Isotope Labelling Studies on the Reactivity of Nα- and Nε- of Lysine in the Presence of Glucose and its Degradation Products

PLAMEN YOULIEV NIKOLOV

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Department of Food Science & Agricultural Chemistry

McGill University

Montreal, Canada

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ABSTRACT

Isotope labelling technique in conjunction with pyrolysis Gas Chromatography/Mass Spectrometry (Py-GC/MS) was utilized to conduct an in-depth investigation into the formation of lysine specific products in the Maillard reaction. Following the pyrolysis of the lysine/glucose models including their labelled counterparts for 20s at 250°C and extensive data analysis it was concluded that lysine can generate piperidine, a reactive secondary amine capable of undergoing Maillard type interactions. The formation of piperidine has been postulated to follow two pathways depending on whether lysine pyrolysis is conducted in the presence or absence of sugars. In the presence of glucose, lysine similar to asparagine and phenylalanine can undergo carbonyl-assisted decarboxylative-deamination reaction to generate Nε-pent-4-ene-1-amine, which is the counterpart of acrylamide - a known food toxicant. Nε-pent-4-ene-1-amine has been shown to cyclize into piperidine. Specifically labelled precursors such as [^{15}N-α]lysine.2HCl, [^{15}N-ε]lysine.2HCl, [U-$^{13}C_6$]lysine.2HCl, [$^{13}C_{-6}$]lysine.2HCl and [U-$^{13}C_6$]glucose were used to confirm the potential adducts of Nε-pent-4-ene-1-amine and piperidine in the model systems which lead to the characterization of two piperidine and one Nε-pent-4-ene-1-amine derivatives. Products simultaneously possessing Nε nitrogen atom and five carbon atoms from lysine (C2' to C6') in addition to either 3 or 6 glucose carbon atoms were targeted for this analysis. The mechanism of formation of the two piperidine derivatives involved the chemical activation of piperidine with formaldehyde followed by aldol addition. The reactivity of piperidine was further demonstrated through detection of various pyridine derivatives postulated to be formed after oxidation reactions. During the course of this study it was also observed that in the presence of lysine, the

glucose moiety was converted into 5-hydroxymethyl-furfural (HMF) and 5-methylfurfural (MF). Analyses of HMF/lysine and glucose/lysine models using high resolution TOF-MS/MS and Py-GS/MS have indicated the formation of Schiff base adducts of HMF with Nε-pent-4-ene-1-amine and piperidine, respectively. The latter being a secondary amine was shown to undergo further stabilization through a vinylogous Amadori rearrangement (vAR) process. Reaction models consisting of HMF with primary and secondary amino acids such as glycine and proline, further confirmed the observed trend that primary amines generated Schiff base adducts and secondary amines resulted in the formation of covalent adducts through vAR. In the absence of amino acids, HMF was discovered to form a dimer through a newly proposed mechanism. The subsequent degradation of the HMF dimer was shown to generate MF and 2,5-furandicarboxaldehyde (FDA), important sugar-specific furans. Furthermore, HMF was shown to form glycosidic linkages with glucose and undergo chain elongation reactions. In addition, reaction of lysine with sugars other than glucose was also explored using ribose/lysine models. These models led to the discovery of furfurylamine, a ribose-specific reactive intermediate whose furfuryl-pyrrole derivatives have been detected in a number of different foods as aroma compounds. The furfuryl-pyrroles were also detected in the model systems generating furfurylamine such as ribose/lysine and in various roasted coffee beans. The formation mechanism of furfuryl-pyrroles was postulated to involve a double adduct of furfurylamine with 3-deoxyribose which was characterized using isotope labelling techniques.

RÉSUMÉ

La formation des produits dérivés de la lysine lors de la réaction de Maillard est analysée par l'entremise d'une technique utilisant des traceurs isotopique en combinaison avec la pyrolyse couplée à la chromatographie en phase gazeuse et spectrométrie de masse (Py-CG/SM). En étudiant la pyrolyse de différents modèles de lysine/glucose ainsi que celle de leur traceurs isotopiques pendant 20s à 250°C, il appert que la lysine peut générer de la pipéridine, un aminé secondaire très réactif pouvant aussi participer à des interactions de type Maillard. Deux mécanismes de formation de la pipéridine ont été démontrés, variant selon la présence ou l'absence de sucres lors de la pyrolyse de la lysine. En présence de glucose, tout comme l'asparagine et la phénylalanine, la lysine peut subir une déamination decarboxylative lieé à un groupement carbonyle, afin de générer le Nε-pent-4-en-1-amine, ce produit étant un homologue de l'acrylamide, un élément toxique alimentaire reconnu. Il a été démontré que le produit Nε-pent-4-en-1-amine peut se «cycliser» afin de former la pipéridine. Des précurseurs isotopiquement marqués tels que [^{15}N-α]lysine.2HCl, [^{15}N-ε]lysine.2HCl, [U-$^{13}C_6$]lysine.2HCl, [^{13}C-$_6$]lysine.2HCl et [U-$^{13}C_6$]glucose ont été utilisés afin de confirmer les composés d'addition potentiels de Nε-pent-4-en-1-amine et de la pipéridine dans les systèmes modèles permettant la caractérisation de deux dérivés de la pipéridine et un dérivé du Nε-pent-4-en-1-amine. Les produits ciblés lors des analyses possédaient un atome d'azote de type Nε et cinq atomes de carbone provenant de la lysine (C2' à C6') ainsi que 3 ou 6 atomes de carbone provenant du glucose. En bref, le mécanisme de formation des deux dérivés de la pipéridine implique l'activation chimique de la pipéridine avec le formaldéhyde suivi d'une addition de type aldol. La réactivité de la pipéridine fut démontrée davantage lors de la détection de plusieurs dérivés de la pyridine quiont été formés suite à des réactions

d'oxydation. De plus, il fut aussi observé au cours de l'étude que la présence de la lysine favorisait la conversion du glucose en 5-hydroxymethylfurfural (HMF) et 5-methylfurfural (MF). Des analyses comparatives de modèles HMF/lysine et glucose/lysine à l'aide d'un TOF-MS/MS à haute résolution et du Py-CG/SM ont indiqué la formation de composés d'addition de base de Schiff du HMF avec le Nε-pent-4-ene-1-amine et la pipéridine. Étant donné que le deuxième composé d'addition est un aminé secondaire, il peut se stabiliser davantage par l'entremise du processus de réarrangement vinylogue d'Amadori (vAR). De plus, la réaction de systèmes modèles combinant le HMF avec des acides aminés primaires et secondaires comme la glycine et la proline confirment qu'il y a une tendance pour que les aminés primaires générant des composés d'addition de base de Schiff et les aminés secondaires mènent à la formation de composés d'addition covalents par le processus de vAR. En l'absence d'acides aminés, cette étude démontre que le HMF forme un dimère par l'entremise d'un nouveau mécanisme proposé. La dégradation subséquente du dimère produit deux furanes spécifiques aux sucres, soit le MF et le 2,5-furandicarboxaldéhyde (FDA). Cette étude démontre aussi que le HMF forme des liens glycosidiques avec le glucose et participe à des réactions d'élongation de la chaine. De plus, la réaction de la lysine avec des sucres autres que le glucose fut aussi explorée en utilisant des modèles de ribose/lysine. Ces modèles ont permis de faire la découverte du furfurylamine, un intermédiaire réactif spécifique au ribose produisant plusieurs dérivés de furfurylpyrrole qui ont été détectés en tant que composés aromatiques dans plusieurs aliments. Dans les systèmes modèles produisant du furfurylamine (par exemple ribose/lysine), ces furfurylpyrroles furent aussi détectés tout comme dans des grains de café rôtis. Le mécanisme de formation des furfurylpyrroles

proposé implique un double composé d'addition du furfurylamine avec le 3-deoxyribose qui fut caractérisé à l'aide de marqueurs isotopiques.

STATEMENT FROM THE THESIS OFFICE

In accordance with the regulations of the Faculty of Graduate Studies and Research of McGill University, the following statement from the Guidelines for Thesis Preparation is included:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

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As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

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When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.

CONTRIBUTION OF AUTHORS

This thesis is presented in manuscript format and consists of eight chapters. A general introduction in Chapter 1 of the Maillard reaction and its significance is presented. The rational, experimental methodology and significance of the investigation are outlined. Chapter 2 provides in depth exploration of the benefits, and detrimental consequences of the Maillard reaction and one of its most reactive precursors - lysine. Chapters 3, 4, 5, 6 and 7 are based on published manuscripts. Chapter 8 encompasses a brief summary of the contributions to knowledge of this investigation. Connecting paragraphs provide rational in order to bridge the concepts of sequential chapters. This dissertation is in accordance with guidelines for thesis preparation as published by the Faculty of Graduate Studies and Research of McGill University.

The present author was responsible for the concepts, design of experiments, experimental work, and manuscript preparation in all the published and submitted papers. Dr. Varoujan A Yaylayan, the thesis supervisor, had direct advisory input into the work as it progressed and as manuscript co-author critically edited the dissertation prior to its submission.

PUBLICATIONS

Nikolov, P.Y. & Yaylayan, V.A. (2010) Formation of pent-4-en-1-amine, the counterpart of acrylamide from lysine and its conversion into piperidine in lysine/glucose reaction mixtures. *Journal of Agricultural and Food Chemistry*, 58, 4456-4462.

Nikolov, P.Y. & Yaylayan, V.A. (2010) Chemical activation of piperidine by formaldehyde and formation of lysine-specific Maillard reaction products. *Food Chemistry*, 123, 684-690.

Nikolov, P.Y. & Yaylayan, V.A. (2011) Reversible and covalent binding of 5-(hydroxymethyl)-2-furaldehyde (HMF) with lysine and selected amino acids. *Journal of Agricultural and Food Chemistry*, 59, 6099 - 6107.

Nikolov, P.Y. & Yaylayan, V.A. (2011) Thermal decomposition of 5-(hydroxymethyl)-2-furaldehyde (HMF) and its further transformations in the presence of glycine. *Journal of Agricultural and Food Chemistry*, 59, 10104-10113.

Nikolov P. Y. & Yaylayan V.A. (2012) The role of ribose-specific marker furfurylamine in the formation of aroma active 1-(furan-2yl-methyl)-1H-pyrrole (or furfuryl-pyrrole) derivatives. *Journal of Agricultural and Food Chemistry*, 60, 10155-10161.

CONFERENCE PRESENTATIONS

Plamen Y. Nikolov. Student competition. CIFST/AAFC joint meeting, Charlottetown, PEI, Canada, May 26-30, 2008.

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LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

ABBREVIATIONS

1-DG	1-Deoxyglucosone
3-DG	3-Deoxyglucosone
3-DR	3-Deoxyribosome
AC	Amadori Compound
AGE	Advanced Glycation Endproducts
Amu	atomic mass units
CML	Carboxymethyllysine
FDA	2,5-Furandicaroxaldehdye
FP	Furfuryl-pyrrole
FFP	Furfurylformyl-pyrrole
FTIR	Fourier Transform Infrared Spectroscopy
GC/MS	Gas Chromatography / Mass Spectrometry
HAA	Heterocyclic aromatic amines
HMF	5- Hydroxymethyl-furfural
IARC	International Agency for Research on Cancer
MF	5- Methylfurfural
MR	Maillard reaction
MRP	Maillard reaction product
MS/MS	Tandem Mass Spectrometry
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
PhIP	2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine
Py-GC/MS	Pyrolysis-Gas Chromatography/Mass Spectrometry
SMF	5-Sulfoxymethyfurfural
vAR	vinylogous Amadori rearrangement
vARP	vinylogous Amadori rearrangement product
TOF MS/MS	Time of Flight Tandem Mass Spectrometry

CHAPTER 1: INTRODUCTION

1.1 General introduction

The Maillard Reaction is a widespread phenomenon in foods encompassing a myriad of reactions that occur during processing. In food the Maillard reaction is practically ubiquitous, especially in high temperature treated foods or foodstuffs exposed to prolonged storage periods. The Maillard reaction generates desirable colours and aromas, essentially infusing unpleasant tasting raw foods with attractive broiled, baked and roasted flavours (Ledl & Schleicher, 1990).

Evidence of the negative effects of the Maillard reaction has been accumulating since its discovery a hundred years ago. Initially, the undesired impact of the Maillard reaction on foodstuffs involved limiting bioavailability of minerals, loss of vitamins and essential amino acids, which lowered the protein quality in processed foods (Erbersdobler & Dummer, 1971). Changes to protein functionality including solubility, heat stability, and emulsifying properties have also been observed (Chevalier et al., 2001a). The Maillard reaction has been demonstrated to take place in the human body. The resulting compounds known as advanced glycation endproducts (AGEs) accumulate with aging and have been correlated with the propagation of a series of chronic disease for example: diabetic complications, kidney failure, and artherosclerosis. Some of these AGEs have also been isolated in foodstuffs suggesting a potential negative biological outcome from the consumption of browned food. Furthermore, the Maillard reaction has been also shown to generate a number of compounds with cytotoxic, mutagenic and carcinogenic characteristics, which are referred to as processed induced toxicants. Common processed induced toxicants include most heterocyclic aromatic amines, furan, acrylamide, styrene, and 5-hydroxymethyl-furfural. As a result of the concern raised by Maillard generated

toxicants the European Union facilitated a comprehensive project to investigate processed induced toxicants. Known as HEATOX (Heat-induced food toxicants, identification, characterization and risk minimization), the project thus far identified approximately 800 compounds resulting from both the Maillard reaction and lipid oxidation (Cotterill et al., 2008).

The Maillard reaction is also associated with a number of beneficial outcomes. Heat treatment is one of the most commonly applied methods for achieving increases to both shelf life and food hygiene through destruction of spoilage-causing bacteria and foodborne pathogens, respectively. The Maillard reaction has also been demonstrated to increase the antioxidant activity, sensory qualities and digestibility of browned food (Borrelli et al., 2003). For example, one of the largest epidemiological studies of coffee, involving 400 000 participants, has observed that although roasting of the coffee bean can generate a number of detrimental compounds, overall daily consumption of coffee lowered the risk of death by 10% (Freedman et al., 2012). Furthermore, some Maillard reaction products (MRP) that have been detected have antiallergenic, antimutagenic, and chemoprotective properties (Lindermeier et al., 2002; Marko et al., 2003).

Although the Maillard reaction has been known for nearly a century, a number of outstanding questions remain unresolved. That is because the Maillard Reaction is an umbrella term that incorporates a large body of unique chemical reactions that occur simultaneously and impact one another. As a result a comprehensive understanding of the reaction behaviour of the reactive intermediates in the Maillard reaction is not yet achieved (Finot et al., 1990). Structural elucidation of large food polymers generated by the Maillard reaction is another area of challenge due to analytical difficulties presented

by the diverse polymeric structures. The large number of unknowns in the Maillard reaction makes a risk/benefit analysis inconclusive. Yet mitigation strategies are crucial for food processors that need to optimize desirable or beneficial components of the Maillard reaction while reducing undesirable or detrimental counterparts. Mitigation strategies can be generated through the continued identification of novel MRP and elucidation of their mechanism of formation.

1.2 Experimental methodology

A number of techniques have been developed that aid in Maillard reaction investigations. Model systems have been in use since Hodge (1953) to simplify analytical requirements for the process of identifying novel MRP. Gas Chromatography / Mass Spectrometry (GC/MS) has revolutionized the detection and characterization of flavour compounds making it possible for the creation of comprehensive compound databases such as the approximately 900 compounds detected in coffee (Buffo & Cardelli-Freire, 2004). A further augmentation of GC/MS is the integration of a pyrolysis unit (Py-GC/MS) that allows for the simultaneous reaction, separation and identification of novel volatile compounds generated in Maillard model systems (Huyghues-Despointes et al., 1994). A pyrolysis unit functions in tandem with GC/MS to provide a thermally controlled micro-reaction chamber to induce chemical reactions and simulate dry Maillard reaction conditions that food encounter when roasted, grilled or baked. Operating at temperature ranges of 150-1000°C pyrolysis can also be utilized for the structural determination as it can reproducibly break down large food polymers into analytically meaningful low molecular weight compounds (Irwin, 1982). Feasibility studies of the character impact compounds generated from aqueous and dry pyrolytic experiments demonstrated that

both conditions generate the same compounds, with identical formation mechanisms (Wnorowski & Yaylayan, 2000). Other techniques analyzing volatile compounds such as headspace and solvent extraction have significant drawbacks that include cumbersome protocols, and solvent induced analyte bias. The advantage of Py-GC/MS is the reduction of the sample preparation time and reaction due to direct injection of volatiles into gas chromatographer after pyrolysis. The need for solvents is also removed as the extraction steps are not required, thus eliminating solvent bias towards compounds of different solubility. Furthermore, in Py-GC/MS individual sample sizes are greatly reduced (approximately in the 1mg weight range) making investigations using expensive labelled precursors feasible.

Isotopic labelling is an identification technique most often utilized in the study of the Maillard reaction to identify the number of carbon and nitrogen atoms in unknown compounds (Tressl et al., 1993a; Keyhani & Yaylayan, 1996). Having the unambiguous number of carbon and nitrogen atoms is a great aid for initial postulations of hypothetical structures of novel MRP. Isotopic labelling can also be utilized as a tracking technique as it can monitor the movements and positions of a target atom within a compound of interest, and thus aid in the elucidation of the mechanism of formation (Yaylayan & Keyhani, 1999; Schieberle, 2005). Isotope labelling techniques are a key strategy to improving the understanding of Maillard reaction by providing important elemental information and mapping of formation mechanisms of unknown compounds.

1.3 Research rationale and objectives

Within the Maillard reaction one of the most reactive amino acids is lysine (Ledl & Schleicher, 1990). Lysine is an essential amino acid that experiences significant

reductions in food during processing or extended period of storage (Bujard & Finot, 1978; Mauron, 1981; Ramirez-Jimenez et al., 2004). This property has made some thermal derivatives of lysine useful as markers of thermal processing or for extended storage (Erbersdobler & Somoza, 2007). The epsilon side chain greatly outnumbers terminal α-amino groups, making it the highest amine source in proteins and also the most modified (Ledl & Schleicher, 1990). The reactivity of lysine and its ability to form advanced glycation endproducts has made it an important candidate for investigating the reaction tendencies of amino acid, peptides and proteins in the Maillard reaction (Henle, 2005).

The majority of literature on the selectivity of the lysine amino groups is focused on pyrazine formation through reactions of Nα (Hwang et al., 1994; Van Lancker et al., 2010). However, much of lysine's reactivity in the Maillard-type reactions is attributed to the epsilon group rather than the Nα (Ledl & Schleicher, 1990; Van Lancker et al., 2011). The epsilon amino group has led to the formation of a number of recently discovered unique lysine degradation products (Murata et al., 2007; Totsuka et al., 2009). However, the reaction mechanisms of a number of these compounds have not been elucidated. Furthermore, the reaction behaviour of lysine-specific compounds has not been the subject of a direct study. Lastly, the loss of lysine due to processing is still larger than the total sum of the known lysine specific degradation products generated in foodstuffs. Henle (2005) has predicted that a large number of unidentified lysine-specific products remain undetected and uncharacterized.

An important product of the Maillard reaction is 5-hydroxymethyl-furfural (HMF), which is one of the most common sugar thermal degradation products in food and its chemical reactivity in foodstuffs is not well explored. HMF is biologically important as it has been

observed to have negative consequences in certain in vitro experiments, including weak mutagenicity. Some HMF derivatives in food have demonstrated a clear negative impact on health, in particular 5-sulfoxymethyfurfural (SMF), which has been shown to be carcinogenic (Monien et al., 2009). HMF concentration in honey is legislated to an upper maximum of 40mg/kg (FSA, 2005). However, the mean range of HMF positive foods is between 1-200mg/kg, with the highest detected concentrations exceeding 3g/kg in certain dried fruits (Bachmann et al., 1997; Abraham et al., 2011). HMF concentrations have been demonstrated to never stabilize in food stored over long periods of time (Ferrer et al., 2005), with the continually observed increase and decrease in concentration resulting from various formation and degradation reactions, with the latter type not well explored.

The main objective of this investigation is therefore to explore, in the presence of reducing sugars or relevant sugar degradation products, lysine-specific thermal degradation products and their mechanisms of formation in order to gain further understanding of the potential reaction behaviour of this amino acid in the Maillard reaction utilizing isotopically labelled precursors, chemical synthesis, Py-GC/MS analysis and other techniques. The specific objectives are:

(1) Elucidation of lysine-specific degradation products in the presence of the most common reducing sugar in food – glucose. Glucose is one of the most frequently used sugars in model system studies of the Maillard reaction.

(2) Investigation of the impact of 5-hydroxymethyl-furfural (HMF) – an important glucose degradation product - in lysine degradation.

(3) Investigation of the reactivity of HMF in the absence of amino acids and generality of the lysine/HMF reactions in the presence of other amino acids.

(4) Investigation of lysine reaction with more reactive pentose sugars such as ribose.

1.4 Significance of the proposed research

Although research into the Maillard reaction has been ongoing since its discovery over a century ago, basic questions about the chemical nature and the biological significance of different MRPs in food remain unresolved. Lysine is an essential amino acid and also one of the most reactive amino acids in the Maillard reaction, thus justifying an in depth understanding of the detailed reaction mechanism of lysine particularly the differences in the reactivity of two amino groups. Furthermore, the nature of lysine-specific products is heavily influenced by the type of the reducing sugar, thus rationalizing the investigation of lysine reaction with different sugars and sugar degradation products. In addition, HMF is one of the most abundant MRP in food, it has a detrimental biological impact and its mechanistic fate during food processing is even less studied than that of lysine. Together lysine and HMF represent arguably the most reactive and abundant degradation precursors in food, respectively. Comprehensive knowledge of the mechanistic behaviour of these two Maillard reaction components alone or in the presence of other reactants is crucial for understanding their role during food processing. Such knowledge may offer food processors essential information for alternative product formulation solutions and/or

mitigation strategies to optimize beneficial and reduce detrimental effects of lysine and HMF specific Maillard reaction products in food.

CHAPTER 2: LITERATURE REVIEW

2.1 The Maillard reaction

When food is subjected to thermal processing or cooking a series of chemical reactions takes place, as a result the colour of the food changes and aroma is generated, this process is referred to as non-enzymatic browning. Within non enzymatic browning the most dominant reaction is the Maillard reaction (Nursten, 2005) and it has become well known that this vast and complex sequence is responsible for generating the rich and complex characteristic flavours, colours and smells of cooked food (Ledl & Schleicher, 1990). When it comes to processed foods the Maillard reaction is ubiquitous, especially in high temperature processed foods or food exposed to prolonged storage periods (Henle et al 2005). The Maillard reaction is initiated by an interaction between a reducing sugar and an amino acid, although many different reactions are involved in the process. The interaction between the amine and carbonyl functional groups is not limited to high temperature settings and its occurrence at ambient conditions has been demonstrated, albeit at much lower rates (Abrams et al., 1955).

Non-enzymatic browning is composed of three separate reaction schemes including the Maillard Reaction, caramelisation, and ascorbic acid oxidation. Caramelisation reactions only occur in reducing sugars, with significant quantities of products forming at elevated temperatures. Coincidentally, some caramelisation reactions that occur with sugar alone can also take place in the presence of sugar and an amino acid, but at much higher concentrations (Hollnagel & Kroh, 2002). The third reaction, ascorbic acid oxidation, is unique because at times it relies on enzymes such as ascorbic acid oxidase, although the enzymes are not required for the reaction to proceed (Nursten, 2005). While the Maillard reaction differs from the above mentioned reactions because it takes place between

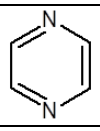
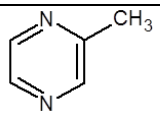
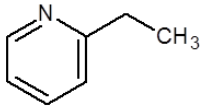
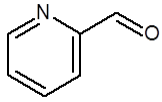
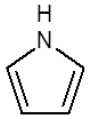
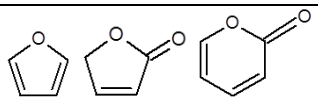
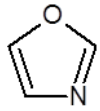
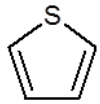
carbonyl compounds, typically being supplied by a reducing sugar, and an amine, from amino acids, peptides, or proteins.

2.1.1 Colour and flavour

One of the largest areas of influence of the Maillard reaction to food industry is in its organoleptic contributions to aroma, flavour and colour. The control of the desirable and undesirable aromas obtained from Maillard reaction during cooking, roasting, baking, and grilling has been of great interest to the flavour/food industry. Determination of the volatile profile of foodstuffs is a complex challenge due to the large number of unique and heterogeneous compounds that can be present in the released aroma of foodstuffs. The utilization of gas chromatomography/mass spectrometry, which has greatly augmented the characterization of various compounds in food aroma, has aided for example in the identification of up to 900 volatile substances in the aroma of coffee (Buffo & Cardelli-Freire, 2004). Some important characteristic coffee aroma compounds generated from the Maillard reaction includes: 2-furfurylthiol, 3-mercapto-3-methylbutylformate, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, and 2-ethyl-3-dimethylpyrazine. In studies with other foods the volatile profile of cooked chicken meat have indicated the presence of over 300 compounds with 14 that have been determined to be key aroma compounds, including 2-methyl-3-furanthiol and decadienal which have meat-like and fatty taste descriptors, respectively. Some of the primary odourants in wheat and barley bread are acetyl-tetrahydropyridine and acetyl-pyrroline (Grosch & Schieberle, 1997), while boiled beef is characterized by methanethiol and dimethyl sulphide (Kerscher & Grosch, 2000). An experiment studying the aroma profiles of the precursors of the Maillard reaction used models composed of any combination of twenty

one amino acids and eight sugars mixed in 1:1 ratios and heated between 100 to 220°C. Aroma descriptors from five to seven of the amino acids were toast, crusty biscuit, bread and cake. In general Maillard reaction generates a wide variety of compounds that have important flavour profiles (Table 2.1).

Table 2.1 Common categories of flavour and aroma compounds found in the Maillard reaction (adapted from van Boekel 2006)

Compound category	General structure	Flavour/aroma	Food examples
Pyrazines		Cooked, roasted, toasted, baked cereals	Heated foods in general
Alkylpyrazines		Nutty, roasted	Coffee
Alkylpyridines		Green, bitter, astringent, burnt	Coffee, barley, malt
Acylpyridines		Cracker-like	Cereal products
Pyrrole		Cereal-like	Cereals, coffee
Furans, furanones, pyranones		Sweet, burnt, pungent, caramel-like	Heated foods in general
Oxazoles		Green, nutty, sweet	Cocoa, coffee, meat
Thiofenes		Meaty	Heated foods

Colour change is an important attribute of the Maillard reaction. The degree of browning is typically measured using absorbance readings which can act as indicators for the extent of the Maillard reaction (Ashood & Zent, 1987). Colour compounds produced from the Maillard reaction are often referred to as melanoidins. The term melanoidins is used to

differentiate from chromophores generated by enzymatic browning, which are known as melanins. Created in the final stage of the Maillard reaction, melanoidins are the result of the polymerization of the reactive intermediates. In practice, the ability to identify and quantify different melanoidins is still largely a work in progress because of the complexity and chemical intractability of dark brown compounds formed in food (Wang et al., 2011). A diverse range of functional groups can be incorporated in browning polymers including furans, carbonyl, carboxyl, amine, amide, pyrrole, indole, azomethine, ester, ether and hydroxyl (Ledl & Schleider, 1990; Ames et al., 1993; Tressl et al., 1998). Melanoidins have also been perceived as cross-links between non-coloured high molecular weight biopolymers, such as proteins, that act as a scaffold for low molecular weight chromophores (Hofmann, 1998).

2.1.2 Negative outcomes

2.1.2.1 Changes to functionality

Decades of Maillard reaction literature has demonstrated that non enzymatic browning of food generates a large number of detrimental effects. One of the most common changes as a result of food processing is in the destruction or the biological inactivation of amino acids, especially essential amino acids such as lysine. In milk, lysine reacts with lactose and the subsequent lysine-Amadori product (fructosyllysine) cannot be utilized as a dietary lysine source (Erbersdobler & Dummer, 1971). The Maillard reaction may inhibit absorption of undamaged amino acids by denaturing digestive enzymes or inhibiting amino acid transport. It particular, studies have found that severely browned milk samples were less readily hydrolyzed by trypsin compared to control (Gothwal & Bhavadasan, 1991), with a digestibility loss of 14-88% in casein-glucose mixtures

(Culver & Swaisgood, 1989). Similar blockages of hydrolysis sites used by trypsin and carboxypeptidase β for enzyme digestion were observed in heated glucose poly-lysine models (Hansen & Millington, 1979). In clinical study with adolescent participants, diets rich in Maillard browning significantly reduced dietary protein digestibility, including a 47% increase in fecal nitrogen excretion (Seiquer et al., 2006). The Maillard reaction also has an impact on several functional properties of food protein. Heating β-lactoglobulin in the presence of a number of reducing monosaccharides at 60°C for 3 days resulted in the modification of the solubility and increasing the heat stability (Chevalier et al., 2001a). The emulsifying properties were improved with arabinose or ribose, and the foaming capacity was augmented with glucose or galactose. Similar conclusions were reached in the emulsifying capacity and emulsion stability from the interaction between the polysaccharide galactomannan (mannose skeleton and galactose side chains) and ovalbumin, lysozyme and α-lactalbumin (Nakamura et al., 1992; 1994). Lastly, improvements in the gel strength and elasticity of soybean proteins reacted with xylose had been postulated to be the result of increased crosslinking via Maillard interactions (Cabodevila et al., 2006).

2.1.2.2 Advanced glycation endproducts (AGEs)

In the late 1960s the first non-enzymatically glycated protein, a glycated hemoglobin in the human body, was discovered (Rahbar et al., 1969). The formation of sugar adducts on proteins under physiological conditions would become one of the major contributors to the expansion of the interest in the Maillard reaction to outside the domain of food. In 2005, Fay and Brevard displayed an increase in interest of the Maillard reaction and its impact on health. Through searching abstracts in Medline and Chemical databases using

the terms “Maillard” and “Food” the authors found that the occurrence had increased from below 5 to 139 during 1965 to 2003.

Some of these investigations into the glycation of proteins with sugars have lead to the discovery of a number of compounds that have been categorized as advanced glycation endproducts, abbreviated as AGEs. The term glycation was introduced to distinguish the Maillard process from the enzymatic equivalent known as glycosylation (Ledl & Scheicher, 1990). AGEs are a heterogeneous group of compounds that are formed by the Maillard reaction through irreversible crosslinking between reducing sugars and the side chains on protein, which are limited to the epsilon and guanidine groups of lysine and arginine, respectively (Henle, 2005). A very important distinction that literature often fails to properly address is the existence of two types of AGEs. The first represents irreversible crosslinks occurring between proteins and sugars in the body, this is known as *in vivo* or physiological AGEs. The second type is an AGEs not formed in the body but inside thermally processed foodstuffs, and are referred to as food or dietary AGEs (Henle, 2005).

In general terms physiological and dietary AGEs can be considered as counterparts because both are formed from the Maillard reaction. However, significant differences exist between physiological and dietary AGEs. The formation conditions are quite dissimilar with *in vivo* AGEs formed over a longer time, at body temperature, and at physiological pH. Food AGEs are typically the result of thermal processing that is characteristic of high temperature and relatively short heating times. Next, most physiological AGEs are bound onto proteins while, dietary AGEs can be formed freely inside the food matrix (Henle, 2005). Furthermore, dietary AGEs must pass the digestive

system and be absorbed by the body. For example, a study reporting a 10% absorption rate of dietary AGEs from heated fructose and egg white meals (Koschinsky, 1997). Furthermore, dietary AGEs access into the body is complicated by their metabolic handling during digestion. Thus, physiological and dietary AGEs must be regarded as different entities.

The physiological AGEs are controversial because their accumulation during aging has been correlated to the pathogenesis of a variety of chronic disease. It is now well established that AGEs are independent risk factors in the complications stemming from diabetes mellitus (nephropathy, retinopathy), kidney diseases, atherosclerosis, cataracts, diseases associated with aging, arthritis and neurodegenerative diseases such as Alzheimer's (Bengmark, 2007). A review of AGE biological impacts in animal and human studies conducted by Sebekova and colleagues (2007) discovered 29 and 7, respectively, unique negative biological effects associated with physiological AGE. Some examples of these effects include: enhanced oxidative stress, vascular dysfunction, renal pathology and shorter survival. In diabetic complication specifically protein glycation and AGEs are implicated because their accumulation is correlated with alteration of intracellular signaling, gene expression, release of pro-inflammatory molecules, and free radicals. Generation of free radicals from AGEs has been shown to fragment proteins and oxidize nucleic acid (Vlassara & Striker, 2011). Glycation of protein can be viewed from the perspective that it belongs to the set of naturally occurring reactions in the body, since the precursor protein and sugars are in abundance. However, recent discoveries have concluded that food-derived AGEs can contribute significantly to AGE-related disorders in the body (Sebekova et al., 2007).

