonyls and glycopeptides were separated and identified after glucosamine autocondensation and glucosamine-fish gelatin peptides conjugation. The potential antimicrobial activity exhibited by glucosamine and glucosamine-peptides suggests that it is feasible to use glucosamine as a functional food ingredient that might serve for food preservation.

Keywords: α-dicarbonyls, glycopeptides, glucosamine, fish gelatin peptides, antimicrobial activity, heat resistant *E. coli*, glycation, glycosylation, iron.

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LIST OF ABBREVIATIONS

AGE´s	Advanced glycation end-products
HMF	Hydroxymethylfurfural
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
ROS	Reactive oxygen species
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
GlcN	Glucosamine
OD	Optical density
UHPLC	Ultra-High performance liquid chromatography
LOD	Lower limit of detection
LOQ	Lower limit of detection
S/N	Signal to noise ratio
RP	Reverse phase
DH	Degree of hydrolysis
TGase	Transglutaminase
FGP	Fish gelatin peptides
FPLC	Fast protein liquid chromatography
CV	Column volume
GCP	Glycated peptides
GSP	Glycosylated peptides
MIC	Minimum inhibitory concentration
UV/Vis	Ultraviolet/Visible

1 LITERATURE REVIEW: AMINO-SUGARS AND MAILLARD REACTION PRODUCTS WITH ANTIMICROBIAL ACTIVITY

1.1 An overview of the Maillard reaction

Non-enzymatic browning reaction, known better as the Maillard reaction, is a well-known phenomenon, although not completely understood. It occurs in a variety of food systems during domestic cooking or industrial food processing conferring particular sensory attributes such as taste, aroma, texture and color to foods. The chemistry of the reaction has been extensively studied and can be divided in three main stages. The earlier stage of the reaction involves the condensation between the carbonyl group of a reducing sugar, aldehyde or ketone and an available amino group in protein, peptide or any nitrogenous compound, forming a Schiff base with the release of water giving place to the formation of an Amadori compound (Figure 1.1) (Hodge et al., 1953).

The intermediate stage begins with the degradation of the Amadori/Heyn's product(s) which can undergo numerous transformations involving different chemical pathways. They include oxidation, fragmentation, enolization, dehydration, acid hydrolysis, and free radical reactions (Friedman, 1996; Ledl et al., 1990). Although some color is produced at this stage, most of it is not produced until the final stage where water-insoluble and nitrogen-containing polymeric compounds referred to “melanoidins” are produced. In this final stage the Amadori products can then form crosslinks between adjacent proteins or with other amino groups forming polymeric aggregates known as advanced glycated end products (AGE´s) (Friedman, 1996).

The Maillard reaction results in hundreds of compounds that are beneficial from the sensory or bioactive point of view, but it can also lead to harmful or toxic compounds during its progress. Heterocyclic amines and acrylamide derived from the Maillard reaction are known to be toxic and probable carcinogenic

compounds produced in the final stage of the reaction (Jinap et al., 2015; El-Hady et al., 2015; Liu et al., 2015). Additionally, advanced glycated end products have been related to diabetes, cardiovascular complications and Alzheimer (Yamaguchi et al., 2014). On the other hand, beneficial compounds produced during the reaction have been reported to possess different bioactivities such as antioxidant, anti-inflammatory, antihypertensive and antimicrobial (del Castillo et al., 2007; Hashemi et al., 2014; Langer et al., 2014). Thus, the beneficial side of the reaction has attracted the attention of many researchers to design different model systems with the aim to further understand and control the reaction for a better utilization and harmless food processing.

The reaction progress is mainly influenced by the water activity, pH, temperature, and heating time of the system as well as the type of proteins and sugars in different food matrices. High temperatures, prolonged time, water activity and neutral pH favor the reaction. For instance, acidic conditions promote the formation of particular compounds such as hydroxymethylfurfural (HMF) and the α-dicarbonyl 3-deoxyglucosone. These conditions can be controlled to diminish or to avoid the formation of more advanced glycation end products such as heterocyclic amines that might be harmful over prolonged consumption (Friedman, 1996). Therefore, controlling the production of harmless bioactive compounds that can be used safely as food additives through the Maillard reaction is a subject of current research interest (Jiang et al., 2010; Wu et al., 2014).

1.2 Antimicrobial compounds from the Maillard reaction

Some microorganisms are used to manufacture foods such as wine, yogurt, beer or bread; however, they can bring illness and even death if they are not properly controlled. Current strategies and new alternatives for the control of pathogens of main concern in food industry such as *Salmonella*, *Campylobacter*, *Escherichia coli* and *Listeria* have become an important research area. Bacteria resistance, side effects and consumer concerns are the major factors for the increased attention in the development of new alternatives to control foodborne

illnesses. New technologies, design strategies and alternative sources of antimicrobials are needed to continue an efficient fight against harmful microorganisms. To date, bioactive peptides from various food sources such as milk, eggs, grains, and meat have been prepared and their bioactive properties have been described (Das et al., 2012).

Thermal processing impacts positively sensory quality, and shelf life of food matrices. Antimicrobial compounds produced from the Maillard reaction have been investigated in foods and models systems to be potential antimicrobials (Maletta et al., 2012). In processed food like coffee and bread antioxidant and antimicrobial compounds are produced due to the Maillard reaction during roasting and baking processes. For instance, several studies showed antioxidant and antimicrobial activity of fractions obtained from roasted cocoa bean against pathogenic bacteria such as *E. coli* and *Enterobacter cloacae* (Summa et al., 2008). Spent coffee has also shown antimicrobial activity mainly against Gram positive bacteria such as *S. aureus, L. monocytogenes, B. subtilis* and the yeast *C. albicans* (Monente et al., 2014). Honey, was used in the past to treat burns, infected wounds and ulcers. This particular product is known to promote microbial control and healing processes due to its antibacterial, antifungal and antiviral properties (Al-Waili et al., 2011).

These studies described a mixture of compounds exerting this bioactivity, but due to the complexity of the reaction few studies have aimed to identify the compounds responsible for the antimicrobial activity. For instance, in some studies melanoidins, pyrazines and α-dicarbonyls are identified as one of the compounds responsible for the antibacterial effect in coffee and honey. Pyrazines derivatives such as methylpyrazine and 2,5-dimethylpyrazine are present in roasted cumin, which is classified as antimicrobial agent in herbal medicine (Kiralan et al., 2012). The main groups identified up to date as antimicrobials derived from the Maillard reaction are glycopeptides, intermediary products such as α-dicarbonyls, and more advanced products such as pyrazines and melanoidins.

1.2.1 Glycopeptides

Carbohydrates play a key role if covalently attached to proteins and peptides; it might change their solubility, stability folding and biological activities (Ferreira et al., 2011). Glycoproteins and glycopeptides are complex structures composed of both protein and sugar moieties, and they vary in many aspects depending on the type of protein and sugar (mono, di or oligo) attached at various sites. Thus, depending on the characteristics of each specific family of glycoforms they may exert different functional and biological properties. Glycopeptides are produced in our body, by microorganisms and some have been isolated from insects and plants (Winans et al., 1999).

Vancomycin and teicoplanin are the most extensively recognized members of the large family of the glycopeptides antibiotics currently used for the treatment of Gram positive infections (Villa et al., 2014). These type I glycopeptides have a heptapeptide backbone consisting of mainly two proteionogenic amino acids, leucine and asparagine. Due to the structural complexity of glycopeptides, the existent ones have been derived by semisynthetic modification of the natural products such as glycosylation. *In vitro* glycosylation have led to a better understanding of the impact of the structural elements like sugar type and protein source and the development of new analogs (Bambeke et al., 2004).

In 2014, small chain glycopeptides were synthetized and tested on *S. aureus, S. caprae, B. subtilis, E. coli, P. aeruginosa* and *S. sonnei*. The glycopeptide His-Asn-lactose was found to be very effective against six Gram-positive and Gram-negative strains (Nagarajan et al., 2014). Furthermore, Hong et al., (2014) and Gottardi et al., (2014) successfully produced glycated and glycosylated peptides from fish by-products and gluten with glucosamine; the formed products improved antimicrobial activities compared to the native hydrolysates. In their studies, the presence of glycopeptides was confirmed by mass spectrometry; however, during glycation several other Maillard reaction products formed might

be responsible for the antibacterial effect. Thus, production of *in vitro* glycopeptides opens the possibility to study and test their range of action against pathogens of concern in food industry serving as potential food additives in food formulations.

1.2.2 α-Dicarbonyls

Through the different stages of the Maillard reaction several hundred of compounds are formed. α-Dicarbonyls are intermediate compounds formed through the Maillard reaction in food and they are highly reactive bioactive compounds (White., 2009; Shibamoto., 2014). α-Dicarbonyls have been found in carbonated soft drinks, honey, wine, syrups, balsamic vinegar, soy sauce, coffee, cheese among many other foodstuffs (Papetti et al., 2013; Papetti et al., 2014). The most studied α-dicarbonyls are diacetyl, glyoxal and methylglyoxal. Glucosone and 3-deoxyglucosone can be formed in some food matrices such as honey and other carbohydrate rich foods. For instance, diacetyl is known as the buttery flavor and listed in the FDA´s as GRAS (generally recognized as safe) (Shibamoto, 2014).

Pure diacetyl, glyoxal and methylglyoxal α-dicarbonyls have been tested against Gram-negative and Gram-positive bacteria such as *E. coli*, *Salmonella*, *S. aureus,* and *S. mutans*, and they were effective to inhibit their growth (Maletta et al., 2012). They have been found in coffee and they contribute to the antibacterial effect of the coffee brew (Mueller et al., 2011). These three α-dicarbonyls have been also reported in meat systems to inhibit bacterial growth over an 8-day period in ground beef and chicken breast (Jay et al., 1982; Maletta et al., 2012). Moreover, honey is widely recognized to possess antibacterial effect since more than five decades ago; recently α-dicarbonyls were extracted and identified from mono-floral and multi-floral honeys. Especially, manuka honey (mono-floral) is one of the most powerful honeys for bacterial growth inhibition due to its high methylglyoxal content which is effective against *E. coli*, *S. aureus* and *B. subtilis* (Mavric et al., 2008; Marshall et al., 2014).

1.2.3 Pyrazines and their derivatives

Pyrazines and their derivatives occur naturally in a wide range of foods such as bakery items, bacon, beef, caramel coloring, cheese, cocoa, coffee, fish paste, baked potato, whiskey, along with others (Coleman et al., 1981; Mottram et al., 1982; Maga et al., 1992). They are known as flavoring additives in foodstuffs in Europe and USA and they are also used as antibiotics. Pyrazines are six-membered ring aromatic heterocyclic compounds containing two nitrogen atoms (Figure 1.2) (Ferreira et al., 2012). They occur in heat-induced food systems through the condensation of amino compounds and α-dicarbonyls at a later stage of the Maillard reaction. Pyrazine and its derivatives can be isolated from natural sources or synthetized by self-condensation of 2-aminoketones or condensation of 1,2-diamines with 1,2-dicarbonyls compounds among other methodologies for their preparation (Sumoto et al., 1991; Maga et al., 1992; Shipar et al., 2006).

Pyrazines derivatives show activity against bacteria and fungi and some of them have become clinically used worldwide as drugs (Ferreira et al., 2012; Foks et al., 2012). Zhang et al., (2014) synthetized twenty Schiff base derivatives containing pyrazine. The resulting pyrazines derivatives were tested on the two Gram-negative bacterial species *Escherichia coli* and *Pseudomonas fluorescence*, and three Gram-positive species *S. aureus*, *B. subtilis* and *B. amyloliquefaciens*, and they showed an effective antibacterial activity. However, pyrazines usage is limited to the pharmacological field; little information of pyrazines in food systems as antimicrobials is available and no pyrazine or its derivatives is used as food preservative.

1.2.4 Melanoidins

Melanoidins are high molecular weight colorful compounds produced in the last stages of the Maillard reaction. These compounds have been identified in heat-treated food such as baked cereals, roasted coffee, cooked meat, honey and dark beers (Delgado-Andrade et al., 2012). Up to date, the chemical structure of melanoidins has not been completely elucidated; however, in general they are

anionic, brown-colored, nitrogen containing compounds (Langner et al., 2014). Melanoidins have been demonstrated to possess antioxidant, antihypertensive, and antiallergenic activities; they are also responsible for the antimicrobial activity in several food matrices and model systems of amino acid-sugars mixtures. Melanoidins isolated from coffee brews, beer and wine effectively reduced the bacterial growth of high thermoresistent food-degradative microorganism, *Geobacillus* (Rufián-Henares et al., 2005). The most effective melanoidins fractions were those from the most heated food products, like higher degree roasted coffee and dark beer.

Several studies about coffee antibacterial effect have demonstrated that melanoidins are the responsible compounds for the inhibition of other bacteria such as *E. coli, S. aureus*, and *L. innocua*. However, in 2011 Mueller and others further investigated the antimicrobial activity of coffee and they discovered that one of the reactive oxygen species (ROS), H_2O_2, present in roasted coffee is also major contributor of antimicrobial activity. It was suggested the synergistic effect among different compounds from the reactions such as α-dicarbonyls, ROS and Melanoidins. Melanoidins mechanism of action against bacteria has been related to their metal chelating properties, which limit the availability of metals necessary for bacterial growth, leading to membrane disruption (Mueller et al., 2011). Food and model systems melanoidins are related to be the highest molecular fractions of the mixtures; nevertheless, proper identification of the compounds within the melanoidins mixture responsible for the antimicrobial effect has not been yet elucidated.

1.3 Antimicrobial compounds in model systems: effect of the Maillard reaction

Model systems facilitate the isolation and characterization of Maillard reaction products possessing bioactivities in order to design new alternatives of functional food ingredients. Different model systems of protein-sugar have been investigated in order to mimic and replicate antimicrobial compounds found in

actual food systems. In 1983, Einarsson and others found that the Maillard products obtained from arginine-xylose and histidine-glucose exert antimicrobial activity against *E. coli, B. subtilis and S. aureus* (Friedman, 1996). They demonstrated that the smaller molecular weight fraction had less antimicrobial effect than the higher fraction. Moreover, lysozyme-xanthan gum conjugate was investigated. Even since lysozyme antimicrobial effect is limited to Gram-positive bacteria, after conjugation with the polysaccharide xanthan gum, lysozyme activity increase by three times against both Gram-negative and Gram-positive bacteria (Hashemi et al., 2014). Recently, mixtures of gluten and fish gelatin peptides conjugated with glucosamine enhance the antimicrobial activity of the native peptides (Gottardi et al., 2014; Hong et al., 2014).

1.4 Amino-sugars: chitosan and glucosamine acting as antimicrobial compounds

Chitosan and its deacetylated form glucosamine are extensively used as dietary supplements; as dietary fiber and for the treatment of osteoarthritis (Qian et al., 2013; Patti et al., 2015). These amino-sugars possess antimicrobial activities when introduced into proteins and peptides the biological properties of the native compounds are enhanced. Chitosan is a polyamine, cationic, nontoxic biopolymer derived from the hydrolysis of chitin present in shells of crustaceans such as shrimps, lobsters and crabs. Chitosan has potential applications in several areas such as food technology, medicine, and food-related industries (Huang et al., 2007; Prashanth et al., 2007; Kandra et al., 2011). Chitosan exhibits antimicrobial and antifungal activities and is used as antibacterial agent in agriculture and winemaking (No et al., 2002; Wu et al., 2014).

The antimicrobial activity of chitosan depends on its molecular weight as stated by No et al., (2002). Furthermore, chitosan is more effective at low pH, suggesting that addition of chitosan to acidic foodstuffs will enhance its potential as food preservative. Chitosan and xylose glycation products were analyzed against *B. subtilis*. Chitosan was compared with its native form and after

conjugated with xylose chitosan improved its antimicrobial activity by five times during the initial stage of the reaction (Huang et al., 2007). In this study the authors demonstrated that chitosan antibacterial activity can be enhanced during the earlier stages of the Maillard reaction, but not necessarily attributed to the formation of melanoidins which are obtained at the last stage of the reaction.

Further studies on chitosan-xylose conjugated products showed bactericidal effect against *B. subtilis*, meanwhile chitosan without conjugation possess only bacteriostatic effect. Recently in 2015, Maillard reacted films from chitosan and corn fiber to improve antimicrobial activity were experimented against *S. aureus* and *E. coli*. The produced Maillard reacted films resulted in up to five times better antimicrobial activity compared with films only from chitosan. They concluded that the enhancement might be due to the formation of Maillard products and these films can be utilized in food industry for packaging or edible films (Kamboj et al., 2015).

Glucosamine the deacetylated form of chitosan has shown antimicrobial activity against Gram positive bacteria when heated in liquid system (Wu et al., 2014). Glucosamine, a highly reactive monosaccharide has carbonyl and amino groups in its structure, two functional groups that lead to glucosamine autocondensation. This autocondensation of GlcN takes place within two molecules of GlcN when condensation between the carbonyl of one molecule occurs with the amino group present in the next molecule (Figure 1.3) (Hrynets et al., 2015). Further rearrangements can occur forming more advanced products during cyclocondensation of GlcN. Moreover, GlcN being a Heyns like compound can form on one hand through enolization the α-dicarbonyl 3-deoxyglucosone, which by retro-aldolization forms the α-dicarbonyl methylglyoxal (Weigel et al., 2003). On the other hand, GlcN can form through oxidation pathway the α-dicarbonyl glucosone which is the precursor of the α-dicarbonyl glycoxal. These α-dicarbonyls can form after more complexes through the condensation of the two carbonyls with the free amino group present in other molecule of GlcN forming more advanced cyclocondensation products. For

instance, formation of pyrazines derivatives from D-glucosamine has been reported in literature (Candiano et al., 1988). These pyrazines are known to possess antibacterial activity (Sumoto et al., 1991). In 2014, chitosan and glucosamine were used to produce antimicrobial compounds. Glucosamine incubated alone was effective against *S. aureus* compared to chitosan; however, it had no antimicrobial activity against *E. coli*. Higher antimicrobial activity was observed when glucosamine was conjugated with xylan at short time of incubation (Wu et al., 2014).

Hong et al., (2014) and Gottardi et al., (2014) conjugated glucosamine with peptides by the Maillard reaction and enzymatic reaction using transglutaminase to produce antioxidant and antimicrobial compounds from gluten and fish hydrolysates. Transglutaminase acyl transfer reaction was previously applied to modify biological activities by covalently linking amine compounds such as spermine, aminated dextran or aminated β-cyclodextrin of some peptides and proteins (Jiang et al., 2010). The potential of amino-sugars as antimicrobial compounds in conjunction with modifications by the Maillard reaction or enzymatic reaction is expected to reinforce their range of action against pathogens increasing their potential to be used in food industry.

1.5 Isolated antimicrobial compounds from the Maillard reaction

Antibacterial compounds from the Maillard reaction have been extensively investigated. Unfortunately clear characterization of the responsible compounds exerting antimicrobial property and their mechanism of action are still not fully understood (Runti et al., 2015). Little information on specific compounds isolated from the Maillard reaction is available up to date. Partial purification of the antimicrobial substances produced during the reaction has been done (Einarsson et al., 1983). In 2009, an aminoreductone (1-(butylamino)-1,2-dehydro-1,4-dideoxy-3-hexulose) produced at the earliest stage of the Maillard reaction of lactose and butylamine was effective against *Helicobacter pylori*, and later tested successfully against *E. coli* and *S. aureus* (Trang et al., 2009; Trang et al., 2013).

However, the complexity of the mixtures has limited the investigation of purified potential food preservatives from this reaction.

Up to date, α-dicarbonyls, melanoidins and pyrazines are identified compounds with antimicrobial activity extracted from the Maillard reaction products mixtures. In the case of melanoidins, for instance, many challenges are described to define their properties due to their complexity and diversity, ending on difficulties for the purification and identification (Langner et al., 2014). Much remains to be done to fully uncover the benefits of the Maillard reaction to define the proper technological conditions to obtain antimicrobial compounds in food matrices and models systems, and to understand the relationship between structure/function of these antibacterial compounds. In this study, α-dicarbonyls and glycopeptides from models systems mixtures of glucosamine and glucosamine with fish gelatin peptides were the main compounds of interest.

1.6 Research hypotheses and objectives

Bacterial resistance and consumer' concerns on current antimicrobial agents used in food industry have become important topics of consideration when new alternatives for food preservation are designed. The literature review on the Maillard reaction and amino-sugars demonstrates the potential of using amino-sugars to produce antimicrobial compounds through the Maillard reaction. Fish gelatin peptides derived from alcalase conjugated with glucosamine by enzymatic and non-enzymatic reactions have been reported previously to enhance antimicrobial activity against *Escherichia coli* of the native hydrolysate.

Glucosamine antimicrobial activity has been demonstrated in Gram-positive bacteria, but antibacterial effect on Gram-negative is still area of investigation. Moreover, the compounds responsible for the antimicrobial activity obtained from glucosamine and fish peptides conjugated with this amino sugar have not been yet identified and characterized. Up to date, limited information, extraction strategies and optimum conditions for the preparation and identification of the Maillard reaction or enzymatic derived antimicrobial compounds has not been yet fully

explored. The work described in our research aimed to produce, separate and identify the antimicrobial compounds derived from glucosamine/iron and glucosamine/peptides during their modification against heat resistant *E. coli* AW 1.7.

In this project the following hypotheses were tested

- Glucosamine modification in the presence of iron compared with glucosamine alone produces faster antimicrobial compounds such as α-dicarbonyls that inhibit the bacterial growth of heat resistant *E. coli*.
- Glycopeptides purified from the mixture of fish gelatin peptides conjugated with glucosamine by glycation and glycosylation increases the antimicrobial activity of the native conjugates.

To prove the hypotheses of this research the following objectives were addressed:

- To modify glucosamine in the presence of iron by heat treatment through Maillard reaction and to assess its antimicrobial activity against heat resistant *E. coli*
- To extract, identify and quantify α-dicarbonyl compounds from glucosamine modified over time and to compare their production in the presence of iron
- To extract, identify and quantify α-dicarbonyls from fish gelatin peptides conjugated with glucosamine by glycation and glycosylation at low temperature and short heating time.
- To separate glycopeptides from glycated and glycosylated mixtures by affinity and reverse phase chromatography and to test their antimicrobial activity against heat resistant *E. coli*

- To confirm glycation and glycosylation of fish gelatin peptides analyzing glucosamine presence in glycopeptides mixtures by LC-MS/MS.

1.7 FIGURES

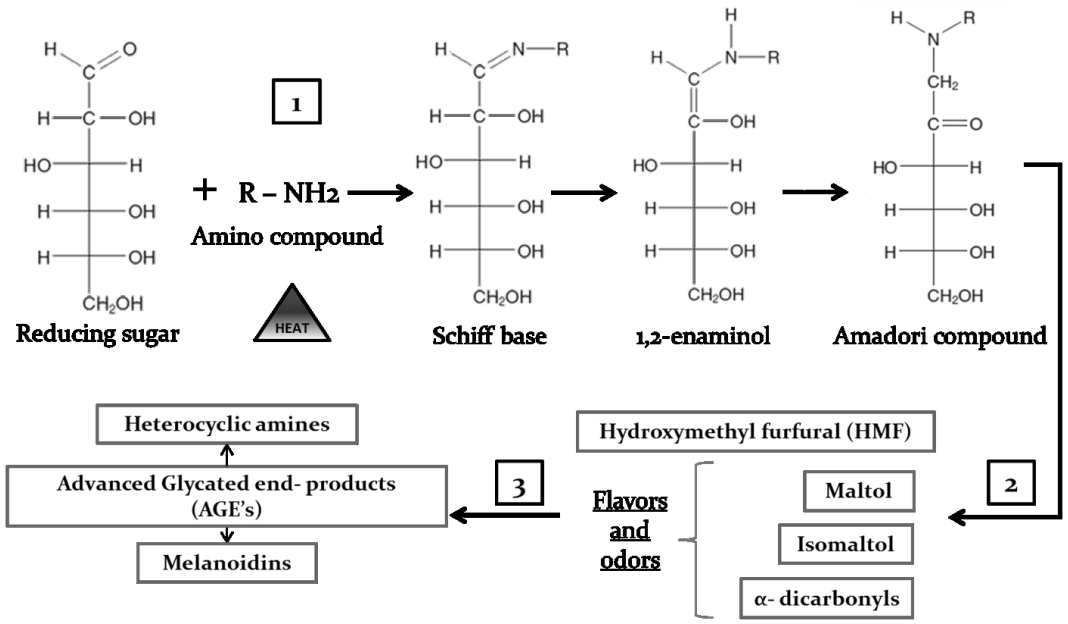


Figure 1.1 Main stages of the Maillard reaction.

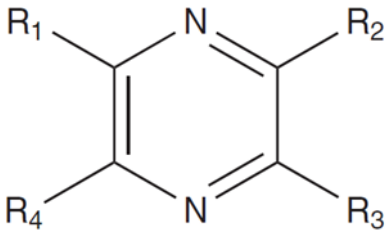


Figure 1.2 General structure of pyrazines in Ferreira et al., 2012.

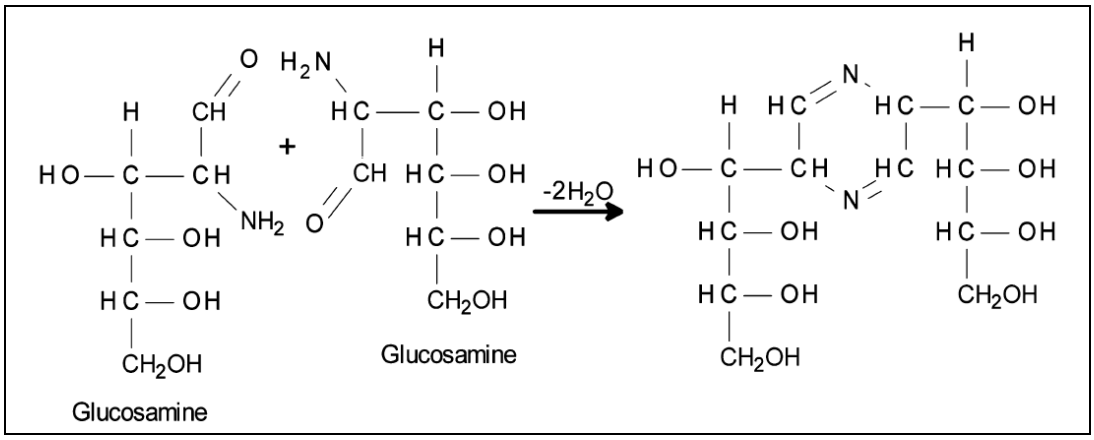


Figure 1.3 Cyclocondensation between GlcN molecules in Hrynets et al., 2015

2 NON-ENZYMATIC MODIFICATION OF GLUCOSAMINE IN PRESENCE OF IRON (Fe^{2+}): α-DICARBONYLS PRODUCTION AND ANTIMICROBIAL ACTIVITY AGAINST *E. COLI*

2.1 INTRODUCTION

Glucosamine (GlcN), 2-amino-2-deoxy-D-glucose is an amino-sugar structural monomer of the polysaccharide chitin which is obtained from crustaceans and some other invertebrate animal by-products. GlcN is normally obtained from the deacetylation of chitin and it is widely used in Europe and the U.S. as dietary supplement for the treatment of osteoarthritis (Block et al., 2010). GlcN is a highly reactive compound due to the presence of the additional free amino group in its structure. The Amadori and Heyns products are important intermediates of the early stage of the Maillard reaction (Kerler et al., 2010). GlcN is an equivalent Heyns compound that generates highly reactive compounds such as α-dicarbonyls precursors of pyrazines and other several advanced more stable condensation products (Candiano et al., 1988; Kraehenbuehl et al., 2008, Hrynets et al., 2015). Among the glucosamine autocondensation products, α-dicarbonyls are aroma-active (Bravo et al., 2008) and possess antimicrobial activity (Olasupo et al., 2003; Adams et al., 2008; Mavric et al., 2008).

Physical and chemical treatments for food preservation may affect the flavor, nutritional value and the texture of food products. Furthermore, some pathogenic strains of Gram-negative bacteria such as *Escherichia coli* can resist heat intervention techniques in beef processing (Dlusskaya et al., 2011). During food elaboration chemical preservatives, such as potassium sorbate, sodium dehydroacetate and calcium propionate are employed (Zhu et al., 2013). However, food industry is particularly interested in using less chemical preservatives to offer “clean label” products, but extending the shelf life of foodstuffs with affordable and practical methodologies. Moreover, the consumer is increasingly calling to obtain safe and non-chemical preservatives added food products (Szűcs et al., 2014; Brown et al., 2015). Thus, novel, food source-derived and affordable antimicrobial compounds seems to be an interesting alternative to cover

consumers and food industry demands. Due to the particular chemistry and reactivity of GlcN as studied by Hrynets et al., 2015, the biological properties and technological functions of this amino-sugar could be of importance. Wu et al., (2014) demonstrated that GlcN possess antimicrobial and antioxidant activities; heated GlcN showed antimicrobial effect against *S. aureus*, but there was no effect against *E. coli*. It has also been observed that chitosan antimicrobial activity was improved with the addition of glucosamine (Blagodatskikh et al., 2013). However, none of these studies have considered if glucosamine itself or some of its autocondensation products such as α-dicarbonyls imparts these activities.

Metal ions can form complexes with Maillard reaction products oxidizing Amadori/Heyns compounds such as GlcN and their derivatives stimulating the reaction (Ramonaitytė et al., 2009). Browning during the Maillard reaction is catalyzed by iron ions acting as pro-oxidants (Kato et al., 1981; Min et al., 2010). Under the presence of metals, Amadori adducts decomposes faster into soluble more reactive dicarbonyls compounds (Hayase et al., 1996; Zhang et al., 2004). In particular, iron promotes the formation of the α-dicarbonyls 3-deoxyglucosone and glyoxal (Fallico et al., 1999; Hayase et al., 1996). Glyoxal is known to possess antimicrobial activity (Weigel et al., 2004). Hence, the addition of metal ions in model systems may induce the production of antimicrobial compounds such as α-dicarbonyls.

The objective of this work was to use iron (Fe^{2+}) as a catalyst to increase the formation/degradation of α-dicarbonyls from GlcN and to assess the antimicrobial activity of GlcN modified on heat resistant *E. coli* AW 1.7. To meet this objectives the production of α-dicarbonyls from GlcN was evaluated over time using liquid chromatography and mass spectrometry. The antimicrobial activity of pure α-dicarbonyls was tested to compare with the amount produced by GlcN. To the best of our knowledge identification and characterization of antimicrobial compounds obtained from GlcN modification has not been reported in the literature.

2.2 EXPERIMENTAL SECTION

2.2.1 Chemicals and Materials

D-Glucosamine hydrochloride (≥99% purity), potassium phosphate monobasic and dibasic (KH_2PO_4 and K_2HPO_4), ammonium ferrous sulfate hexahydrate ($(NH4)_2Fe(SO_4)_2$ $6H_2O$; 99% purity; MW 390.14 g mol^{-1}), HPLC-grade solvents (acetonitrile, methanol and formic acid), glucosone (2-keto-D-glucose; ≥98.0% purity; MW 178.14 g mol^{-1}), glyoxal (ethanedial; 40% in H_2O; MW 58.04 g mol^{-1}), methylglyoxal (2-oxopropanal; 40% in H_2O; MW 72.06 g mol^{-1}), diacetyl (butane-2,3-dione; ≥95.0% purity; MW 86.09 g mol^{-1}) and 1,2-diaminobenzene were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-deoxyglucosone (3-Deoxy-D-erythro-hexosulose; ≥95% purity; MW 162.14 g mol^{-1}) was obtained from Cayman Chemical (Ann Arbor, MI, USA). SPE tC-18 Sep-Pak Vac 6 cc columns were obtained from Waters (Milford, MA, USA). Filtration membranes (0.22 μm) were from Millipore (Billerica, MA, USA), Difco Luria-Bertani (LB) media obtained from BD (Mississauga, ON, Canada)

2.2.2 Bacterial strain and culture conditions

Escherichia coli AW 1.7 isolated from beef (Dlusskaya et al., 2011) was grown in Difco Luria-Bertani (LB) media containing: 10.0 g tryptone, 5.0 g yeast extract, 10.0 g sodium chloride (NaCl), and 15.0 g agar per litre of media. Both, agar and broth medium were prepared with distilled water and final pH 7.0 ± 0.2, before autoclaving (121 °C x 20 min). Cells were incubated at 37 °C under aerobic conditions for 24 h for further utilization in growth inhibition analyses.

2.2.3 Experimental Design

In this study the non-enzymatic degradation of glucosamine (GlcN) and glucosamine in presence of iron ($GlcN/Fe^{2+}$) was evaluated at 0, 3, 12, 24 and 48 h in phosphate buffer at 50 °C using UV/Vis spectroscopy analyses, α-dicarbonyls production and pH evaluation. Further study was done to analyze their antimicrobial activity against *E. coli* AW 1.7. A total of 16 tubes (2 tubes ×

treatment × incubation time) were prepared at 150 g/L, incubated and collected over time (3, 12, 24 and 48 h). Four control tubes were left aside representing 0 h per treatment. Three independent trials were conducted resulting in a total of n = 60 tubes for further analyses. For the first part of the study, UV/Vis profiles were assessed at 0, 3 and 48 h to understand the major changes occurring at the earliest (3 h) and latest (48 h) stages of GlcN and GlcN/Fe^{2+} reactions. Moreover, pH measurements were taken after incubation to analyze the changes during the development of the reaction.

On the second part, α-dicarbonyls were identified and quantified to follow up the degradation of GlcN and GlcN/Fe^{2+} over time at 0, 3, 12, 24, and 48 h. Reverse-phase Liquid chromatography and mass spectrometry approaches were used for the separation, identification and quantification of the main α-dicarbonyls produced in the different treatments. In the last part of this study, antimicrobial activity of the GlcN and GlcN/Fe^{2+} samples were evaluated at 0, 3, 12, 24 and 48 h by critical dilution assay and percentage of bacterial growth reduction against *E. coli* AW 1.7 was determined by the development of turbidity using vertical photometry, optical density (OD_{630nm}).

2.2.4 Preparation of GlcN and GlcN/Fe^{+2} treatments

Modification of glucosamine (GlcN) and GlcN in the presence of iron (GlcN/Fe^{2+}) in liquid system was induced by heat and time promoting the Maillard reaction. Briefly, GlcN concentrations of 15, 30, 50, 75, 100 and 150 g/L in phosphate buffer 50 mM at pH 7.5 were incubated at 50 °C for 0, 3, 12, 24 or 48 h. Each treatment was carried out in triplicate. The effect of Fe^{2+} in GlcN modification was evaluated at 0.5 mM. The pH value was not adjusted during the reaction to enable a natural course for the generation of intermediate Maillard reaction products as described by Chen et al., 2011.

2.2.5 Development of the reaction: UV/Vis and pH measurements

Modification of GlcN was followed by absorbance in the UV/Vis region and pH measurements. UV/Vis absorbance spectrum screening of GlcN and GlcN/Fe^{2+} solutions at 15 g/L incubated over time was analyzed using a microplate reader (Spectramax Molecular Devices, CA, USA). Absorbance screening was analyzed in the range of 200-500 nm. Phosphate buffer was used as blank and subtracted by the system to obtain all the readings. Samples were diluted 1:20 v/v ratio with 50 mM phosphate buffer at pH 7.5, and readings were taken using 1 cm-Quartz cuvettes. The pH values of GlcN and GlcN/Fe^{2+} systems of the highest concentration prepared at 150 g/L were taken for each incubation time with a pH meter (Orion 2-star, Thermo scientific, CA).

2.2.6 Solid-Phase Extraction (SPE) of free α-dicarbonyls

α-Dicarbonyls extraction, separation and identification were performed following the method described by Papetti et al., (2013) and Papetti et al., (2014) with some modifications. Briefly, for the extraction of α-dicarbonyls SPE tC-18 Sep-Pak cartridges (Waters, Milford, MA, USA) were conditioned with 10 mL of methanol followed by 20 mL of water previously filtrated at a flow rate of 2 mL/min. After conditioning, a volume of 6 mL of sample were passed through the column and washed with 2 mL of water. In the second step, the polar fraction eluted of 8.00 mL in total and α-dicarbonyl standards were spiked with 6 mg of 1,2-diaminobenzene and the pH adjusted to 3.00 ± 0.02 with 4 N HCl, followed by incubation at 37 °C for 1 h protected from light with aluminum foil. The derivatized solution was passed through a new pre-conditioned SPE cartridge (tC-18 Sep-Pak). The polar fraction was discarded and the cartridge was washed with 2 mL of water; the non-polar fraction corresponding to the quinoxalines derivatives were eluted with 4 mL of a MeOH/H_2O mixture of 90/10 v/v ratio at a flow rate close to 2 mL/min. The first 1 mL was discarded, and the next 2 mL recovered for further identification of α-dicarbonyls using an ultra-high-performance liquid chromatography (UHPLC) apparatus (Shimadzu, MD, USA).

Derivatization was employed in order to obtain quinoxalines derivatives more stable compounds making possible further analysis in liquid chromatography. Complete derivatization reaction is accomplished at low temperatures less than 60 °C and short times less than 3 h (Barros et al., 1999; de Revel et al., 2000; Nemet et al., 2004). Derivatization was performed at 37 °C for 1 h to avoid changes in the samples due to further development of Maillard reaction. Moreover, the samples were adjusted to low pH of 3.00 ± 0.02 since the derivative agent contains amino groups that catalyze α-dicarbonyls formation under basic pH conditions, thus further producing potentially inaccurate results (Chen et al., 2011; Ruiz-Matute et al., 2014).