2.1.2.3 Dietary advanced glycation endproducts

The similarity of physiologically formed protein glycation adducts to those generated by the Maillard reaction in food has resulted in a number of articles attempting to determine whether AGEs formed from thermal processing in food contribute to the accumulation of physiological AGEs in the body. Selected literature into the effects of exaggerated intake of thermally processed food has strengthened the connections between the two types of AGEs (Sebekova et al., 2007). Studies with rats found that animals fed diets containing high AGEs for 6 weeks had an increased plasma level of three different AGEs including carboxymethyllysine (CML, 32.9%), carboxyethyllysine (CEL, 24.5%) and pentosidine (82.4%) (Sebekova et al, 2005). While the AGE plasma concentrations were increased by 1.5 orders of magnitude in a similar study that fed rats for a 6 month duration (Sandu et al., 2005).

In clinical AGE trials twenty one volunteers were fed a protein diet which was either heated or unheated. Both diets contained similar composition of fat, carbohydrates and proteins. Individuals consuming the heated protein or AGE high diet had a significant increase of the CML serum level during fasting (Uribarri et al., 2005). Other human studies have also confirmed that AGEs circulating in the plasma were influenced by the consumption of heated fructose and egg white meals, with 10% of these dietary AGEs being actually absorbed (Koschinsky et al., 1997). A recent study tested the impact of reducing the AGE content of food by 50% on forty healthy individuals and nine individuals with kidney diseases. After four months of consumption, as much as 60% decline was observed in blood AGE levels, lipid peroxides, inflammatory markers, and biomarkers of blood vessel health. The improvements were observed to a similar degree in both healthy individuals and kidney patients (Vlassara et al., 2009). The adverse

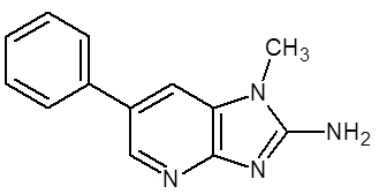
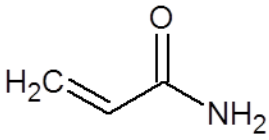
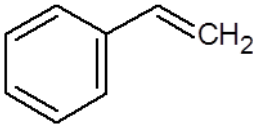
influence of AGEs is most pronounced in diabetic individuals. An ethical clinical trial involving human diabetic patients divided the participants into AGE high and low diets. Diabetic patients in the high AGE group diet had on average of 64.5% increased plasma AGE levels, while the low AGE diet group had a 30% decrease of their plasma AGE concentration. There were also further increases in inflammatory markers and markers of vascular dysfunction in the high dietary AGE group (Vlassara et al., 2002). Another study has demonstrated that significant changes of the above parameters in diabetic patients can be detected after the consumption of only a single high AGE meal (Negrean et al., 2005).

2.1.2.4 Processed induced toxicants

The Maillard reaction has been attributed for the generation of a large number of compounds in foods that are considered processed induced toxicants (Cotterill et al., 2008). The impact is based on discoveries of cytotoxic, mutagenic and carcinogenic characteristics of MRP. For example, tests for mutagenicity using *S. typhimurium* TA100 were positive for instant and caffeine-free coffee as well as a number of teas (Nagao et al., 1979). In a clinical study consisting of a cohort of 170 individuals diagnosed with stomach cancer and 500 without, it was concluded that habitual consumers of medium well or well done beef had a threefold increased risk of stomach cancer relative to individuals consuming it rare or medium-rare (Ward et al, 1997). The risk was doubled when individuals consumed beef more than 4 times a week. The cause of this health outcome is attributed to heterocyclic aromatic amines (HAA), which are carcinogenic (Bogen et al., 1994). In animal studies the consumption of 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine or PhIP (Table 2.2), one of the most studied HAA,

induces tumours in 50% of the animals at doses as low as 31.3mg/kg/day of the animal (Stavric et al., 1994).

Table 2.2 Processed induced toxicants formed by the Maillard reaction

PhIP	Furan	Acrylamide	Styrene
			

One common toxicant found in food is furan (Table 2.2), which is carcinogenic to rats and mice and is classified as 'probably carcinogenic to humans' by the International Agency for Research on Cancer (IARC, 1995). In animal models, consumption of a dose of 30mg/kg furan for 13 weeks resulted in the detection of tumours, while lower doses caused loss of body weight (NTP, 1993). Quantification of furan content has been done in a large number of foods, in particular baby foods, canned vegetables, fruit, meat, pasta sauces and coffee (Crews & Castle, 2007). At a limit of quantification of 5µg/kg many of the tested foods contained furan, with the highest being canned or jarred vegetables such as beans, squash, and sweet potato. A concerning finding was that nearly all baby foods contained furan. Some foods not cooked in closed containers such as - potato crisps, crackers and crisp breads had substantial levels of furan ranging from 20-200µg/kg (Hoenicke et al., 2004). The estimated dietary exposure was based on a limited quantity of data however it was particularly pertinent to baby foods since it can constitute the sole diet source of some infants. Approximate consumption for a 6 month old baby weighing 7.5kg ranged between <0.2 to 26µg furan/day or 0.03 to 3.5µg/kg bw/day. The daily intake for adults was approximated to 2.4 to 111µg/person, 1.1 to 23 µg/person, and 1.3-

50 μg/person from coffee, canned vegetables and beer, respectively (Crews & Castle, 2007).

In 2003 another type of process induced toxicant, acrylamide Table 2.2, was discovered in a number of processed foods including: potato chips, french fries, bread, and cereals (Svensoon et al, 2003). This was a concern because this process induced toxicant has been demonstrated by many studies to be an animal and human neurotoxin, a reproductive toxicant, and a carcinogen in rodents and thus considered "probably carcinogenic to humans" by the IARC (European Commission, 2002; Shipp et al., 2006, IARC 1994). Consistent estimates of dietary exposure to acrylamide have been challenging due to the great variations in population consumption habits. The current range has been suggested to be from 0.5μg/kg body wt to 2.0μg/kg body wt, with outliers in the 99^{th} percentile consuming up to 5.1μg/kg body wt (Dybing et al., 2005; WHO, 2005). One of the main pathways of formation of the heat-induced contaminant acrylamide is through the Maillard reaction of free asparagine. The yield of acrylamide is greatly enhanced in the presence of reducing sugars where asparagine experiences a carbonyl assisted thermal decarboxylation and deamination (Yaylayan et al., 2003). The requirement of the carbonyl source justifies the observation of elevated levels of acrylamide in reducing sugar rich foods such as fried potatoes and bakery products (Svensoon et al, 2003). Another process-induced toxicant is styrene, and it is generated from phenylalanine and reducing sugars, Table 2.2. Evidence from mutagenic and carcinogen tests has also lead to a "probably carcinogenic to humans" label for styrene (Cotterill et al, 2008). The formation similarities between acrylamide and styrene have allowed them to be categorized as vinylogous compounds (Goldmann et al, 2009). The

potential of other amino acids to form vinylogous compounds through the Maillard reaction has not been thoroughly explored, limited to a study with aspartic acid, glutamine and glutamic acid (Stadler et al., 2003).

2.1.3 Benefits of the Maillard reaction

The dilemma of the Maillard reaction is that it generates compounds that are either harmful or beneficial to human health. MRP have been shown to have antioxidant activity. A study that investigated the antioxidant activity of lysine models with three sugars (glucose, fructose, and ribose) generated at 120°C, for 2 hours and divided the resulting MRP into low and high molecular weight fractions. Using several different antioxidant or radical scavenging tests it was demonstrated that all lysine-sugar models induced equivalent protection, with the high molecular weight fraction possessing greater antioxidant activity relative to lower molecular weight fractions (Chen & Kitts, 2011). *In vitro* investigations using protein (gluten) and glucose model and a biscuit matrix found strong antioxidant activity from soluble melanoidins (Borelli et al., 2003). However, a number of other *in vitro* studies have detected antioxidant activity in Maillard reaction products (Shizuuchi and Hayase, 2003; Daglia et al., 2004). *In vivo* investigations with rats consuming coffee melanoidins observed an increased antioxidant capacity in their plasma, with the effect attributed to N-methylpyridinium (Somoza et al., 2003). The impact of the antioxidant properties of Maillard reaction products on human consumption is unclear. However, a recent epidemiological study examined the association between mortality and coffee consumption, one of the highest sources for Maillard reaction products in our diet. After adjusting for confounding variables it was concluded that coffee consumption in the cohort of nearly 400 000 older participants (50-71years of age)

during a thirteen year period lowered the risk of death by approximately 10% (Freedman et al., 2012). Furthermore, the consumption of coffee lead to an inverse association of mortality from heart disease, respiratory disease, strokes but not for deaths from cancers. However, a number of Maillard reaction compounds have been found that exhibit potential chemopreventive activity (Lindenmeier et al., 2002) and inhibiting tumor cell growth (Marko et al., 2002; Marko et al., 2003).

2.2 Mechanism of the Maillard reaction

Even though the initial reaction between sugar and amino acids was discovered by Louis Maillard the reaction scheme that is associated with the Maillard reaction was produced in 1953 by John E. Hodge. The coherent scheme has been augmented and updated in Figure 2.1. For simplification purposes in the past, the Maillard reaction has been segmented into three sections, what was known as the early, advanced, and final stages. In brief, the early stage encompassed the formation of a Schiff base from a carbonyl-amine interaction, followed by Amadori rearrangement. In the advanced stage the products that were formed began to degrade leading into an array of compounds that may react further with themselves. The final stage of the Maillard reaction sees the polymerization of the reactive intermediates to form the large molecular weight compounds melanoidins. The three stages occur simultaneously and will exert influence over each other (Friedman, 1996).

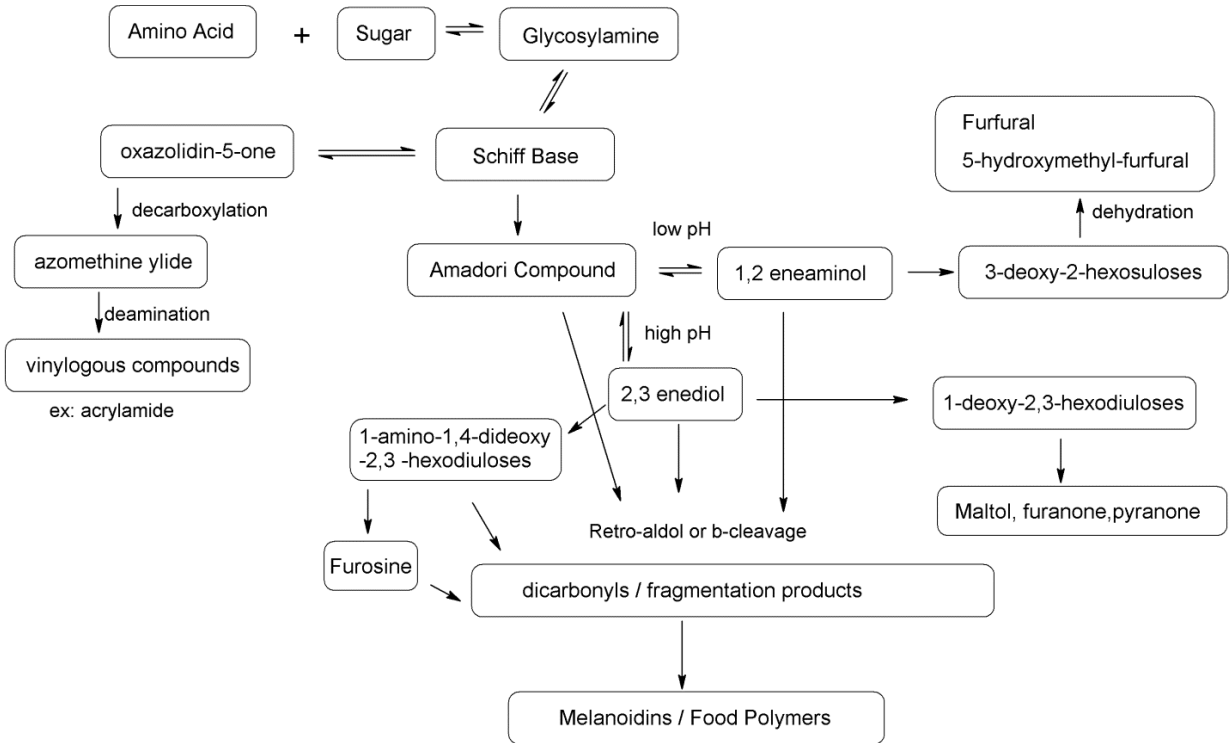


Figure 2.1 Generalization of the Maillard reaction (adopted from Hodge, 1953; Tressl et al., 1995; Yaylayan, 2009)

2.2.1 Amadori formation

The Amadori compound (AC) is an important precursor because it is typically the main pathway of sugar and amino acid degradation in food and under physiological conditions (Yaylayan & Huyghues-Despointes, 1994). The basic mechanistic steps in the formation of the AC were determined in 1930s by Kuhn and Dansi. The initial stage of the Maillard reaction begins with the interaction of a reducing sugar and an amino group. This stage can only proceed when an aldose sugar molecule is in its open chain form. The interaction between the anomeric position of the aldose and the amino group yields the chair confirmation carbonyl-amine intermediate glycosylamine. N-glycosylamine is unstable and subsequent dehydradation will generate the Schiff's base in Figure 2.2 with the equilibrium generally favouring the reversal of the intermediate back into the starting precursors (Ge & Lee, 1997). The enol will stabilize and undergo ring closure generating

1-amino-1-deoxy-ketose (AC). Amadori rearrangement is an acid catalyzed process, with the amino acid acting as a sufficient internal acid catalyst. Recent evidence suggests that Schiff base formation and not the Amadori rearrangement is the rate-limiting step in a phenylalanine-glucose model, thus potentially raising the importance of pre-Amadori intermediates (Ge & Lee, 1997).

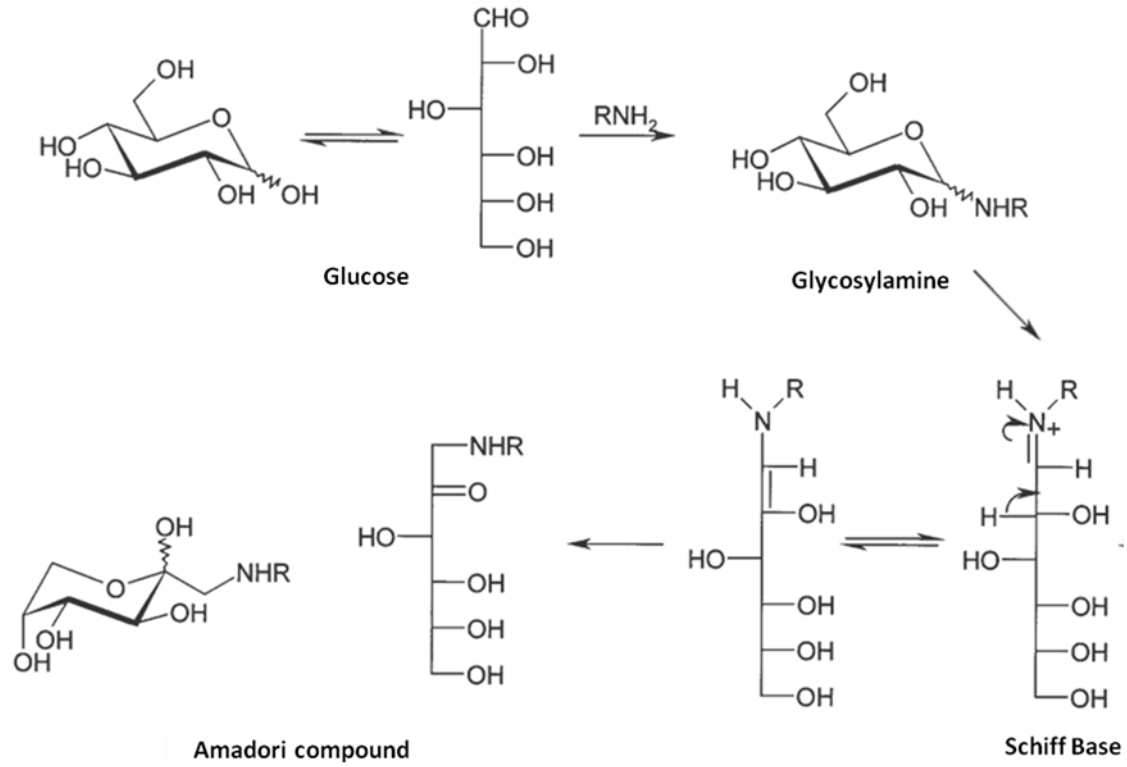


Figure 2.2 Formation of the Amadori compound from aldohexose sugar and amine (adapted from Wrodnigg & Eder, 2001)

The type of sugar, aldose or ketose, will dictate whether the rearrangement leads to Amadori or Heyns products, respectively. The Amadori rearrangement involves the enolization of N-glycosylamine to 1-amino-2-deoxy-2-ketose (AC), which represents a transformation from aldose, such as glucose, to a ketose. On the other hand Heyn's rearrangement in Figure 2.3, takes a ketosylamine and following an enolization reaction the 2-amino-2-deoxyaldose, also known as Heyns product, is formed. Thus, the Heyn's

rearrangement converts a ketose, such as fructose, to an aldose, contrary to the Amadori rearrangement. Furthermore, Heyns product can interconvert to the Amadori product in the presence of free amino acids through a pathway similar to the Lobry de Bruyn-Alberda van Ekenstein transformation of glucose into fructose. Overall, the reaction rate of Heyns product is slower than Amadori compound in the absence of an amino acid (Pilkova et al., 1990).

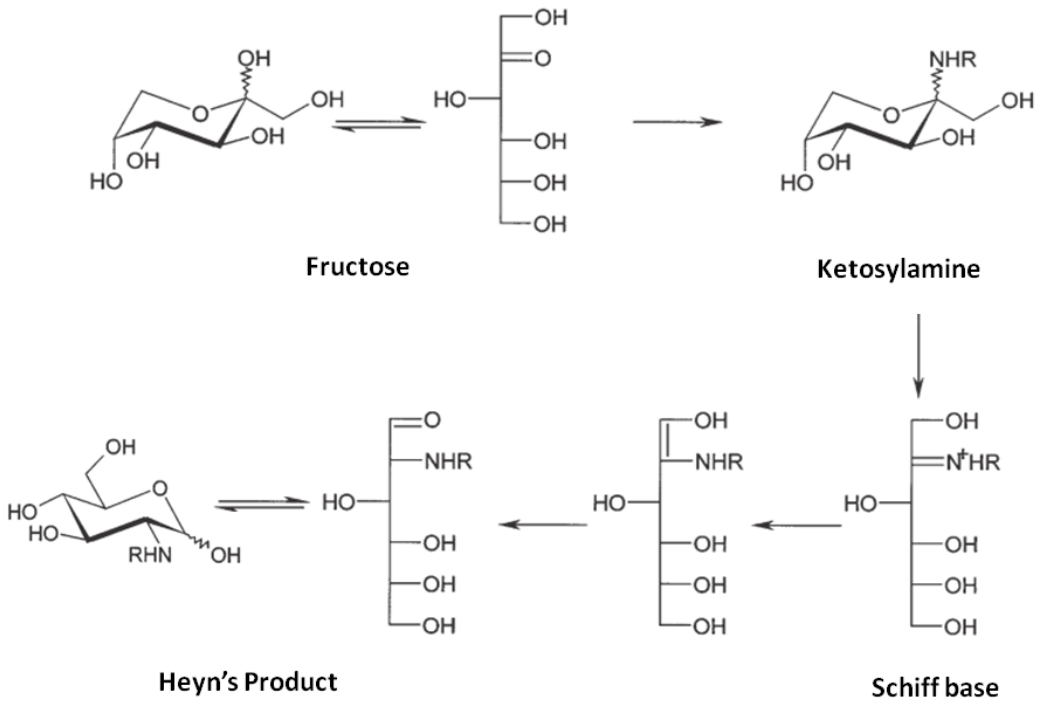


Figure 2.3 Formation of the Heyns product from ketohexose sugar and amine source (adapted from Wrodnigg & Eder, 2001)

2.2.2 Fragmentations of the Amadori intermediate

The study of the mechanisms of degradation of Amadori compounds is an integral part of the Maillard reaction because it offers one of most efficient pathways, due to lower activation energy, for the degradation of sugars and amino acids (Yaylayan & Forage, 1991). The most characteristic AC transformation is the ability to undergo enolization,

with the main possibilities being 1,2 or 2,3-enolizations. A low pH will lead to the 1,2-enolization route that will generate 3-deoxy-2-hexosulose (3-DG). A high pH favours 2,3-enolization, which yields 1-deoxy-2,3-hexodiulose (1-DG) in Figure 2.4. The 1,2 enolization pathway leads to creation of an enaminol moiety, an arrangement consisting of a double bond and aldehyde functional groups, occurring between the C-1 and C-2. The reaction pathway continues with a β-elimination of hydroxyl group on C-3 giving the double bond between C-2 and C-3, which destabilizes the amine bond becoming susceptible to hydrolytic scission resulting in the formation of 3-deoxy-2-hexosuloses, better known as 3-deoxyglucosone (3-DG) (Anet, 1964). 3-DG has been detected *in vivo* in human urine and plasma (Knech et al., 1992). In foodstuffs 3-DG is an important intermediate leading to the formation of furfurals such as 5-hydroxymethyl-furfural, with a total loss of three water molecules, and eventually melanoidins in the presence of amines.

At basic pH the electron density is increased at the C-1 sugar residue, decreasing the likelihood of 1,2 enolization (Anet, 1964). Instead the 2,3-enolization pathway occurs, resulting in an enediol formation occurring between C-2 and C-3 of the sugar residue. The double bond formation will instigate the elimination of the C-1 amino group, which after enolization will yield 1-deoxyglucosone (1-DG). However, an elimination occurring on the C-4 hydroxyl group can instead produce 1-amino-1,4-dideoxy-2,3-hexodiulose, a reactive dicarbonyl intermediate (Feather, 1989). 1-DG is an important intermediate that can lead to the formation of an array of compounds, including α-dicarbonyls, acetylfurnan, furanones, and reductones (Feather, 1989). Reductones differ from furfurals as they only lose two water molecules, compared to the 3 lost in furfural formation, and

they also contain the –C(OH);C(OH)- function. Reductones are important because they tend to account for some of the reductive power generated by the Maillard reaction.

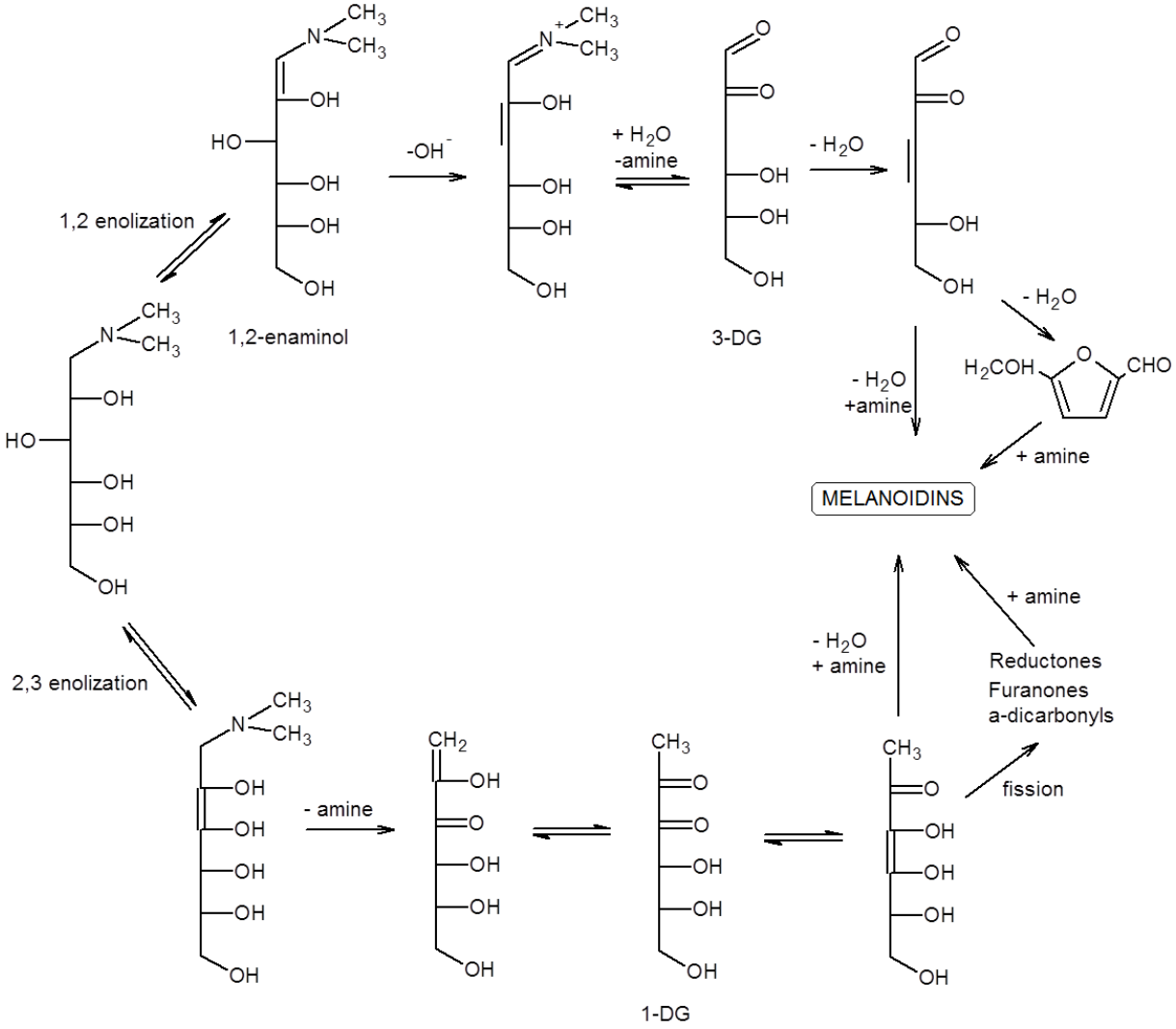


Figure 2.4 Two main mechanism of sugar dehydration (adapted from Nursten, 2005)

Amadori rearrangement can be perceived as a distabilization pathway for sugars, paving the way for their heterogeneous decomposition. The main mechanism through which this is carried out is retro-aldolisation, with oxidative fission potentially having a role as well (Weenen & Apeldoorn, 1996). The fragmentation of sugars derivatives results in the formation of a handful of different derivatives. The cleavage that generates these

derivatives is often categorized as C_5/C_1, C_4/C_2, and C_3/C_3. Often referred to as α-dicarbonyl and α-hydroxymethylcarbonyl, large numbers of permutations of these compounds have been found in model systems and food. One of the main variables that influences the type of dicarbonyls generated in a system is the nature of the precursors (Weenen & Apeldoorn, 1996).

2.2.3 Melanoidin formation

What is often referred to the final Maillard reaction stage involves the condensation between aldehyde and amine intermediates and the formation of brown pigments, melanoidins. This stage relies on breakdown products from the previous stages, which react with each other resulting in an array of compounds (Strecker decomposition). Upon further decomposition these compounds, especially α,β-unsaturated aldehydes, can polymerize at low temperatures into high molecular mass compounds, often of unknown structure and variable solubility. In fact the constitution of melanoidins often varies depending on the nature of the starting materials. For example, melanoidins formed from furfural and glycine have been described as having high ether content. Those from glucose and glycine have high alcoholic hydroxyl content. Models from 2-oxopropanal and glycine have produced melanoidins with high enolic hydroxyl and low ether characteristics (Nursten, 2005).

Temperature has a strong influence on the profile of melanoidins. Benzing-Purdie and colleagues (1985) incubated xylose and glycine at three different temperature and time conditions; - 22°C for 9 months, 68°C for 6 weeks, and 100°C for 38h. After the application of dialysis with a membrane cut-off of 12kDa it was demonstrated that higher temperatures or longer periods of incubation increased the non-dialysable characteristics

of the melanoidins. Although it seems that the impact of temperature on the amount of high molecular mass compounds is much more significant than that of time. The study also concluded that the incubation resulted in the loss of material, which was mainly H_2O and CO_2, with this loss being much greater as temperature rose: 54% vs. 30% loss at 100°C and 68°C, respectively. When it came to determining the influence of time on melanoidin development, a study was able to make two main conclusions using ^{15}N-NMR. Firstly, the increase in incubation times was mirrored by an increase in unsaturated, carbonyl, and total carbon in the formed melanoidins. Secondly, it was demonstrated that the number of pyrrole moieties increased.

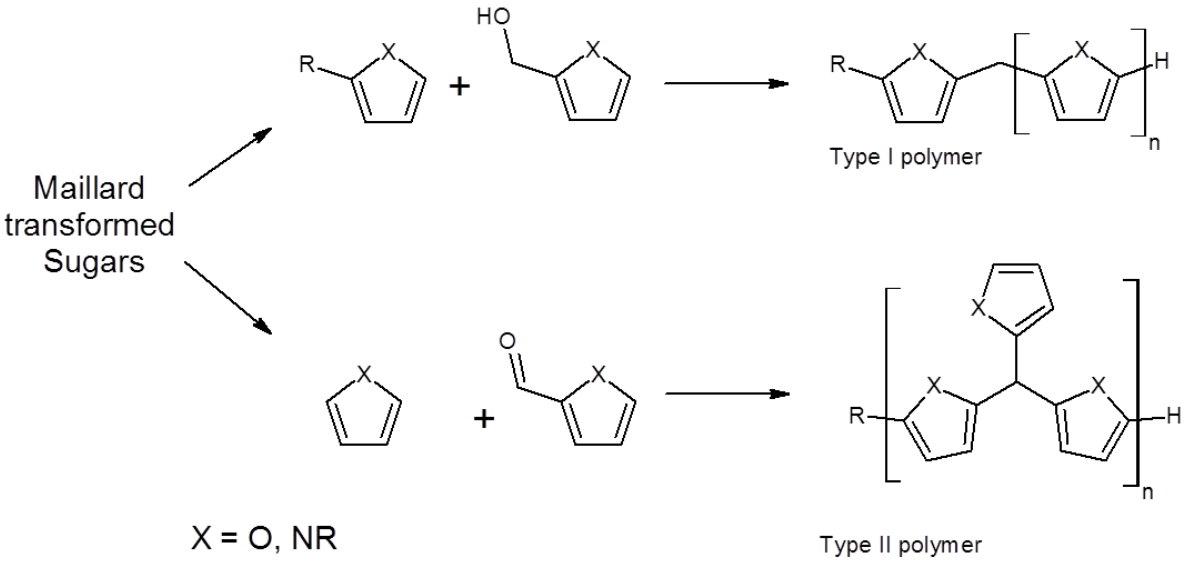


Figure 2.5 A proposed mechanism for the formation of melanoidins (Tressl et al., 1998a; Tressl et al., 1998b)

The events surrounding the formation and prorogation of the polymers that become melanoidins have many unanswered questions. One stumbling block has been the wide range of potential precursors involved in melanoidin formation. To illustrate the importance of precursors on length of melanoidins and monomeric ratios, different

incubation studies have been conducted (Tressl et al., 1998a; 1998b). When a mixture containing N-methylpyrrole and N-methyl-2-formylpyrrole was incubated a branched oligomer (or Type II polymer) of 26 units was found (Figure 2.5). Substitution of the methylamine with the methyl ester of β-alanine resulted in the multiple formation of oligomers up to a tetradecamer, a complex of four subunits, in size. Mixtures of furfural with N-methylpyrrole generated branched oligomers of more than 30 units, with the ratios of the precursor in the oligomers being 1:2, 2:3, and 3:4, respectively. When N-(2-methoxycarbonylethyl)-pyrrole was mixed with furfural, the oligomers identified were branched and reached up to 25 units in length. However, mixtures of N-methyl-2-formylpyrrole and 2-methylfuran did not result in the incorporation of the methylfuran in the oligomeric structure. Another important parameter is the water content of a mixture. Macromolecules formed in aqueous-heating mixtures of glucose and glycine were water soluble, with a tiny percentage having a molecular mass higher than 3kDa. Furthermore, polymers formed under dry-heating conditions generated a significant proportion, 13.6%, with a molecular mass higher than 30kDa (Hofmann, 2001).