2.2.7 UHPLC-UV identification of quinoxaline derivatives:

The α-dicarbonyls separation was achieved using an Ascentis Express ES-C18 column (150 × 4.6 mm, 2.7 μm particles; Sigma-Aldrich, MO, USA) operating at 25.0 ± 0.5 °C and at flow rate of 0.3 mL/min. The binary mobile phase consisted of (A) 0.1% formic acid in water and (B) methanol. The gradient is described as follows: 0–5 min (90–85% A), 5–13 min (85–80% A), 13–40 min (80% A), 40–65 min (80–70% A), 65–90 min (70–50% A), 90–100 min (50–0% A), 100–105 min (0% A), and 105–110 min (0–90% A). Before injection, the samples were filtered (PVDF, 13 mm, 0.22 μm; Millipore Millex, Billerica, MA, USA) and 5 μL were injected. The photo-diode array detector (PAD, SPD-M20A) was set at 314 nm. Identification of extracted α-dicarbonyls was based on comparison with the retention time of known reference compounds.

2.2.8 Quantification of quinoxaline derivatives

Finally, for α-dicarbonyls quantification, external calibration curves were used. Each quinoxaline derivative was diluted to final concentrations ranging between 0.5 - 16.5 (glucosone), 0.12 - 2 (3-deoxyglucosone), 0.2 - 0.002 (glyoxal), 0.05 0.001 (methylglyoxal), and 0.05 - 0.005 mM (diacetyl). Calibration curves were prepared for each standard and concentrations were analyzed in triplicate. The peak area was plotted against concentration in mM and

the regression equations were calculated. The correlation coefficients for all calibration curves were $R^2 \geq 0.99$. The average limit of detection (LOD) was calculated as 2.15 ± 0.07 (glucosone), 0.27 ± 0.00 (3-deoxyglucosone), 0.13 ± 0.00 (glyoxal), 0.09 ± 0.00 (methylglyoxal) and 0.18 ± 0.00 μM (diacetyl). The average limit of quantification (LOQ) was determined as 6.52 ± 0.21 (glucosone), 0.81 ± 0.01 (3-deoxyglucosone), 0.39 ± 0.01 (glyoxal), 0.27 ± 0.01 (methylglyoxal), and 0.55 ± 0.02 μM (diacetyl) by assuming a signal-to-noise ratio (S/N) of 3:1 for LOD and S/N of 10:1 for LOQ. The 5 α-dicarbonyls of interest detected by UHPLC were manually collected and subjected to mass spectrometry analyses (Hrynets et al., 2015). The accurate mass and the respective fragments were compared with the standards reference data provided in previous studies.

2.2.9 Determination of antimicrobial activity

The antimicrobial activity of GlcN and GlcN/Fe^{2+} samples was determined by critical dilution assay described by Parente et al., (1995) and Gänzle et al., (1999). Sterilization of samples was achieved by filtration through sterile 0.22 μm membrane filters (Millipore, MA, USA). *E. coli* was subcultured twice in liquid media under conditions described by Sánchez-Maldonado et al., (2011). Briefly, 100 μL of GlcN or GlcN/Fe^{2+} solutions and 100 μL of LB broth media were mixed, and two fold serial dilutions of the mixtures were prepared on 96-wells microtitre plates. Finally, 50 μL of overnight inoculated media was added to microtitre wells. The final concentration of GlcN and GlcN/Fe^{2+} samples in growth media ranged from 50.0 g/L to 0.048 g/L and the initial cell count of about 10^6 CFU/mL. The final pH ranged from 5.83 ± 0.04 to 5.12 ± 0.03 for 0 h and 48 h treatments, respectively. Microtitre plates were incubated for 16 h at 37 °C. After incubation, antimicrobial activity of GlcN treatments was evaluated by the development of turbidity by vertical photometry, optical density (OD_{630nm}). All the experiments were carried out in triplicate. The percentage of inhibition was determined according to Pritchard et al., (2010) by:

$$\% \, Inhibition = \frac{(A_{sample} - A_{negative\ control})}{(A_{positive\ control} - A_{negative\ control})} \times 100$$

Bacterial growth inhibition of standard α-dicarbonyls was evaluated as described above and minimal inhibitory concentration (MIC) values were calculated by mathematical extrapolation using Origin 2015 software.

2.2.10 Statistical analysis

Percentage of bacterial growth reduction is expressed as mean ± standard deviation of the three independent experiments. The effect of incubation time at 0, 3, 12, 24 and 48 h in presence and absence of iron on the concentration of α-dicarbonyls and percentage of inhibition on *E. coli* was analyzed by two-way ANOVA using the general linear model of Minitab considering interactions between the factors treatment and time of incubation at a significant level of $\alpha = 0.05$. Differences between means were determined by Tukey´s multiple range test with $p<0.05$ using Minitab software package v.17 (Minitab Inc. PA, U.S.).

2.3 RESULTS AND DISCUSSION

2.3.1 UV/Vis profiles and pH variations of non-enzymatic modification of GlcN and GlcN/Fe^{2+}

During Maillard reaction a wide variety of aromatic, carbonyls, and more advanced complex mixture of poorly characterized compounds are produced. In this study, absorbance screening in the UV/Vis region was useful to understand changes during modification of GlcN over time. UV/Vis absorbance was monitored in GlcN and GlcN/Fe^{2+} solutions of 15 g/L at the beginning at 3 h and at the end at 48 h of incubation in order to analyze the major changes within the two systems (Figure 2.1 (a) and (b)). Maximum absorbance was found to be at wavelengths of $\lambda = 274$ and $\lambda = 320 - 360$. After 3 h of incubation at 50 °C a steady increase in absorbance at these wavelengths was observed in both systems. However, the increase was higher in GlcN/Fe^{2+} compared with GlcN treatment, which indicates that major changes occurred when iron is added to the system.

The increase of absorbance at these wavelengths in GlcN has been attributed to the formation of different Maillard reaction products. For instance, Zhang et al., (2004) ascribed the increase at 275 nm to GlcN autocondensation and Maillard reaction products generated in the mixture of GlcN/fibrinogen and GlcN/human serum albumin. Other authors have shown that the increase in UV absorption at around 274 nm is due to the formation of pyrazines formed as one of the products of autocondensation of GlcN (Candiano et al., 1988; Horowitz et al., 1991). The increase observed at 320 nm can be associated with the formation of advanced products in the reaction such as soluble pre-melanoidins as according to Fogliano et al., (1999).

After 3h of incubation GlcN/Fe^{2+} turned yellowish and then darker-brown over time. The color development of GlcN and GlcN/Fe^{2+} was observed at 420 nm of 3 and 48 h of incubation compared to time 0 h (Figure A.2) as described by Liu et al., (2007). The presence of Fe^{2+} and Fe^{3+} has been found to promote browning reactions in ovalbumin (Kato et al., 1981); in the GlcN system, the color development was less strong than with iron. Moreover, the pH values of the treatments at the highest concentration prepared of 150 g/L dropped during the progress of the reaction from 5.5 to 2.5 in GlcN and GlcN/Fe^{2+} treatments with no significant difference among them. As reported in literature it is expected during the development of the reaction to observe a decrease on pH (Liu et al., 2007; Chen et al., 2011) for instance, due to α-dicarbonyls cleavage forming acidic compounds (Davidek et al., 2005).

We concluded by this preliminary analyses that main changes over the spectra 274 - 420 nm and pH decrease during GlcN incubation are associated with the production of a wide variety of Maillard reaction products. Furthermore, the presence of iron led to faster variations on UV and color absorbances during the reaction.

2.3.2 Identification and quantification of free α-dicarbonyls in GlcN and GlcN/Fe^{2+}

As explained previously, UV/Vis profiles of modified GlcN suggested the generation of Maillard reaction products, such as α-dicarbonyls, pyrazines and melanoidins which possess antimicrobial activity. In this study, we evaluated α-dicarbonyls presence during GlcN and GlcN/Fe^{2+} modification over time. α-Dicarbonyls are intermediates in the Maillard reaction containing two carbonyl groups in two adjacent carbons. They are present in diverse food products such as honey, beer, and wine, contributing to organoleptic characteristics and bioactive properties (Olasupo et al., 2003; Adams et al., 2008; Mavric et al., 2008; da Silva Ferreira et al., 2007; Bravo et al., 2008; Tan., et al 2008; Marceau et al., 2009; Rincon-Delgadillo et al., 2012; Spanneberg et al 2012).

After extraction and UHPLC separation five main α-dicarbonyls in GlcN and GlcN/Fe^{2+} were identified and quantified after derivatization. Figure 2.2 (a) shows UHPLC chromatograms of five standard commercially available α-dicarbonyls. The α-dicarbonyls produced from the GlcN and GlcN/Fe^{2+} systems after 48 h of incubation are presented in Figure 2.2 (b) and 2.2 (c) as representative chromatograms. The peaks found in GlcN and GlcN/Fe^{2+} correspond to glucosone, 3-deoxyglucosone, glyoxal, methylglyoxal and diacetyl according to the retention time of commercial standards. Their presence was fully confirmed by the pseudo molecular weight of manually collected peaks analyzed by mass spectrometry (Table 2.1). The main fragments of the parent ions of the α-dicarbonyls identified were in agreement with those reported previously by Papetti et al., (2014), and Gensberger et al., (2012).

The presence of other peaks was noticed during the separation with UHPLC but identification was not conducted. The focus of this study was on those α-dicarbonyls which were denoted in literature to possess antimicrobial activity. The concentration of each of the α-dicarbonyls produced by GlcN and GlcN/Fe^{2+} was determined over time from 0 to 48 h and results are presented in Table 2.2.

Glucosone and glyoxal were present at time 0 h, which can be explained by the high reactivity of GlcN (Kraehenbuehl et al., 2008; Hrynets et al., 2015). 3-Deoxyglucosone was the most abundant α-dicarbonyl present when glucosamine is heated with and without iron. The highest concentration of 3-deoxyglucosone was determined at 3 h in the GlcN/Fe^{2+} system while the development of 3-deoxyglucosone in the GlcN system reached the highest amount only after 12 h. A significant decrease of 3-deoxyglucosone in GlcN/Fe^{2+} was noticed from 3 h to 48 h. It has been reported that iron triggers the production of 3-deoxyglucosone, which is a precursor of advanced Maillard compounds, such as pyrazines (Fallico et al., 1999).

The predominance of 3-deoxyglucosone in our systems can be also explained by the low pH in the samples since acidic conditions also favors enolization to produce 3-deoxyglucosone (Chen et al., 2011). The production of glucosone in the GlcN/Fe^{2+} system followed a similar trend as 3-deoxyglucosone. The maximum concentration of glucosone was reached at 3 h, and then decreased over time; the same behavior was determined in the GlcN system. At 48 h the amount of glucosone and methylglyoxal was significantly lower in the presence of iron compared to GlcN without iron. However, the concentration of 3-deoxyglucosone, glyoxal and diacetyl was not significantly different at 48 h between the two systems. Overall, there was a higher production of glucosone, 3-deoxyglucosone, glyoxal and diacetyl in the GlcN/Fe^{2+} system compared to GlcN, especially at 3 h of incubation.

It was also noticed that the production of diacetyl was faster in GlcN/Fe^{2+}, reaching a maximum concentration at 12 h compared to GlcN, which reached the maximum just after 24 h. On the contrary, methylglyoxal production over time was dominant in the GlcN system. Methylglyoxal had a steady increase for GlcN in presence or absence of iron. It is known that retro-aldolization of 3-deoxyglucosone produces methylglyoxal (Weigel et al., 2003); hence, the decrease of 3-deoxyglucosone observed in our samples can be explained by sustained increase of methylglyoxal. α-Dicarbonyls in the Maillard reaction will

generate a variety of condensation and further rearranged products since they are highly reactive compounds, especially, in the presence of metals acting as catalysts such as Fe^{2+}.

2.3.3 Antimicrobial activity of GlcN and GlcN/Fe^{2+}

The antimicrobial activity of GlcN and GlcN/Fe^{2+} of the 150 g/L solutions were analyzed at 0, 3, 12, 24 and 48 h; but only the samples of GlcN and GlcN/Fe^{2+} after 3 h of incubation showed reduction on *E. coli* growth (Figure 2.3). After 12 and 24 h the antimicrobial activity was significantly higher than 3 h. Finally, the statistical analysis showed that there was a significant difference within treatments, being GlcN/Fe^{2+} incubated for 48 h the most effective for the reduction of *E. coli* growth at a concentration of 50 g/L. The pH of the samples ranged from 5.8 to 5.1 for 0 h to 48 h, respectively, after mixing each sample with media. This pH found in the samples is low enough to influence the bacterial growth reduction.

Besides the impact of pH, free α-dicarbonyls formed during glucosamine degradation over time might also contribute to the antimicrobial activity. These compounds possess antibacterial effect against Gram-positive bacteria such as *Salmonella* (Maletta et al., 2012) and Gram-negative such as *E. coli* (Olasupo et al., 2003). For instance, in a previous study diacetyl addition to ground beef and poultry delayed the bacterial growth at 5-7 °C to up to five months (Jay J. 1982). In a recent study glyoxal and methylglyoxal were mixed with coffee filtrate with the aim to reduce the *Salmonella* growth in chicken breast; as a result the growth was delayed during the first day and total inhibition was achieved during storage at 8 °C (Maletta et al., 2012). In addition, methylglyoxal is considered the major contributor for the high antimicrobial capacity of Manuka honeys which also contains glyoxal and 3-deoxyglucosone, being the last one predominant (Marshall et al., 2014; Mavric et al., 2008).

In order to understand the concentration effect of α-dicarbonyls in GlcN and GlcN/Fe^{2+}, pure commercial standards were tested against *E. coli* AW 1.7 for

further comparisons (Figure 2.4). Methylglyoxal standard was the most effective α-dicarbonyl to inhibit bacterial growth followed by diacetyl, glyoxal, 3-deoxyglucosone and glucosone. The concentration to inhibit the bacterial growth for each standard was 0.065 (methylglyoxal), 0.168 (diacetyl), 0.259 (glyoxal), 1.011 (3-deoxyglucosone), and 26.90 (glucosone) g/L. Glyoxal, methylglyoxal and diacetyl have been previously reported in the literature approximately the same concentration as found in our study (Ferguson et al., 1998; Olasupo et al., 2003; Lee et al., 2010). However, glucosone and 3-deoxyglucosone have not been reported previously to possess antimicrobial activity. The amount of glucosone, 3-deoxyglucosone, glyoxal, methylglyoxal and diacetyl found in the most effective treatment GlcN/Fe^{2+} was 0.36, 0.72, 0.004, 0.004 and 0.01 g/L, respectively. In GlcN and GlcN/Fe^{2+} at 48 h of incubation the amount of total free α-dicarbonyls contributes to the antimicrobial activity, specially, 3-deoxyglucosone inhibitory concentration is close to the commercial standard.

Some possible mechanisms of action of α-dicarbonyls against bacteria have been discussed in the literature. For instance, inhibition of *E. coli* growth can be related to the interaction of α-dicarbonyls with the protective thiol groups in cysteine residues in the bacteria causing cell damage (Ozyamak et al, 2013; Feyaerts et al., 2015). Diacetyl has been shown to inhibit *E. coli* and *S. aureus* by avoiding the utilization of arginine by the cells in the periplasmic space between inner and outer membrane of the bacteria (Tan et al., 2012). Methylglyoxal exposure to bacteria causes damage via covalent modification of specific proteins, and DNA bases such as guanine in the cell interfering with protein synthesis and initiation of DNA replication (Fraval et al 1980; Fergusson et al., 1998; Ozyamak et al., 2013).

Glucosone and 3-deoxyglucosone antimicrobial activity have not been discussed in the literature. However, we hypothesize that the carbonyls groups present in glucosone and 3-deoxyglucosone interact with amino acids in the cell structure of the bacteria; thus inducing disruption in the cell membrane. Moreover, 3-deoxyglucosone is the precursor of methylglyoxal which possess the

highest antimicrobial activity that during contact with bacteria can be further produced. In our samples, α-dicarbonyls together might exert a synergistic effect among them accompanied by the low pH disturbing in this way the growth of *E. coli*. However, α-dicarbonyls in GlcN/Fe^{2+} 48 h system were present at the same or lower amount compared to GlcN without iron at the same time of incubation (Table 2.2). Therefore, the bacterial growth reduction by GlcN/Fe^{2+} 48 h cannot be only attributed to the presence of the identified α-dicarbonyls.

Other non-identified compounds formed during the reaction can also complement the antimicrobial activity of modified GlcN/Fe^{2+}. The enhanced activity might be also related to other autocondensation and advanced reaction products that were formed faster in the presence of iron. 3-Deoxyglucosone is known to be the precursor of other compounds such as pyrazines, thiazoles, carboxilic compounds, etc. (Weenen et al., 1992). Pyrazines derivatives are known to possess a wide range of biological activities including antibacterial (Ferreira et al., 2012; Foks et al., 2012). Further investigation on the identification and characterization of other antimicrobial compounds produced by glucosamine modification is an interesting research area.

2.4 CONCLUSIONS

This study demonstrated that glucosamine modification at 50 °C for up to 48 h in the presence of iron resulted in the production of antimicrobial compounds such as α-dicarbonyls that inhibit *E. coli* AW 1.7 growth at a concentration of 5% (w/v). UV/Vis absorbance screening of the different systems showed an increase in absorbance at λ = 274 and 320 – 420 nm compared to glucosamine without modification (0 h). Changes in UV/Vis absorbance and pH values confirmed the progress of the reaction over time and a faster reaction occurs when iron is added.

Free α-dicarbonyls such as glucosone, 3-deoxyglucosone, glyoxal, methylglyoxal and diacetyl are produced from glucosamine autocondensation under mild heat conditions. The addition of iron into the system resulted in faster production of 3-deoxyglucosone, glyoxal and diacetyl. The low pH present

naturally in all the samples might explain partially the reduction of the bacterial growth; however, the highest percentage of inhibition was found in GlcN/Fe^{2+} at 48 h of incubation where the pH was not significantly different from GlcN treatment at 48 h.

As shown in this study standard α-dicarbonyls possess antimicrobial activity against *E. coli,* and the activity of modified glucosamine can be also explained by the presence of the five identified α-dicarbonyls, specially, 3-deoxyglucosone. We hypothesize that the faster production of α-dicarbonyls with iron gives place to faster formation of GlcN autocondensation and more advanced Maillard reaction products with antimicrobial activity such as pyrazines. The formation of these products might enhanced the antimicrobial effect of modified GlcN/Fe^{2+}.