2.3 Pyrolytic and aqueous model systems for studying the Maillard reaction

One of most successful strategies used to elucidate Maillard reaction products and their mechanism of formation is the model systems approach. Food is a very complex and heterogeneous matrix that generates too many volatile from too many precursors for current analytical methodologies to unambiguously characterize. Model systems investigate the interactions between simple precursors found in food to determine the reaction behaviour, for example by isolating two compounds a reducing sugar and an amino acid to study the Maillard reaction. Model systems consisting of two or more

precursors can still generate large numbers of MRP and have numerous advantages including simplifying the analytical requirements for isolation and characterization of compounds. Compared to the number of compounds generated in heated foods model systems result in reducing the complexity and amount of volatiles, thus increasing the chance of successful identification (Parliament, 1989).

A pyrolysis unit functions as a thermal micro-reaction chamber for volatile materials that is suitable to investigate the Maillard reaction. A pyrolysis unit can work in tandem with a GC/MS providing controllable heating source with a range of 150-1000°C. The thermal environment induces chemical reactions but also aids in the structural determination of compounds by decomposing large complex molecules into smaller and thus more analytically useful fragments (Irwin, 1982). Pyrolysis has been applied to rapid characterization of whole foods including roasted coffee, tea, biscuits, chocolate and milk powder (Halket et al., 1988) and beef and soy protein (Raghavan et al., 1986). Pyrolysis has been used on a variety of amino acid and sugar models in determining reaction products and mechanisms in the Maillard reaction (Huyghues-Despointes et al., 1994; Keyhani & Yaylayan, 1996). A study comparing the key aroma notes from dry and aqueous cysteine and ribose/rhamnose models displayed that the most important aroma compounds were present under both conditions, with most concentration increases observed in dry models (Schieberle & Hofmann, 1998). Feasibility studies comparing the aqueous and pyrolytically generated Maillard products agree with Schieberle & Hofmann, 1998 that in general pyrolytic or dry conditions generally created a higher number of products. This is due in part by the removal of oxygen and its substitution with helium in the pyrolytic chamber. Most of the products identified in the aqueous model are also

identified in the pyrolytic models and more importantly also share a matching label distribution, Table 2.3 (Yaylayan & Keyhani, 1999).

Table 2.3 Comparison of ^{13}C label distribution in compounds from pyrolytic or aqueous conditions.

Compound	Pyrolysis (250°C, 20s)	Aqueous
Methylpyrazine[b]	0%	0%
2,5-dimethylpyrazine[b]	0%	0%
2,6-dimethylpyrazine[b]	0%	0%
2-ethyl-5-methylpyrazine[b]	72% at C-2 of ethyl	70% at C-2 of ethyl
3,5-diethyl-2-methylpyrazine[b]	65% s, 23% d at C-2 of ethyl(s)	70% s, 23% d at C-2 of ethyl(s)
1-(1'-pyrrolidinyl)-2-propanone[c]	45% at C-3	40% at C-3
2-acetylpyrrole[d]	97% at C-5	98% at C-5
1-formyl-5-methylpyrrole[d]	98% at CHO	98% at CHO

[b] Incorporation of C-3 atom in model system heated at 180°C, pH 5.6, for 7 min (Amrani-Hemaimi et al., 1995).
[c] Incorporation of C-1 atom at 150°C, 1.5 h, in water (Tressl et al., 1993b).
[d] Incorporation of C-1 atom at 150°C, 1.5 h, in water (Tressl et al., 1995).

This observation indicates a similarity of formation mechanisms under the two reaction phases. This hypothesis was confirmed with a direct comparison of the ^{13}C label incorporation in glucose-glycine models reacted by both pyrolytic (250°C for 20 seconds) and aqueous (120°C for 3 hours) conditions. Each of the nine compounds in the aqueous model had an identical mechanism of formation as their counterparts in the pyrolytic models (Wnorowski & Yaylayan, 2000). Furthermore, such observations suggest that mechanistic conclusions determined in pyrolytic models can be applicable to aqueous models.

2.4 Isotope labelling technique for the study of the Maillard reaction

Isotopically labelled molecules are compounds that have one or more of their atoms replaced by a stable isotope of the same element. The commonly used stable isotopes in

Maillard models are ^{13}C and ^{15}N, which allows the unambiguous determination of the number of labelled atoms incorporated in an analyte typically through the use of mass spectrometry (Schieberle, 2005). For example, an isotopic labelling experiment involves having a standard model reacted under Maillard-type conditions and a model where one of the precursors of the former model has a carbon replaced by a ^{13}C isotope and reacted under the same conditions. Following the analysis of the analytes in question an increase in the molecular mass by one unit is a proof of the incorporation of a single labelled atom in the analyte of interest. In this manner, isotopic labelling can aid in the determination of mechanistic pathways of novel compounds as it traces the location of particular atoms during the formation of MRPs. Furthermore, having precursors with all their carbon or nitrogen atoms labelled, for example $^{13}U6$-glucose or ^{15}N-glycine, allows for the complete isotope mapping of the carbon and/or nitrogen atoms in unidentified MRPs (Schieberle, 2005).

The first investigations to utilize stable isotopes in the study of the Maillard mechanism were done in the 1990s. For example, 4-aminobutyric models reacted with ^{13}C-1 labelled glucose, arabinose and fructose were used to elucidate the intermediates and formation pathways of a number of pyrroles and 2-pyrrolidones (Tressl et al., 1993a). Hwang and colleagues (1995 a, b) used a sugar and two amino acids model, where glycine was always kept standard while the other amino acid was rotated, to elucidate the formation of flavour compounds in Maillard models. By using ^{15}N glycine, the authors could determine the precise contribution that the glycine made to the resulting end products, in this case pyrazines. For example, in models with phenylalanine, glycine would only account for 29% of the nitrogen in the pyrazines, while with glutamic acid, glycine

contributed half. Label studies have also been used in amino acids that have two nitrogen groups to differentiate between the behaviour of the alpha and epsilon groups of lysine (Hwang et al., 1994). Label studies with carcinogenic compounds such as furans have determined the existence of multiple formation pathways including a mechanism during which the sugar skeleton remained intact and another where sugar C_2 and/or C_3 fragments merged together (Limacher et al., 2008). Using entirely labelled glucose ([U-$^{13}C_6$] glucose) the authors demonstrated that under roasting conditions in the absence of amino acids, furan formed from unbroken sugar backbone. However, with inclusion of amino acids, and their label counterparts [U-$^{13}C_3$] alanine, the major mechanism of furan formation changed and was the result of the addition of reactive C_2 fragments. These fragments, acetaldehyde and glycoaldehyde, are common in the Maillard reaction, however label isotopes demonstrated that their precursors were both sugars and amino acids.

Limacher et al., (2008) used a particular isotopic method known as CAMOLA or carbon module labelling. CAMOLA was designed to clarify the observation that even when starting with one precursor a number of different pathways can occur simultaneously and yield the same end product. For example, a study of the mechanistic origin of 2,3-pentanedione from glucose/alanine models found that five different mechanisms contribute to the formation of the dicarbonyl (Yaylayan & Keyhani, 1999). The principle of CAMOLE is to use a set ratio of unlabelled and fully labelled carbohydrate precursors. When a model is heated, sugars will undergo degradation and recombination resulting in a mixture of isotopemers of the target compound. Mass spectroscopic data and statistical analysis are used to discern the importance of each isotopemers, thus the significance of

the multiple mechanistic pathways involved in the formation of the same target compound can be determined (Schieberle, 2005).

2.5 Lysine

2.5.1 Nutritionally blocked lysine

Lysine is an essential amino acid, thus it must be obtained from the diet. The availability is particularly important for infants as their daily requirement for lysine is much higher than in adults, 103 and 12mg/kg/d, respectively. Thus, in western countries, processing techniques such as roller drying are no longer used for infant formula even though milk from cows has 20% more lysine than human milk (Bujard & Finot, 1978). The damage caused by processing techniques on lysine is displayed in Table 2.4, including a 50% loss during roller dried processing, with up to 80% reported in certain cases (Hurrell & Carpenter, 1981).

Table 2.4 The nutritional loss of lysine from different processing techniques (adapted from Bujard & Finot, 1978; Mauron, 1981)

Food Sample	%
Raw or freeze-dried milk	0
Pasteurised (74C, 40s)	0-2
HTST pasteurised (135-150, a few seconds)	0-3
HTST sterilized	5-10.
UHT	0-2
Spray dried powder	0-3
Sweetened condensed	0-3
Sterilised fluid	8-15
Roller dried (w/o precondensation)	10-15
Evaporated	15-20
Roller dried (precondensation)	20-50

The loss of lysine in cereal foods is particularly concerning to food processors because it is commonly the limiting amino acid and thus important for the overall protein quality of cereal products. In an example of a high shelf-life food, up to 2 years, such as infant cereals, lysine losses between 4.26-23.2% were observed, depending on storage temperature and maximum storage duration of 12 months (Ramirez-Jimenez et al., 2004). The blocking of lysine seems to occur during the formation of the Amadori product because lysine that is a part of ε-glycosylamine, before the onset of Amadori rearrangement, appears to be still nutritionally available (Mauron, 1981). To demonstrate the potential impact of lysine loss in an animal study, the impact of consumption of the heat damaged protein on the animal's growth was observed. Ingestion of the scorched skim milk powder, containing only 2.7% available lysine, lead to an improper weight gain in the rats, the main cause being inadequate available lysine. However, the potential impact of the newly formed lysine and Maillard reaction products on weight gain was not evaluated. The protein quality and weight gain of the animals was improved when the scorched skim milk powder was supplemented with lysine (Erbersdobler & Dummer, 1971).

Lysine's sensitivity to heat treatment has made the amino acid and its thermal derivatives prime candidates as markers of thermal exposure of foodstuffs. The Amadori products of lysine with fructose, lactose and maltose - fructosyl-lysine, lactulosyl-lysine and maltulosyl-lysine, respectively, have been suggested as possible control parameters for nutitional quality of foods (Erbersdobler & Hupe, 1991). One of the main modification products of lysine in processed foods is furosine. Furosine forms though an acid catalyzed conversion of the lysine-glucose AC (Nε-deoxyfructosyllysine) and its

correlation with lysine loss has been known for decades (Ferrer et al., 2003; Erbersdobler & Somoza, 2007). As a result, furosine has been used as an indicator for heat damage of protein during the thermal processing of foods (Erbersdobler & Somoza, 2007). In dairy products, furosine is among the main processing indicator compounds along with lactulosyl-lysine (Vinale et al., 1999). Combining furosine levels with lysine content, through amino acid analysis, has been used for the calculation of total and blocked lysine (Bujard & Finot et al., 1978). Furosine's application as an indicator of heat damage is not limited to dairy products. There has been varying successes achieved as a thermal marker in cereals, pastas, honey, and cheese (Resmimi & Pelegrino, 1994; Delgado-Andrade et al., 2007; Sanz et al., 2003). Although lysine loss and furosine gain are correlated, studies show that lysine loss is much greater than the parallel increase in furosine, thus lysine's thermal fate is much broader than furosine (Ferrer et al., 2003).

2.5.2 Lysine reactivity compared to other amino acids

Lysine is considered one the most reactive amino acids in the Maillard reaction, with its side chain known to be the most modified during the Maillard reaction involving proteins (Hwang et al., 1995a; Ledl & Schleicher, 1990). Ashoor and Zent (1987) studied the intensity of browning formation at 420nm as a result of the Maillard reaction among 21 amino acids, including hydroxyproline, and the reducing sugars glucose, fructose, ribose and lactose. Lysine was the second most reactive amino acid in generating Maillard browning behind glycine, with lysine having the highest absorbance relative to other amino acids in glucose models at 0.947. However, another study found lysine generated the highest colour intensity at 420nm in models with five different reducing sugars (maltose, fructose, glucose, arabinose, xylose), with the absorbance often being twice as

much as the next highest amino acid model. The study compared the reactivity of twelve amino acids (aspartic acid, glutamic acid, alanine, leucine, isoleucine, valine, proline, serine, cysteine, phenylalanine, arginine, and lysine) at 100°C for 3 hours (Kwak & Lim, 2004). Furthermore, authors found that glucose-lysine models had the least amount of glucose concentrations remaining after 3-12 hours incubations.

In a glucose and ^{15}N glycine investigation, reacted at 180°C for 1 hour, lysine was found to have the highest synergistic effect on the concentration and on the number of pyrazine derivatives (Hwang et al., 1995a). From the list of 7 other amino acids (glutamine, glutamic acid, asparagine, aspartic acid, arginine, phenylalanine, and isoleucine) the lysine models had the highest pyrazine concentrations in 9 out of 56 types of pyrazines, with the overall yield (μg/g of glucose) more than double in all the models studied except the next closest. A parallel study using glucose and a 50:50 mixture ^{15}N Glycine with the same list of eight amino acids, investigated the impact on the formation of pyridines, pyrroles and oxazoles. Among amino acids with nitrogen side chains lysine generated the highest totals in all three types of compounds, with aspartic having the highest overall amounts. Lysine models generated only a single pyridine, with the formation mechanism favouring α-amino groups. In regards to the generation of pyrroles and oxazoles, lysine models were the second highest contributors in both groups.

In the reaction four amino acids (glycine, lysine, cysteine and methionine) individually with ribose, lysine again demonstrated the highest quantities of nitrogen containing heterocyclic compounds such as pyrazines (Meynier & Mottram, 1995). As a result the organoleptic properties of lysine containing models were described as caramel, nutty, sweet and roasted. Lastly, Shinohara and colleagues (1983) investigated the mutagen

forming potential of 20 amino acids when heated for 100°C for 10h with various reducing sugars. By monitoring the mutation of *Salmonella typhimurium* TA100 and/or TA98 cells it was displayed that lysine models had one of the highest activities. Lysine's reactivity is a result of the combined nucleophilicity of its alpha and epsilon amine groups in free amino acids, and the epsilon amine in protein or bound lysine. The nonionized amino groups are strong nucleophiles, with pKa values for the alpha and epsilon being 8.95 and 10.53 (CRC, 1974) respectively, with the latter being the highest value for an amino acid side chain group. The only exception is arginine's strongly basic guanidino group with the nonionized version only found in small amounts at normal pH values (Creighton, 1984).

In proteins, the number of accessible amine groups from the epsilon side chain easily outnumbers the alpha terminal amino groups (Van Lancker, et al 2011). Lysine's epsilon side chain has the highest reactivity among amino acids with amine containing side chains. An investigation that compared the stability of protein bound tryptophan in different food models systems using alkaline hydrolysis and also in rat assays displayed that the losses of protein bound lysine were much higher than tryptophan (Nielsen et al., 1985). Particularly in oxidizing lipids and whole milk powder (reducing sugars) tryptophan had minor or insignificant losses, respectively, while lysine degradation was extensive. The reactivity of primary amines is higher than in secondary amines (Ledl & Schleicher, 1990) as has been observed in dipeptides containing proline (Van Lancker et al., 2012). Thus, it is not surprising that tryptophan and histamine are less reactive than lysine. Another amino acid with an amine containing side chain is arginine and it has a strongly basic 6-guanidino side group. To compare the impact of a second amino acid on

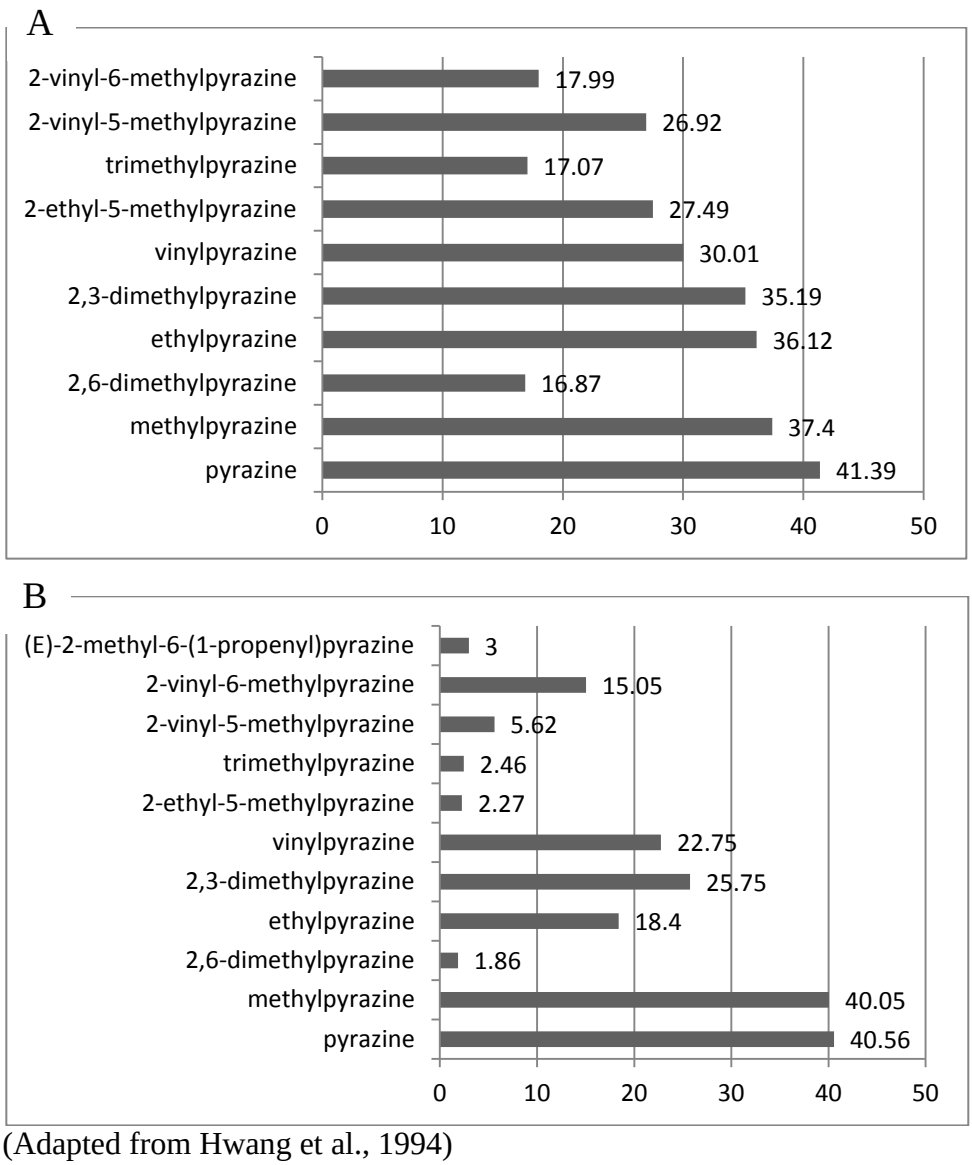
the pyrazine formation ability of glucose-glycine models a collection of amino acids, including arginine and lysine, were spiked into the original glucose-glycine model (Hwang et al., 1995a). The reactivity of glycine was decreased by the addition of arginine and increased in the presence of lysine. This result was rationalized to be due to the unreactive nature of the strongly basic side chain of arginine, with a pKa 12.5 (CRC, 1974). Furthermore, when Nα-acetylarginine was heated in the presence of glucose the concentration of the reducing sugar remained largely unchanged and no browning was observed. Thus, the guanidino side chain group cannot participate in the Maillard reaction unless it is in the presence of reactive Maillard degradation products such as α/β dicarbonyls. This was observed in newer findings demonstrating that arginine is a suitable trap for carbonyls such as glyceraldehyde (Usui et al., 2008) and dicarbonyls including glyoxal (Glomb, 2001) and methylglyoxal (Klopfer et al., 2011), resulting in the formation of imidazoline derivatives.

2.5.3 Reactivity of alpha and epsilon nitrogen atoms of lysine

Lysine has two amino groups that participate in a multitude of reactions in food during processing or long storage periods. A comparison between percent contributions of the two nitrogen groups towards the formation of pyrazines was investigated through a ^{15}Nα-isotopically labelled lysine (Hwang et al., 1994). After calculating the relative contribution from ^{15}Nα it was demonstrated that both α and ε participated. The study also analyzed changes to the relative contribution of each nitrogen source under two different model conditions: dry and aqueous at 180°C for 1 hour. In the dry model the pyrazine experiencing the highest contribution of Nε is the unsubstituted pyrazine at approximately 41%, while the most substituted pyrazine (2-ethyl-6-methylpyrazine) had

the least content at 18% (Table 2.5-A). It is clear that in all situations the Nε makes a smaller contribution than Nα. Furthermore, it is apparent that the degree of Nε input is significantly altered in nearly all pyrazines from the dry and aqueous models. The rational for the observed inequality of amine group contribution to pyrazine formation are outlined in the mechanism of formation in Figure 2.6.

Table 2.5 Relative contribution of percent Nε in formation of pyrazines in A = dry, B = aqueous models



(Adapted from Hwang et al., 1994)

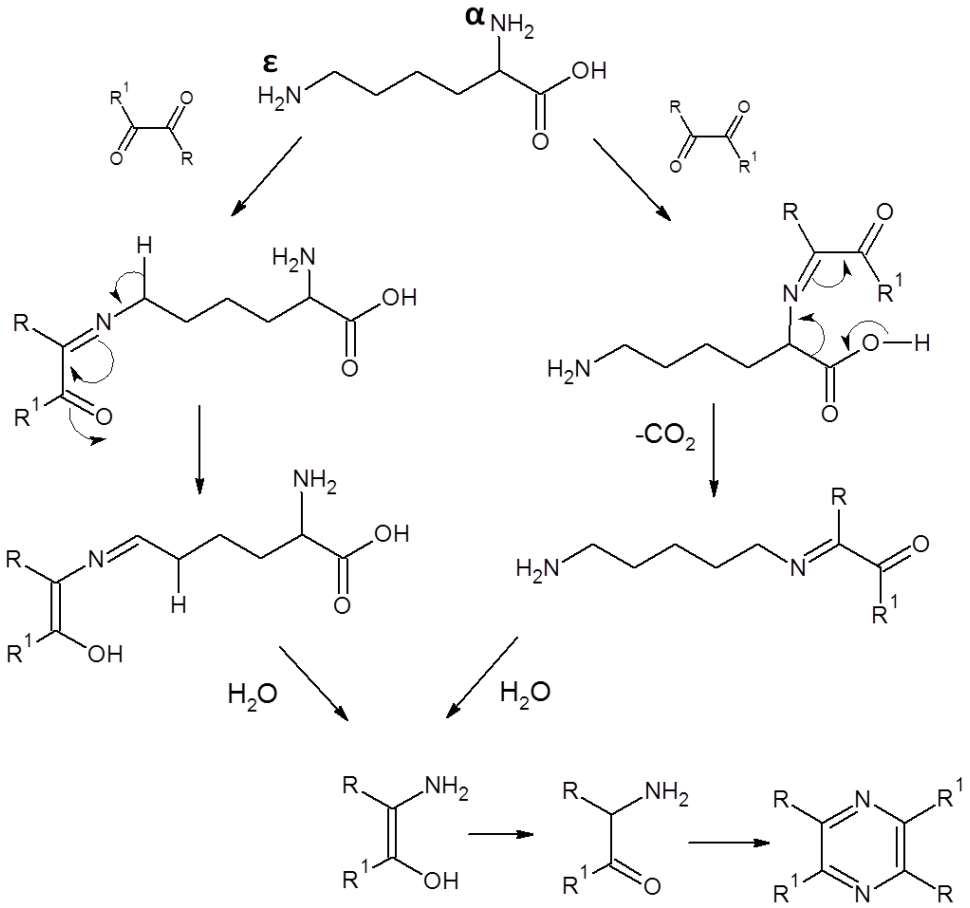


Figure 2.6 Proposed mechanism for pyrazine formation by both Nε and Nα (adapted from Hwang et al., 1994)

In short, it appears that the Schiff base formed from Nα is more efficient because it comes from α-aminoketones through Strecker degradation, while the Nε counterpart forms via intermolecular rearrangement and a hydration reaction. Also higher substituted pyrazines, 2,6-dimethylpyrazine and trimethylpyrazine have the lowest contribution from Nε because these come from relatively large carbonyl fragments the two three-carbon, and one three and one four-carbon fragments, respectively. Thus, during intermolecular rearrangement of their intermediate the longer chain has significantly higher steric hindrance. However, in a study of 56 pyrazines the authors demonstrated that the influence of side chain nitrogen was most apparent not in multi-substituted pyrazine but

in high order complex pyrazines such as bicyclic pyrazine (Hwang et al., 1995a), with little influence observed in alkylpyrazines or pyrazines. Generally the higher order group includes pyrazines with furanyl, phenyl or cyclopentyl substitutes. Among the tested amino acids possessing nitrogen side chains - glutamine, asparagine and lysine made the largest contribution to bicyclic pyrazines coming from the epsilon of lysine. Even though the Nα-terminal end is more reactive in pyrazine formation (Hwang et al., 1994) the epsilon side chain is the best candidate for reaction with bound lysine that is responsible for the formation of advanced glycation endproducts and a number of other important crosslink products.

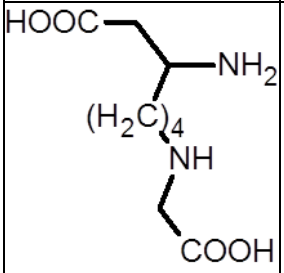
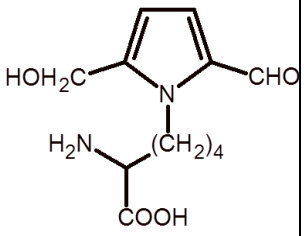
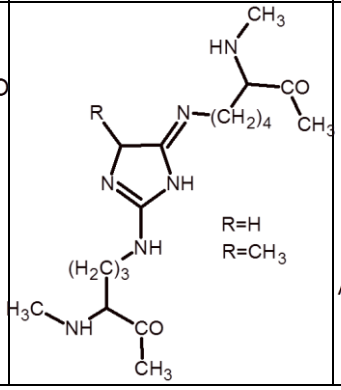
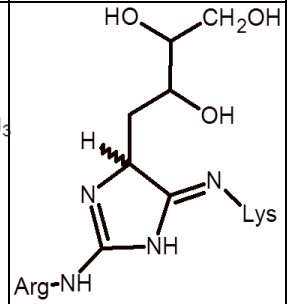
2.5.4 The behaviour of lysine N-epsilon nitrogen in model systems and in vivo

One of the most important modifications of lysine in thermally processed food and *in vivo* is the formation of carboxymethyl-lysine (CML) as showing in Table 2.6. CML is the most studied AGE and arguably the most important as it is utilized as a biomarker for oxidative stress and long-term protein damage in normal aging and diabetes. This compound has also been used to evaluate the progression of the Maillard reaction in food (Hewedy et al., 1994; Hartkopf & Erbersdobler, 1995). The formation of CML is indicative of the complexity and ultimately the challenge of controlling AGE in foods in that CML has multiple formation pathways, with the prevalence of each closely linked to the reaction setting. The major pathway of CML formation is through oxidative cleavage of the Amadori product (Ahmed et al., 1986). In short, during the progress of the Maillard reaction glycated lysine forms Nα-formyl-Nε-fructoselysine that is oxidatively cleaved between the C-2 and C-3 of the glucose portion. This will form CML, while the remnants of the glucose moiety give the four carbon erythronic acid, which has been detected.

However, CML can also be generated through a host of different methods including autoxidative glycosylation in lysine and glyoxal models (Alabed & Bucala, 1995). This occurs through a simple addition reaction of lysine and glyoxal, with the degradation of Maillard reaction products acting as the source of the α-dicarbonyl. The Schiff base degradation via the Namiki pathway (Wellsknecht et al., 1995) is similar to the autoxidative glycosylation in that glyoxal reacts with lysine. However, this mechanism is unique in that glyoxal is proposed to originate from the degradation of the Schiff base and without forming the Amadori product as is the case in the previous mechanism. Other potential mechanisms of CML formation include the reactions between lysine and ascorbate, and metal induced oxidation of fatty acids in the presence of proteins (Fu et al., 1996).

Pyrraline is another AGE and has also been evaluated as a potential indicator of heat damaged proteins in whey proteins and enteral formulas (Rufian-Henares et al., 2004). Pyrraline is an adduct of lysine ε side group and 3-DG, and its concentration has been correlated, in physiological studies, with elevated levels in cataractous lenses compared to corresponding age controlled normal lenses (Naharaj & Sady, 1996). Some peculiar lysine-arginine crosslinks with 3-DG/glyoxal/methylglyoxal were discovered in bovine serum albumin incubations abbreviated as DODIC, GODIC, and MODIC, respectively, Table 2.6 (Lederer & Klaiber, 1999; Lederer & Buhler, 1999). In a 2001 study these crosslinks were detected in five foodstuffs including butter biscuits, salt sticks and egg whites. Quantitatively MODIC and DODIC were determined to be the most important (Biemel et al., 2001). Potentially suspected to be AGEs, a year later these crosslinks were discovered in human lens and serum albumin proteins (Biemel et al., 2002).

Table 2.6 List of lysine AGEs found food

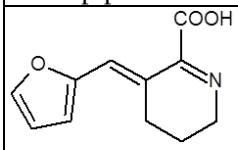
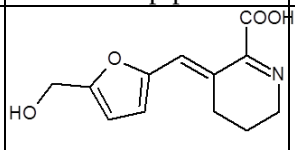
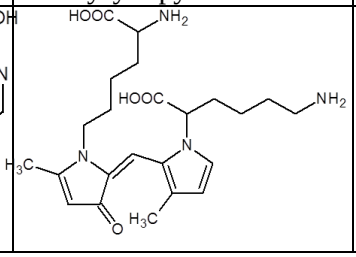
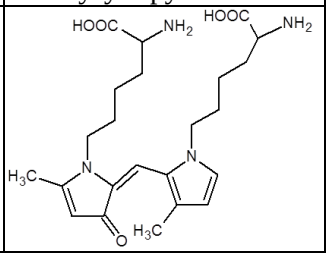
Carboxymethyl-lysine (CML)	Pyrraline	GODIC (R=H) MODIC (R=CH_3)	DODIC
			

2.5.5 Selected novel compounds from lysine model systems

Having explored the mechanistic differences between models with lysine-glucose/glucose degradation products from the perspective of reactivity of Nα and Nε atoms, the focus will be shifted onto lysine behaviour with another important sugar category – pentoses. Compared with glucose-lysine, pentose reaction models are less studied but a number of lysine studies with xylose, ribose, furfural (degradation product of xylose/ribose) have been reported. In a recent study, lysine was reacted with glucose, HMF, arabinose, xylose and furfural in a slightly acidic aqueous solution heated at 95°C for 1 hour (Murata et al., 2007). Browning measurements had xylose, arabinose and furfural as the highest contributors. Lysine models with furfural yielded a new novel yellow compound furpipate (compound **1**, Table 2.7) that contributed 25% to total colour of the model. The involvement of the epsilon group was confirmed when models using Boc-Lys-OH (where the alpha nitrogen is blocked by a Boc group) also generated furpipate. The formation mechanism was not elucidated but could involve deamination, dehydration and cyclization. The same research group used a HMF-lysine model to identify the furpipate's

HMF counterpart, hydroxymethyl-furpipate compound **2**, Table 2.7 (Totsuka et al., 2009). Furthermore, the group was able to identify the decarboxylated versions of both furpipate and the HMF counterpart. Furpipate and its decarboxylated version in the furfural-lysine models contributed 25% and 3%, respectively to the total colour, while the HMF-furpipate and the decarboxylated counterpart made a 43% and 18% contribution to colour in the HMF-lysine models.

Table 2.7 Colour compounds from furfural and HMF models with lysine

1.Furpipate	2.HMF-furpipate	3.Dilysyldipyrrolones A	4.Dilysyldipyrrolones B
			

Reaction behaviour of xylose and lysine heated at 95°C for 3 hours yield crosslinks **3**, **4** (Table 2.7). Named dilysyldipyrrolones A and B the formation mechanism of the low molecular weight pigments was not determined (Sakamoto et al., 2009). The compounds have interesting structural characteristics including a methylidene bridge between dihydropyrrolone and hydroxypyrrole moieties that appear to be a product of two xylose molecules, which attached and then cyclised with the two available amine groups. The difference between the A and B derivative is that the A has both alpha and epsilon links to the sugar moieties while the B derivative has two epsilon connections. To further illustrate the potential complexity of lysine specific products, a melanoidin type mixture was formed in a heated incubation of casein and furfural for 1 hour at 70°C (Hofmann, 1998). Complete enzymatic hydrolysis was used to overcome the analytical challenge of

melanoidin analysis and resulted in detection of two multi-ringed compounds. The mechanism of formation consisted of the epsilon side chain of lysine acting as an anchor to multiple furfural adducts, Figure 2.7. The complexity of the furfural moiety in this study demonstrates that sugar thermal degradation needs to be also explored in the investigation of the formation of Maillard reaction products.

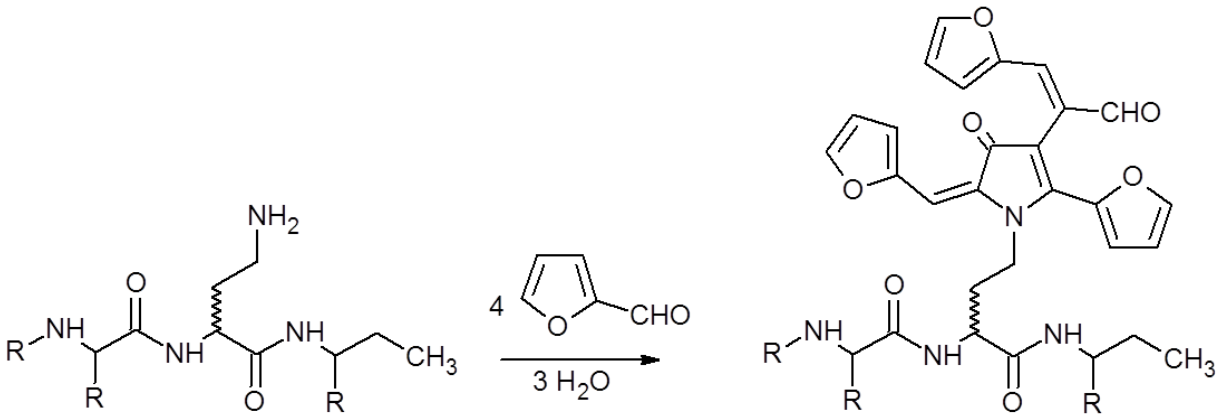


Figure 2.7 The anchor behaviour of lysine in casein and furan-2-carboxaldehyde model (adapted from Hofmann, 1998)

2.6 Degradation of glucose and ribose and their role in the Maillard reaction

Simple reducing sugars and their degradation products are crucial for the development of the Maillard reaction. Some of the recent focus on the biological significance of sugars is related to the observed correlation to the obesity epidemic which has resulted in discussion of potential taxes on sugar sweetened beverages (Finkelstein et al., 2010). The two most abundant categories of sugars found in food that participate in the Maillard reaction are hexoses and pentoses, with the former being the most common (Southgate et al., 1978). Glucose is one of the most abundant and also the most studied sugar in food and thus its interaction behaviour with lysine is well documented. The interaction of lysine in glucose models systems results in the formation of a variety of characteristic

Maillard reaction compounds including pyrazine, pyrrole, and furan derivatives (Hwang et al., 1994; Hwang et al., 1995b; D'Agostina et al., 1998).

Aldose sugars (glucose) are considered to have higher reactivity than ketoses (fructose) because their terminal aldehyde group has less steric hindrance and this accessibility increases the likelihood of carbonyl/amine bond formation (O'Brien & Morrissey, 1989). Similarly aldose sugars have higher electrophilicity at the aldehyde group and thus are a better target for nucleophiles in the Maillard reaction than ketoses (Bunn & Higgins, 1981). Also unlike aldoses, ketose sugars are not typically in a reactive form and require an extra degradation or an initiation to become active (Brands & van Boekel, 2002). Evidence has been presented that the Amadori compounds generated from aldoses have faster browning rates than Heyns products from ketose sugars (Kwak & Lin, 2004). Lastly, aldoses favour higher pyrazine production over ketoses (Koehler & Odell, 1970).

2.6.1 HMF an important glucose degradation products

Glucose is one of the least reactive and also most abundant monosaccharides in food. Glucose's non reactivity is hypothesized to be an important evolutionary reason for its selection as the primary metabolic energy source because this limits detrimental protein glycation reactions (Bunn & Higgins, 1981). However, this stability trend ends when glucose is exposed to thermal heating resulting in the formation of an array of reactive products which are important for the propagation of the Maillard reaction. One of the most abundant glucose thermal degradation products in food is 5-hydoxymethyl-2-furancarboxaldehyde (HMF) (Abraham et al., 2011). Interest in the biological significance of HMF originated from the high levels of HMF consumption with estimates ranging from 30 to 60 mg/person (Janzowski et al., 2000) and 150 mg/person (Ulbricht et

al., 1984). Animal models involving rats and mice have discovered that HMF acts as an initiator and promoter of colonic aberrant crypt foci (ACF) (Zhang et al., 1993), a type of carcinogenic lesion in the intestine. Another study had rats consuming 200mg/kg body weight of HMF and resulted in developed tumours in the kidney (Schoental et al., 1971). *In vitro* experiments to determine cytotoxicity demonstrated a narrow range of LC_{50} values of 115-118mM when HMF was incubated in two different cells lines for one hour (Janzowski et al., 2000). HMF has also been demonstrated to have weak positive genotoxicity assays during *in vitro* studies (Glatt et al., 2005) as well as a DNA strand-breaking ability (Omura et al., 1983).

HMF is known to undergo a variety of metabolic transformations resulting in derivatives with varying degrees of biological significance, including HMF derivatives having acid, glycine, dicarboxylic acid, or sulphooxymethyl groups (Glatt & Sommer, 2007). The most biologically significant of these 5-(sulphooxymethyl)-2-furfural (SMF) is now perceived as a form of bioactivation of HMF. The metabolic pathway involves sulphotransferase enzyme to convert HMF into a reactive allyl ester SMF and has been observed in *in vivo* animal models (Monien et al., 2009) after intravenous introduction of HMF. SMF is known to be carcinogenic (Monien et al., 2009), and mutagenic in both bacteria and mammalian cells *in vitro* (Surh & Tannenbaum, 1994; Lee et al., 1995) with the highly electrophilic allyl carbocation responsible for these effects.

2.6.2 HMF content in food

HMF is found in aqueous food matrices consisting of simple monosaccharides in acidic medium, although it is practically absent in fresh foods, it is found in a variety of thermally processed foods. In honey, HMF concentration is legislated to an upper

maximum of 40mg/kg (FSA, 2005). A number of different studies have quantified HMF content in food (Murkovic & Pichler, 2006; Husoy et al., 2008) with one study alone analyzing 500 different foods samples (Bachmann et al., 1997). In a recent study estimating concentration levels of HMF in foodstuffs from Germany presented in Table 2.8, the largest concentrations were found in dried fruits ranging between 5.5 to 1350 mg/kg (Abraham et al., 2011). Coffee is one of the highest contributors to daily consumption of HMF with the intake in Spanish populations estimated at 8.57 mg/day/person. The HMF content in coffee varied significantly, with the concentration range of four analyzed coffee types ranging between 100 and 2480 mg/kg (Arribas-Lorenzo & Morales, 2010). Correspondingly, the contribution of HMF intake from coffee is 50% of the total exposure in the Spanish study and 63% from a Norway study (Husoy et al., 2008).

Table 2.8 HMF content in food (adapted from Abraham et al., 2011)

Food	Number of samples	Mean concentration (mg/kg)	< Limit of quantification (%)
Honey	726	9.1	10
Apple juice	234	7.4	12
Orange juice	10	0.4	0
Multivitamin nectar	16	40.9	19
Pineapple juice	29	2.6	24
Grape juice	80	6.3	9
Plum juice beverage	11	707.7	0
Plum butter/jam	174	410.9	0
Cereal bar	117	43.2	9
Chocolates/praline	13	273.8	0
Dried Plums	153	350.8	0
Beverage powder with coffee	23	286.1	0
Rye-wheat bread	20	44.5	10
Almond, roasted and coated	28	155.5	0
Cocoa-containing beverage powder	14	503.8	0
Beverage from dried plums	71	1022.1	0

HMF concentration can have great variation with one of the highest HMF contents found in any food being dried apples and pears where levels of 3500 mg/kg were measured (Bachmann et al., 1997).

2.6.3 HMF as an indicator of thermal processing

HMF's prevalence in thermally processed foods has made it useful for determining the extent of browning or as an indicator of the severity of heat treatment. HMF determination is very common in the fruit juice industry to test for potential temperature abuse (Tu et al., 1992). Quantification of both free (Van Boekel & Rehman, 1987) and bound HMF (Morales et al., 1997) has also been used as an index of the Maillard reaction in dairy products. Free HMF refers to HMF formed entirely from sugar degradation, while bound HMF originates from the degradation of the sugar-amino acid Amadori product. Lastly, the presence of HMF has been used to determine the severity of heat processing and the inappropriate storage conditions for jams and infant foods (Rada-Mendoza et al., 2004).

2.6.4 Mechanism of formation of HMF

The general requirements for HMF formation is the thermal treatment of hexose sugars in the presence of amino acids or proteins. The general formation mechanism is an acid-catalysed thermal degradation of glucose. The mechanism resembles the early Maillard reaction initiated with a Schiff base formation between glucose and amine, followed by 1,2 enolization and three dehydration steps (Figure 2.8). The key intermediate is 3-deoxyglucosone which after dehydration generates HMF. HMF has been demonstrated to be able to form even at low temperature (50°C) while in acidic media however, its concentration is drastically increased at higher temperatures (Lee & Nagy, 1990). It was

demonstrated that the favoured precursor for HMF is fructose followed by sucrose as the latter two generated 31.2 and 18.5 fold higher HMF concentrations than glucose (Lee & Nagy, 1990). The identification of a very reactive fructofuranosyl cation using ^{13}C sucrose at the C-1 fructose moiety by Locas & Yaylayan (2008) provided a rational for fructose's superior HMF production.

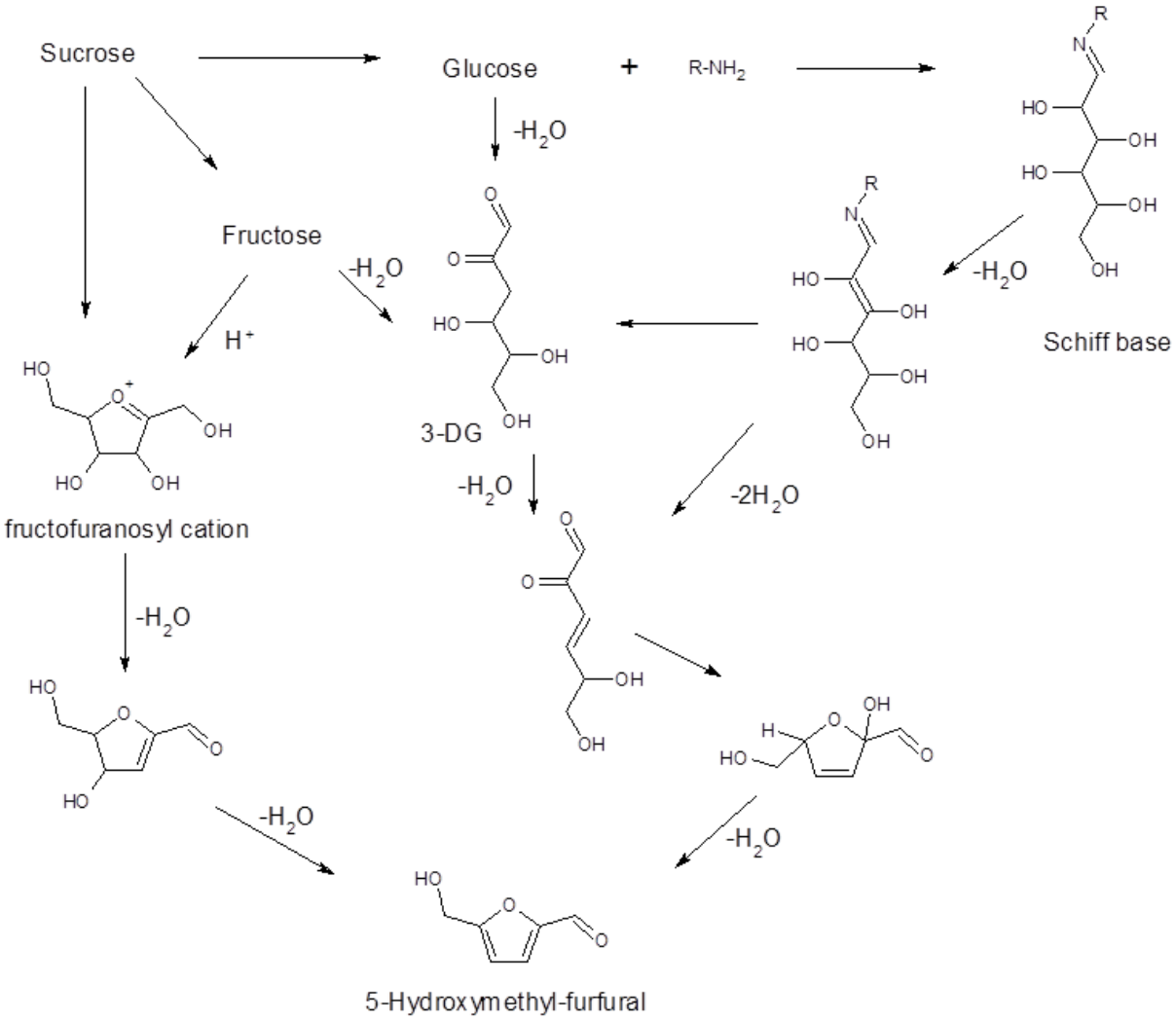


Figure 2.8 Formation of HMF from glucose, fructose, and sucrose (adapted from Perez-Locas & Yaylayan, 2008; Capuano & Fogliano, 2011)

The high concentration of HMF in food, its potential biological significance and its chemical reactivity from the alcohol and α, β-unsaturated aldehyde contribute to the importance of this compound in foodstuffs. Thus, it is surprising that the thermal fate of HMF during processing or long storage intervals has not been explored in food. The majority of literature elucidating HMF related mechanisms has focused on organic syntheses, which use solvents and catalysts to achieve desired reactions (Rosatella et al., 2011). These conditions are not representative of the food matrix and thus limit the applicability of such studies to the Maillard reaction of HMF. A study focusing on the concentration of HMF during storage of infant formula displayed that the levels are in a flux, constantly increasing and decreasing (Ferrer et al., 2005). These changes could suggest that HMF is constantly being broken down, polymerizing or reacting with other molecules such as amine from amino acids or proteins. The extent of HMF reactivity has been demonstrated recently with the compound shown to react and form a stable adduct with human hemoglobin *in vivo* (Davies et al., 2009). Lastly, it has been suggested that HMF can form the structural skeleton of melanoidins through aldol condensation (Guan et al., 2011). These novel mechanistic possibilities and the fluctuating HMF concentration during storage suggest that the already high levels of HMF in food could be underestimated. This merits a further investigation of HMF-specific reaction products. This may result in HMF-specific polymers and reaction derivatives being included in future assessments of HMF content in food.

2.6.5 Ribose

Even though the most common free sugars in foodstuffs are hexoses such as glucose and fructose, the five carbon pentoses are considered more reactive (Shibamoto & Bernhard,

1977; Southgate et al., 1978). Ashoor and Zent (1987) demonstrated that the Maillard reaction intensity, measured by degree of browning at 420nm, with lysine had the highest absorbance in ribose 1.22A, followed by 1.04A and 0.947A for fructose and glucose, respectively. This trend was observed in 13 of the 21 amino acids, including hydroxyproline. Similarly with ribose the reaction also generated the highest absorbance in 12 of the 15 amino acids categorized in high and medium browning groups. Ribose is an important sugar for the development of flavour compounds, especially in meat (Meynier & Mottram, 1994). A kinetic study (Laroque et al., 2008) compared the reactivity of reducing sugars in the Maillard reaction of five sugars including: ribose, xylose, glucose, fructose and arabinose. The methodology involved the sugars reacting with shrimp hydrolysates at 55°C and pH 6.5, and the models were monitored for changes in sugar consumption, browning intensity and rate of free amino group uptake. The results demonstrated that pentose sugars (arabinose, ribose and xylose) had greater Maillard reactivity potential than hexoses and that among the pentoses, ribose has the highest reactivity. A comparison of the antioxidant activity in lysine models with three sugars (glucose, fructose, and ribose) observed that the relative reaction rates and degree of polymerization was highest in MRP generated from the ribose-lysine models (Jing & Kitts et al., 2004). In the following *in vitro* scavenging assays DPPH (1,1,-diphenyl-2-picryl-hydrazyl radical) and the Fenton reaction-induced hydroxyl radical, the high molecular weight MRP from the ribose-lysine model had the highest free radical scavenging affinity of all three models. Chevalier and colleagues (2001b) observed that β-lactoglobulin heated in solution at 60°C had the highest degree of modification with

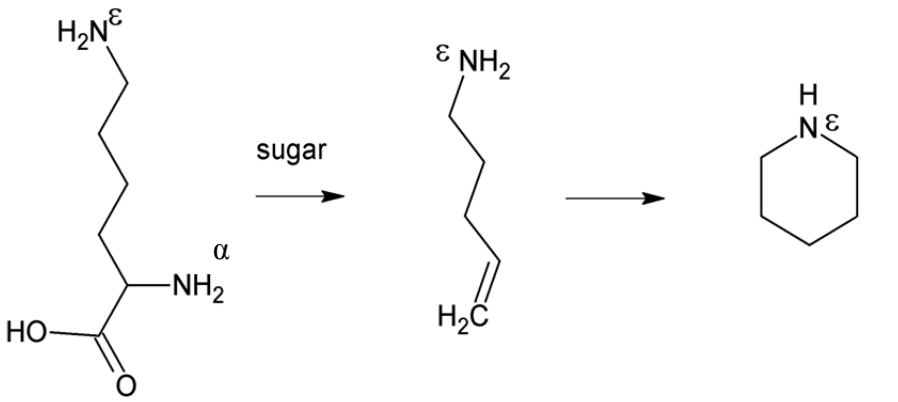
ribose and arabinose, from a list of sugars that also included: galactose, glucose, lactose and rhamnose.

A number of characteristics are credited for the reactive superiority of pentose over hexose sugars. Pentoses such as arabinose, xylose and ribose have shorter chains thus resulting in less steric hindrance (Jing & Kitts et al., 2004) than hexoses. Sugars molecules that participate in the Maillard reaction must be in their open chain form where their carbonyl groups are available for nucleophilic attack by amines. Pentose sugars have higher percentage of the open-chain form, for example the availability of open chain ribose at 20°C is 25 fold higher than glucose which has only 0.002% open form (Hayward et al., 1977). Structurally, this can be rationalized by the higher tension and thus lower stability of furanose (5 atoms) rings of pentoses relative to the more stable pyranose (6 atoms) ring of hexose sugars.

CONNECTING PARAGRAPH

Chapter 2 provided the merit for the investigation into lysine-specific products through the exploration of the reactivity of lysine in the Maillard reaction. Chapter 3 investigates the nature of reactive intermediates generated from lysine's epsilon group. The focus will be on the characterization of the reaction products formed from these intermediates. Furthermore, the chapter also highlights the influence of sugars on the formation of lysine-specific intermediates. The most common hexose sugar, glucose, will be utilized to study these interactions in glucose/lysine models. Lastly, the chapter will explore if lysine can also generate amino acid specific processed induced toxicants such as acrylamide from asparagine. Chapter 3 was published in the Journal of Agricultural and Food Chemistry. (Nikolov, P.Y., Yaylayan, V.A. (2010). Formation of pent-4-en-1-amine, the counterpart of acrylamide from lysine and its conversion into piperidine in lysine/glucose reaction mixtures. *Journal of Agricultural and Food Chemistry*, 58, 4456-4462)

CHAPTER 3: FORMATION OF Nε-PENT-4-ENE-1-AMINE, THE COUNTERPART OF ACRYLAMIDE FROM LYSINE AND ITS CONVERSION INTO PIPERIDINE IN LYSINE/GLUCOSE REACTION MIXTURES



Lysine **Pent-4-ene-1-amine** **Piperidine**

3.1 Abstract

Isotope labelling studies performed using lysine/glucose model systems have indicated that lysine can generate piperidine a reactive amine capable of undergoing Maillard type interactions. Two possible mechanisms were identified for the formation of piperidine; one arising through decarboxylation of lysine alone to generate cadaverine (1,5-diaminopentane) followed by deamination to form pent-4-ene-1-amine which in turn can cyclise into piperidine where both Nε and Nα atoms of lysine can be equally involved in its generation due to the symmetrical nature of the precursor diamine. On the other hand, in the presence of sugars, lysine similar to asparagine and phenylalanine can undergo carbonyl-assisted decarboxylative deamination reaction to generate Nε-pent-4-ene-1-amine the counterpart of acrylamide from lysine. The Nε-pent-4-ene-1-amine, can then cyclize to form piperidine through Nε atom of lysine. To confirm the formation of Nε-pent-4-ene-1-amine in lysine/glucose model system a useful strategy based on Py-GC/MS analysis was developed using isotope labelling technique to identify sugar adducts of Nε-pent-4-ene-1-amine. Products simultaneously possessing five lysine carbon atoms (C2'-C6') and the Nε-amino group from lysine in addition to glucose carbon atoms were targeted using specifically labelled precursors such as [^{15}Nα]lysine.2HCl, [^{15}Nε]lysine.2HCl, [U-$^{13}C_6$]lysine.2HCl, [^{13}C-6]lysine.2HCl and [U-$^{13}C_6$]glucose. The complete labeling studies along with structural analysis using synthetic and other available precursors have shown the presence of a peak that satisfies the above criteria and the peak was tentatively identified as *N*-(5-methylfuran-2-yl)methylidene]penta-1,3-dien-1-amine incorporating Nε-pent-4-ene-1-amine in its structure.

3.2 Introduction

The Maillard reaction products that are formed in a similar fashion to that of acrylamide have been termed as "vinylogous compounds" (Stadler et al., 2003). Only two such compounds have been studied so far the acrylamide itself and styrene (Goldmann et al., 2009) (Figure 3.1).

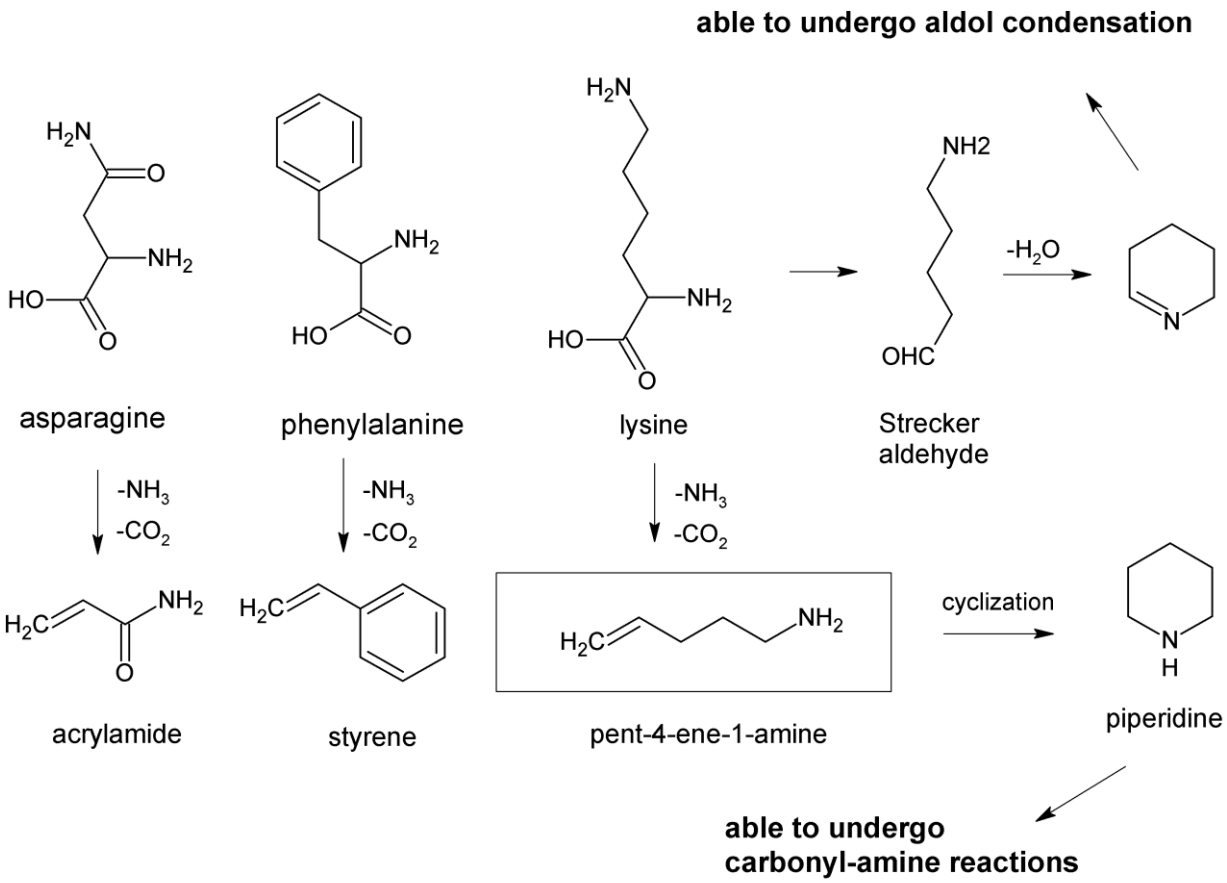


Figure 3.1 Formation of amino acid-derived vinylic compounds acrylamide, styrene and pent-4-ene-1-amine from asparagine, phenylalanine and lysine respectively.

The terminology used to describe this category of Maillard reaction products could be misleading since the term "vinylogous" implies an analogy in reactivity behaviour due to

conjugation rather than similarity in structure i.e. possessing a double bond. It is preferable that this type of Maillard-generated compounds be rather referred to as amino acid derived "vinylic compounds". All amino acids except glycine are capable of undergoing such decarboxylative-deamination reactions in the presence of carbonyls and generating amino acid-specific vinylic compounds such as acrylic acid from aspartic acid (Stadler et al., 2003) and 3-butenamide from glutamine (Stadler et al., 2004). However, the most studied amino acids in this respect are asparagine (Perez-Locas & Yaylayan, 2008) and phenylalanine (Goldmann et al., 2009) due to the potential safety concerns of the resulting products in heated foods. Studies performed in asparagine model systems (Perez-Locas & Yaylayan, 2008) have indicated that the initially formed decarboxylated Schiff base can either directly generate the vinylic compound (pathway D in Figure 3.2) or indirectly through hydrolysis (pathway C) and formation of decarboxylated amino acid (Granvogl et al., 2006). The relative importance of these pathways (D or C in Figure 3.2) will very much depend on the reaction matrix and conditions and the type of amino acid involved (Perez-Locas & Yaylayan, 2008).

Lysine on the other hand, is considered to be one of the important amino acids in the Maillard reaction and its ability to undergo such decarboxylative-deamination reaction to generate Nε-pent-4-ene-1-amine the counterpart of acrylamide has not been explored. In this chapter we demonstrate that Nε-pent-4-ene-1-amine can form in lysine model systems and can exists as piperidine a reactive secondary amine capable of interacting with furfural to form yellow pigments as demonstrated by Hofmann (1998). Related structures such as tetrahydropyridine has been also identified in glucose/lysine mixtures (Miller et al., 1984) and presumed to be formed through intramolecular cyclization of the

Strecker aldehyde of lysine (Figure 3.1). Yaylayan & Sporns (1989) found that piperidinium cation at m/z 84 constitutes the base peak in the mass spectrum of lysine Amadori product. Mills et al., (1969) have synthesized piperidine Amadori product and studied its degradations. Piperidine is classified as toxic and can also be formed enzymatically from lysine in biological systems and has been detected in human urine and in various animal organs (Schmidtglenewinkel, 1977). Due to its reactivity, piperidine may also be involved in the generation of dietary advanced glycation end-products. The purpose of this chapter was to demonstrate that lysine similar to asparagine can undergo decarboxylative deamination reaction and generate Nε-pent-4-ene-1-amine the counterpart of acrylamide.

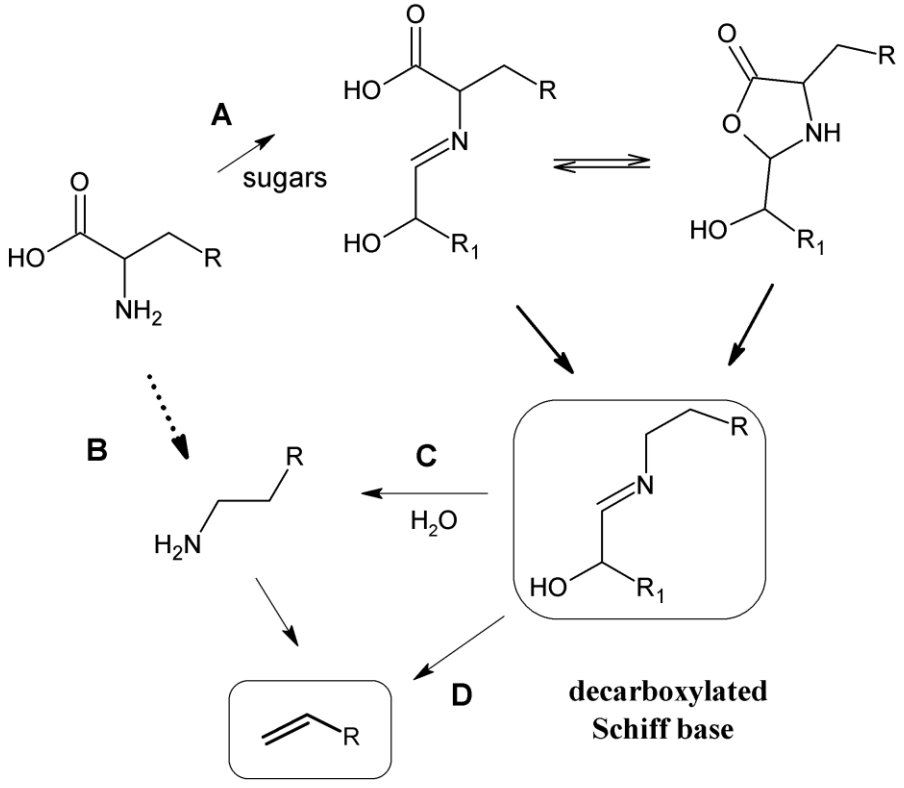


Figure 3.2 Pathways of formation of amino acid-derived vinylic compounds based on (Perez-Locas & Yaylayan, 2008 and Granvogl et al., 2006).
Pathway **A** in the presence of reducing sugars, pathway **B** in the absence of reducing sugars. R = amino acid side chain; R_1= sugar moiety

3.3 Materials and Methods

All chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. [^{15}Nα]lysine.2HCl, [^{15}Nε]lysine.2HCl, [U-$^{13}C_6$]lysine.2HCl, [^{13}C-6]lysine.2HCl, [U-$^{13}C_6$]glucose, [^{13}C-1]glucose, [^{13}C-2]glucose, [^{13}C-3]glucose, [^{13}C-4]glucose, [^{13}C-5]glucose and [^{13}C-6]glucose were purchased from CIL (Andover, MA). Melting points were determined on OptiMelt automated melting point system (Sunnyvale, Ca). The ^{13}C- and ^{1}H-NMR spectra were acquired in CD_3OD on a 300 MHz Varian Unity spectrometer. Infrared spectra were recorded on a Bruker Alpha-P spectrometer (Bruker Optic GmbH, Ettlingen, Germany) equipped with a deuterated triglycine sulphate (DTGS) detector, a temperature controlled single bounce diamond Attenuated Total Reflectance (ATR) crystal and a pressure application device for solid samples.

3.3.1 Sample Preparation

The dihydrochloride salts of the commercially available isotopically labelled lysines were unreactive when pyrolyzed as such, however, mixing the salts with an equimolar amounts of unlabelled free lysine resulted in increased reactivity when pyrolyzed. Consequently, equimolar amounts of unlabelled DL-lysine and specifically labelled DL-lysine.2HCl were mixed and homogenized before mixing with equimolar amount of D-glucose. For isotope labelling studies [^{15}Nα]lysine.2HCl, [^{15}Nε]lysine.2HCl, [U-$^{13}C_6$]lysine.2HCl, [^{13}C-6]lysine.2HCl, [U-$^{13}C_6$]glucose, [^{13}C-1]glucose, [^{13}C-2]glucose, [^{13}C-3]glucose, [^{13}C-4]glucose, [^{13}C-5]glucose and [^{13}C-6]glucose were used. Similar experiments with glycolaldehyde dimer, glyceraldehydes, 2,3-butanedione and pyruvic acid were conducted only to confirm the formation of piperidine in these systems.

3.3.2 ESI-TOF MS samples

Glucose (10 mg) and lysine.2HCl (16 mg) were dissolved in distilled water (0.8 mL) and heated in an open vial at 110^{o}C for 45 min or until dryness generating a brown powder. The experiments were repeated with [U-$^{13}C_6$]glucose and [$^{15}N\alpha$]lysine. The pyrolysis of this powder generated a similar profile to that of lysine glucose samples pyrolyzed without prior heating as described above.