Our findings opened the possibility to consider the use of GlcN in food industry as a novel preservative especially in meat processing industry. In meat products, iron presence can play an important role with GlcN to produce antimicrobial compounds during the heat processing helping to the reduction of pathogens of main concern. Identification of other compounds with antimicrobial activity produced by glucosamine autocondensation with proteins can be further topic of investigation. In addition, the relationship between dose and toxic effects of modified glucosamine might be a motivating further topic of research.

2.5 TABLES

Table 2.1 Retention time, MS and MS/MS data of the α-dicarbonyls quinoxaline derivatives identified in modified treatments.

Characterization of derivatized α-dicarbonyl compounds

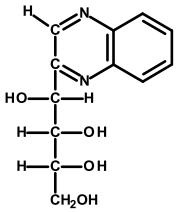
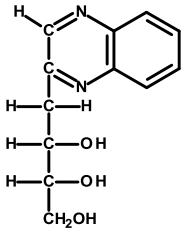
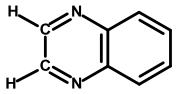
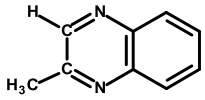
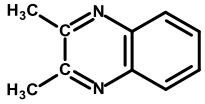
Corresponding dicarbonyl	Retention time (min)	Structure of Quinoxaline derivative	Mass $[M+H]^+$ *(m/z)*	MS/MS Fragments
Glucosone	21.5		251.1032	145.0553, 151.0710, 173.0710, 187.0867, 197.0712, 215.0817, 233.0924
3-deoxyglucosone	33.2		235.1081	130.0436, 145.0761, 157.0761, 171.0918, 187.0868, 199.0869, 217.0957
Glyoxal	67.1		131.0611	77.0164, 104.0492
Methylglyoxal	79.9		145.0767	77.1615, 92.0192, 102.4849, 118.0445
Diacetyl	87.4		159.0925	102.0488, 131.0602, 145.0761

Table 2.2 Concentration of free α-dicarbonyls in mg/L produced from 150 g/L of GlcN and GlcN/Fe^{2+} solutions incubated from 0 to 48 h, and the interaction between treatments and time of reaction.

Free α-dicarbonyl compounds in mg/L produced from 150 g/L of GlcN and GlcN/Fe^{2+} solutions						
		Glucosone	**3-Deoxyglucosone**	**Glyoxal**	**Methylglyoxal**	**Diacetyl**
Interaction (T*t) (n = 3)						
GlcN/Fe^{2+}	0h	343.83 ± 1.05^{de}	nd	0.87 ± 0.08^{f}	nd	nd
	3h	532.60 ± 18.25^{a}	941.85 ± 15.33^{a}	5.96 ± 0.12^{b}	1.01 ± 0.08^{f}	3.66 ± 0.48^{e}
	12h	364.97 ± 2.82^{cd}	910.34 ± 11.30^{a}	6.77 ± 0.19^{a}	2.31 ± 0.19^{e}	12.10 ± 0.08^{a}
	24h	386.30 ± 4.72^{c}	770.38 ± 14.96^{b}	4.74 ± 0.05^{c}	3.84 ± 0.11^{c}	10.84 ± 0.19^{b}
	48h	358.20 ± 5.14^{d}	725.24 ± 8.98^{bc}	3.89 ± 0.09^{de}	4.21 ± 0.10^{b}	9.80 ± 0.20^{cd}
GlcN	0h	324.05 ± 5.09^{e}	nd	0.62 ± 0.05^{f}	nd	nd
	3h	444.70 ± 18.16^{b}	660.82 ± 15.16^{cd}	3.71 ± 0.05^{e}	0.80 ± 0.09^{f}	3.11 ± 0.14^{e}
	12h	388.75 ± 1.90^{c}	921.38 ± 1.11^{a}	7.31 ± 0.37^{a}	2.98 ± 0.18^{d}	9.77 ± 0.47^{cd}
	24h	343.86 ± 7.65^{de}	594.72 ± 82.19^{d}	4.21 ± 0.68^{cde}	4.09 ± 0.12^{bc}	10.28 ± 0.13^{bc}
	48h	388.37 ± 2.95^{c}	779.21 ± 15.17^{b}	4.63 ± 0.06^{cd}	6.12 ± 0.10^{a}	9.39 ± 0.19^{d}
Source of variation		**P-value**				
T		< 0.0001	< 0.0001	0.002	< 0.0001	< 0.0001
t		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
T x t		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Means that do not share an uppercase letter are significantly different ($p<0.05$). nd, indicates non detected.

2.6 FIGURES

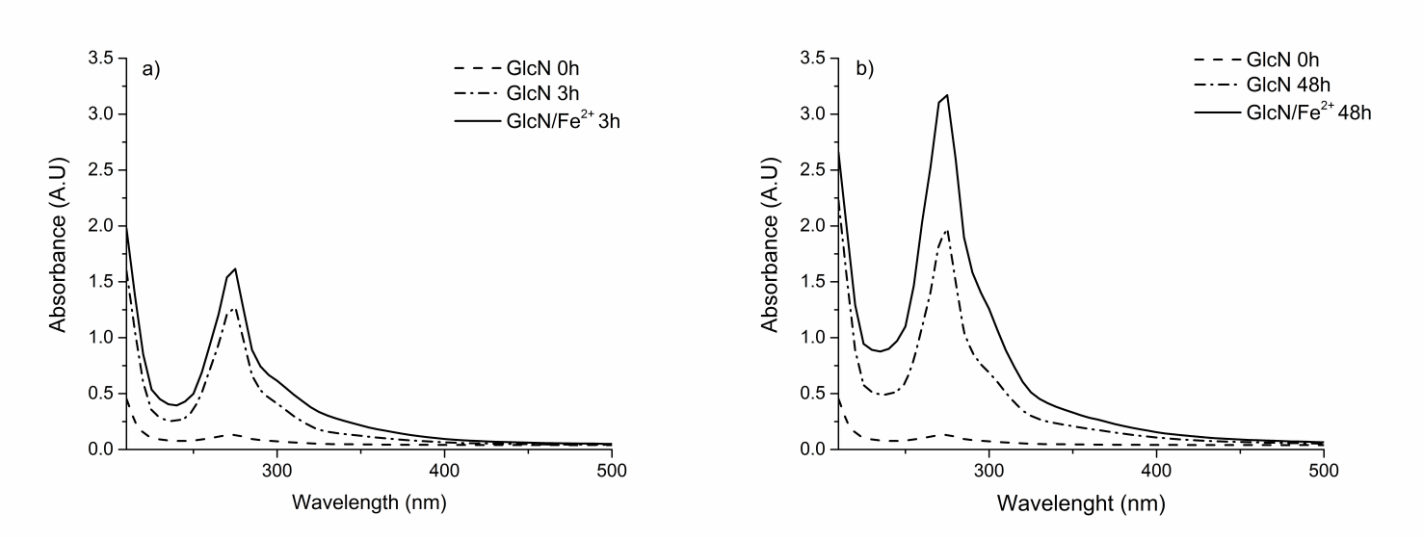


Figure 2.1 Spectrophotometric changes (UV/Vis absorbance) of GlcN and GlcN/Fe^{2+} as function of heating time at the earlier and latest stages of the reaction. (a) 3 h of incubation, and (b) 48 h of incubation. The values are expressed in arbitrary units (A.U).

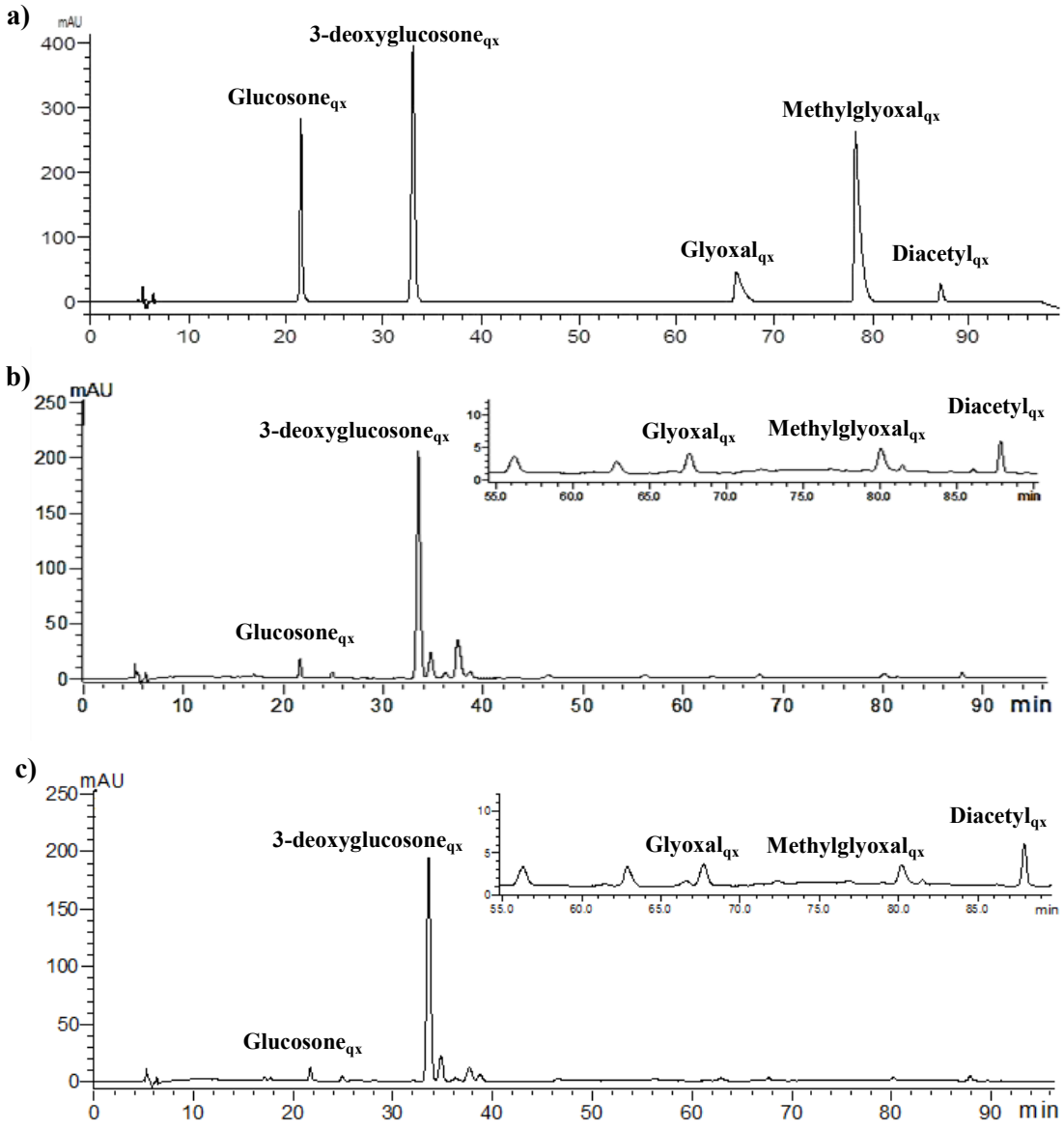


Figure 2.2 UHPLC separation of α-dicarbonyls quinoxaline derivatives. (a) Standard references of glucosone, 3-deoxyglucosone, glyoxal, methylglyoxal, and diacetyl, (b) GlcN α-dicarbonyls separation after 48 h of incubation and, (c) GlcN/Fe^{2+} α-dicarbonyls separation after 48 h of incubation. Inset shows a zoomed-in view of the peaks eluted at 60 to 90 min glyoxal, methylglyoxal and diacetyl.

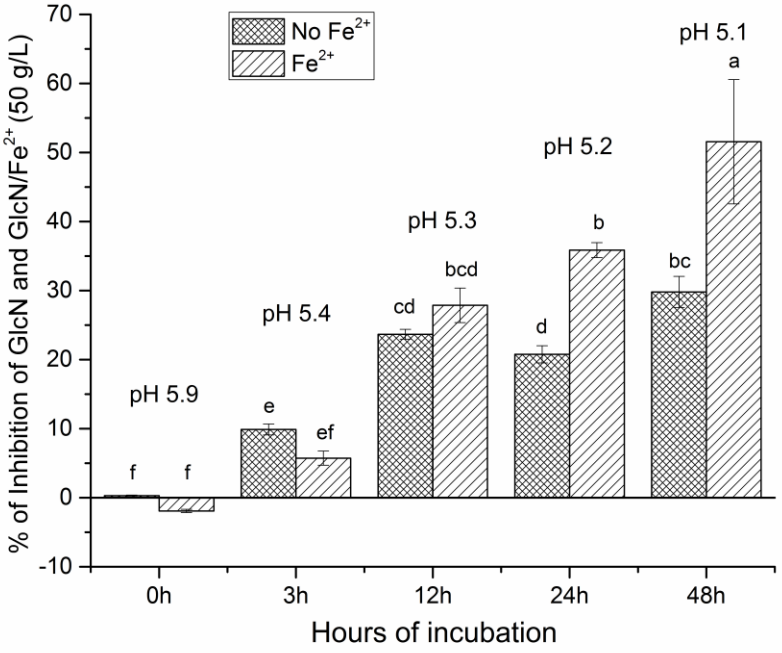


Figure 2.3 Inhibitory effect on the growth of *E. coli* by GlcN and GlcN/Fe^{2+} treatments at a concentration of 50 g/L. Vertical bars represent means and standard deviation values (n = 3).

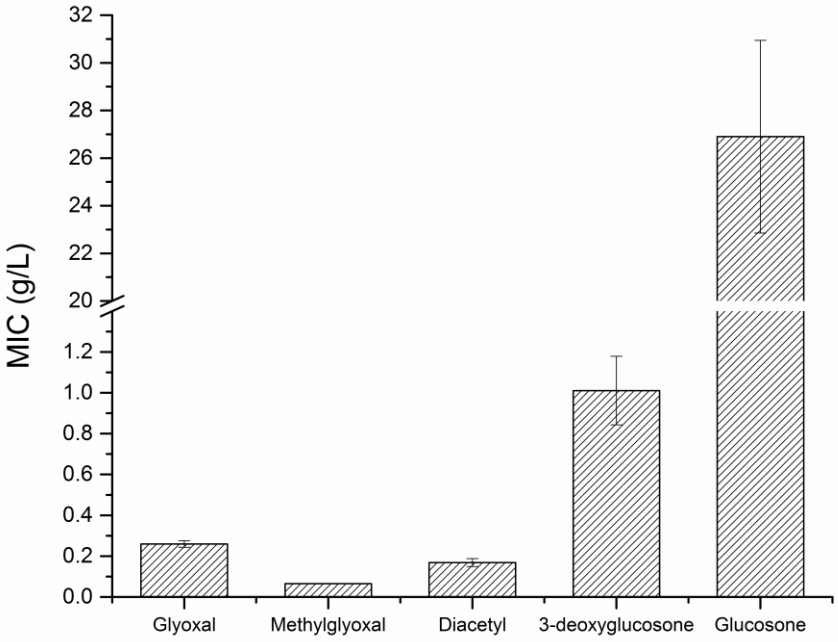


Figure 2.4 Minimum inhibitory concentration of glyoxal, methylglyoxal, diacetyl, 3-deoxyglucosone and glucosone for growth inhibition of *E. coli*. Vertical bars represent means and standard deviation values (n = 3).

3 GLUCOSAMINE AND FISH GELATIN PEPTIDES ANTIMICROBIAL COMPOUNDS: SEPARATION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY AGAINST *E. COLI.*

3.1 INTRODUCTION

Some peptides obtained from foods such as eggs, cheese, sesame, meat and fish by-products, possess biological activities (Pritchard et al., 2010; Ting et al., 2010; Das et al., 2012; Doyen et al., 2012). Fish by-products such as skin, bones, heads and liver are mainly used for the elaboration of fish meal and oil; they are also used to produce gelatin. Gelatin from fish has inferior rheological properties compared to porcine and bovine, and imparts unpleasant odor and color limiting its use in food industry (Karim et al., 2009; Alfaro et al., 2014). Peptides from fish by-products such as hepcidin and cathelicidins have been reported to impart antioxidant and antimicrobial activities (Uzzell et al., 2003; Chen et al., 2005). To date, food industry besides thermal treatments for food preservation mostly relies on the use of chemical preservatives, such as potassium sorbate, sodium dehydroacetate and calcium propionate (Zhu et al., 2013). However, consumers' concerns towards the presence of chemicals in processed foods have challenged the food industry to develop new alternatives (Szűcs et al., 2014; Brown et al., 2015)

The Maillard reaction is a complex network of reactions which naturally occurs during processing of foods between reducing sugars and free amino groups of proteins/peptides. The Maillard reaction has been reported as an industrially feasible method to produce safe and nontoxic glycoconjugates with optimum functionality in food systems (Sanmartín et al., 2009). Additionally, it has been reported to improve bioactivity of proteins and peptides (Jiang et al., 2010). D-glucosamine (GlcN, 2-amino-2-deoxy-D-glucose) is an amino-sugar structural monomer of the polysaccharide chitosan obtained mainly from crustaceans. Recently, GlcN has been used to generate antimicrobial and antioxidant

compounds when conjugated with gluten and fish peptides (Gottardi et al., 2014; Hong et al., 2014). Antimicrobial activity of these peptides was achieved by the Maillard reaction known as glycation and transglutaminase-catalyzed reaction known as glycosylation with glucosamine. Therefore, glycation and glycosylation are promising approaches to generate active compounds that might serve as potential food preservatives.

Among the products generated during the Maillard reaction α-dicarbonyls and glycopeptides have been evaluated as antimicrobial compounds. α-Dicarbonyls such as diacetyl, glyoxal and methylglyoxal are known to possess antimicrobial activity (Olasupo et al., 2003; Adams et al., 2008; Mavric et al., 2008; Marceau et al., 2009; Chen et al., 2010). On the other hand, glycopeptides are noted for their efficacy and wide spectrum of action as antibiotics (Van-Bambeke et al., 2006; Yang et al., 2009). Glycopeptides have been naturally found and isolated from insects and plants and their antimicrobial activity has been reported in literature. During glycation and glycosylation the resulting mixtures contains hundreds of other rearranged compounds that might interfere with α-dicarbonyls and glycopeptides bioactivities. Therefore, efficient fractionation/isolation steps are required in order to purify these active compounds.

The objective of this work was to produce, fractionate and characterize antimicrobial compounds from fish gelatin hydrolysates conjugated with glucosamine (GlcN) at low temperature and short time by glycation and glycosylation. In order to fractionate the compounds of interest α-dicarbonyls and glycopeptides, liquid chromatography techniques such as size exclusion, affinity and reverse phase were used. After each fractionation step the antimicrobial activity of the compounds obtained was assessed against heat resistant *E. coli* AW 1.7. Finally, characterization of α-dicarbonyls and glycopeptides was investigated by LC-MS/MS. To date, separation and characterization of antimicrobial compounds from fish gelatin peptides conjugated with glucosamine has not been reported in the literature.