3.3.3 Pyrolysis-GC/MS

Analyses were conducted using a Varian CP-3800 GC coupled with a Saturn 2000 Ion Trap Mass Spectrometer (Varian, Walnut Creek, USA). The pyrolysis unit included a CDS Pyroprobe 2000 and a CDS 1500 valved interface (CDS Analytical, Oxford, USA) installed onto the GC injection port. About 2.5 milligram of sample mixture was packed inside a quartz tubes (0.3mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed for 20 seconds at a temperature of 250°C. The sample separation was carried out on a DB-5MS (5% diphenyl, 95% dimethyl polysiloxane) capillary column with dimensions of 50 m length by 0.2 mm internal diameter and 0.33 μm film thickness (J&W Scientific, ON, Canada), using helium as the carrier gas. The GC column flow rate was regulated by an electronic flow controller (EFC) and set at a pressure pulse of 70psi for the first 4 minutes and later maintained with a constant flow of 1.5 mL/minute for the remainder of the run. The GC oven temperature was set at -5°C for 5 minutes using CO_2 as the cryogenic cooling source. The temperature was increased to 50°C at a rate of 50°C/minute and then to 270°C at a rate of 8^{o}C/minute, and kept at 270°C for 5 minutes. The samples were detected by using an ion-trap mass spectrometer with a scan range of 20-650m/z. The MS transfer-line temperature was set at 250°C,

manifold temperature was set at 50°C, and the ion-trap temperature was set at 175°C. The ionization voltage of 70 eV was used, and EMV was set at 1700 V. The number of compounds was calculated using the peak area of analytes integrated by Varian MS Data Review software with the following parameters: peak width 2.0 seconds; slope sensitivity 4 (SN); tangent 10%; and peak size reject 10000 counts. Compound identification was performed using AMDIS (ver 2.65) and NIST Standard Reference Databases (data version 05 and software ver 2.0d) to compare the target compounds with the existing mass spectral libraries or by injecting commercially available standards. The reported percent label incorporation values (corrected for natural abundance and for percent enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

3.3.4 ESI-TOF MS analysis

Samples were diluted in 1 mL water and then again 1/100 with 50% methanol and 0.1% formic acid. Each sample (5 μL injections) was directly analyzed by liquid chromatography-mass spectrometry (LC-MS), on a 1200 series Agilent rapid resolution LC system coupled to an Agilent 6210 time of flight (ESI-TOF) instrument. Mobile phase consisted of 50% methanol, 0.1% formic acid at a flow rate of 0.3 ml/min. Data were acquired in positive electrospray mode with an acquisition mass range of m/z 100-1000 and internal calibration using *m/z* 121.050873 and *m/z* 922.009798 (Agilent ESI tuning mix) for accurate mass measurements with a dual sprayer ESI source and constant infusion of calibrant ions. Source conditions were as follows: Gas Temp 350°C, ESI voltage 4000 V, Dry gas flow (nitrogen) 12 L/min, nebulizer gas pressure 35 psig, fragmentor and skimmer voltages of 100 and 60 V, respectively.

3.3.5 Synthesis of 1,1'-[(5-methylfuran-2-yl)methanediyl]dipiperidine (2)

The 5-methylfurfural (50 mg) was added to an excess piperidine solution in the absence of any solvent and stirred at room temperature for 10 min or until heavy precipitate forms. The resulting solid was filtered and crystallized from acetonitrile to give white needles in almost quantitative yield. MP 61.2^{o}C, ^{1}H-NMR: δ 1.45 - 1.56 (m, 12H, H-3'to H-5' and H-3''to H-5''), 2.27 (s, 3H, H-1), 2.55(t, 2H, H-2'), 2.65 (t, 2H, H-2"), 2.76 (t, 4H, H-6',6"), 4.64 (s, 1H, H-6), 5.98 (s, 1H, H-4), 6.20 (s, 1H, H-3). ^{13}C-NMR: δ 151.7(C-2), 149.3(C-5), 109.2(C-3), 105.5(C-4), 92.6 (C-6), 48.5(C-6',6"), 46.2(C-2',2"), 25.9(C-3',3") 25.3(C-5',5") 24.4 (C-4") 24.3(C-4') 12.0(C-1). FTIR (solid) 2965-2700 cm^{-1} (alkyl), 1000 cm^{-1} (C-N stretch), 1108 cm^{-1} (C-N stretch), 790 cm^{-1} (C=CH). MS m/z (% abundance): 39 (13.2), 41 (11.5), 43 (1.35), 84 (16.4), 95 (100), 96 (16.9), 97 (27.8), 98 (18.9), 136 (15.9), 178 (42.9), 179 (48.5) 180 (38.4).

3.4 Results and discussion

Analyses of the reaction mixtures generated through pyrolysis experiments from lysine and various sugars (glucose, glyceraldehydes and glycolaldehyde) and carbonyl compounds (2,3-butanedione, pyruvic acid) have indicated that under the experimental conditions all the model systems produced piperidine including lysine alone (see Figure 3.3). The identity of the piperidine was confirmed through mass spectral library searches and by comparison of its retention time to an authentic sample (11.6 vs 11.5 min) and through spiking experiments where piperidine was co-pyrolyzed with lysine generating a more intense peak relative to the un-spiked sample at the same retention time. In addition, labelling experiments (see subsequent sections) have also confirmed the exact origin of all the atoms originating from lysine. Furthermore, under the experimental conditions

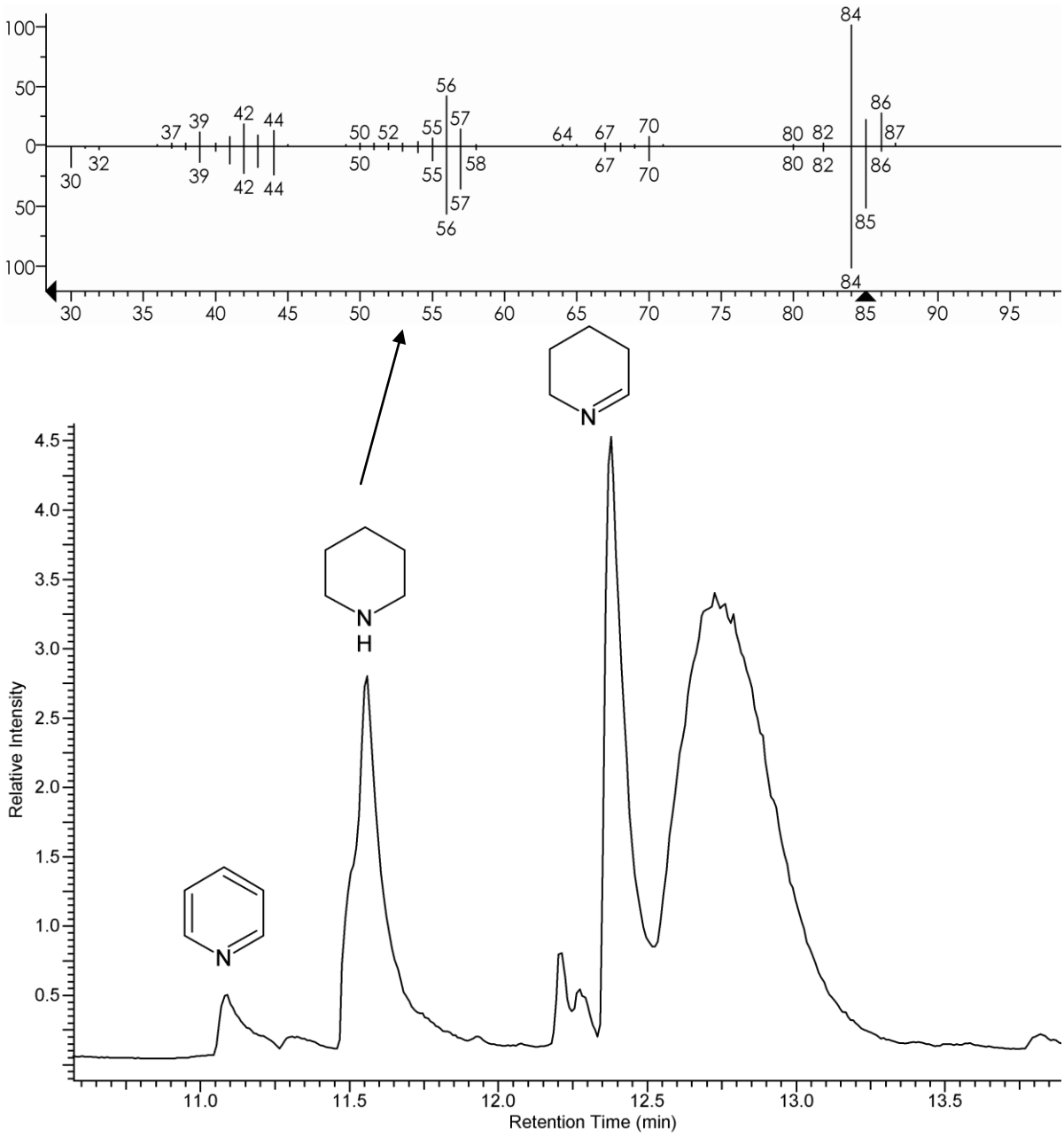


Figure 3.3 Partial GC chromatogram of lysine generated through pyrolysis at 250^{o}C showing the peak of piperidine at 11.5 min and its mass spectrum compared with authentic NIST library spectrum in a head-to-tail fashion

piperidine was the major product of lysine/glycolaldehyde mixture indicating its possible importance as an intermediate in lysine containing foods during roasting, baking, toasting, grilling and other processes associated with pyrolysis (Baltes, 1985).

3.4.1 The origin and mechanism of formation of piperidine in lysine/glucose model systems

To investigate the origin of piperidine in lysine/glucose model systems, labelling studies were carried out using [^{15}Nα]lysine, [^{15}Nε]lysine, [U-$^{13}C_6$]lysine, [^{13}C-6]lysine and [U-$^{13}C_6$]glucose. When lysine/glucose model systems were analyzed, piperidine was shown to incorporate predominantly (> 90%) Nε atom of lysine and the five carbon atoms of piperidine originated solely from C2-C3-C4-C5-C6 carbon chain of lysine as shown in Table 3.1.

Table 3.1 Percent and number of labelled atom incorporation in piperidine[1] generated through the interaction of labelled lysine in the presence of glucose.

	[^{15}Nα]Lysine	[^{15}Nε]lysine	[U-$^{13}C_6$]Lysine	[^{13}C-6]Lysine
% Label incorporation	**10%**	**90%**	**100%**	**100%**
Number of labelled atoms	**1**	**1**	**5**	**1**

[1] retention time 11.56 min (11.55 min standard)

Accordingly, no sugar carbon atom incorporation was observed eliminating the possibility of sugar carbon contribution to the structure of piperidine. The predominant formation of Nε-piperidine on the expense of Nα-piperidine in glucose/lysine model system is significant in terms of determining its mechanistic origin. As shown in Figure 3.4, piperidine can be formed by the cyclization of pent-4-ene-1-amine generated from lysine. In fact, pent-4-en-1-amine intermediate can be considered as the only logical precursor of piperidine that allows cyclization through a nucleophilic addition reaction

(Schlummer et al., 2002) to form piperidine. The formation of piperidine from lysine alone can be rationalized by proposing decarboxylation of lysine to generate the symmetrical 1,5-diaminopentane or cadaverine (pathway B in Figure 3.4). This diamine can first deaminate into a 50% mixture of Nε- and Nα-pent-4-ene-1-amines and then cyclize into piperidine in turn generating a 50% mixture of nitrogen isotopomers due to

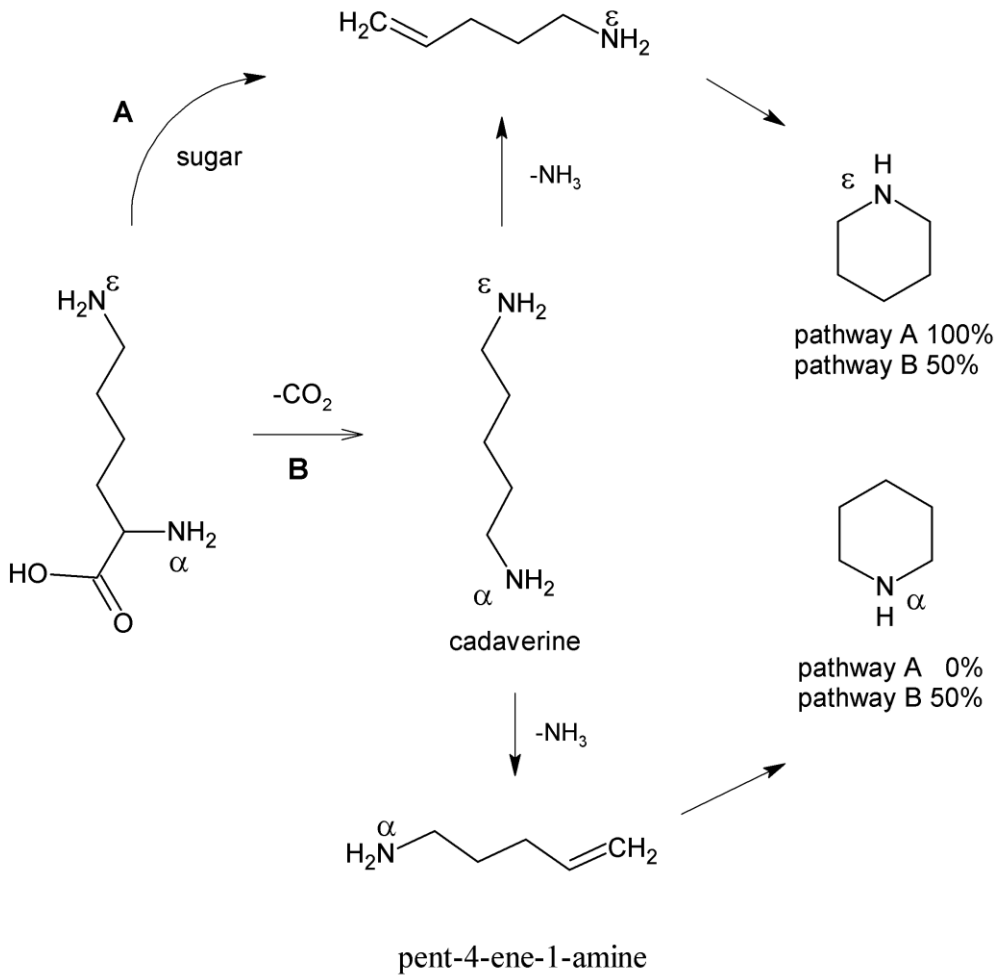


Figure 3.4 Proposed formation pathways of piperidine from lysine**.**
Pathway A in the presence of sugars (to form decarboxylated Schiff base followed by pathway D) as shown in Figure 3.2 where R = $-CH_2CH_2CH_2CH_2CH_2NH_2$ and pathway B in the absence of sugar through formation of cadaverine the decarboxylated lysine.

the symmetrical nature of the precursor. In fact, the addition of commercially available cadaverine to lysine enhanced the formation of piperidine by 15 fold implicating cadeverine as a precursor of piperidine in lysine model system. If piperidine originated only from cadaverine (decarboxylated lysine) in glucose/lysine system then the observed incorporation of Nε atom would have been 50% and not 90%. To justify the predominant formation of Nε-piperidine in lysine/glucose system a pathway (Figure 3.4 pathway A) that favours the exclusive deamination of Nα nitrogen over the Nε of lysine should be identified. One such pathway is shown in Figures 3.2 and 3.5 (pathway A followed by D). This pathway, based on the mechanism of acrylamide formation (Locas & Yaylayan, 2008; Granvogl & Schieberle, 2006; Chu & Yaylayan, 2009a) ensures deamination of only Nα nitrogen and exclusive generation of Nε-piperidine from the intermediate Nε-pent-4-ene-1-amine (see Figure 3.5).

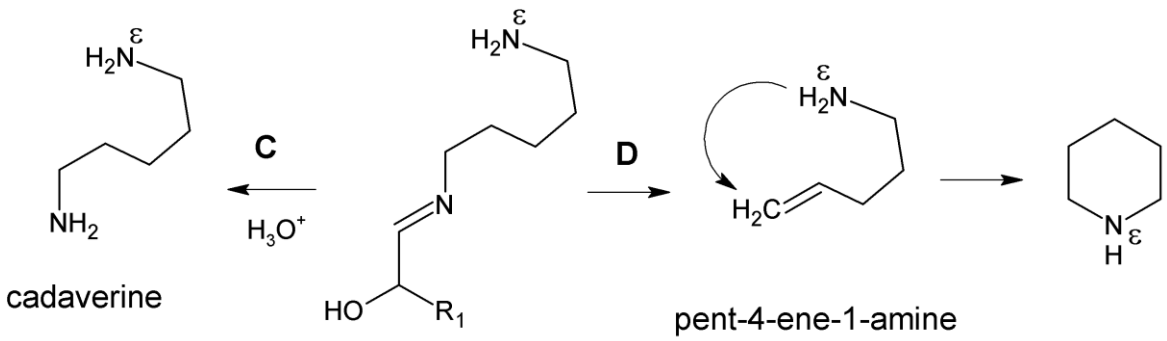


Figure 3.5 Proposed formation pathway of piperidine from the decraboxylated Schiff base shown in Figures 3.2 (pathway D)**.**

Such intramolecular hydroamination reactions of alkenylamines similar to Nε-pent-4-ene-1-amines have already been shown to be catalyzed by different agents including Brønsted acids at 100^{o}C in toluene to generate pyrrolidines and piperidines (Schlummer et al., 2002). In lysine/glucose model systems the formation of ~10% Nα-piperidine can be attributed to its direct formation from free lysine.

3.4.2 Isotope labelling studies for the identification of Nε-pent-4-en-1-amine adducts

Only indirect evidence is presented above for the formation of Nε-pent-4-ene-1-amine in glucose/lysine system, through detection of its possible cyclization product the piperidine. However, due to the known reactivity of amines with aldehydes its further reaction products were also sought in glucose/lysine reaction mixtures using specifically labelled precursors such as [^{15}Nα]lysine.2HCl, [^{15}Nε]lysine.2HCl, [U-$^{13}C_6$]lysine.2HCl, [^{13}C-6]lysine.2HCl and [U-$^{13}C_6$]glucose. Products simultaneously possessing Nε nitrogen atom and five carbon atoms from lysine (C2' to C6') in addition to glucose carbon atoms were specifically investigated to identify Nε-pent-4-ene-1-amine related sugar adducts.

Table 3.2 Number and percent distribution of peak areas incorporating Nα and Nε-nitrogens and carbon atoms in the pyrolysis products of lysine/glucose model system

	Number of Peaks	Percentage of Total Area
^{15}Nα+[^{13}C]glucose	11	18.1%
^{15}Nε+[^{13}C]glucose	15	10.6%
^{15}Nα+^{15}Nε+[^{13}C]glucose	3	1.9%
[^{13}C]glucose atoms only	41	68.5%
Undetermined		0.9%

Analysis of lysine/glucose model systems yielded a total of 87 potential compounds. Table 3.2 summarizes the percent distribution of Maillard reaction products possessing

specific atoms from each reactant in the system. The labelling studies confirmed the presence of eleven compounds containing Nα atom of lysine, fifteen compounds possessing Nε atom and three compounds were identified containing both nitrogens. Although the Nα atom incorporated into fewer peaks, however the total area of these peaks represented a much higher percentage contribution to the total abundance relative to compounds incorporating the Nε atom, which is in agreement with previous observations (Hwang et al., 1994). Table 3.2 also summarizes the percent distribution of Maillard reaction products in lysine/glucose system containing glucose and lysine carbon atoms.

Three compounds containing Nε and five carbon atoms of lysine (C2'to C6') in addition to glucose carbon atoms were detected. The label distribution is shown in Table 3.3. Such interaction products of lysine and glucose listed in Table 3.3 can lead to the detection of Nε-pent-4-ene-1-amine or piperidine-related adducts. It was observed that in these products glucose contributed either three or six carbons. In addition no product was identified possessing all six carbon atoms from lysine and the loss was always the C-1 atom most probably through decarboxylation. The incorporation of C-6' of lysine was verified due to labelling experiments with [^{13}C-6]lysine.

Table 3.3 Percent label distribution[1] in selected products generated from lysine/glucose model system

Retention time(min)	Molecular weight	M	M+1	M+2	M+3	M+4	M+5	M+6
19.435	141							
[^{15}Nα]Lysine		0	10	0	0	0	0	0
[^{15}Nε]Lysine		0	90	0	0	0	0	0
[^{13}C-6]Lysine		0	100	0	0	0	0	0
[$^{13}U_6$]Lysine		0	0	0	0	0	100	0
[$^{13}U_6$]Glucose		0	0	0	100	0	0	0
24.03	137							
[^{15}Nα]Lysine		0	10	0	0	0	0	0
[^{15}Nε]Lysine		0	90	0	0	0	0	0
[^{13}C-6]Lysine		0	100	0	0	0	0	0
[$^{13}U_6$]Lysine		0	0	0	0	0	100	0
[$^{13}U_6$]Glucose		0	0	0	100	0	0	0
24.317	175							
[^{15}Nα]Lysine		0	10	0	0	0	0	0
[^{15}Nε]Lysine		0	90	0	0	0	0	0
[^{13}C-6]Lysine		0	100	0	0	0	0	0
[$^{13}U_6$]Lysine		0	0	0	0	0	100	0
[$^{13}U_6$]Glucose		0	0	0	0	0	0	100

[1]Corrected and adjusted for the 50% mix

3.4.3 Confirmation of Nε-pent-4-ene-1-amine generation: formation of proposed *N*-(5-methylfuran-2-yl)methylidene]penta-1,3-dien-1-amine (1).

One of the reaction products possessing simultaneously one Nε nitrogen atom (mainly) and five carbon atoms (C2'-C5') from lysine in addition to six glucose carbon atoms was observed at the retention time of 24.317 min with nominal molecular weight of m/z 175. The molecular weight and the labelling data (Table 3.3) suggested that the compound could arise from the interaction of a six carbon glucose moiety such as 5-methylfurfural (5-MF) or 5-hydroxymethyl-furfural (HMF) with piperidine to form the structure (**1'**) shown in Figure 3.6. Furthermore, a peak possessing similar properties was generated from the LC-TOF-MS analysis of an aqueous model system consisting of glucose and

lysine heated to dryness at 110^{o}C for 45 min in an open vial. The $[M+H]^{+}$ ion had a molecular weight of 176.1068 amu corresponding to elemental composition of $C_{11}H_{13}NO$ for the unprotonated species. This was consistent with the label incorporation data of 6 (glucose) + 5 (lysine) carbons and a nitrogen atom generated from Py-GC/MS analysis. The spiking experiments with 5-MF yielded a significant increase in the abundance of the peak at 24.317 min in lysine/glucose model system. Similar spiking experiments with HMF did not change significantly the intensity of the peak at 24.317 min. On the other hand, if this unknown peak was indeed the result of piperidine reaction with 5-MF the interaction of the two should generate the same compound. In an attempt to synthesize this adduct we obtained instead a solid product (see under Materials and Methods) that matched the structural properties of 1,1'-[(5-methylfuran-2-yl)methanediyl]dipiperidine (**2)** based on ^{13}C- and ^{1}H-NMR analysis (see Figure 3.6). However, the pyrolysis of (**2)** did not generate compound (**1)** or any peak with m/z value of 175. In addition, spiking of glucose/lysine model system with excess piperidine also did not enhance the intensity of the peak in question. Similarly, model systems of piperidine/HMF or piperidine/5-MF did not generate the peak in question. Based on the elemental composition of compound **1** and the number and origin of carbon and nitrogen atoms from glucose and lysine (see Table 3.4) and based on the fact that 5-MF significantly enhances the intensity of this peak in the presence of glucose we propose that the Schiff base adduct is formed between 5-MF and Nε-pent-4-ene-1-amine instead of piperidine and undergoes oxidation to generate the extensively conjugated compound (**1)** as depicted in Figure 3.6.

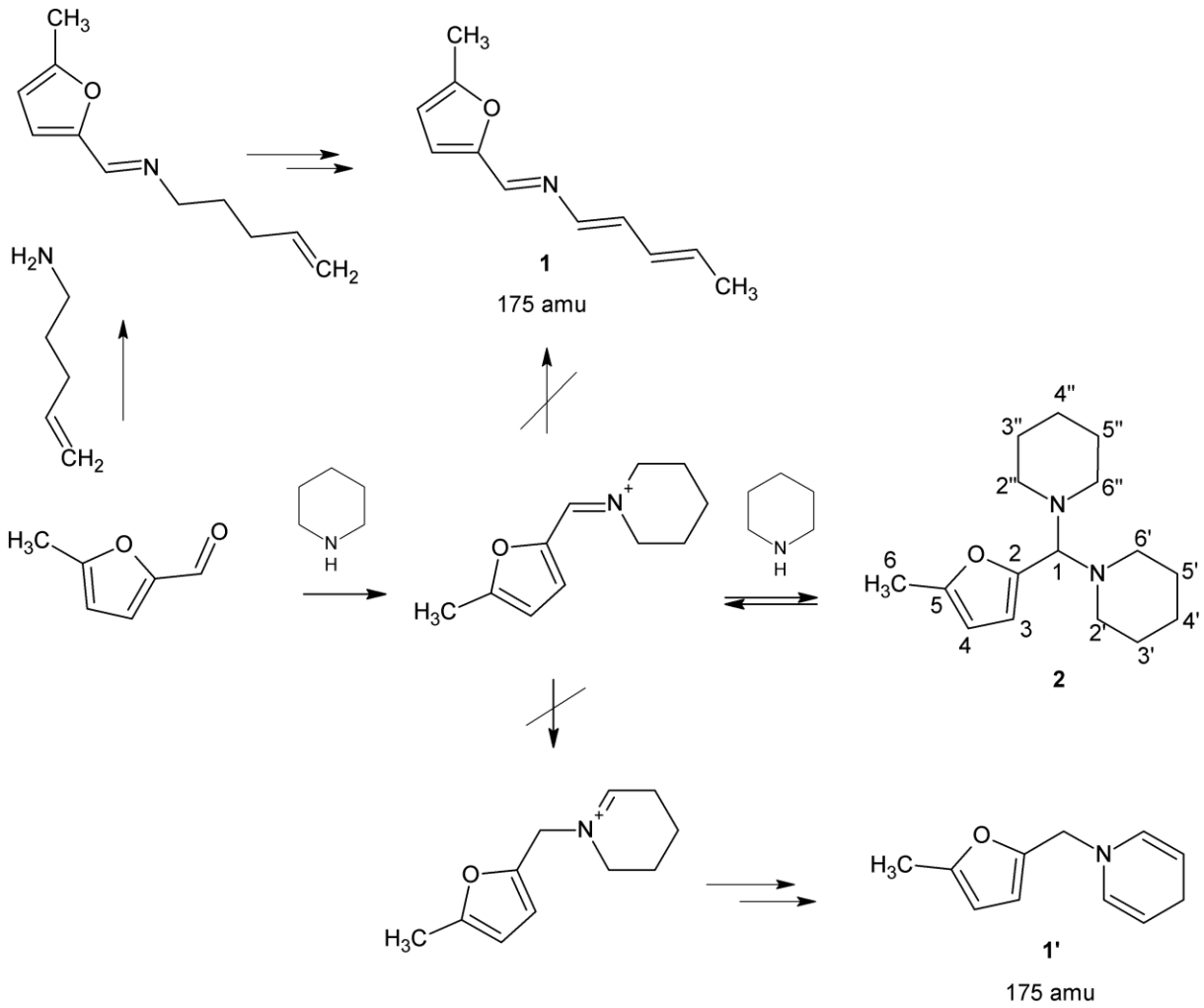


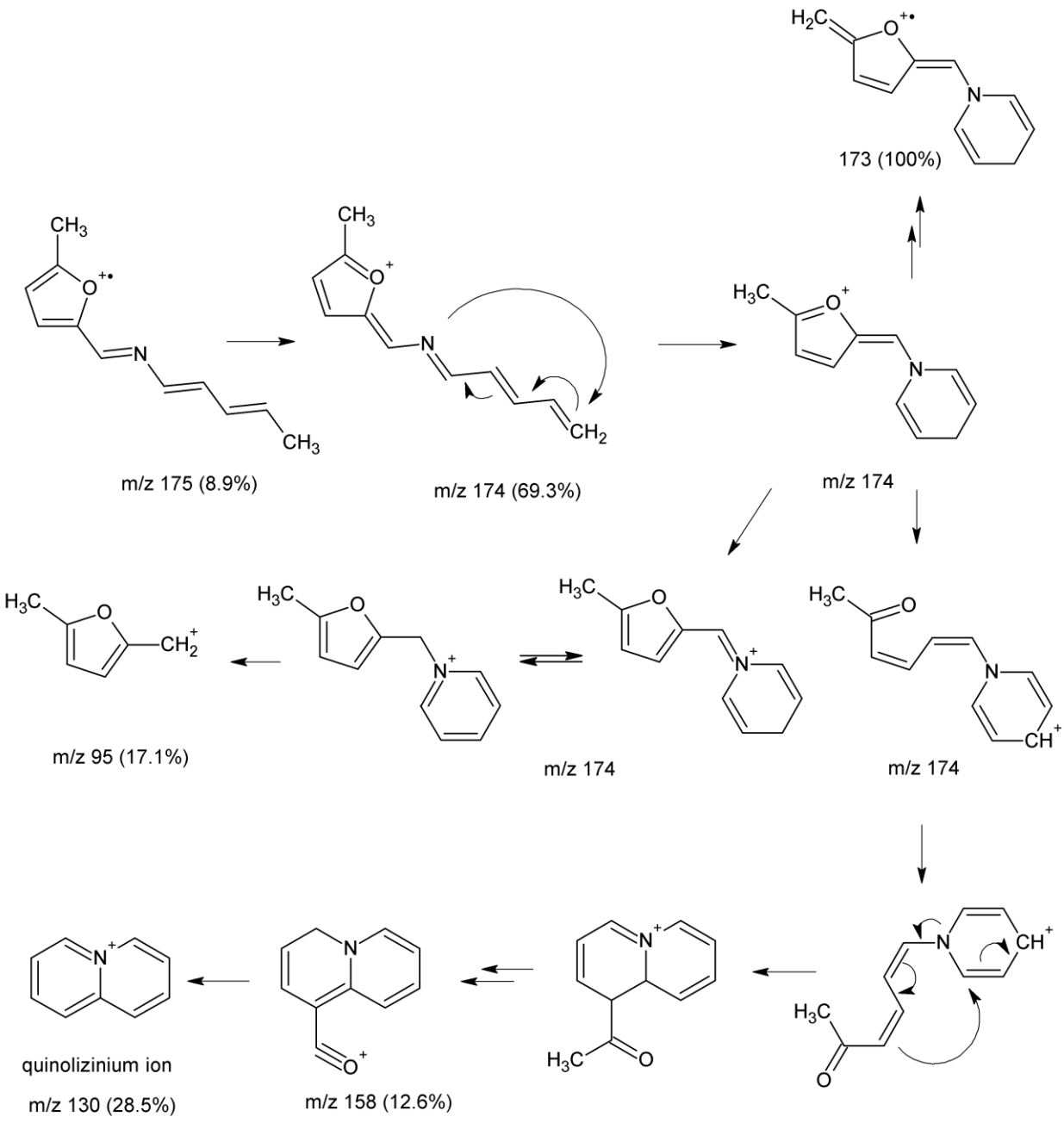
Figure 3.6 Proposed reaction of 5-methylfurfural with Nε-pent-4-ene-1-amine and piperidine

Table 3.4 Summary of structural information for the proposed structure *N*-(5-methylfuran-2-yl)methylidene]penta-1,3-dien-1-amine (**1**).