3.2 EXPERIMENTAL SECTION

3.2.1 Chemicals and Materials

D-Glucosamine hydrochloride (≥99% purity), gelatin (from cold water fish skin, Type A), alcalase (Alc, EC 3.4.21.14, from *Bacillus licheniformis*), transglutaminase from guinea pig liver, HPLC-grade solvents (acetonitrile, water, methanol, formic acid, and trifluoroacetic acid), glucosone (2-keto-D-glucose; ≥98.0% purity), glyoxal (ethanedial; 40% in H_2O), methylglyoxal (2-oxopropanal; 40% in H_2O), diacetyl (butane-2,3-dione; ≥95.0% purity) and 1,2-diaminobenzene were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-deoxyglucosone (3-Deoxy-D-erythro-hexosulose; ≥95% purity) was obtained from Cayman Chemical (Ann Arbor, MI, USA). SPE tC-18 Sep-Pak Vac 6cc columns were obtained from Waters (Milford, MA, USA). Filtration membranes 0.22 μm were from Millipore (Billerica, MA, USA), Difco Luria-Bertani (LB) media obtained from BD (Mississauga, ON, Canada). Ultrafiltration membranes (Minimate TFF Capsule) were purchased from PALL Life Sciences Corporation (Quebec, Canada). Lectin affinity column 5 mL (HiTrap Con A 4B, from *Canavalia ensiformis*) were obtained from GE Healthcare Life Sciences (Mississauga, ON, Canada)

3.2.2 Bacterial strain and culture conditions

Heat resistant *Escherichia coli* AW 1.7, a beef isolate (Dlusskaya et al., 2011) was grown in Luria-Bertani (LB) media containing: 10.0 g tryptone, 5.0 g yeast extract, 10.0 g sodium chloride (NaCl), and 15.0 g agar per litre of media. Both, agar and broth medium were prepared with deionized water with pH 7.0 ± 0.2 before autoclaving (121 °C x 20 min). Cells were incubated at 37 °C under aerobic conditions for 24 h for further utilization in growth inhibition analyses.

3.2.3 Experimental design

In this study glycation and glycosylation were induced in the mixture of fish gelatin peptides with glucosamine at 25 °C for 3.5 h. The main objectives were to isolate and identify antimicrobial compounds produced from both reactions such

as α-dicarbonyls and glycopeptides and to analyze their antimicrobial activities against *E. coli* AW 1.7. In the first part of the study peptides were obtained by hydrolysis of fish gelatin with alcalase, followed by separation of smallest peptides using molecular weight cut off of 3 kDa. The resulting peptides were conjugated by glycation and glycosylation, and the conjugated mixtures were tested against heat resistant *E. coli* AW 1.7 and compared with the native hydrolysates.

In the second part, RP-liquid chromatography for the identification and quantification of the main α-dicarbonyl compounds produced was performed. Moreover, lectin affinity chromatography was employed for the enrichment of glycopeptides and their antimicrobial activity was tested against *E. coli*. The glycopeptides profiles were obtained by using size exclusion and reverse phase liquid chromatography. In the last part of the experiment the identification of α-dicarbonyls and glucosamine presence in glycopeptides was performed by LC-MS/MS. Figure 3.1 shows the schematic representation of the experimental design.

3.2.4 Preparation of fish skin gelatin peptides and their molecular weight distribution

Fish skin gelatin was enzymatically hydrolyzed as described by Hong et al., (2014). Briefly, fish skin gelatin at 5% w/v was dissolved in 0.05 M $(NH_4)HCO_3/NH_4OH$ buffer, the final pH of the mixture was 7.8 ± 0.2. To achieve pasteurization the mixture was heated at 80 °C for 10 min followed by cooling down to 50 °C before adding alcalase at 1:10 ratio enzyme/buffer. The mixture was incubated for 3.5 h at 50 °C in the presence of alcalase followed by inactivation of the enzyme at 80 °C for 15 min. The resulting hydrolysates (peptides) were filtered by using Whatman No 1 filter paper. The filtrate equivalent to fish gelatin peptides was collected and kept frozen at -20 °C.

The degree of hydrolysis (DH) was assessed by *o*-phthaldialdehyde (OPA) method according to Nielsen et al., (2001) by using leucine as a standard for

percentage of hydrolysis determination. Gelatin hydrolysates molecular weight distribution was analyzed by MALDI-TOF MS, the samples were diluted in 50% acetonitrile/water and 0.1% trifluoroacetic acid. One µL of each sample was mixed with 1 µL of α-cyano-4-hydroxycinnamic acid (4-HCCA, 10 g/L in 50% acetonitrile/water and 0.1% trifluoroacetic acid). One µL of the sample/matrix solution was placed on 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. Mass spectrometry services were provided by the Institute of Biomolecular design of the University of Alberta.

3.2.5 Preparation of glycated and glycosylated peptides with glucosamine

The experimental conditions were selected based on the results obtained from previous study conducted by Hong et al., (2014) with some modifications. Two different approaches for conjugation were used to produce antimicrobial compounds from fish gelatin peptides with glucosamine. The first approach is based on the non-enzymatic reaction, known as glycation, within carbonyl compounds from glucosamine and amino groups present in the fish gelatin peptides. For the second approach, enzymatic reaction known as glycosylation was achieved with TGase which catalyzes acyl transfer reaction between the γ-carboxyamide group of the peptide-bound in glutamine residues as the acyl donor with primary amines in GlcN as acyl acceptor (Lorand et al., 2003; Jaros et al., 2006). Before conjugation fish gelatin peptides were fractionated by using molecular weight cut off membrane of 3 kDa (5,000 × g, 35 min, 4 °C, Amicon ultra centrifugal tubes 15 mL (Millipore, Cork, Ireland)). Fish gelatin peptides were mixed at 5% w/v with GlcN at the w/w ratio 1:1.

Each of the mixtures was incubated at 25 °C for 3.5 h with and without the addition of TGase at 2 units/g peptides previously activated with 5 mM calcium chloride with a final pH of 6.8 ± 0.05. TGase inactivation was achieved by molecular weight cut off of 10 kDa after incubation. All the samples were passed through 0.2 µm PVDF syringe filter (13 mm, Mandel, Ontario) for sterilization.

To remove the unreacted GlcN, dialysis was performed using a molecular weight cut off membrane from 100-500 Da (Spectrum Laboratories, Inc., Rancho Dominguez, CA). The fish gelatin peptides and GlcN were prepared at the same conditions as controls. Each of the dialyzed samples were collected, freeze-dried and stored at -20 °C for further analyses. Each treatment was carried out by triplicate.

3.2.6 Extraction, identification, and quantification of free α-dicarbonyls quinoxaline derivatives

α-Dicarbonyls extraction, identification and quantification of fish gelatin peptides, glycated and glycosylated peptides were performed as described in Chapter 2, Sections 2.2.6 – 2.2.8 as according to Papetti et al., (2013), and Papetti et al., (2014).

3.2.7 Isolation of glycopeptides by using lectin affinity chromatography

For the enrichment of glycopeptides lectin affinity chromatography was used according to Alvarez-Manilla et al., (2010) with some modifications. The type of lectin used was ConA which is purified from the seeds of the legume C*anavalia ensiformis* (Jack bean) and immobilized on a sepharose bed. This lectin binds specifically branched mannoses, carbohydrates with terminal mannose, glucose or sterically related residues. Affinity chromatography was conducted on an Agilent 1100 series LC system equipped with a fraction collector. Lectin affinity columns (HiTrap ConA, 4B, 5 mL) were attached to the FPLC system. The mobile phases consisted of (A) binding buffer: 20 mM Tris-HCl, 0.5 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 7.4 and (B) elution buffer: 0.1 M sodium acetate and 1 M NaCl, pH 6. Two extra mobile phases for the regeneration and storage of the lectin columns were used.

A 4-stepwise isocratic elution was performed for fish gelatin peptides, GlcN, glycated, and glycosylated peptides solutions. In the first step, equilibration of the column was achieved by passing 10 column volumes (CV) of binding buffer (A) through the affinity column. After equilibration, 100 µL of sample was

injected. The column was washed with approximately 5 CV with binding buffer (A) or until no material appears in the eluent monitored by UV absorption at A_{214nm} at 0.5 mL/min. In the second step, the retentate was desorbed by 5 CV of buffer (B) or until no material appears. The two collected fractions corresponded to ConA-eluent and ConA-retentate per each treatment. After separation the fractions were desalted by using ultrafiltration membranes of 650 Da molecular weight cut off monitored by conductivity measurements. The desalted fractions were freeze-dried and stored at -20 °C and reconstituted for subsequent antimicrobial activity, and size exclusion and reverse phase liquid chromatography analyses.

3.2.8 Liquid affinity chromatography fractions profiles

After enrichment of glycated and glycosylated peptides the profiles of both collected fractions ConA-eluent and ConA-retentate were analyzed by using two different chromatography techniques. Size exclusion and reverse phase were used to analyze the apparent molecular size distribution and the abundance of glycopeptides in both fractions. First, the fractions were separated through a 120 mL HiLoad 16/60 Superdex 200 pg size exclusion column (GE Healthcare Amersham Biosciences, Canada) connected to a fast protein liquid chromatography (FPLC) system at 0.5 mL/min using filtrated water. Aliquots of 100 µL with a concentration of 1 g/L of each fraction were injected, and separation was monitored at 214 nm. Mass calibration was performed using a standard mixture of compounds ranging from 0.2 to 12 kDa under the same conditions.

To obtain reverse phase liquid chromatography profiles the samples were separated through Ascentis Express ES-C18 column (150 × 4.6 mm, 2.7 µm particles; Sigma-Aldrich, MO, USA) operating at 60.0 ± 0.5 °C and at flow rate of 0.5 mL/min. The binary mobile phase consisted of (A) 0.05% trifluoroacetic acid in water and (B) 0.01% trifluoroacetic acid in acetonitrile. The gradient is described as follow: 0–3 min (0% B), 3–120 min (0–30% B), and 120–129 min (100% A). Before injection, the sample solutions were filtrated with 0.22 µm

PVDF filter (13 mm, Millipore Millex, Billerica, MA, USA). The eluent compounds were monitored by UV absorption at $A_{214\ nm}$ using an ultra-high performance liquid chromatography (UHPLC) apparatus (Shimadzu, MD, USA).

3.2.9 RP-liquid chromatography for further purification of glycopeptides

The ConA-retentate which corresponds to the glycopeptides fraction was further purified by separation of fluorescent molecules from the non-fluorescent ones. Briefly, the collected fraction ConA-retentate was separated through Ascentis Express ES-C18 column (150 × 4.6 mm, 2.7 µm particles; Sigma-Aldrich, MO, USA) at the same conditions used to obtain the profiles (See section 3.2.8. However, the separation was monitored at excitation and emission wavelengths of $\lambda_{exc} = 350$, $\lambda_{em} = 430$ nm for total fluorescence according to Henle et al., (1999). The collected fractions were freeze-dried and stored at -20°C for further antimicrobial and mass spectrometry analyses.

3.2.10 LC-MS/MS analysis for the characterization of glycopeptides

The isolated fluorescent compounds were subject to LC-MS/MS analysis on a q-Tof premier mass spectrometer (Waters, Milford, MA) coupled with a nanoAcquity UPLC system (Waters, Milford, MA). Five µL of the peptides was loaded onto a nano trap column (180µm x 20mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA) followed by a nano analytical column (75 µm × 150 mm, Atlantis™ dC18 nanoAcquity™ column, Waters, Milford, MA). Desalting on the peptide trap was achieved by flushing trap with 2% acetonitrile, 0.1% formic acid in water at a flow rate of 5 µL/min for 4 min. Peptides were separated with a gradient of 2 - 75% solvent B (acetonitrile with 0.1% formic acid) over 55 min at a flow rate of 350 nL/min. The column was connected to a q-Tof premier (Waters Corporation) for ESI-MS/MS analysis. Mass spectrometry analysis services were provided by mass spectrometry facility of the Department of Chemistry at the University of Alberta. Presence of 162 and 163 Da GlcN fragments were analyzed for each of the peaks identified, in search of glucosamine presence in the peptides.

3.2.11 Determination of antimicrobial activity

The antimicrobial activity of fish gelatin peptides, GlcN, conjugated peptides and fractionated samples was determined by critical dilution assay as described by Parente et al., (1995) and Gänzle et al., (1999). The antimicrobial activity was considered before and after each of the different steps of fractionation and purification. A first screening of antimicrobial activity of fish gelatin peptides (FGP), glucosamine (GlcN), glycated (GCP) and glycosylated (GSP) peptides was assessed. Further analysis was performed on the fractions obtaining after affinity chromatography from glycated and glycosylated peptides being ConA-eluent and ConA-retentate.

Finally, antimicrobial activity of the fluorescent and non-fluorescent compounds separated on reverse phase liquid chromatography from glycosylated peptides treatment was analyzed. Sterilization of samples was achieved by filtration through sterilized 0.22 µm membrane filters (Millipore, MA, USA). *E. coli* was subcultured twice in liquid media under conditions described by Sánchez-Maldonado et al., (2011). Briefly, 100 µL of sample solutions and 100 µL of LB broth media were mixed, and two fold serial dilutions of mixtures were prepared on 96-wells microtitre plates. Finally, 50 µL of inoculated media was added to microtitre wells. The pH value of the final mixtures was measured with a pH meter (Orion 2-star, Thermo scientific, CA). The initial cell count was about 10^6 CFU/mL. All the experiments were carried out in triplicate. Antimicrobial activity of standard α-dicarbonyls and the different samples solutions were evaluated by the development of turbidity by vertical photometry, optical density (OD_{630nm}). MIC values were calculated by mathematical extrapolation of the concentration needed to reduce 50% of the bacterial growth using Origin 2015 software.

3.2.12 Statistical analysis

MIC values, α-dicarbonyls concentration and Degree of Hydrolysis (DH) are expressed as mean ± standard deviation of the three independent experiments.

Liquid chromatographic profiles obtained from UV and fluorescence detections of the different trials were injected at least two times and are reported as chromatograms. The effect of glycation and glycosylation on the production of α-dicarbonyls and antimicrobial activity was analyzed by one-way ANOVA considering significant difference between the treatments at a significant level of $\alpha = 0.05$. Differences between means were determined by Tukey´s multiple range test ($p<0.05$) using Minitab software package v.17 (Minitab Inc. PA, U.S.).

3.3 RESULTS AND DISCUSSION

3.3.1 Degree of hydrolysis and mass distribution of fish gelatin peptides

Alcalase a non-specific endopeptidase of microbial origin (*Bacillus licheniformis*) has been used widely for the hydrolysis of collagen because preferentially cleavage at the carboxyl termini of Glu, Met, Leu, Tyr and Lys (Fu et al., 2015). The degree of hydrolysis of fish gelatin subjected to alcalase was 27 ± 2.4 % of cleaved peptide bonds, where 100% is the total number of fish gelatin peptide bonds (Nielsen et al., 2001). In this study alcalase was used to prepare fish gelatin peptides since alcalase leads to a higher degree of hydrolysis in shorter time compared to other enzymes releasing peptides with bioactive properties (Gómez-Guillén et al., 2011).

The molecular weight distribution of gelatin peptides was analyzed by MALDI-TOF MS; the resulting peptides ranged from 500 to 2000 Da (Figure 3.2). Moreover, the hydrolysates were analyzed by reverse phase liquid chromatography and the profile showed that more than hundred peaks or peptides are produced from fish gelatin hydrolysis using alcalase. The gradient for separation was composed by 0 - 30% of acetonitrile, the non-polar phase; therefore, these highly hydrophilic conditions indicate that most of the peptides were mainly polar (See Appendix. Figure A.3).

3.3.2 Identification and quantification of free α-dicarbonyls

α-Dicarbonyls are present in diverse proteinous food systems like dairy products and soy sauce, and they are known to be antimicrobials (Adams et al., 2008.; Rincon-Delgadillo et al., 2012; Spanneberg et al., 2012). In our study, mild conditions of 25 °C and 3.5 h incubation applied in glycation and glycosylation reactions produced α-dicarbonyl compounds. α-Dicarbonyls presence was evaluated in fish gelatin peptides, glycated and glycosylated peptides, and isolated glycopeptides. After extraction, derivatization and separation by liquid chromatography five α-dicarbonyls were identified and quantified. Glucosone, 3-deoxyglucosone, glyoxal, methylglyoxal and diacetyl were found in glycated and glycosylated peptides. The retention time of each of the separated dicarbonyls was compared with a commercially available standard previously derivatized (Figure 2.2 (a)). This was confirmed by the pseudo molecular weight of manually collected peaks analyzed by mass spectrometry as shown in Table 2.1.

The concentration of each of the α-dicarbonyls found in the glycated and glycosylated treatments was quantified and results are presented in Table 3.1. Glucosone and 3-deoxyglucosone were the most abundant α-dicarbonyls found in both systems. Glyoxal, methylglyoxal and diacetyl α-dicarbonyls were found at smaller concentrations in both systems. There was no significant difference between glycation and glycosylation treatments in the production of glucosone, glyoxal, methylglyoxal and diacetyl; however, the production of 3-deoxyglucosone by glycosylation was significantly higher compared to glycation. It is worth to notice that production of glyoxal in the presence of peptides was six times higher than glucosamine autocondensation as stated in the in Chapter 1. Table 2.2. Fish gelatin peptides and isolated glycopeptides did not contain any of the dicarbonyls of investigation (data not shown).

3.3.3 Antimicrobial activity of fish gelatin peptides vs. glycated and glycosylated peptides

The antimicrobial activity of fish gelatin peptides (FGP) and the respective conjugated peptides with GlcN by glycation and glycosylation reactions was

analyzed to determine their minimal inhibitory concentration (MIC). The final pH of the samples tested was 6.7 ± 0.1. The conjugated peptides by glycation and glycosylation showed higher antimicrobial activity against heat resistant *E. coli* AW 1.7 compared with the fish gelatin peptides in accordance with Hong et al., (2014) (Figure 3.3). However, the MIC of the glycated and glycosylated peptides was higher compared to 40 g/L found in previous study (Hong et al., 2014). There was no significant difference between glycation and glycosylation treatments for the reduction of bacterial growth.

Antibacterial effect enhancement of gelatin peptides might be attributed to the several compounds that are formed during the reaction with glucosamine. For instance, α-dicarbonyl compounds found in both treatments contribute to the antimicrobial activity exerted. α-Dicarbonyls commercially available glucosone, 3-deoxyglucosone, glyoxal, methylglyoxal and diacetyl were tested against *E. coli* AW 1.7 and results are shown in Chapter 2. Figure 2.4. The concentration of 3-deoxyglucosone found in the glycated and glycosylated peptides was 0.9 and 1.1 g/L, respectively. These values are comparable with the concentration of commercial standard α-dicarbonyl 3-deoxyglucosone of 1 g/L for the inhibition of bacterial growth. Other antimicrobial compounds can be produced during the reactions and they can also contribute to the overall reduction of bacterial growth. In our study we also investigated the presence and the antimicrobial effect of glycopeptides formed in both glycation and glycosylation.