Molecular weight $[M+H]^+$	176.1068 amu	TOF-MS
Elemental formula	$C_{11}H_{13}NO$	TOF-MS
Number of C atoms from glucose	C-1, C-2, C-3, C-4, C-5, C-6	Labelling studies with MS
Number and identity of lysine atoms	Nε, C-2', C-3', C-4', C-5', C-6' (90%) Nα, C-2', C-3', C-4', C-5', C-6' (10%)	Labelling studies with Py-GC/MS
Partial elemental formula	C_{11}Nε (90%) C_{11}Nα (10%)	Labelling studies with Py-GC/MS
Precursor studies - Enhanced signal	Glucose, 5-MF, Lysine	Py-GC/MS
Precursor studies- No change in signal	HMF, piperidine, 1,1'-[(5-methylfuran-2-yl)methanediyl]dipiperidine (**2**)	Py-GC/MS, synthesis
Mass spectrum	Labelled mass spectral fragments consistent with proposed structure	Py-GC/MS (see Figure 3.7)
Necessary precursor moieties	5-MF and Nε-pent-4-ene-1-amine	Labelling studies with MS Precursor studies Elemental formula & MW

5-MF = 5-methylfurfural, HMF = 5-hydroxymethyl-furfural

This proposed structure is not only consistent with the isotope labelling data incorporating a non-piperidine moiety possessing C2'-C'6 atoms including Nε of lysine and six carbons of glucose in the form of 5-MF but also with the spiking experiments with piperidine and 5-MF. Furthermore, Figure 3.7 shows the mass spectral fragmentation of compound (**1)** consistent with the labelling data. In addition, experiments carried out with specifically labelled glucoses have also indicated that the fragment at m/z 158 showed no incorporation of C-6 atom of glucose and the fragment at m/z 130 showed no incorporation of both C-5 and C-6 atoms of glucose as expected from the proposed fragmentation pattern shown in Figure 3.7.



m/z	175	174	173	158	130	95
[$^{13}U_6$]Glucose	+6	+6	+6	+5	+4	+6
[$^{13}U_6$]Lysine	+5	+5	+5	+5	+5	0
[^{13}C-6]lysine	+1	+1	+1	+1	+1	0
[^{15}Nε] Lysine	+1	+1	+1	+1	+1	0
[^{15}Nα] Lysine	+0	+0	+0	+0	+0	0

Figure 3.7 Mass spectral fragmentation pattern of compound **1** and the number of labelled atoms incorporated into the major mass spectral fragments

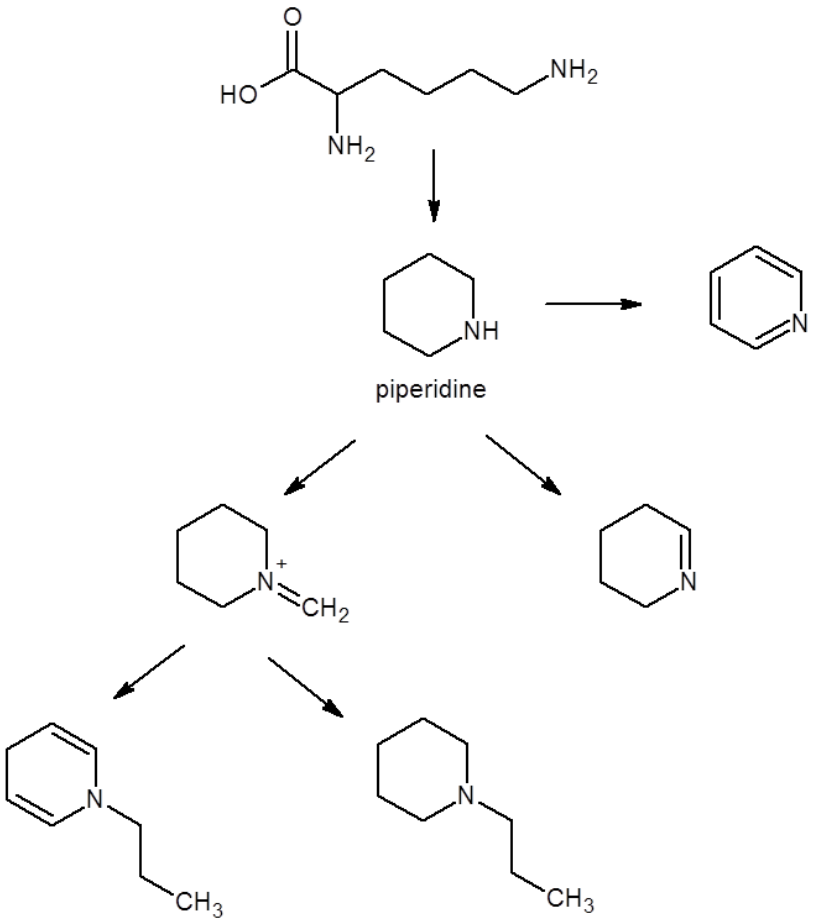
3.5 Conclusions

Complete isotope mapping and precursor studies of the unknown compound (**1)** together with other relevant information shown in Table 3.4 have indicated that the compound in question is composed of both glucose and lysine moieties. Furthermore, the lysine moiety can only arise from Nε-pent-4-ene-1-amine or one of its non-cyclic derivatives such as penta-1,3-dien-1-amine and not from piperidine, confirming the ability of lysine to undergo a similar chemical transformation to that of asparagine in generating an amino acid derived vinylic compound. The second lysine intermediate, piperidine, was demonstrated to form a stable dipiperidine adduct with a glucose degradation product, 5-methyl furfural, at room temperature.

CONNECTING PARAGRAPH

Chapter 3 established the existence of the lysine-specific intermediates piperidine and Nε-pent-ene-amine in glucose/lysine models. The structures of a piperidine and Nε-pent-ene-amine reaction products were successfully characterized. In Chapter 4 the reaction behaviour of the commercially available piperidine will be investigated. The focus of this chapter will be to determine the types of compounds formed in piperidine models with glucose or glucose degradation products and their mechanism of formation. Chapter 4 was published in the Journal of Food Chemistry. (Nikolov, P.Y., Yaylayan, V.A. (2010). Chemical activation of piperidine by formaldehyde and formation of lysine-specific Maillard reaction products. *Food Chemistry*, 123, 684-690)

CHAPTER 4: CHEMICAL ACTIVATION OF PIPERIDINE BY FORMALDEHYDE AND FORMATION OF LYSINE-SPECIFIC MAILLARD REACTION PRODUCTS



4.1 Abstract

Piperidine is considered as a lysine-specific Maillard reaction product that can be formed from free lysine through decarboxylation and deamination reactions or through cyclization of Nε-pent-4-ene-1-amine the counterpart of acrylamide from lysine. Due to the importance and reactivity of piperidine its further interaction products in glucose/lysine model system was investigated. A useful strategy based on Py-GC/MS analysis was developed using isotope labelling technique to identify reaction products incorporating piperidine moieties. Products simultaneously possessing five lysine carbon atoms (C2'-C6') and the Nε-amino group from lysine in addition to glucose carbon atoms were targeted using specifically labelled precursors such as [^{15}Nα]Lysine.2HCl, [^{15}Nε]lysine.2HCl, [U-$^{13}C_6$]Lysine.2HCl, [^{13}C-6]Lysine.2HCl and [U-$^{13}C_6$]Glucose. Detailed labeling studies using specifically ^{13}C-enriched sugars have shown that the piperidine can form reactive 1-methylidenepiperidinium ion with formaldehyde which is able to undergo further aldol addition reactions to form compounds such as 3-(piperidin-1-yl)propanal and 3-(pyridin-1(4*H*)-yl)propanal. Furthermore, these studies have also demonstrated that oxidation of piperidine into di- and tetrahydropyridine derivatives can generate reactive eneamine moieties capable of nucleophilic attack at carbonyl groups and formation of pyridine derivatives.

4.2 Introduction

Discovery of acrylamide as one of the thermally generated toxicants formed in foods not only contributed to our further understanding of the mechanism of the Maillard reaction in general (Yaylayan, 2009) but also allowed the identification of the corresponding products originating from different amino acids other than asparagine such as styrene

from phenylalanine (Goldmann et al., 2009) and Nε-pent-4-ene-1-amine from lysine (Nikolov & Yaylayan, 2010a). Acrylamide and styrene are relatively stable end-products although they are known to undergo further reactions under specific conditions such as the reaction of amino acids with acrylamide (Zamaro et al., 2010) and polymerization reactions of styrene. On the other hand, the chemical reactions of Nε-pent-4-ene-1-amine, the counterpart of acrylamide from lysine, are relatively unknown due to its recent discovery (Nikolov and Yaylayan, 2010a). However, it has been shown for example that Nε-pent-4-ene-1-amine can react with 5-methylfurfural and form an adduct which has been identified as *N*-(5-methylfuran-2-yl)methylidene]penta-1,3-dien-1-amine (Structure **1**, Figure 4.1). In addition, pent-4-ene-1-amine has been shown to exist mainly as its intramolecular cyclization product the piperidine (Structure **2**, Figure 4.1) a reactive secondary amine capable of interacting with furfural to form yellow pigments as demonstrated by Hofmann (1998). On the other hand, the cyclization of Nε-pent-4-ene-1-amine to produce 2-methylpyrrolidine is less likely to occur due to the higher stability of the competing six-membered ring system. Related structures such as 2,3,4,5-tetrahydripyridine has been also identified in glucose/lysine mixtures (Miller et al., 1984) and presumed to be formed through intramolecular cyclization of the Strecker aldehyde of lysine (Figure 4.1). In addition, piperidinium cation at m/z 84 was found to constitute the base peak in the mass spectrum of lysine Amadori product (Yaylayan & Sporns, 1989). Mills and colleagues (1969) have synthesized piperidine Amadori product and studied its degradation.

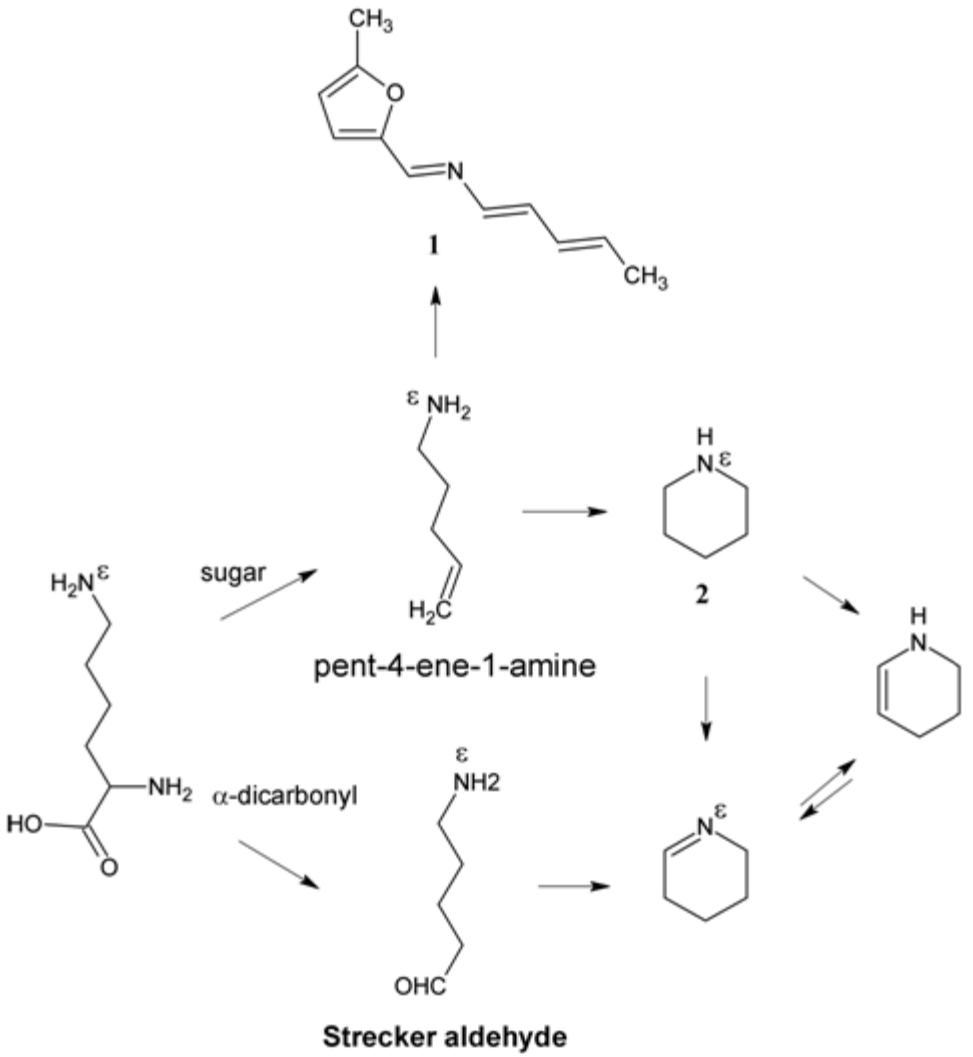


Figure 4.1 Formation of lysine-specific degradation products (α-dicarbonyls from the Maillard reaction can initiate Strecker reaction).

In addition, it has been shown (Nikolov and Yaylayan, 2010a) that lysine alone can also produce piperidine through decarboxylation and deamination reaction sequence. Piperidine is classified as toxic and can also be formed enzymatically from lysine in biological systems and has been detected in human urine and in various animal organs (Schmidtglenewinkel et al., 1977). Due to its reactivity, piperidine may also be involved in the generation of dietary advanced glycation end-products. In this chapter, further

reactions of piperidine initiated by formaldehyde are explored as lysine specific Maillard reaction products.

4.3 Materials and methods

All chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. [^{15}Nα]Lysine.2HCl, [^{15}Nε]lysine.2HCl, [U-$^{13}C_6$]Lysine.2HCl, [^{13}C-6]Lysine.2HCl, [U-$^{13}C_6$]Glucose, [^{13}C-1]Glucose, [^{13}C-2]Glucose, [^{13}C-3]Glucose, [^{13}C-4]Glucose, [^{13}C-5]Glucose and [^{13}C-6]Glucose purchased from CIL (Andover, MA).

4.3.1 Sample preparation

The dihydrochloride salts of the commercially available isotopically labelled lysines were unreactive when pyrolyzed as such, however, mixing the salts with an equimolar amounts of unlabelled free lysine resulted in increased reactivity when pyrolyzed. Consequently, equimolar amounts of unlabelled DL-lysine and specifically labelled DL-lysine.2HCl were mixed and homogenized before mixing with equimolar amount of D-glucose. For isotope labelling studies [^{15}Nα]Lysine.2HCl, [^{15}Nε]lysine.2HCl, [U-$^{13}C_6$]Lysine.2HCl, [^{13}C-6]Lysine.2HCl, [U-$^{13}C_6$]Glucose, [^{13}C-1]Glucose, [^{13}C-2]Glucose, [^{13}C-3]Glucose, [^{13}C-4]Glucose, [^{13}C-5]Glucose and [^{13}C-6]Glucose were used.

4.3.2 Pyrolysis-GC/MS

Analyses were conducted using a Varian CP-3800 GC coupled with a Saturn 2000 Ion Trap Mass Spectrometer (Varian, Walnut Creek, USA). The pyrolysis unit included a CDS Pyroprobe 2000 and a CDS 1500 valved interface (CDS Analytical, Oxford, USA) installed onto the GC injection port. About 2.5 milligram of sample mixture was packed

inside a quartz tubes (0.3mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed for 20 seconds at a temperature of 250°C. The sample separation was carried out on a DB-5MS (5% diphenyl, 95% dimethyl polysiloxane) capillary column with dimensions of 50 m length by 0.2 mm internal diameter and 0.33 μm film thickness (J&W Scientific, ON, Canada), using helium as the carrier gas. The GC column flow rate was regulated by an electronic flow controller (EFC) and set at a pressure pulse of 70psi for the first 4 minutes and later maintained with a constant flow of 1.5 mL/minute for the remainder of the run. The GC oven temperature was set at -5°C for 5 minutes using CO_2 as the cryogenic cooling source. The temperature was increased to 50°C at a rate of 50°C/minute and then to 270°C at a rate of 8°C/minute, and kept at 270°C for 5 minutes. The samples were detected by using an ion-trap mass spectrometer with a scan range of 20-650m/z. The MS transfer-line temperature was set at 250°C, manifold temperature was set at 50°C, and the ion-trap temperature was set at 175°C. The ionization voltage of 70 eV was used, and EMV was set at 1700 V. The number of compounds was calculated using the peak area of analytes integrated by Varian MS Data Review software with the following parameters: peak width 2.0 seconds; slope sensitivity 4 (SN); tangent 10%; and peak size reject 10000 counts. A Hewlett-Packard GC with a mass selective detector (5890 GC/5971B MSD) interfaced to a CDS pyroprobe 2000 unit was used for the Py-GC/MS analysis of piperidine with formaldehyde. The column used was a PLOT-Q with dimensions of 30 m length by 0.320 mm internal diameter and 20 μm film thickness (J&W Scientific, ON, Canada), using helium as the carrier gas. The pyroprobe interface temperature was set at 250°C. The capillary direct MS interface temperature was 250°C, and the ion source temperature was 130°C. Ionization voltage

was 70eV, and the electron multiplier voltage was 1871V. Initial temperature of the GC oven was set at 35°C for 2 min. The temperature was increased to 175°C at a rate of 30°C/minute and then to 250°C at a rate of 10°C/minute and held for 20 minutes. Compound identification was performed using AMDIS (ver 2.65) and NIST Standard Reference Databases (data version 05 and software ver 2.0d) to compare the target compounds with the existing mass spectral libraries or by injecting commercially available standards. The reported percent label incorporation values (corrected for natural abundance and for percent enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

4.4 Results and discussion

Lysine can be converted into two azine derivatives, piperidine (**2** in Figure 4.1) through intramolecular cyclization of Nε-pent-4-ene-1-amine (Nikolov & Yaylayan, 2010a) and 2,3,4,5-tetrahydropyridine (Miller et al., 1984) through intramolecular cyclization of the Strecker aldehyde (see Figure 4.1). Although piperidine can be converted into 2,3,4,5-tetrahydropyridine through oxidation, however, the reverse reaction is not known to occur freely under Maillard reaction conditions. Figure 4.2 shows the major products of the reaction of lysine with honey indicating the importance of these two cyclic amines in lysine containing food systems. Due to the known reactivity of secondary amines with aldehydes their further reaction products were investigated in glucose/lysine reaction mixtures using specifically labelled precursors such as [^{15}Nα]lysine.2HCl, [^{15}Nε]lysine.2HCl, [U-$^{13}C_6$]lysine.2HCl, [^{13}C-6]lysine.2HCl and [U-$^{13}C_6$]glucose. Products simultaneously possessing Nε nitrogen atom and five carbon atoms from lysine (C2' to C6') in addition to glucose carbon atoms were specifically investigated to identify

piperidine related sugar adducts. Table 4.1 lists two such products at retention times of 19.43 and 24.03 min satisfying the above requirements. Detailed isotope labelling studies with specifically ^{13}C-enriched glucoses have also indicated that formaldehyde played a key role in their formation as elaborated below.

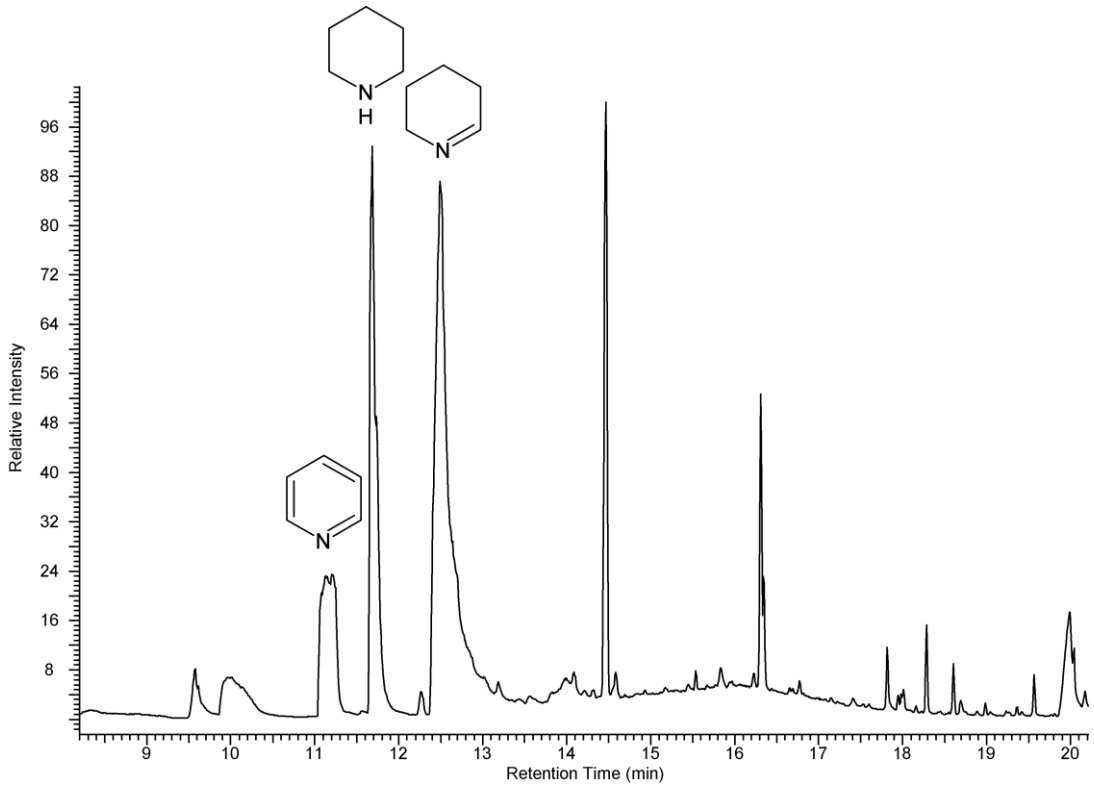


Figure 4.2 Py-GC/MS chromatogram of lysine/honey mixture (1:1 w/w) heated at 250 ^{o}C for 20 seconds, showing the major peaks (piperidine and 2,3,4,5-tetrahydropyridine)

Table 4.1 Percent label distribution[1] (± 5%) in selected products generated from lysine/glucose model system

Retention time(min)	Molecular weight	M	M+1	M+2	M+3	M+4	M+5	M+6
19.435	141							
[^{15}Nα]Lysine		0	0	0	0	0	0	0
[^{15}Nε]Lysine		0	100	0	0	0	0	0
[^{13}C-6]Lysine		0	100	0	0	0	0	0
[$^{13}U_6$]Lysine		0	0	0	0	0	100	0
[$^{13}U_6$]Glucose		0	0	0	100	0	0	0
24.03	137							
[^{15}Nα]Lysine		0	0	0	0	0	0	0
[^{15}Nε]Lysine		0	100	0	0	0	0	0
[^{13}C-6]Lysine		0	100	0	0	0	0	0
[$^{13}U_6$]Lysine		0	0	0	0	0	100	0
[$^{13}U_6$]Glucose		0	0	0	100	0	0	0

[1]Corrected and adjusted for the 50% mix

4.4.1 Formation of proposed 3-(piperidin-1-yl)propanal (3) and 3-(pyridin-1(4H)-yl)propanal (4)

The two proposed piperidine adducts (**3** & **4**) are shown in Figure 4.3. Both structures possessed five carbons (C2' to C6') and one Nε-atom from lysine in addition to three carbon atoms from glucose **(**Table 4.1). They differed only with 4 atomic mass units (amu) one having a nominal molecular weight of 141 amu and the other 137 amu. Detailed investigation of the origin of the glucose carbon atoms in compound **4** using specifically labelled glucoses showed a mixed origin and scrambling of labels in the 3-carbon sugar fragment (see Tables 4.2 and 4.3).

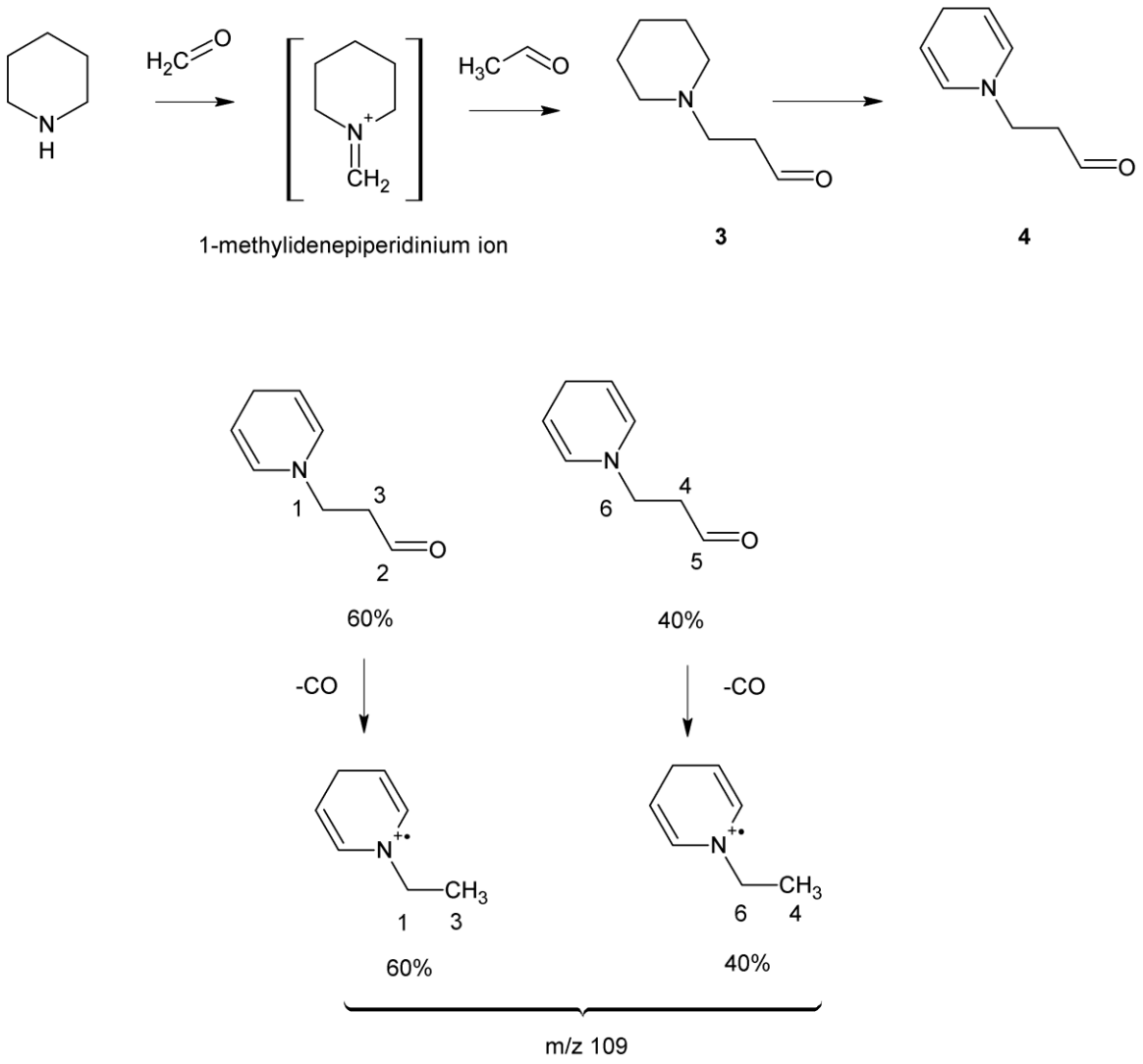


Figure 4.3 Proposed mechanism of formation of compounds **3** & **4** and the origin of carbon atoms from glucose

According to Table 4.3, the three carbon sugar moiety involved the incorporation of C1-C3-C2 (60%) and C6-C4-C5 (40%) carbon atom sequences from glucose (see Figure 4.3). Spiking experiments with piperidine significantly enhanced the intensities of the peaks at retention times 24.03 and 19.43 min (compounds **3** and **4**) confirming the incorporation of piperidine. From the labelling and spiking data it can be concluded that piperidine in the first step reacts with a one carbon sugar moiety formaldehyde (generated from C-1 or

Table 4.2 Number of labelled atoms incorporated in the molecular ion at m/z 137 and selected mass spectral fragments[1] of compound **4**

m/z	137	136	109	108	94	81	80
[$^{13}U_6$]Glucose	+3	+3	+2	+2	+2 (50%) +1 (50%)	+1	+1
[$^{13}U_6$]Lysine	+5	+5	+5	+5	+4 (50%) +5 (50%)	+4	+4
[^{13}C-6]lysine	+1	+1	+1	+1	+1 (50%) +0 (50%)	+1	+1 (80%) +0 (20%)
[^{15}Nε] Lysine	+1	+1	+1	+1	+1	+1	+1
[^{15}Nα] Lysine	+0	+0	+0	+0	+0	+0	+0

[1]see Figure 4.4

Table 4.3 Percent label incorporation in the molecular ion at m/z 137 and selected mass spectral fragments[1] of compound **4.**

m/z	137	138	109	110	108	109
	M	M+1	M	M+1	M	M+1
[^{13}C-1]Glucose	40%	60%	40%	60%	40%	60%
[^{13}C-2]Glucose	40%	60%	0	0	0	0
[^{13}C-3]Glucose	40%	60%	40%	60%	40%	60%
[^{13}C-4]Glucose	60%	40%	60%	40%	60%	40%
[^{13}C-5]Glucose	60%	40%	0	0	0	0
[^{13}C-6]Glucose	60%	40%	60%	40%	60%	40%

[1]see Figure 4.4

C-6 of glucose) and forms the 1-methylidenepiperidinium ion as shown in Figure 4.3, followed by aldol addition of acetaldehyde (C2-C3 or C4-C5 sugar atoms) to generate 3-(piperidin-1-yl)propanal the proposed structure for the peak at retention time of 19.43 min (structure **3** in Figure 4.3). This structure can undergo further oxidation to generate 3-(pyridin-1(4*H*)-yl)propanal (proposed structure **4** for peak at 24.03 min). It seems that under the experimental conditions formaldehyde was generated from the two terminal carbon atoms C-1and C-6 of glucose and acetaldehyde was generated from a C3-C2 and C4-C5 glucose fragments. The origin of formaldehyde as arising from C-1 and C-6 glucose carbon atoms is consistent with the literature data (Paine III et al., 2008) where

flash pyrolysis of specifically labelled glucoses generated formaldehyde mainly from C-1 and C-6 carbon atoms. Furthermore, the mass spectral fragmentation pattern shown in Figure 4.4 indicates a loss of CO from fragment at m/z 136 to generate the base peak at m/z 109. This fragmentation step can be used to confirm the isotopic origin of the carbonyl carbon atom in structure **4** and at the same time identify the exact carbon sequence of the acetaldehyde reacting with 1-methylidenepiperidinium ion as shown in Figure 4.3. The isotope labelling data shown in Table 4.3 indicate that the ion at m/z 109 (or m/z 108) is generated through the loss of CO either from C-2 or from C-5 atoms of

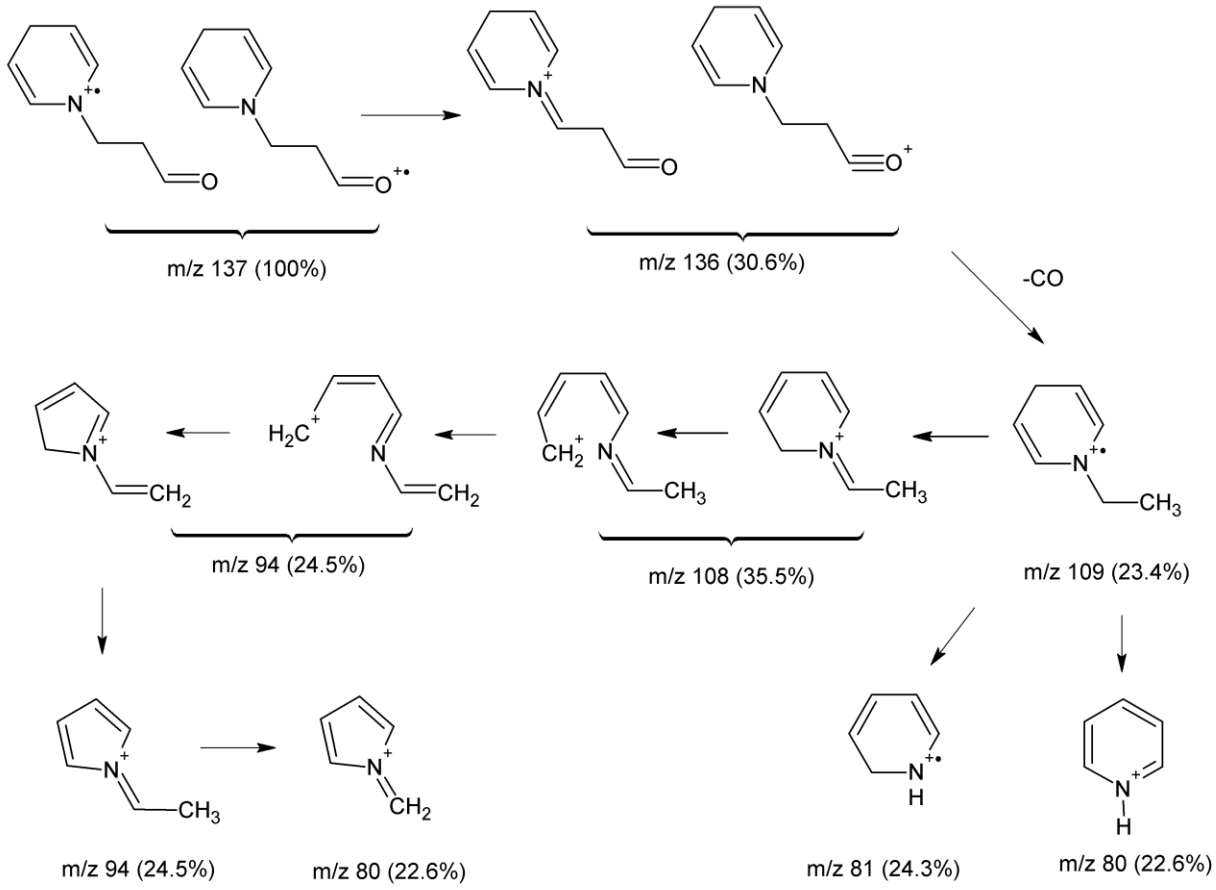


Figure 4.4 Mass spectral fragmentation pattern of compound **4**

glucose thus confirming the sequence of isotopically labelled carbon atoms in structure **4** as shown in Figure 4.3. The observed glucose carbon atom distribution and sequence in acetaldehyde and formaldehyde can be rationalized by the assumption that they can be formed through α-dicarbonyl cleavage of methylglyoxal. The methylglyoxal is known (Gobert, & Glomb, 2009) to arise through retro-aldol reaction of 3-deoxyglucosone (3-DG) as shown in Figure 4.5. However, due to the carbonyl group migration in glucose (Yaylayan & Ismail, 1995) or the formation of Lederer's glucosone (Reihl et al., 2004) methylglyoxal can also arise from C6-C5-C4 carbon sequence (40%) in addition to C1-C2-C3 sequence (60%) as shown in Figure 4.5. A recent study (Jenny & Glomb, 2009) has confirmed the importance of Lederer's glucosone formation in glucose/lysine model system.