3.3.4 Glycopeptides enrichment by lectin affinity chromatography

Lectin affinity chromatography is widely used to separate and isolate glycoproteins/glycopeptides from complex mixtures by reversible interaction of the sugar attached to the protein/peptide with the lectin (Kaji et al., 2003; Zhang et al., 2008). The affinity of glycopeptides for the lectin may vary depending on the type of lectin used and the sugar that is attached on the backbone of the peptide. For instance, mannose/glucose binding lectins have more affinity for glucose than for mannose. The rationale for choosing lectin-ConA affinity chromatography in our experiment originated from the similarity of GlcN with

glucose and mannose structure. These three sugars share the same free hydroxyl groups (OH) at carbons C-3, C-4, and C-5 needed for reversible interaction with ConA (Figure 3.4). Therefore, we hypothesized that peptides containing the main core of glucosamine could be successfully captured by ConA lectin. Figure 3.5 shows the 4 elution-desorption steps for the separation of glycopeptides compounds from conjugated peptides. The yield of glycopeptides was about 30-50 mg per gram of glycated and glycosylated peptides. The ConA column was stable after regeneration and could be used repeatedly for at least ten times.

An off-line desalting step was required to remove the large amounts of salts collected in the ConA-retentate (glycopeptides) molecules of interest prior to antimicrobial analysis to avoid any interference (Zhang et al., 2008). This off-line desalting leads to losses up to 50% of the samples and might explain the low recovery of ConA-retentate fraction. After extraction, two fractions per each treatment were recovered, the first one after three washes applied was the ConA-eluent fraction and the second after desorption was ConA-retentate which corresponds to the molecules of interest, glycopeptides. Fish gelatin peptides were subjected to separation as controls and the 4 step elution-desorption run showed that no attachment occurred when fish gelatin peptides are passed through affinity chromatography column (data not shown).

To investigate the differences of ConA-eluent and ConA-retentate after separation, size exclusion and reverse phase liquid chromatography were conducted. The profile obtained from size exclusion indicated that two main peaks monitored at 214 nm were present in the ConA-retentate fraction while the ConA-eluent fraction showed five main peaks (Figure 3.6). ConA-eluent profile ranged from 0.5 to 10 kDa apparent molecular weight; however, the two main peaks of the ConA-retentate fraction where present in the low molecular weight region from 0.5 to 2 kDa approximately. Moreover, the profiles obtained from reverse phase chromatography analysis indicated main differences in the abundance of peaks separated from ConA-eluent and ConA-retentate (Figure 3.7 (a) and (b)). On one hand, the ConA-eluent showed more than hundred peaks

separated in the hydrophilic region, while the ConA-retentate fraction showed only around 20 peaks. These profiles confirmed that the fraction retained by lectin affinity chromatography is less complex fraction that may contain glycopeptides molecules of interest.

3.3.5 LC MS/MS analyses for glucosamine presence in isolated glycopeptides

LC-MS/MS is widely used for the identification of glycoproteins/glycopeptides. Normally, the first step to accomplish the identification of these molecules is based on tryptic digestion followed by enrichment of obtained glycopeptides (Kaji et al., 2003). In our experiment, the mixture of compounds was hydrolyzed and enriched by affinity chromatography which makes them suitable to be analyzed by mass spectrometry. During MS/MS analysis there is a cleavage of the carbohydrate moiety from the peptide leaving the saccharide portion intact at the low molecular weight region (Alving et al., 1999; Medzihradszky et al., 2005; Fentabil., 2010; Dodds et al., 2012; Vékey et al., 2013; Halim et al., 2014).

In our experiment, liquid chromatography separation of the glycopeptides fraction resulted in 14 main peaks eluted (Figure 3.8). The MS/MS data for each peak was analyzed by searching the sugar moiety de-attached in the low molecular weight region of the spectra. Moreover, it has been studied that the Amadori product, glycopeptide in this experiment, is easily altered when dissociation by MS/MS occurred; it promptly dehydrates losing up to 4 water molecules (72 Da) as neutral loses and 30 Da corresponding to formaldehyde (HCHO) (Frolov et al., 2006; Zhang et al., 2008; Yamaguchi et al., 2014). A fragment ion of 72.1 in the low molecular region of the MS/MS spectra was also considered as a key fragment indicating the presence of GlcN. Molecular weight, glucosamine parent ions and fragments found on the separated peaks of the ConA-retentate fraction are presented in Table 3.2.

Two representative examples of the MS/MS manual analysis of the peaks with the presence of glucosamine ion 162 and 163 $[M+H]^+$ are shown in Figure 3.9 and Figure 3.10. During the Maillard reaction the condensation of the peptide with glucosamine will release 18 Da corresponding to a molecule of water. On the other hand, when glucosamine is incorporated through transglutaminase by acyl transfer reaction one molecule of ammonia (17 Da) is released. This mass spectrometry information may serve as complementary information for glycopeptides analysis.

Data acquisition during LC-MS/MS of complex glycopeptides mixtures is never fully completed and the process is unable to collect tandem mass spectra for all the peptides eluted (Alvarez-Manilla et al., 2010). After analysis of the 14 separated peaks only 5 of the peaks presented glucosamine at the low molecular region by MS/MS. Even though, fully confirmation of glycopeptides structure was not accomplished, it is worth to notice that the glycopeptides reported in this study were enriched by affinity chromatography.

3.3.6 Antimicrobial activity of the separated glycopeptides

Glycopeptides have been widely investigated and they possess antimicrobial activity since carbohydrates play an important role in the bioactivity of the peptides. To follow up the effect on the separation of glycopeptides from conjugated mixtures of peptides by glycation and glycosylation, the antimicrobial activity of each fraction after affinity chromatography was tested against *E. coli*. The MIC of ConA-retentate from glycation and glycosylation treatments was 16.5 and 9 g/L respectively, with no significant difference among treatments (Figure 3.11). The ConA-eluent fraction recovered was tested up to 120 g/L and no antibacterial effect was detected.

Separated glycopeptides showed better antimicrobial activity compared with fish gelatin peptides and glucosamine controls. Thus, separation of glycopeptides from the mixture increased the antimicrobial activity by ten times. There is no general mechanism of action attributed to glycopeptides since they may vary in

type of sugar, site of glycation and peptide sequence. One of the proposed mechanisms of action of some glycopeptides is based in the obstruction of the outer membrane of the bacterial barrier. This obstruction generates ion-permeable channels in the cell membrane and leaking of cytoplasm once the glycopeptides combine with the peptides on the cell membrane (Yang et al., 2009).

3.3.7 Reverse phase liquid chromatography for further purification of glycopeptides

In the Maillard reaction fluorescent compounds are some of the compounds produced at the latest stage known as advanced glycated end products (AGE´s) (Birlouez-Aragon et al., 2004; Ferrer et al., 2005). In our study we have presented α-dicarbonyls and glycopeptides produced from the reactions at mild conditions. In addition, reverse phase liquid chromatography was used to separate the fluorescent compounds present in the ConA-retentate from those non-fluorescent. The fluorescent fraction was collected up to 6 min of the run (Figure 3.7 (c)) and non-fluorescent fraction was collected after 6 min of the run until no material appear (Figure 3.7 (b)). Regardless the mild temperature and time conditions applied to the treatments it was also noticed the presence of fluorescent more advance glycated compounds. These findings indicate that during the reactions a wide variety of different compounds are produced. Both fluorescent and non-fluorescent fractions were analyzed against *E. coli* and the fluorescent fractions did not show bacterial growth reduction. On the other hand, non-fluorescent fraction recovered reduced the bacterial growth at the same concentration than separated glycopeptides from affinity chromatography but no improvement was noticed.

3.3.8 Glycation vs. glycosylation

Despite of the use of TGase to catalyze the attachment of glucosamine into peptides, the Maillard reaction or glycation was predominant and its development took place. In our study we found that α-dicarbonyls and glycopeptides were produced during both reactions; however, 3-deoxyglucosone was predominant during glycosylation. Glycopeptides from both systems did not present significant

difference in their antimicrobial activity. Regardless the utilization of TGase to avoid strong development of the Maillard reaction, advanced glycated fluorescent products were still produced. Therefore, it can be concluded that the addition of transglutaminase did not improve the antimicrobial activity of glycopeptides and did not differ from those produced from the sole Maillard reaction.

3.4 CONCLUSIONS

It was described in a previous study that conjugation of fish gelatin peptides with glucosamine increases antimicrobial activity (Hong et al., 2014). However, on their study compounds responsible for the antimicrobial activity that resulted from the conjugation were not investigated. As reported in literature, the Maillard reaction produced an immense mixture of different compounds poorly identified, but providing bioactive properties. In the present study glucosamine and fish gelatin peptides-derived antimicrobial compounds by glycation and glycosylation were successfully characterized. The antimicrobial activity of fish gelatin peptides significantly increased when conjugated with GlcN. However, there was no significant difference between glycation and glycosylation treatments on the antimicrobial activity against *E. coli*.

In this study, α-dicarbonyls and glycopeptides produced during the non-enzymatic and enzymatic reactions were isolated and identified. Glucosone, 3-deoxyglucosone, glyoxal, methylglyoxal and diacetyl were found in the conjugates and 3-deoxyglucosone concentration found in our samples was around 1 g/L, which was in accordance with the MIC of the pure standard. Moreover, enrichment of glycopeptides was achieved by affinity chromatography and the antimicrobial activity increased by 10 times compared to the whole mixture of conjugates. Finally, it was possible to provide information to support the incorporation of sugar onto peptides by the presence of GlcN core using mass spectrometry.

However, to elucidate the structure of a glycopeptide, information on the peptide sequence, glycosylation sites, and glycan final structures must be further

investigated (Ferreira et al., 2011; Dodds et al., 2012). Therefore, further studies are needed to evaluate the glycopeptides structure produced from GlcN derived antimicrobial compounds from fish peptides in order to synthetize them and analyze their mode of action. Our findings open the possibility to use GlcN as functional ingredient that forms antimicrobial agents during food processing when this amino-sugar interacts with proteins and peptides in food systems.

3.5 TABLES

Table 3.1 Concentration of derivatized α-dicarbonyls identified in fish gelatin peptides (FGP), glycated (GCP) and glycosylated peptides (GSP) in mg/L

Free α-dicarbonyl compounds in mg/L produced from 150 g/L of GCP and GSP solutions

Treatment (n = 3)	Glucosone	3-deoxyglucosone	Glyoxal	Methylglyoxal	Diacetyl
FGP	nd	nd	nd	nd	nd
GCP	858 ± 14[a]	872 ± 37[b]	22 ± 8.1[a]	1.6 ± 0.1[a]	6.0 ± 0.6[a]
GSP	913 ± 19[a]	1125 ± 70[a]	24 ± 2.6[a]	1.8 ± 0.3[a]	6.3 ± 1.5[a]

nd; non detected

Table 3.2 MS and MS/MS data analysis of peaks obtained from LC-MS/MS separation of isolated glycopeptides from glycated peptides treatment. Ammonia, water and formaldehyde releases are shown for each of the detected peaks.

MS and MS/MS analyses of glycopeptides from fish gelatin peptides conjugated with glucosamine via enzymatic reaction

Peak #	RT (min)	MW (Da)	Loss H_2O (18 Da)	Formaldehyde (30 Da)	Loss NH_3 (17 Da)	GlcN Residue (m/z)	GlcN Fragment
1	12.77	707.3			2		72.1
2	13.65	601.3	3	1			72.1
3	14.94	1469.8			2		
4	16.45	822.5	1		3		
5	17.83	840.4	1		3		
6	19.03	587.3					72.1
7	22.34	1913					72.1
8	23.18	2168	3		2	162	
9	25.35	2621.4	2	2		162, 163	72.1
10	27.92	2961.6	4				72.1
11	31.95	819.4	4			162	72.2
12	39.38	540.4	3	1		162	
13	40.76	360.3	5		2	163	72.1
14	49.21	750.5	-	-	-	-	

3.6 FIGURES

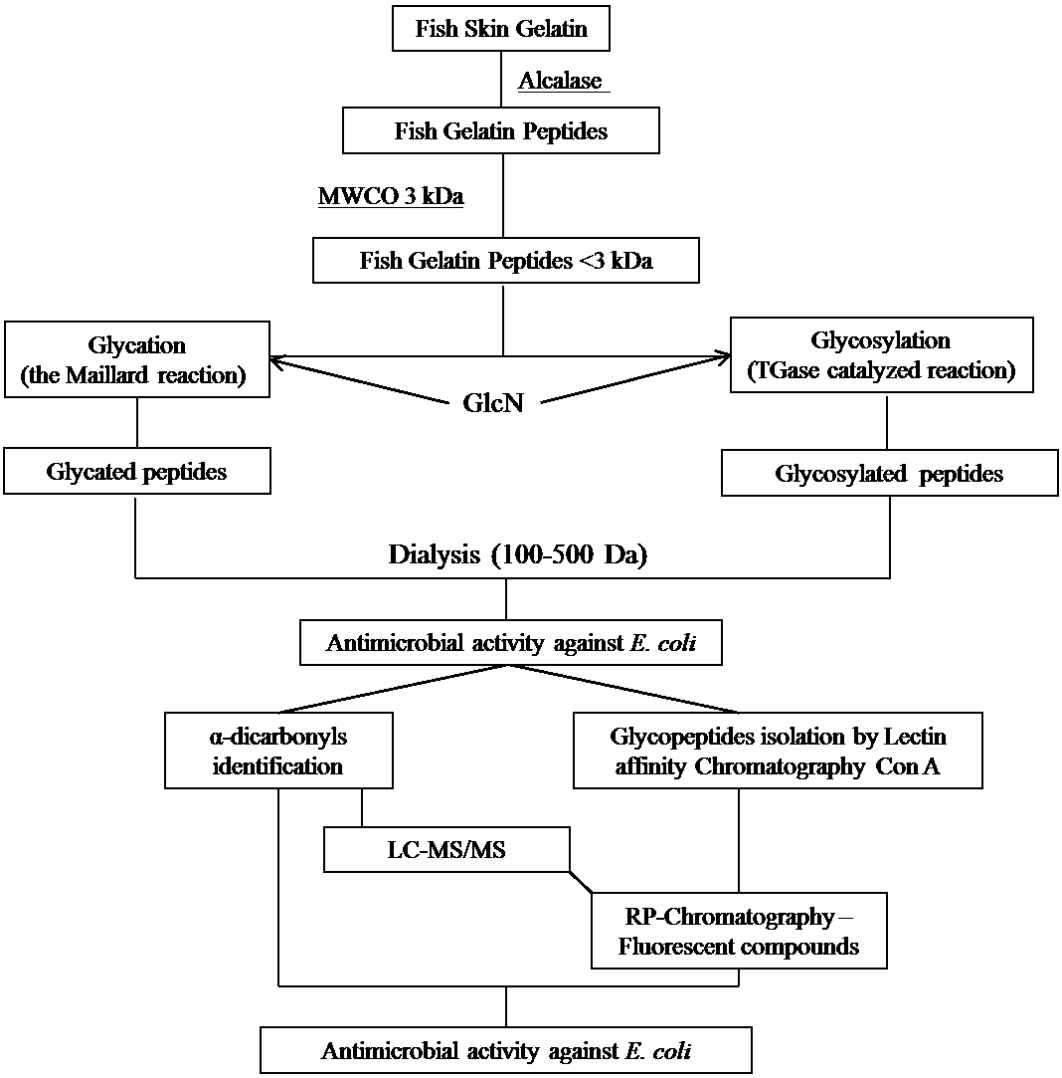


Figure 3.1 Schematic representation of the experimental design employed for the production of peptides and conjugates, and the different techniques used for the isolation and characterization of α-dicarbonyl compounds and glycopeptides.

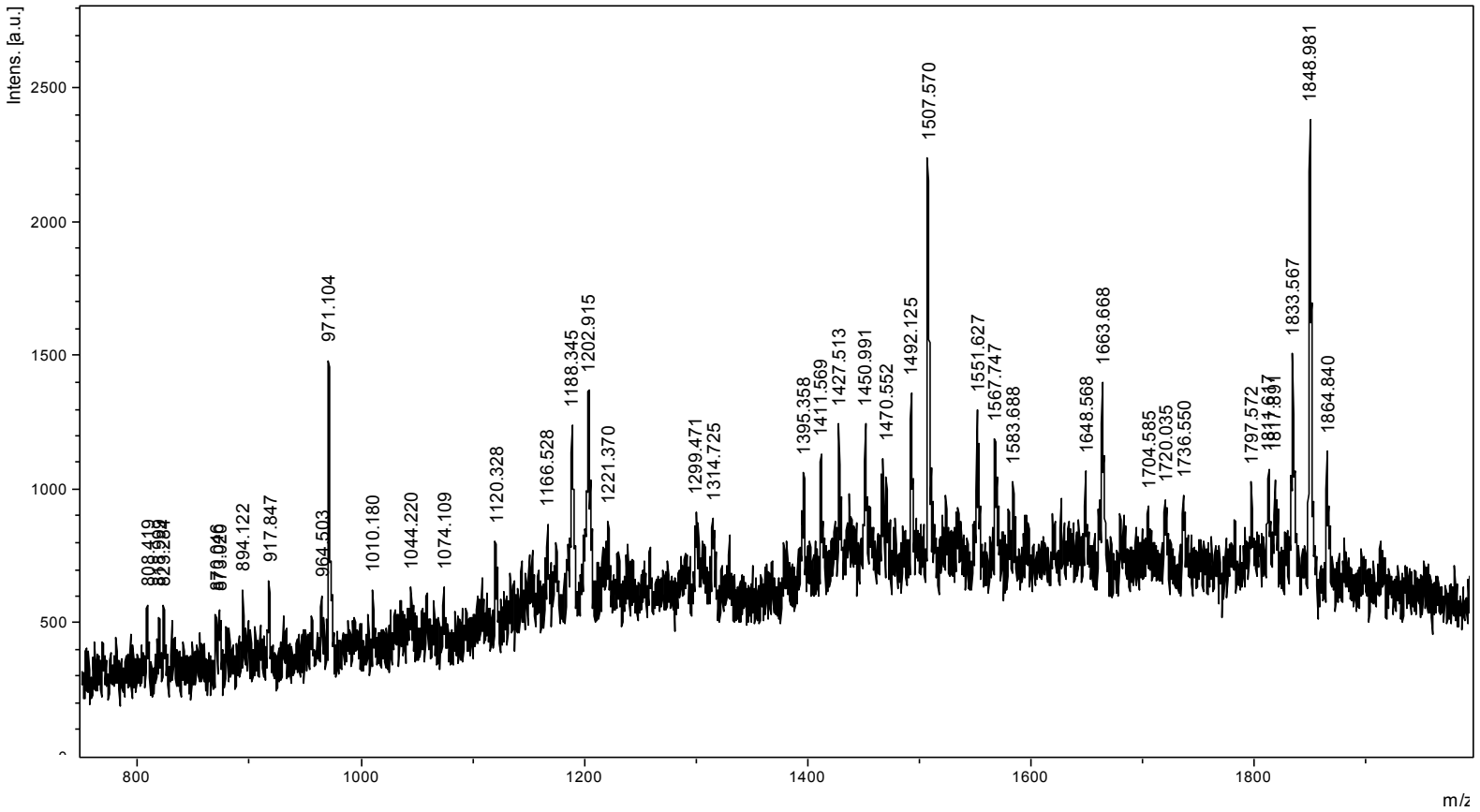


Figure 3.2 Molecular weight distribution of fish gelatin peptides using alcalase by MALDI-TOF MS.