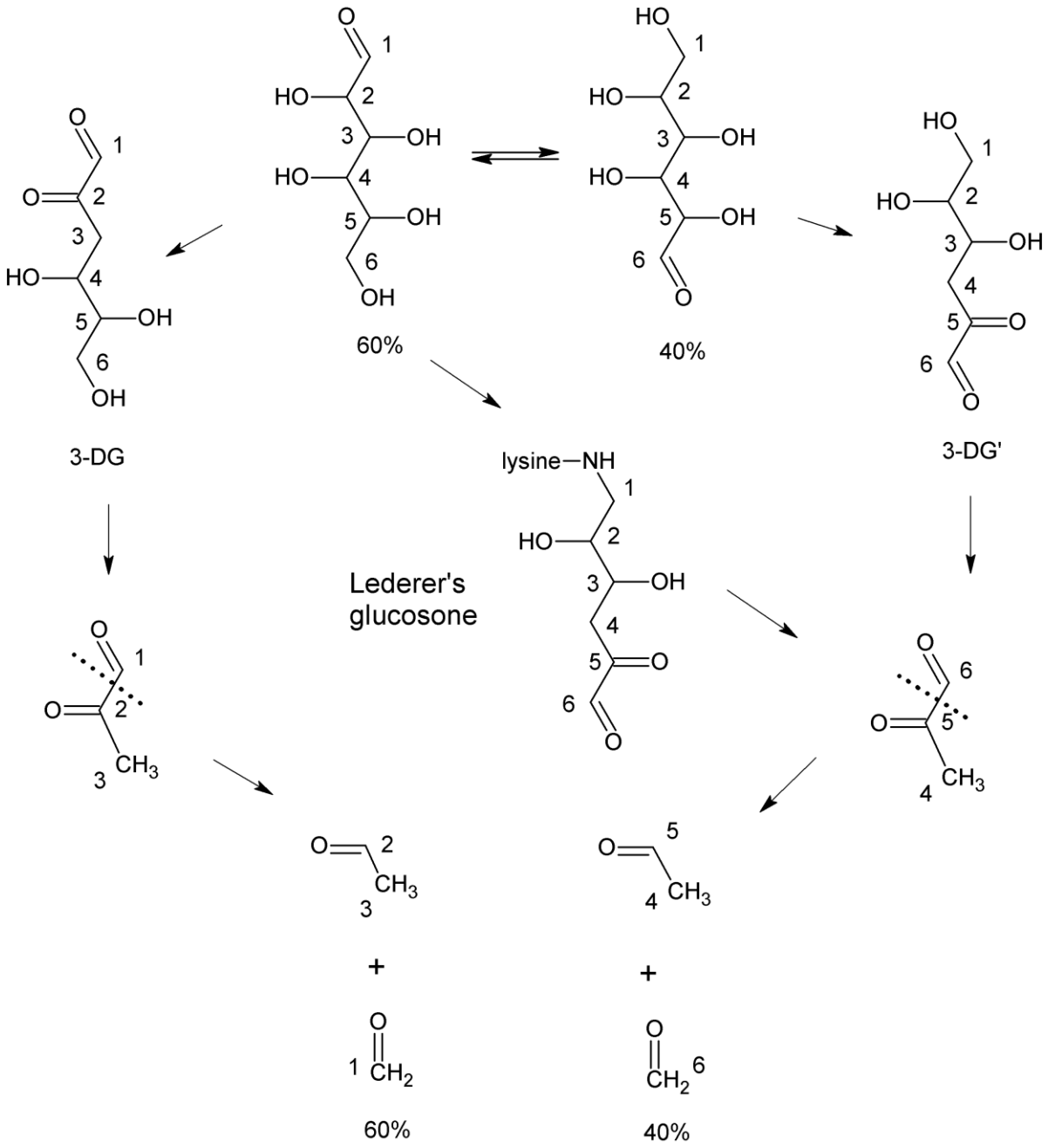


Figure 4.5 Proposed mechanism of formation of formaldehyde and acetaldehyde from glucose

4.4.2 Reaction of piperidine with formaldehyde – activation towards nucleophilic attack

To investigate further the piperidine formaldehyde interaction which constitutes the first step of formation of structures **3** and **4**, this simple model was studied using Py-GC/MS. The reaction mixture produced four major products 1,1'-methanediyldipiperidine, pyridine, 3-methylpyridine and 3,5-dimethylpyridine shown in Figure 4.6 tentatively identified through NIST library search. The product distribution indicated that piperidine is able to undergo Schiff base formation (a reactive imminum ion) which can be stabilized through nucleophilic reaction with a second mole of piperidine to produce the dipiperidine structure (see Figure 4.6) or with any other amino acid. In addition, piperidine can undergo oxidation to produce dihydro- and tetrahydropiperidines able to react as an enamine with formaldehyde and form methylated pyridine derivatives shown in Figure 4.6. These results are consistent with the mechanism of formation of **4**.

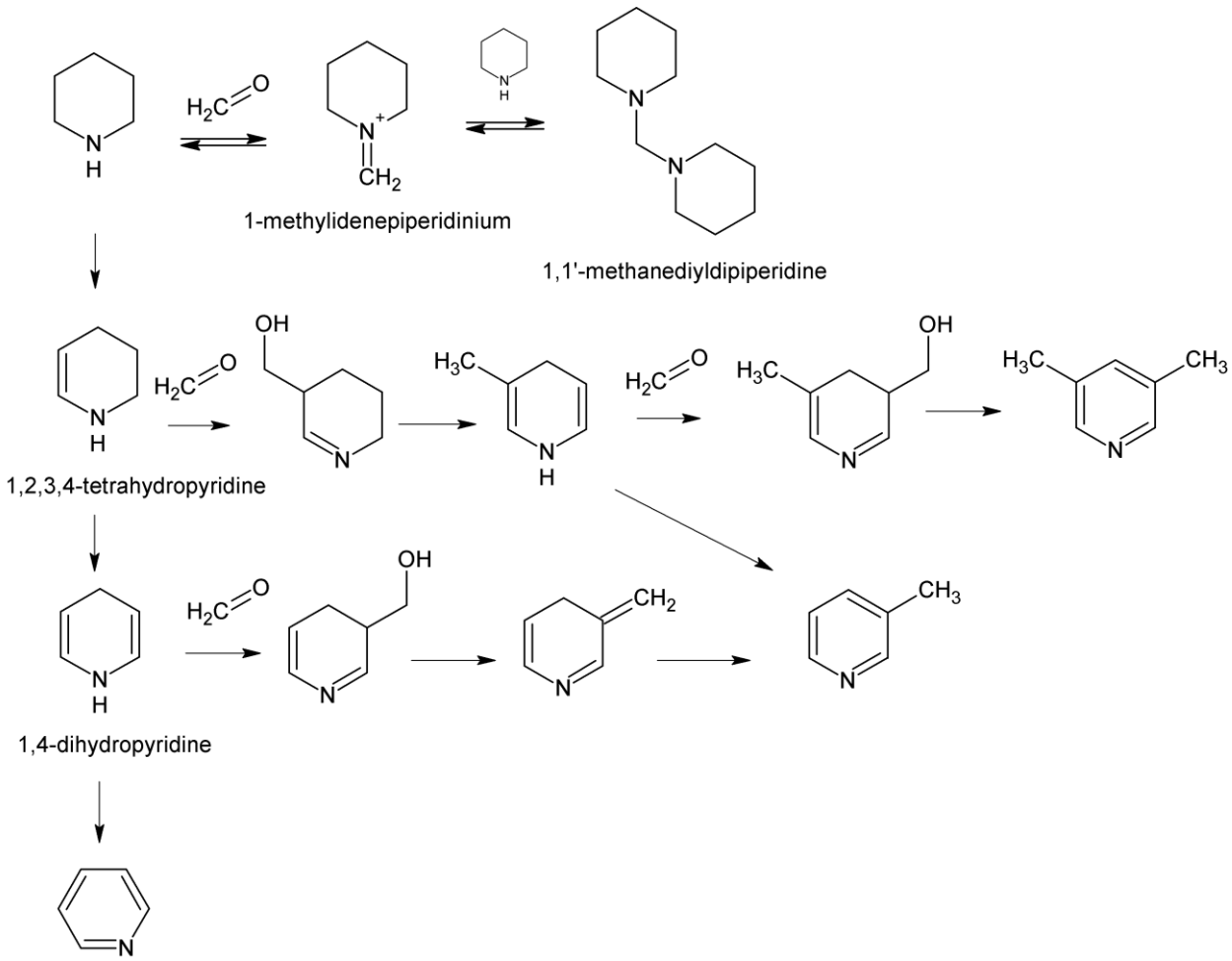


Figure 4.6 Reaction of piperidine with formaldehyde and formation of pyridine derivatives

4.5 Conclusions

Piperidine can be considered a lysine specific reactive amine originating from Nε-pent-4-ene-1-amine, the acrylamide counterpart of lysine. It is able to undergo carbonyl-amine reactions with formaldehyde to form the activated intermediate 1-methylidenepiperidinium ion. This chemically activated ion serves as a potent trap for carbonyls, with subsequent additions of acetaldehyde leading to two novel piperidine/sugar degradation products. A second mechanistic pathway involves piperidine undergoing enamine-carbonyl interactions generating lysine specific Maillard products, such as various pyridine derivatives, summarized in Figure 4.7.

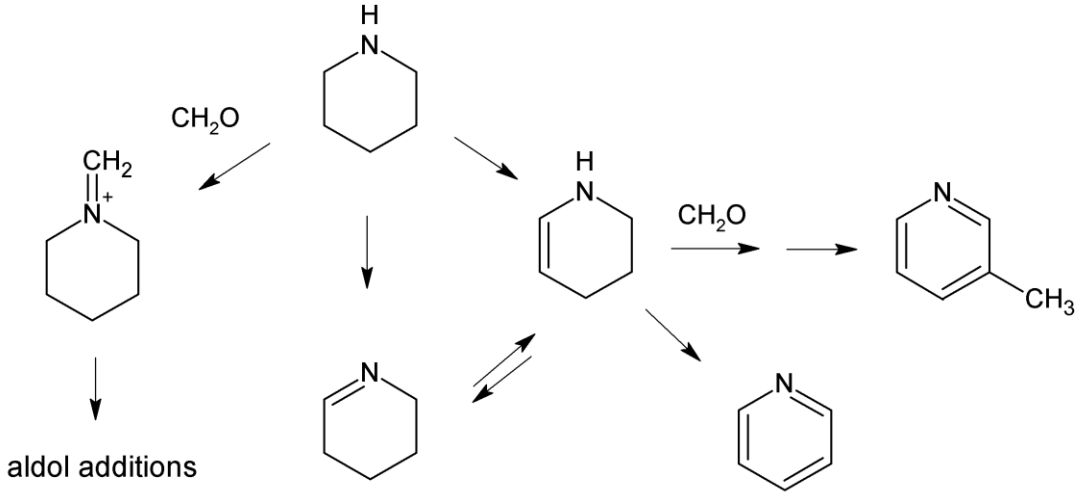
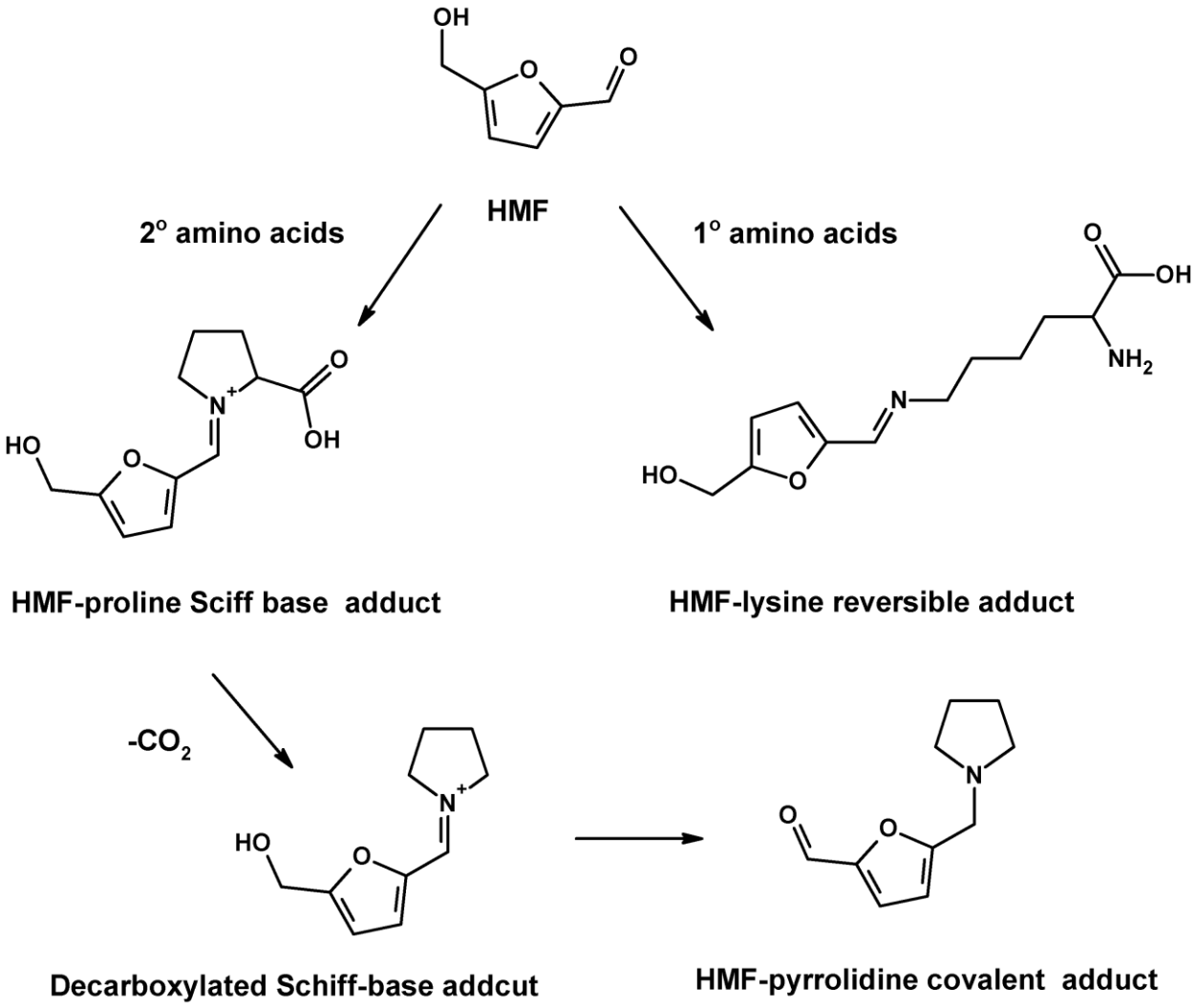


Figure 4.7 Summary of piperidine interaction with formaldehyde

CONNECTING PARAGRAPH

In Chapters 3 and 4, the reaction of glucose degradation products such as methylfurfural and acetaldehyde with lysine-derived reactive intermediates were investigated. Chapter 5 investigates the reaction behaviour of one of the most common thermal degradation products of glucose in food, 5-hydroxylmethyl-furfural (HMF) with lysine. The focus was to study the interaction between the lysine-derived intermediates, piperidine and Nε-pent-4-ene-1-amine and HMF. Furthermore, to confirm the generality of the reaction behaviour of HMF with lysine other models containing different amino acids were also studied. Chapter 5 was published in the Journal of Agricultural and Food Chemistry (Nikolov, P.Y. & Yaylayan, V.A. (2011) Reversible and Covalent Binding of 5-(hydroxymethyl)-2-furaldehyde (HMF) with Lysine and selected Amino Acids. *Journal of Agricultural and Food Chemistry*, 59, 6099 – 6107).

CHAPTER 5: REVERSIBLE AND COVALENT BINDING OF 5-(HYDROXYMETHYL)-2-FURALDEHYDE (HMF) WITH LYSINE AND SELECTED AMINO ACIDS



5.1 Abstract

The chemical reactivity of 5-(hydroxymethyl)-2-furaldehyde (HMF) with lysine, glycine and proline was studied using isotope labelling technique. To confirm the formation of HMF adducts in glucose amino acid model systems a useful strategy was developed in which products simultaneously possessing six glucose (HMF moiety) and any number of amino acid carbon atoms in addition to nitrogen were targeted using specifically labelled precursors such as [^{15}Nα]lysine.2HCl, [^{15}Nε]lysine.2HCl, [U-$^{13}C_6$]lysine.2HCl, [^{13}C-6]lysine.2HCl and [U-$^{13}C_6$]glucose in the case of lysine model system. In addition, model systems containing HMF and amino acids were also studied to confirm specific adduct formation. The complete labelling studies along with structural analysis using appropriate synthetic precursors such as HMF Schiff base adducts of piperidine and glycine have indicated that HMF generated in the glucose/amino acid model systems initially forms a Schiff base adduct that can undergo decarboxylation through oxazolidin-5-one intermediate and form two isomeric decarboxylated Schiff bases. Unlike the Schiff bases resulting from primary amines or amino acids such as glycine or lysine, those resulting from secondary amino acids such as proline or secondary amines such as piperidine can further undergo vinylogous Amadori rearrangement forming N-substituted 5-(aminomethyl)furan-2-carbaldehyde derivatives.

5.2 Introduction

The chemical reactivity of one of the most abundant Maillard reaction products the 5-(hydroxymethyl)-2-furaldehyde (HMF) is also one of the least studied. Although its origin and formation mechanism is well understood, however its fate remains to be

investigated specially due to its abundant formation in many foods including honey (White, 1979; Jeuring & Kuppers, 1980; Anklam, 1998), bread (Ramirez-Jimenez et al., 2000), infant formulas (Ferrer et al., 2005) citrus juices, freeze-dried pears, grape juice, tomato products and syrup (Kim et al., 1992) to name a few. Fructose and sucrose are considered to be the most efficient precursors in food that can be transformed into HMF through the formation of reactive fructofuranosyl cation (Perez- Locas & Yaylayan, 2008). According to Bachmann et al. (1997) the average content of HMF in various foods exceeds 1g/kg levels with estimated daily consumption of 150 mg/person or 2.5mg/kg body weight (Ulbrich et al., 1984). Its prevalence in thermally processed foods has made it a useful indicator of the severity of heat treatment or storage time (Sanz et al., 2003; Porretta, 1991; Rada-Mendoza et al., 1991). Due to its demonstrated ability to break DNA strands (Omura & Jahan, 1983) and its weakly positive genotoxicity response (Glatt & Sommer, 2006) HMF has been the subject of intense study. Although some of its toxic metabolites have been identified, such as 5-hydroxymethyl-2-furoic acid and 5-sulfoxymethyl-furfural, however, its reactivity with amino groups of proteins or peptides has only been investigated under physiological conditions. Janzowski et al. (2000) demonstrated its reactivity with glutathione in different cell cultures. Abdulmalik et al. (2005) showed reversible binding with human hemoglobin in transgenic mouse and only recently that direct evidence for its ability to bind to N-terminal valine groups of human hemoglobin through Schiff base adduct formation has been demonstrated (Davies et al., 2009). This study has shown that in some human blood samples background levels of 10-35pmol/g of globin of HMF-Schiff base adduct has been detected. On the other hand, using [U-^{14}C]-HMF treated rats and mice Godfrey et al. (1999) concluded that HMF may

also bind covalently with tissue proteins since extensive washing of tissue homogenates did not remove the radioactivity. Although reversible binding can be explained through Schiff base formation, however, non-reversible covalent binding of HMF has not been rationalized. Understanding the reactions of HMF with amino acids may provide further insight into its chemical reactivity in addition to its known ability to polymerize.

5.3 Materials and methods

All chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. L-[^{15}Nα]lysine.2HCl, L-[^{15}Nε]lysine.2HCl, L-[U-$^{13}C_6$]lysine.2HCl, L-[^{13}C-6]lysine.2HCl, D-[U-$^{13}C_6$]glucose, D-[^{13}C-1]glucose, D-[^{13}C-2]glucose, D-[^{13}C-3]glucose, D-[^{13}C-4]glucose, D-[^{13}C-5]glucose, D-[^{13}C-6]glucose, [^{13}C-1]glycine, [^{13}C-2]glycine, [^{15}N]glycine, [^{13}C-1]proline, and [U-$^{13}C_6$,^{15}N] proline were purchased from CIL (Andover, MA), Melting points were determined on OptiMelt automated melting point system (Sunnyvale, Ca). The ^{13}C- and ^{1}H-NMR spectra were acquired in CD_3OD on a Varian VNMRS 500 MHz spectrometer. Infrared spectra were recorded on a Bruker Alpha-P spectrometer (Bruker Optic GmbH, Ettlingen, Germany) equipped with a deuterated triglycine sulphate (DTGS) detector, a temperature controlled single bounce diamond Attenuated Total Reflectance (ATR) crystal and a pressure application device for solid samples.

5.3.1 Sample Preparation

The dihydrochloride salts of the commercially available isotopically labelled lysines were unreactive when pyrolyzed as such, however, mixing the salts with an equimolar amounts of unlabelled free lysine resulted in increased reactivity when pyrolyzed. Consequently, equimolar amounts of unlabelled DL-lysine and specifically labelled DL-lysine.2HCl were

mixed and homogenized before mixing with equimolar amount of D-glucose (see Table 5.1).

5.3.2 Preparation of ESI-TOF MS samples

Glucose (10 mg) and lysine.2HCl (14 mg) were dissolved in distilled water (0.8 mL) and heated in an open vial (3mL) at 110^{o}C for 45 min or until dryness generating a brown powder. The experiments were repeated with [U-$^{13}C_6$]glucose and [$^{15}N\alpha$]lysine. The pyrolysis of this powder generated a similar but not identical profile to that of lysine glucose samples pyrolyzed without prior heating as described above.

5.3.3 Pyrolysis-GC/MS

Analyses were conducted using a Varian CP-3800 GC coupled with a Saturn 2000 Ion Trap Mass Spectrometer (Varian, Walnut Creek, USA). The pyrolysis unit included a CDS Pyroprobe 2000 and a CDS 1500 valved interface (CDS Analytical, Oxford, USA) installed onto the GC injection port. About 2.5 milligram of a sample mixture (see Table 5.1) was packed inside a quartz tubes (0.3mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed for 20 seconds at a temperature of 250°C. The sample separation was carried out on a DB-5MS (5% diphenyl, 95% dimethyl polysiloxane) capillary column with dimensions of 50 m length by 0.2 mm internal diameter and 0.33 μm film thickness (J&W Scientific, ON, Canada), using helium as the carrier gas. The GC column flow rate was regulated by an electronic flow controller (EFC) and set at a pressure pulse of 70psi for the first 4 minutes and later maintained with a constant flow of 1.5 mL/minute for the remainder of the run. The GC oven temperature was set at -5°C for 5 minutes using CO_2 as the cryogenic cooling source. The temperature was increased to 50°C at a rate of 50°C/minute and then to 270°C at a rate of 8^{o}C/minute,

and kept at 270°C for 5 minutes. The samples were detected by using an ion-trap mass spectrometer with a scan range of 20-650m/z. The MS transfer-line temperature was set at 250°C, manifold temperature was set at 50°C, and the ion-trap temperature was set at 175°C. The ionization voltage of 70 eV was used, and EMV was set at 1700 V. Compound identification was performed using AMDIS (ver 2.65) and NIST Standard Reference Databases (data version 05 and software ver 2.0d) to compare the target compounds with the existing mass spectral libraries or by injecting commercially available standards. The reported percent label incorporation values (corrected for natural abundance and for percent enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

5.3.4 ESI-TOF MS analysis

Samples were diluted in 1 mL water and then again 1/100 with 50% methanol and 0.1% formic acid. Each sample (5 μL injections) was directly analyzed by liquid chromatography-mass spectrometry (LC-MS), on a 1200 series Agilent rapid resolution LC system coupled to an Agilent 6210 time of flight (ESI-TOF) instrument. Mobile phase consisted of 50% methanol, 0.1% formic acid at a flow rate of 0.3 ml/min. Data were acquired in positive electrospray mode with an acquisition mass range of m/z 100-1000 and internal calibration using *m/z* 121.050873 and *m/z* 922.009798 (Agilent ESI tuning mix) for accurate mass measurements with a dual sprayer ESI source and constant infusion of calibrant ions. Source conditions were as follows: Gas Temp 350°C, ESI voltage 4000 V, Dry gas flow (nitrogen) 12 L/min, nebulizer gas pressure 35 psig, fragmentor and skimmer voltages of 100 and 60 V, respectively. In the MS/MS mode the

CID spectra of the selected ions were similarly acquired in the positive ion mode with collision energy of 30V.

5.3.5 [5-(dipiperidin-1-ylmethyl)furan-2-yl]methanol (3)

HMF (124 mg) was dissolved in 2x excess piperidine and mixed thoroughly and left at room temperature without solvent for 24h. Acetonitrile (0.2mL) was then added to the mixture and the resulting powder was filtered and washed with excess acetonitrile. The resulting solid had a chemical purity of 98% based on ^{1}H-NMR. Mp 80.6 - 81.6°C. ^{1}H-NMR: δ 1.44 -1.57 (m, 12H, H-3' to 5' and H-3" to H-5"), 2.57 (t, 2H, H-2'), 2.65 (t, 2H, H-2"), 2.74 (t, 4H, H-6', 6") 3.30 (s, 1H, H-1), 4.49 (s, 2H, H-6), 6.28 (s, 2H, H-3,4). ^{13}C NMR: δ 154.4 (C-5), 151.2 (C-2), 109.0 (C-4), 107.4 (C-3), 92.6 (C-1), 56.0 (C-6), 48.5 (C-6',6"), 46.2 (C-2',2"), 25.9 (C-3',3"), 25.4 (C-5',5"), 24.3 (C-4"), 24.2 (C-4'). FTIR (solid): 3226 cm^{-1}(C-OH stretch), 3122 cm^{-1}(=C-H stretch), 2927 cm^{-1}(CH_2-CH_2 stretch), 2855 cm^{-1}(CH_2-CH_2 stretch), 1011cm^{-1}(C-O-C stretch). MS *m/z* (% abundance): 39 (8.6), 42 (11.5), 53 (15.2), 81 (19.8), 84 (11.0), 109 (68.8), 136 (6.4), 164 (24.3), 192 (100), 193 (45.7), 194 (12.7).

5.3.6 Sodium ({[5-(hydroxymethyl)furan-2-yl]methylidene}amino)acetate (7)

HMF (124 mg) and sodium glycinate (97 mg) was mixed at room temperature without a solvent until a homogenous mixture was produced as a light brown oil (15 min). ^{1}H-NMR analysis indicated the presence of two sets of peaks one set was specific to HMF (25%) and the second set was consistent with the title compound (75%). ^{1}H NMR: δ 4.2 (s, 2H, H-2'), 4.6 (s, 2H, H-6), 6.4 (d, 1H, H-3), 6.9 (d, 1H, H-4), 8.0 (s, 1H, H-1). ^{13}C NMR: δ 176.0 (C-1'), 157.8 (C-5), 152.5 (C-1), 150.8 (C-2), 116.2 (C-4), 109.0 (C-3), 63.7 (C-2'), 56.2 (C-6). FTIR (solid): 3243 cm^{-1} (C-OH stretch), 3122 cm^{-1} (=C-H

stretch), 1646 cm^{-1} (C=N stretch), 1582 cm^{-1} (COO^- stretch), 1017 cm^{-1} (C-O-C stretch). MS m/z (% abundance): 39 (30.4), 41 (24.7), 42 (59.2), 51 (19.7), 69 (27.0), 81 (17.3), 97 (23.9), 110 (35.7), 122 (20.5), 138 (57.1), 139 (100), 140 (17.8).

Table 5.1 Composition of model systems analyzed by Py-GC/MS[a]

Target ions	**Model systems studied**
m/z 191 m/z 353	Glucose + [^{15}Nα]lysine.2HCl[b] (1:1) Glucose + lysine.2HCl[b] (1:1) Glucose[U-$^{13}C_6$] + lysine.2HCl[b] (1:1)
m/z 193 m/z 191	Glucose or sucrose + [^{15}Nα]lysine.2HCl + lysine Glucose or sucrose + [^{15}Nε]lysine.2HCl + lysine Glucose or sucrose + lysine + [^{13}C-6]lysine.2HCl Glucose or sucrose + lysine + [U-$^{13}C_6$]lysine.2HCl [U-$^{13}C_6$]glucose+ lysine+ lysine.2HCl HMF + piperidine [5-(dipiperidin-1-ylmethyl)furan-2-yl]methanol (**3**)
m/z139	HMF + [$^{13}C_1$]glycine HMF + [$^{13}C_{2]}$glycine HMF + [^{15}N]glycine HMF + glycine [U-$^{13}C_6$]glucose+ methylamine-HCl + glycine [U-$^{13}C_6$]glucose + methylamine-HCl Glucose + methylamine.HCl + glycine Glucose + methylamine.HCl HMF + methylamine.HCl Sodium ({[5-(hydroxymethyl)furan-2-yl]methylidene}amino)acetate (**7**)
m/z 161 m/z 179	HMF + [U-$^{13}C_5$,^{15}N]proline HMF + [^{13}C-1]proline HMF + proline HMF + pyrrolidine

[a] unless otherwise specified, [b] analyzed by TOF-MS (see section 5.3.4)

5.4 Results and discussion

The chemical reactivity of HMF with amino acids has not been investigated in detail although Schiff base formation could logically be assumed to be a viable end product due to the stability of the resulting conjugated system. The 5-methylfurfural (MF) for example has been shown to form a Schiff base adduct (**1**) with Nε-pent-4-ene-1-amine the counterpart of acrylamide from lysine (see Figure 5.1) in glucose lysine model systems (Nikolov & Yaylayan, 2010a). Similar adducts could be expected to be formed with HMF, however, the latter is also considered as α, β-unsaturated aldehyde derivative that can undergo Michael addition with sulfur nucleophiles or vinylogous Amadori rearrangement with nitrogen nucleophiles (Yaylayan & Locas, 2007; Wondrak et al., 1997). However, the fact that α, β-unsaturated moiety constitutes a part of the HMF aromatic ring system drastically reduces its reactivity requiring a driving force to overcome the activation energy needed to disrupt the conjugated system. To explore the chemistry of HMF reactions with various amino acids and particularly with lysine appropriate model systems were investigated through isotope labelling technique (see Table 5.1).