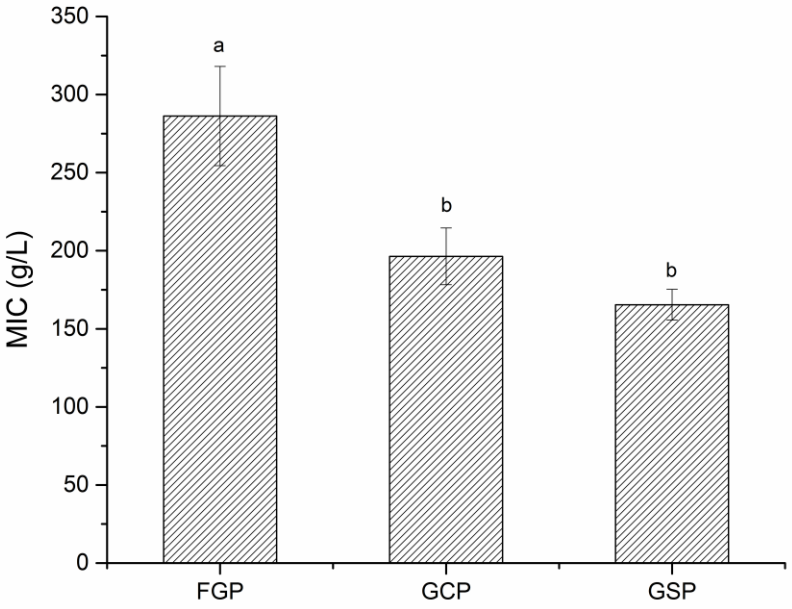


Figure 3.3 Minimum inhibitory concentration of fish gelatin peptides (FGP) and glycated (GCP) and glycosylated peptides (GSP) with glucosamine against heat resistant *Escherichia coli* AW 1.7.

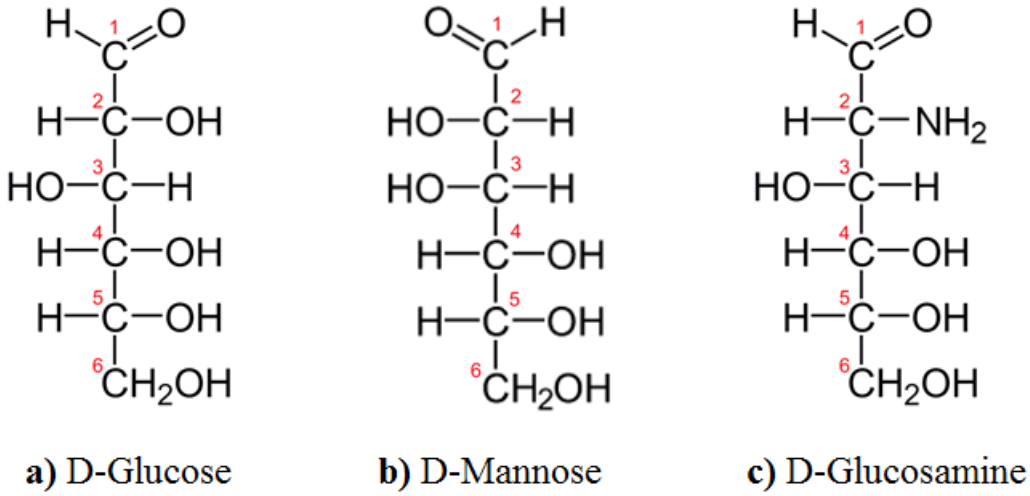


Figure 3.4 Open chain structures of monosaccharides with affinity for lectin carbohydrate-binding protein ConA. **a)** D-Mannose, **b)** D-Glucose and, **c)** D-Glucosamine free hydroxyl groups in positions C-3, C-4 and C-5 are required for reversible reaction with ConA binding lectin.

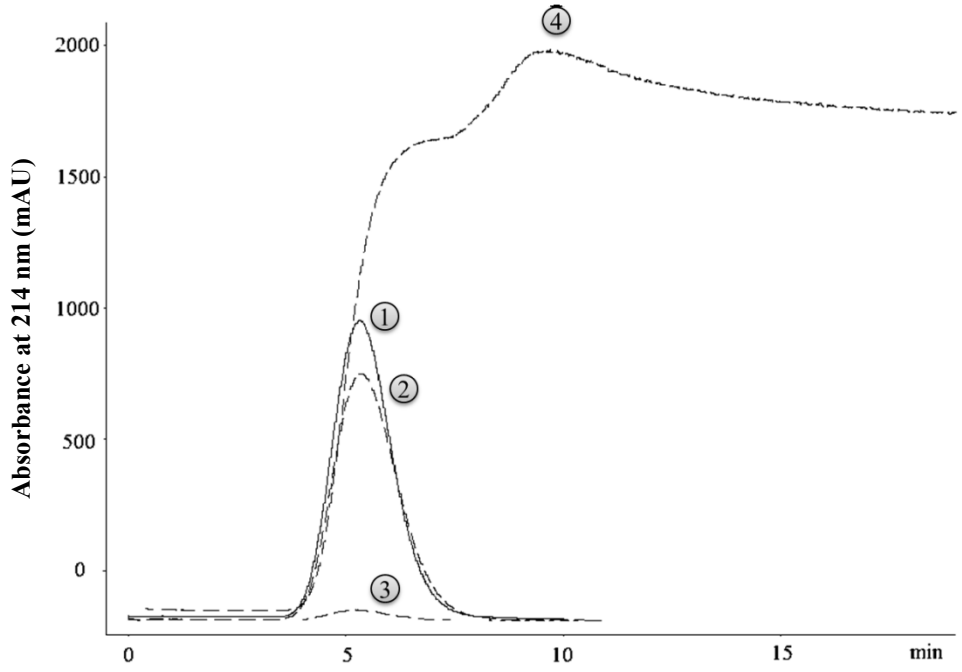


Figure 3.5 Isolation of glycopeptides on ConA sepharose 4B lectin affinity in FPLC system. **(1)** ConA-eluent first wash. **(2)** ConA-eluent second wash. **(3)** ConA-eluent third wash. **(4)** ConA-retentate desorption.

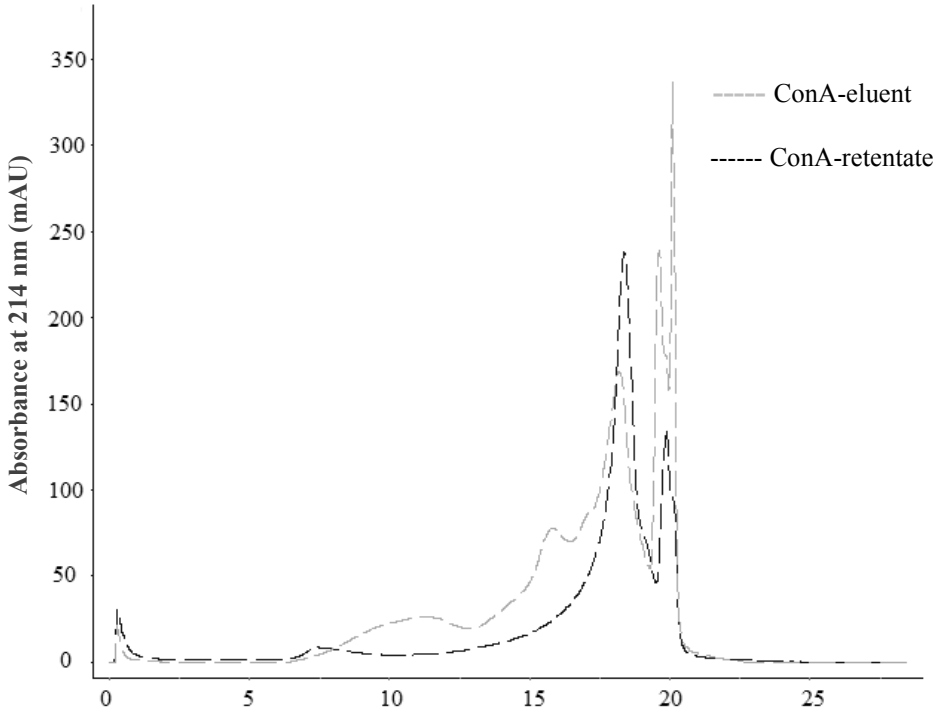


Figure 3.6 Size exclusion chromatography profiles of ConA-eluent and ConA-retentate fractions from glycosylated peptides on Superdex peptide column.

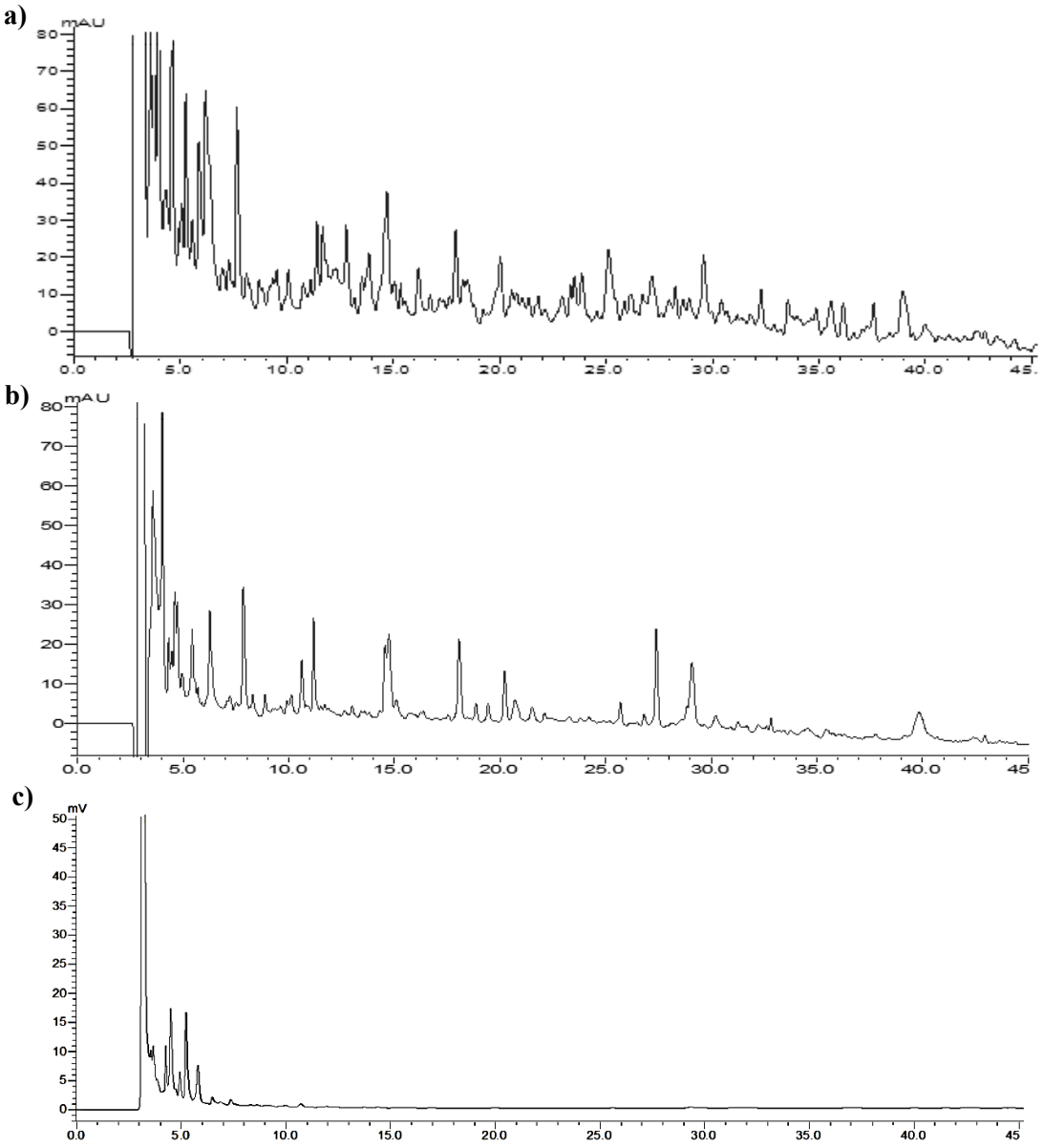


Figure 3.7 RP-UHPLC chromatograms of fractions obtained from affinity chromatography of glycosylated peptides. **a)** ConA-eluent detected at 214 nm. **b)** ConA-retentate detected at 214 nm. **c)** ConA-retentate detected by fluoresence (λ_{exc} = 350 nm, λ_{em} = 430 nm).

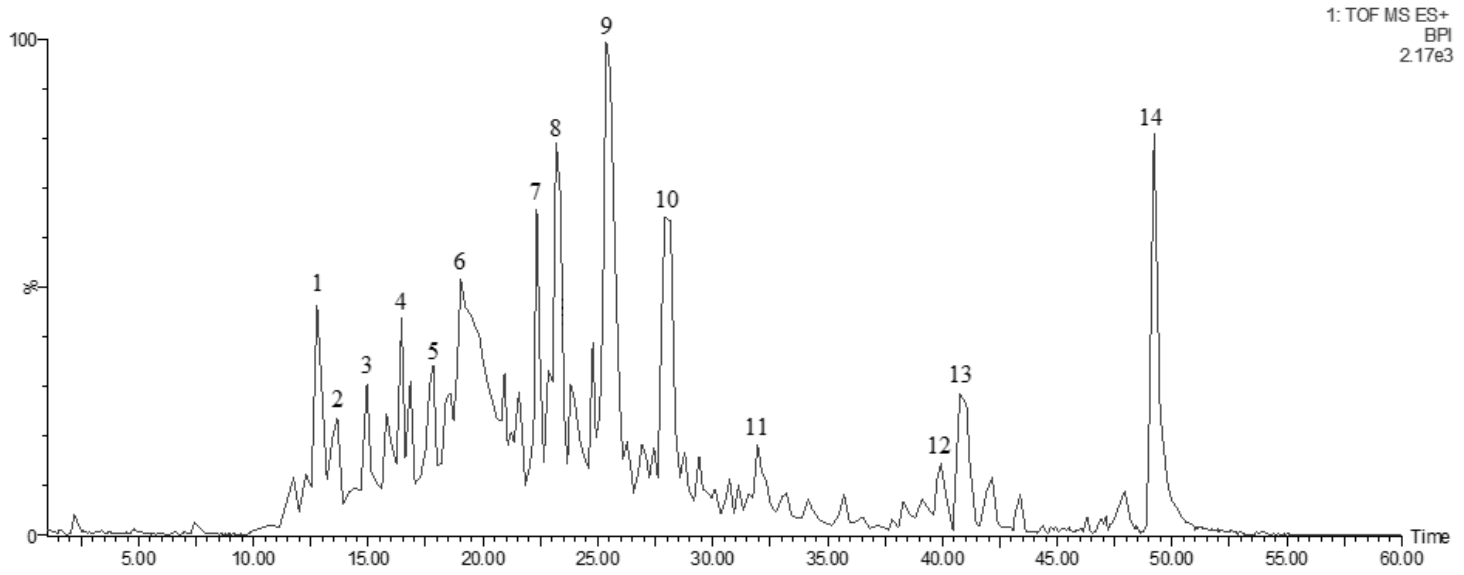


Figure 3.8 LC-MS chromatogram of non-fluorescent compounds corresponding to the ConA-retentate isolated glycopeptides.

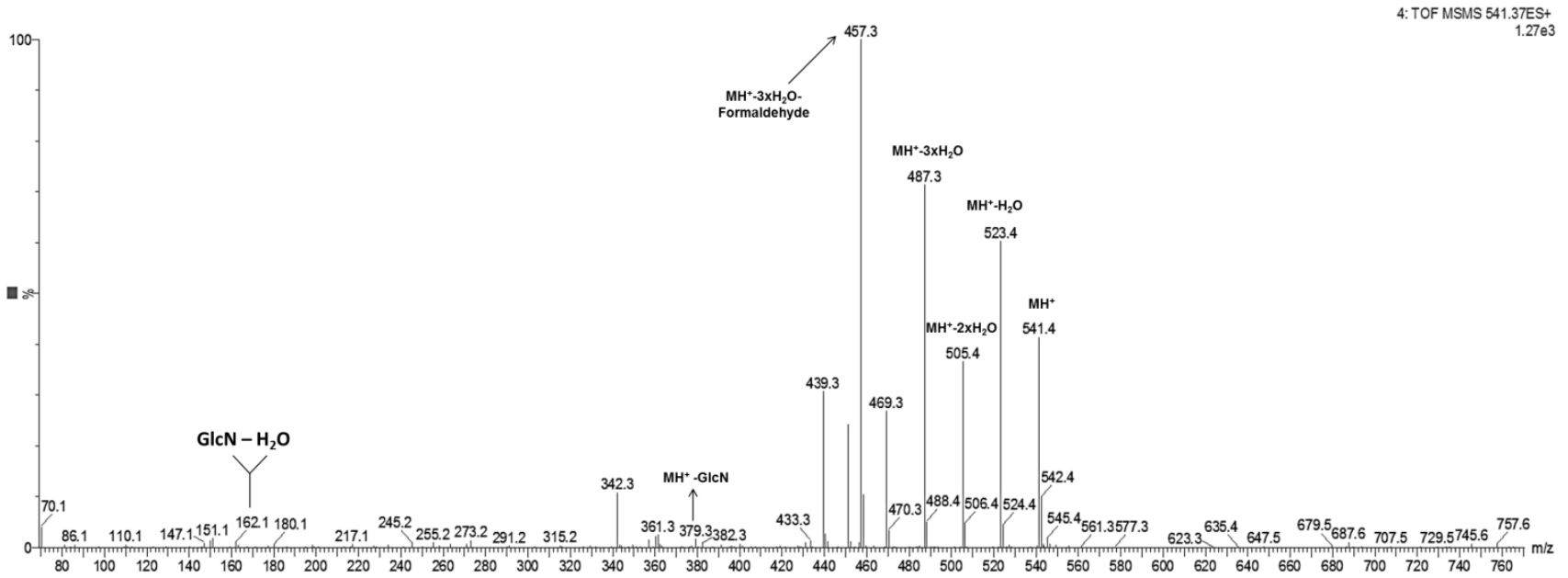


Figure 3.9 MS/MS spectra of peak 12. Example of a glycopeptide obtained from glycation.

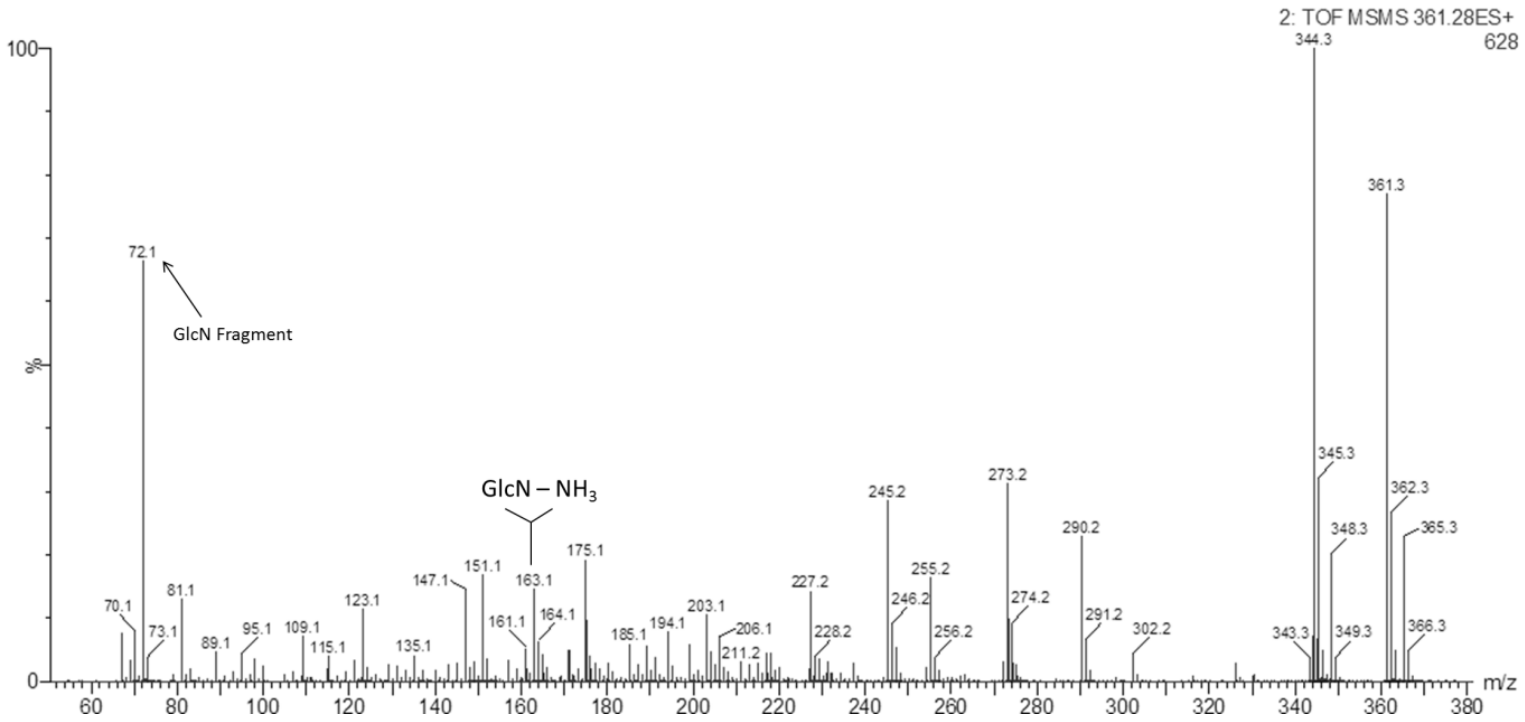


Figure 3.10 MS/MS spectra of peak 13. Example of glycopeptide obtained from glycosylation.

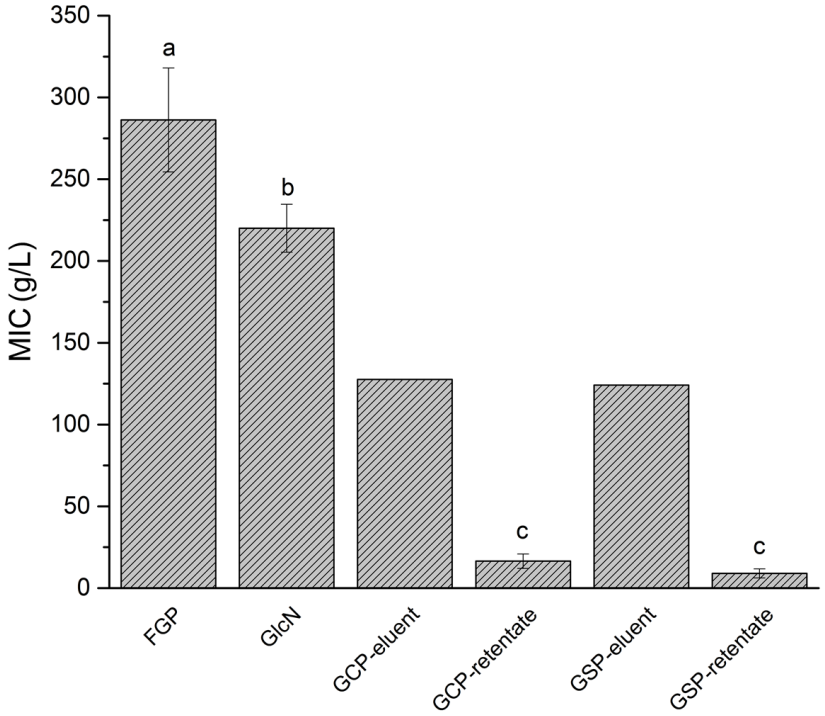


Figure 3.11 Minimum inhibitory concentration of ConA-retentate from glycation (GCP_retentate) and from glycosylation (GSP_retentate) against *E. coli* AW 1.7. ConA-eluent fractions from both treatments did not present inhibition around 120 g/L (GCP-eluent and GSP-eluent). Fish gelatin peptides (FGP) and Glucosamine (GlcN) were analyzed as controls.

4 CONCLUSIONS AND FUTURE WORK

The objectives of this research were to produce, separate and characterize antimicrobial compounds generated by the modification of glucosamine or glucosamine with fish gelatin peptides. The modification was done by glycation or the Maillard reaction and glycosylation catalyzed by transglutaminase. Even though, the Maillard reaction produces a complex mixture of compounds, in this study we were able to extract and identify α-dicarbonyl compounds and glycopeptides using liquid chromatography. In overall glucosamine modification and separation steps resulted in increasing antimicrobial activity against heat resistant *Escherichia coli*.

In the first part of the study, the modification of glucosamine gave rise to α-dicarbonyls such as, glucosone, 3-deoxyglucosone, glyoxal, methylglyoxal and diacetyl. 3-deoxyglucosone was the highest α-dicarbonyl produced by glucosamine modification. A faster production rate was found in the presence of iron. After modification of glucosamine with iron, 5% of modified glucosamine solution was needed to inhibit the growth of the Gram-negative heat resistant *E. coli* AW 1.7. It was found that α-dicarbonyls partially contributed to the antimicrobial activity as well as the low pH found at the end of the reaction. In the second part of this study, fish gelatin peptides were conjugated with glucosamine. α-Dicarbonyls compounds and glycopeptides were found in the resultant mixtures. Two different approaches for the incorporation of glucosamine into peptides were used; glycation and glycosylation. There was no significant difference ($p>0.05$) between the treatments; neither antimicrobial activity nor α-dicarbonyls production, except for 3-deoxyglucosone concentration which was higher when glycosylation was induced.

As in the modification of glucosamine, the five α-dicarbonyls found were also present in glucosamine-fish gelatin peptides conjugates. However, the amount of glyoxal was 10 times higher than the amount present in glucosamine autocondensation. Moreover, glycopeptides were isolated from the mixtures of glycated and glycosylated peptides by using lectin affinity chromatography.

Glycopeptides were tested against *E. coli* and we found that their antimicrobial activity was higher than the original mixture. After extraction from the mixture, the antibacterial effect of glycopeptides from glycosylation and from the Maillard reaction or glycation was comparable.

Due to the complexity of glycopeptides formed which is dependent of the type of glycan, peptide sequence and glycation sites, additional mass spectrometry analyses using synthetic glucosamine-glycopeptides is a further topic of investigation. Furthermore, as demonstrated in the literature review, the Maillard reaction produces hundreds of different compounds that might also possess antimicrobial activity. Therefore, the presence of other antimicrobial compounds such as pyrazines from glucosamine-Maillard products is an interesting topic of study. This evaluation opens the possibility to use glucosamine as food ingredient to create functional compounds that might exert antibacterial effect along with the heat processing.

Overall, this research demonstrated that glucosamine can be used as a potential functional ingredient. Its antimicrobial activity can be enhanced in food systems containing peptides and metals due to the formation of α-dicarbonyls and glycopeptides during heat processing. In addition, flavor compounds developed during the Maillard reaction progress in the presence of glucosamine may confer properties as salt taste modifier in foods. Therefore, the addition of glucosamine in food systems can play a dual role, as flavoring and antimicrobial functional ingredient. Our findings provide valuable information for further investigation in the functional properties of glucosamine and fish peptides to continue in the path of creating new alternatives of food preservatives and functional ingredients from food sources.

APPENDIX

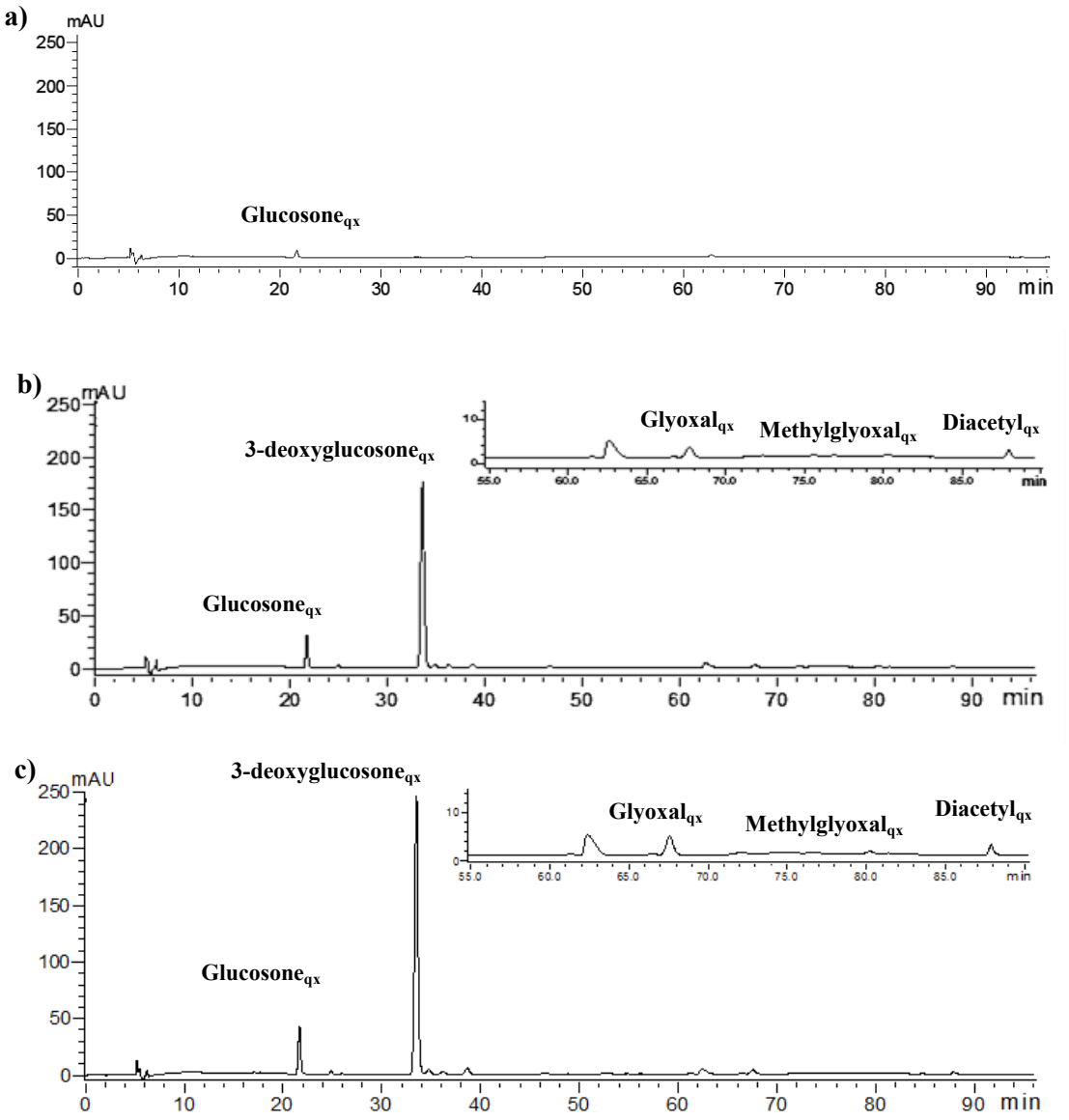


Figure A.1 UHPLC separation chromatograms of derivatized α-dicarbonyls from GlcN and GlcN/Fe^{2+} at 0 and 3 h of incubation. (a) GlcN α-dicarbonyls separation at 0 h of incubation, (b) GlcN α-dicarbonyls separation at 3 h of incubation, and (c) GlcN/Fe^{2+} α-dicarbonyls separation at 3 h of incubation.

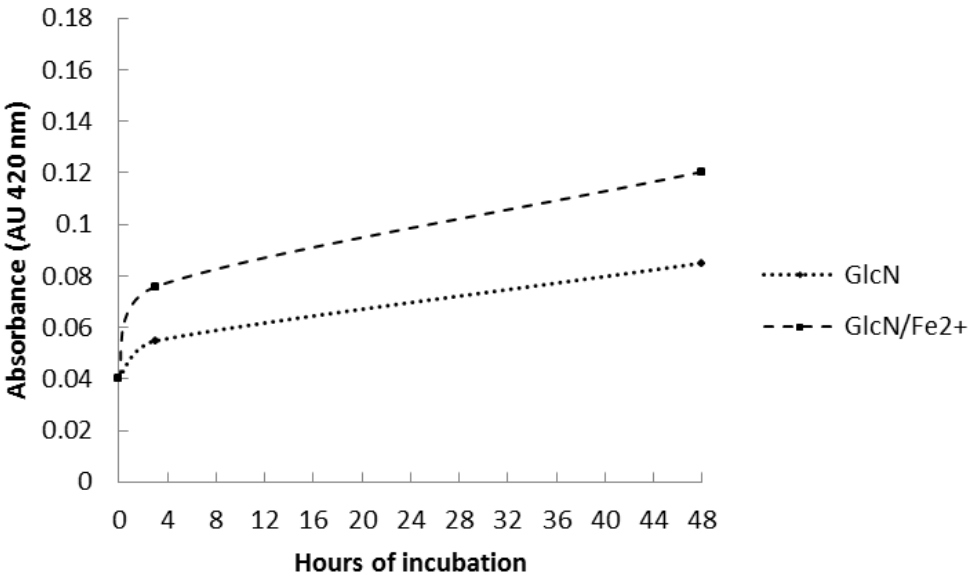


Figure A.2 Color changes at 420 nm in GlcN and GlcN/Fe^{2+} systems incubated at 50°C for 0, 3 and 48 h.

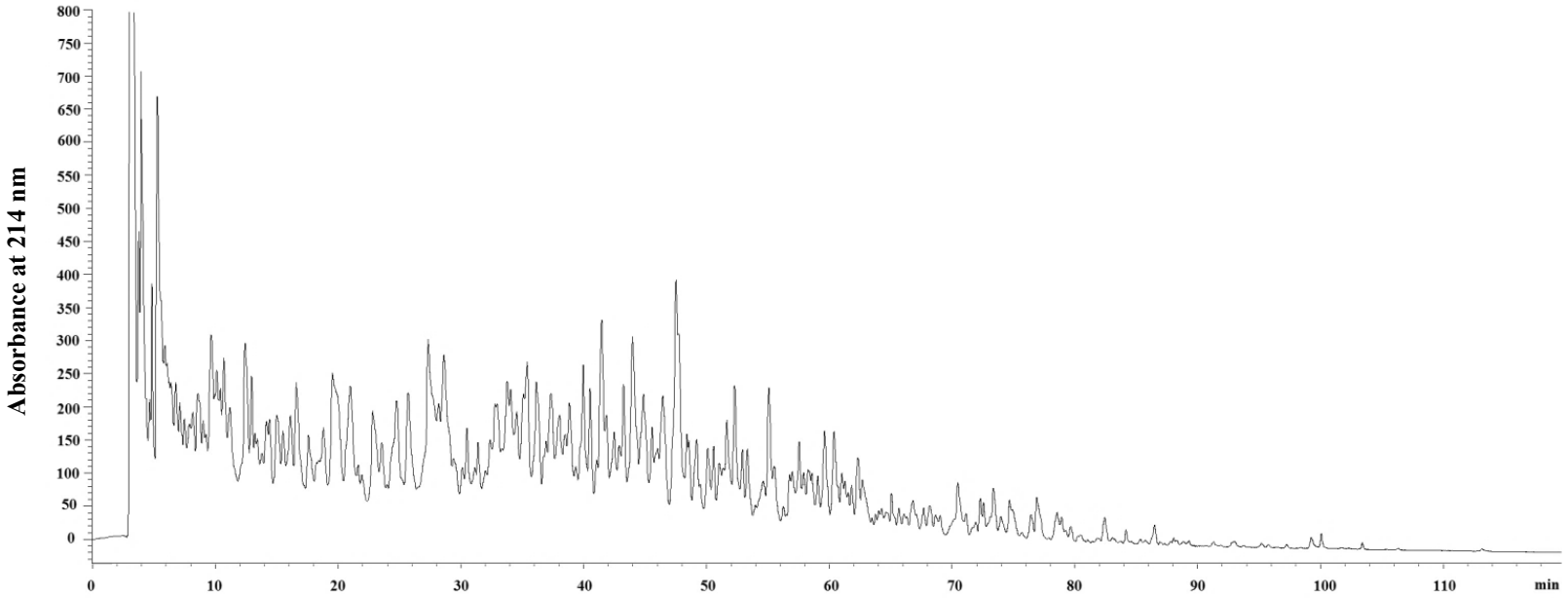


Figure A.3 Reverse phase chromatography profile of fish gelatin peptides by using alcalase.

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