5.4.1 Reaction of glucose or sucrose with lysine

Sugar/lysine model systems were prepared by two methods, in one approach glucose/lysine mixtures were dissolved in distilled water and heated in an oven at 110^{o}C using an open vial for 45 min or until dryness generating a free flowing brown powder. The powder was analyzed through high resolution TOF-MS using mainly [U-$^{13}C_6$]glucose labelled precursors. In the second approach glucose/lysine and sucrose/lysine model systems were pyrolyzed at 250^{o}C for 20s using variously labelled

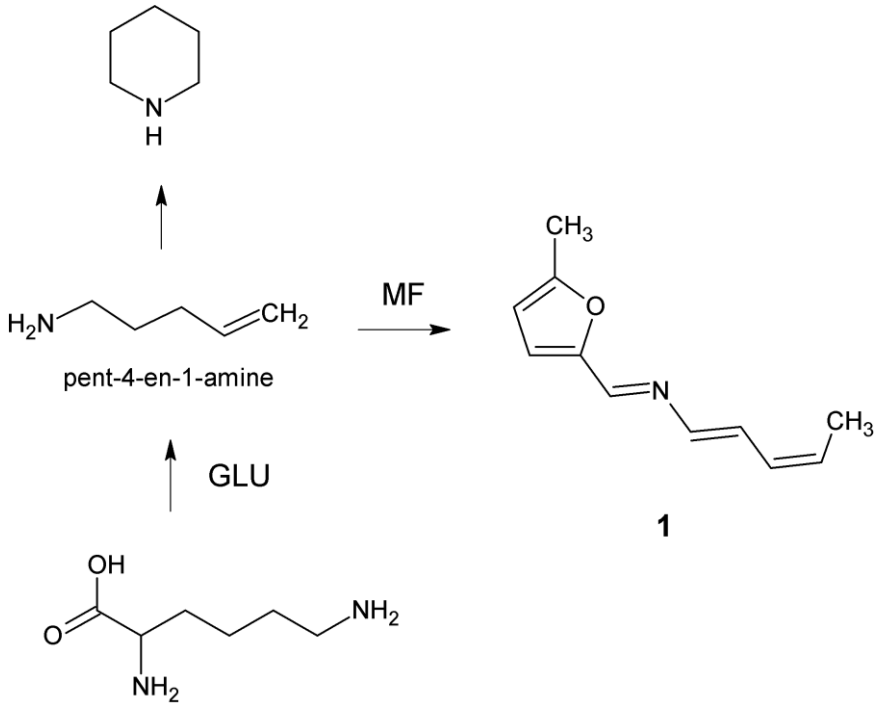


Figure 5.1. Formation of Nε-pent-4-ene-1-amine and its Schiff base adduct with 5-methylfurfural (MF) based on Chapter 3.GLU = Glucose

lysine precursors (see Table 5.1). Sucrose lysine model systems were used to enhance the formation of HMF generated products. Furthermore, to confirm the formation of HMF adducts a useful strategy based on isotope labelling technique was developed in which products simultaneously possessing six glucose (HMF moiety) and any number of lysine carbon atoms in addition to nitrogen were targeted using specifically labelled precursors such as [^{15}Nα]lysine.2HCl, [^{15}Nε]lysine.2HCl, [U-$^{13}C_6$]lysine.2HCl, [^{13}C-6]lysine.2HCl and [U-$^{13}C_6$]glucose. In addition, model systems containing HMF and lysine or HMF and piperidine were also studied to confirm specific adduct formation. The complete labelling studies along with structural analysis using synthetic and other available precursors have shown the presence of two such peaks at retention times 26.9 (m/z 193) and 30.9 min (m/z 191) that satisfied the above criteria. The peak at retention time of 26.9 min was detected only in the pyrolyzed sucrose/lysine samples however the peak at retention time

of 30.9 min was detected in both pyrolyzed glucose/lysine and in the aqueous heated samples that was analyzed by TOF-MS/MS. Furthermore, the aqueous heated sample also generated a related compound possessing 12 carbon atoms from glucose ($[M+H]^+$ = 354.1547; Molecular formula = $C_{17}H_{24}NO_7$).

5.4.2 Identification of compound (2) eluting at 26.9 min

This compound was detected only in sucrose/lysine and piperidine/HMF pyrolyzed samples. In fact the major peak in the model system of HMF/piperidine eluted at retention time of 26.9 min. Preliminary analysis of the isotope labelling pattern generated from the sucrose/lysine model systems have indicated the incorporation of five lysine carbon atoms (including C-6) and one Nε atom. This pattern of lysine atom distribution is indicative of either piperidine or Nε-pent-4-ene-1-amine moiety (Nikolov & Yaylayan, 2010a). In a previous study (Nikolov & Yaylayan, 2010a) lysine was found to release Nε-pent-4-ene-1-amine through interaction with glucose and isotope labelling studies confirmed its conversion into piperidine (see Figure 5.1). The precursors of compound **2** were confirmed when the reaction of HMF with piperidine produced the major peak at the same retention time and with an identical mass spectrum. Piperidine being a secondary amine can initially form an imminium ion when reacted with HMF (see Figure 5.2). This ion can be stabilized either through its reaction with a second mole of piperidine to form [5-(dipiperidin-1-ylmethyl)furan-2-yl]methanol (**3**) or undergo vinylogous Amadori rearrangement to form a covalent adduct (**2**). Although the dipiperidine adduct **3** (equivalent to the Schiff base) was not detected however, when a synthetic sample was pyrolyzed it generated an identical compound eluting at the same retention time and with an identical mass spectrum to that of **2**. Formation of compound **2**

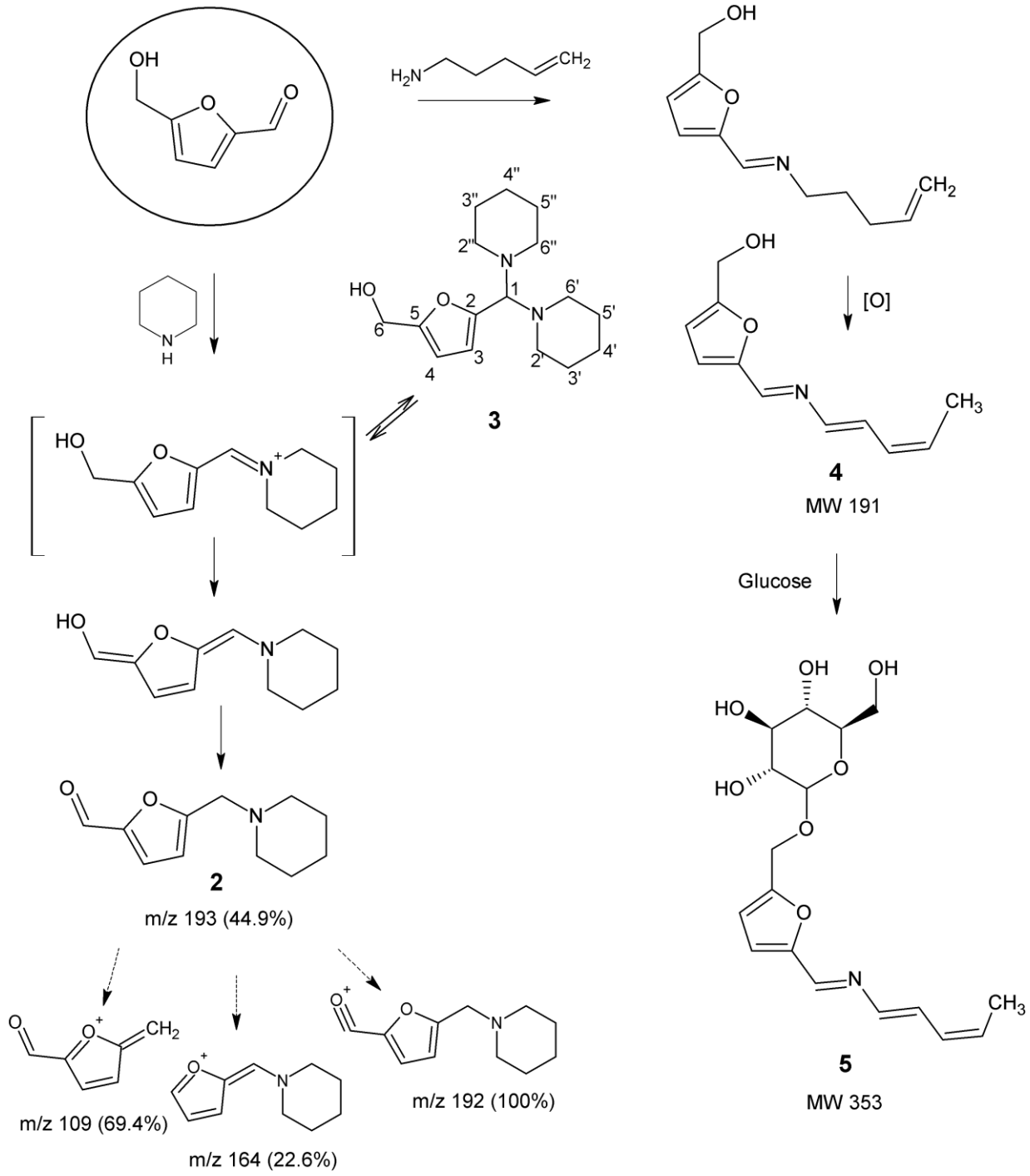


Figure 5.2 Schiff base formation of 5-(hydroxymethyl)-2-furaldehyde (HMF) with Nε-pent-4-ene-1-amine and its vinylogous Amadori rearrangement with piperidine.
For compound **2** (m/z 193) important EI mass spectral fragments are also shown. [O] = oxidation; MW = molecular weight

indicates secondary amines such as piperidine that can form imminum ions are also able to provide the necessary driving force for its isomerisation into the corresponding Amadori product to neutralize the positive charge.

The main mass spectral fragments of **2** are also shown in Figure 5.2 and the corresponding label incorporation patterns are listed in Table 5.2. The fragmentation pattern observed is consistent with presence of an aldehyde functionality and the label incorporations in the fragment ions are also consistent with the proposed structure.

Table 5.2 Number of labelled atoms incorporated in the major mass spectral fragments of compound **2**[a] shown in Figure 5.2

	m/z 193	**m/z 192**	**m/z 164**	**m/z 109**
[$^{15}N_{\alpha}$]lysine	0	0	0	0
[$^{15}N_{\varepsilon}$]lysine	1	1	1	0
[$^{13}U_{6}$]lysine	5	5	5	0
[^{13}C-6]lysine	1	1	1	0

[a] generated through pyrolysis of glucose/lysine or sucrose/lysine model systems

On the other hand, the primary amine Nε-pent-4-ene-1-amine is also expected to be formed in glucose/lysine model system (Nikolov & Yaylayan, 2010a) and subsequently generate a stable Schiff base adduct with HMF (expected MW 191 amu) similar to 5-methylfurfural (**1**). A peak eluting at 30.92 min (nominal molecular weight m/z 191) and incorporating six glucose carbon atoms, five lysine carbon atoms (including C-6) and one Nε atom was detected in glucose/lysine model systems and also in the aqueous heated glucose/lysine sample analyzed by TOF MS/MS (Table 5.3).

Table 5.3 Number of labelled atoms incorporated in the major EI and ESI fragments of compound **4** shown in Figure 5.2

EI[a]						
[M+] ions	**m/z 191**	**m/z 176**	**m/z 163**	**m/z 135**	**m/z 107**	**m/z 93**
[$^{15}N_{\alpha}$]lysine	0	0	0	0	0	0
[$^{15}N_{\varepsilon}$]lysine	1	1	1	1	0	1
[$^{13}U_6$]lysine	5	4	5	5	5	2
[^{13}C-6]lysine	1	0	1	1	0	0
[^{13}U6]glucose	6	6	4	3	2	3
ESI[b]						
[M+H] ions	**m/z 192**	**m/z 175**	**m/z 146**	**m/z 132**		
[$^{15}N_{\alpha}$]lysine	0	0	0	0		
[^{13}U6]glucose	6	6	5	4		

[a] From pyrolysis experiments [b]from aqueous model system using TOF MS/MS, $[M+H]^+$ = 192.1043 and molecular formula of $C_{11}H_{13}NO_2$

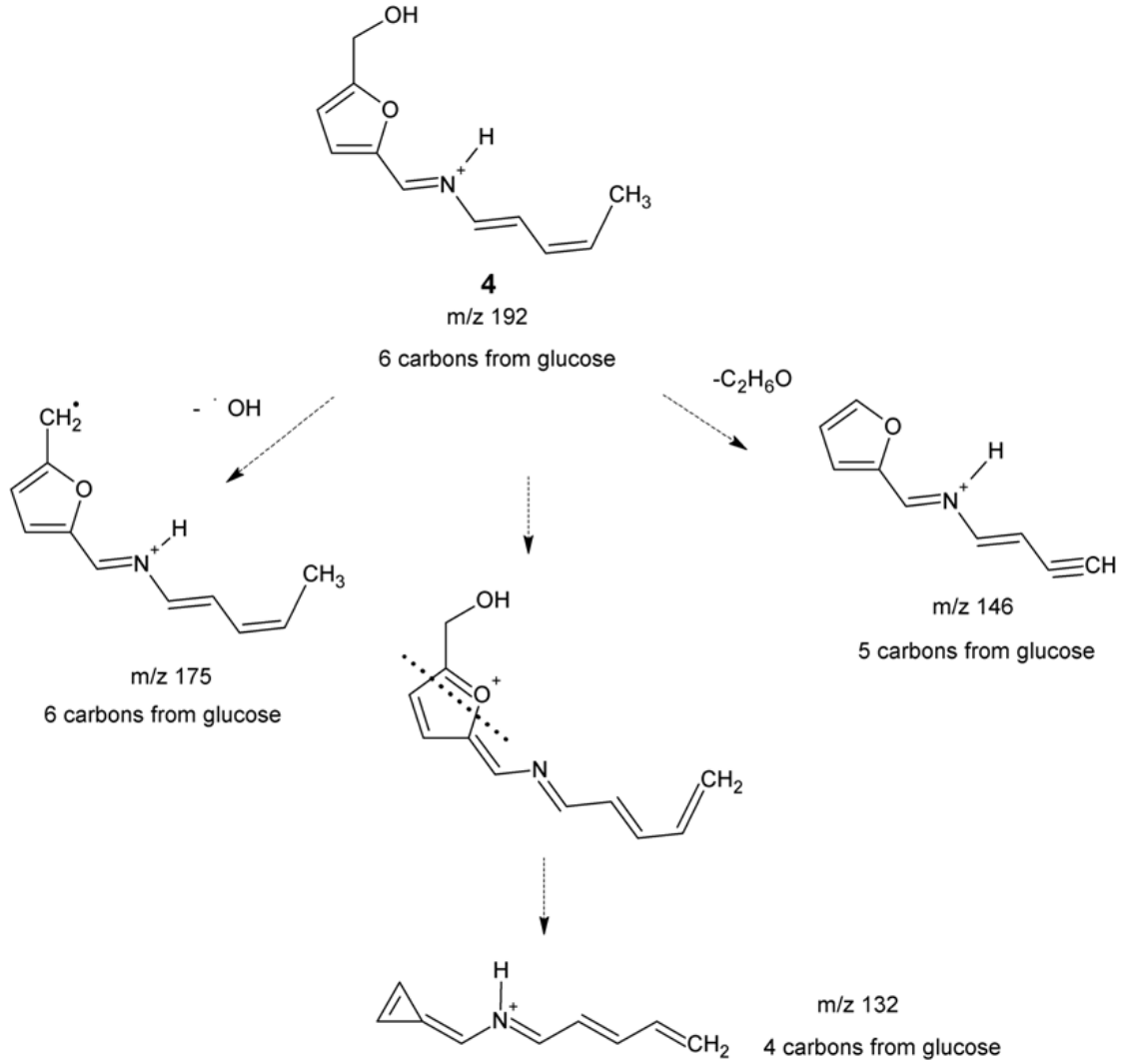


Figure 5.3 TOF-MS/MS fragmentations of structure **4** (see also Table 5.3)

5.4.3 Tentative identification of compound (4) eluting at 30.9 min

As mentioned above this compound was detected in both pyrolyzed and aqueous heated samples of glucose and lysine. Analysis of the label incorporation pattern from the TOF-MS analysis indicated the incorporation of six carbon atoms from glucose and showed a consistent molecular formula ($C_{11}H_{13}NO_2$) and MS/MS data (Table 5.3). Similarly, analysis of the label incorporation pattern from pyrolysis experiments indicated the incorporation of six carbon atoms from glucose, five lysine carbon atoms (including C-6) and one Nε atom. Since this pattern of label incorporation is indicative of either piperidine or Nε-pent-4-ene-1-amine moiety (Nikolov & Yaylayan, 2010a) and since the peak was not generated in the piperidine/HMF model, the evidence therefore points to a structure equivalent to the Schiff base formed between 5-methylfurfural (MF) and Nε-pent-4-ene-1-amine shown in Figure 5.1 and detected in the same model system of glucose and lysine (Nikolov & Yaylayan, 2010a). The TOF MS/MS fragmentations shown in Figure 5.3 provide further evidence to the proposed structure **4**. Tentative identification of the glycosylated derivative (**5**) of structure **4** in the same aqueous heated sample provides further evidence for its formation. Isotope labelling experiments using TOF-MS analysis indicated the presence of 12 carbon atoms from glucose (see Table 5.4) with a molecular weight of $[M+H]^+$ = 354.1547 and a calculated molecular formula of $C_{17}H_{24}NO_7$ consistent with the proposed structure **5**. The remaining 5 carbons and the one nitrogen atom should therefore originate from the lysine component. In fact a fragment ion (m/z 192) indicative of structure **4** incorporating six carbon atoms from glucose (see Figure 5.4) was the major daughter ion in MS/MS spectrum of **5** (see Table 5.4 and

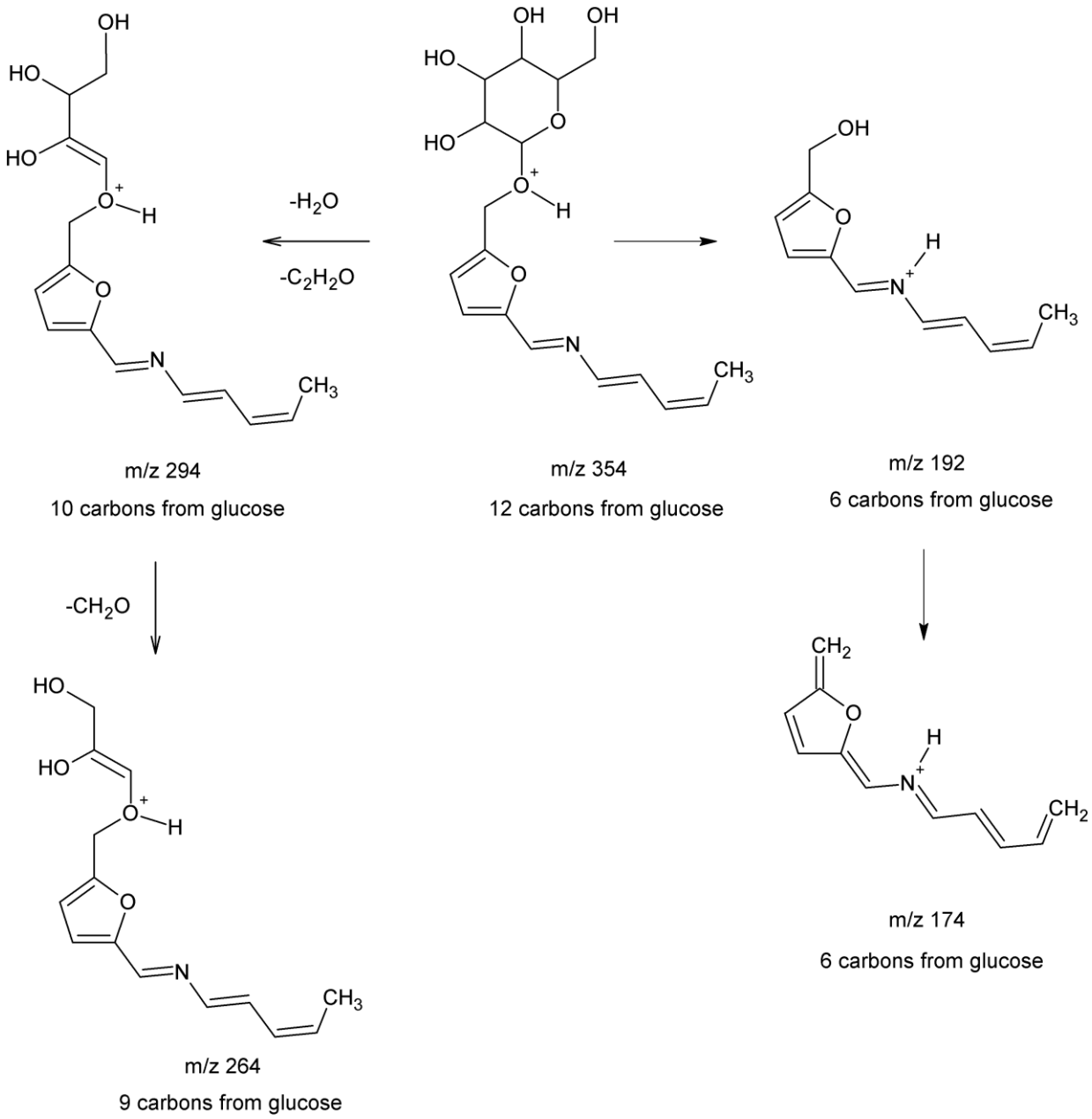


Figure 5.4 TOF-MS/MS fragmentations of structure **5** (see also Table 5.4)

Table 5.4 TOF MS/MS[a] ESI fragment ions [M+H] of compound **5** shown in Figure 5.2

	m/z 354	**m/z 294**	**m/z 264**	**m/z 192**	**m/z 174**
[$^{15}N_{\alpha}$]lysine	0	0	0	0	0
[^{13}U6]glucose	12	10	9	6	6

[a] from aqueous model system, $[M+H]^+$ = 354.1547 and molecular formula of $C_{17}H_{24}NO_7$

Figure 5.4). All the proposed ESI fragments shown in Figure 5.4 were consistent with the proposed structure and the labelling data.

5.4.4 Reaction of HMF with glycine and proline

To confirm the assertion that secondary amines or secondary amino acids undergo vinylogous Amadori rearrangement and primary amino acids or primary amines form only Schiff bases with HMF, the reaction of HMF with glycine and proline was investigated. When glycine was pyrolyzed with HMF a peak corresponding to the specification of a decarboxylated Schiff base adduct (**6**) and eluting at retention time of 21.44 min was observed (see Figure 5.5). The peak incorporated one nitrogen atom and one C-2 atom of glycine and no C-1 atom from glycine (see Table 5.5). The same peak was also generated from a mixture of HMF/methylamine and glucose/methylamine. The latter model system allowed the confirmation of incorporation of six glucose carbon atoms into the structure of the peak eluting at 21.44 min when glucose was replaced with [$^{13}U_6$]glucose. Furthermore, when synthetic HMF-glycine Schiff base adduct (**7**) was pyrolyzed, a peak eluting at the same retention time and having identical mass spectrum to that of **6** was also generated confirming unequivocally the structure **6** shown in Figure 5.5. Furthermore, loss of an OH radical to form the ion at m/z 122 confirms the presence of an alcohol moiety in **6**.

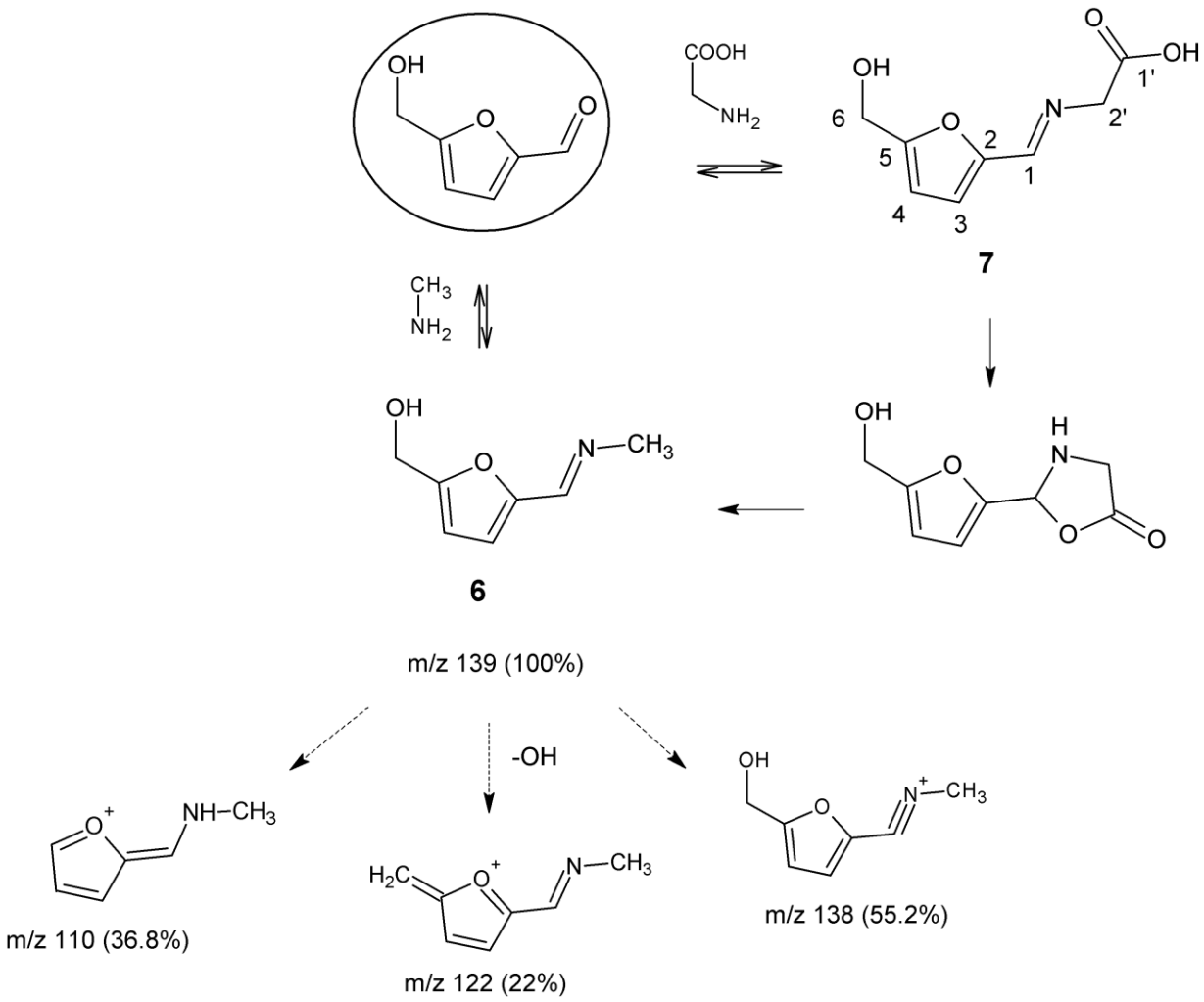


Figure 5.5 Reaction of 5-(hydroxymethyl)-2-furaldehyde (HMF) with glycine (see also Table 5.5). For compound **6** (m/z 139) important EI mass spectral fragments are also shown.

Table 5.5 Number of labelled atoms incorporated in the major EI fragments of compound **6**[a] shown in Figure 5.5

	m/z 139	**m/z 138**	**m/z 122**	**m/z 110**
[$^{13}C_1$]glycine	0	0	0	0
[$^{13}C_2$]glycine	1	1	1	1
[^{15}N]glycine	1	1	1	1
[$^{13}U_6$]glucose	6	6	6	5

[a] generated through pyrolysis of HMF/glycine and glucose/methylamine.HCl model systems

On the other hand, the reaction of secondary amino acid proline with HMF generated two relevant adducts one eluting at 24.5 min tentatively identified as structure **8** and the other at 25.5 min tentatively identified as structure **9** (see Figure 5.6). Labelling studies using [U-$^{13}C_5$,^{15}N]proline and [^{13}C-1]proline indicated the incorporation of a pyrrolidine moiety in both structures (see Table 5.6) and a subsequent study on the reaction of pyrrolidine with HMF confirmed the generation of mainly the peak eluting at 25.5 min (structure **9**) and a trace amount of **8**. The Schiff base adducts of HMF with amino acids are prone to undergo decarboxylation through oxazolidin-5-one intermediate and formation of two isomeric imminium ions, one conjugated that can undergo vinylogous Amadori rearrangement to generate **9**, and the other not conjugated that can be stabilized through dehydration to form **8** as shown in Figure 5.6. The fact that the reaction of HMF with proline generates comparable amounts of **8** and **9** but only trace amounts of **8** when pyrrolidine was used further confirms the formation of oxazolidin-5-one intermediate whose formation is possible only with amino acids. The formation of **8** in pyrrolidine models can be rationalized through less desirable trans-amination reaction that requires basic conditions (Yaylayan & Wnorowski, 2002). As expected the major mass spectral fragments of **9** shown in Figure 5.6 are generated through identical fragmentation pathways to that of its piperidine analog (structure **2**) shown in Figure 5.2**.**

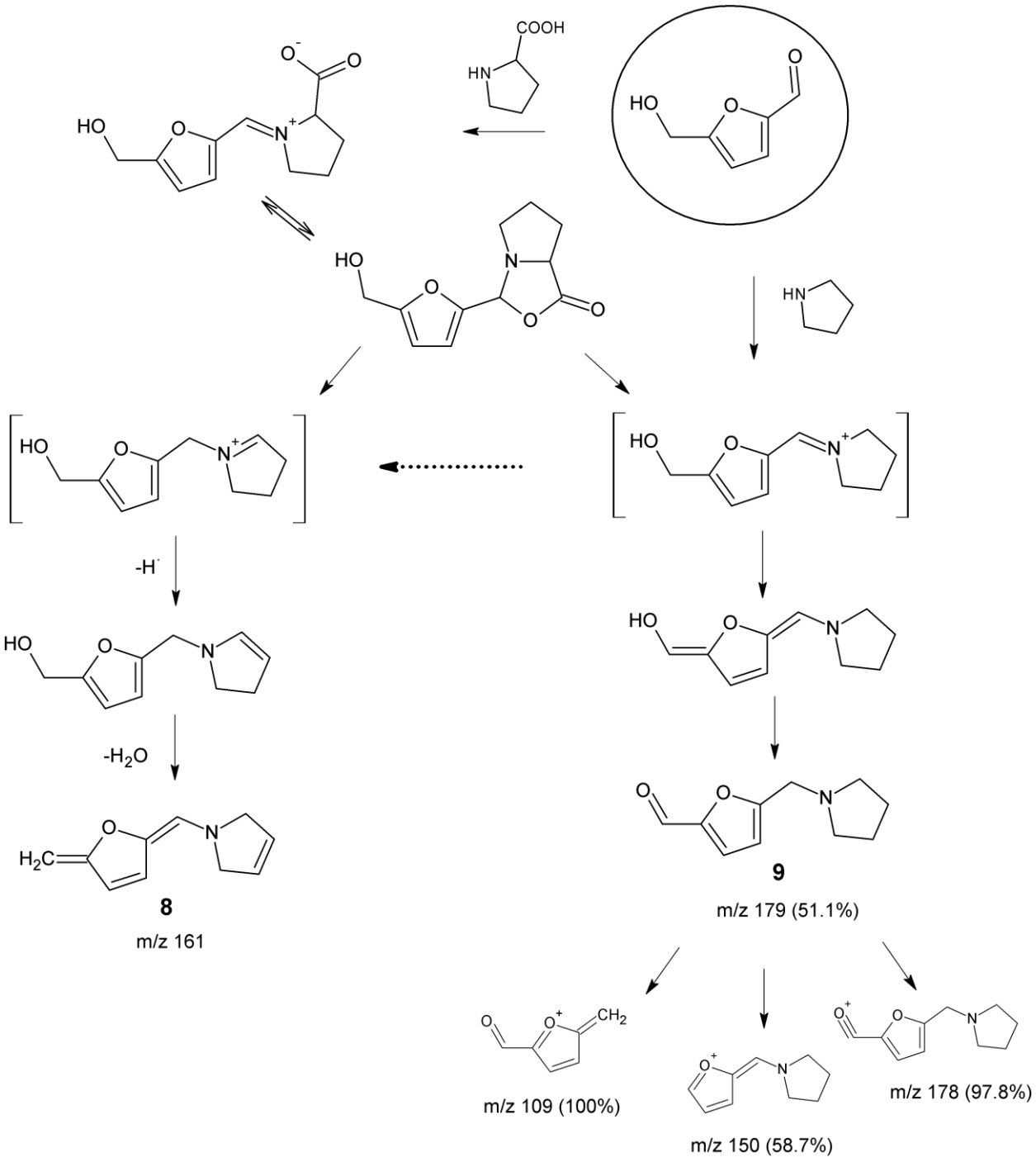


Figure 5.6 Reaction of 5-(hydroxymethyl)-2-furaldehyde (HMF) with proline (see also Table 5.6) for compound **9** (m/z 179) important EI mass spectral fragments are also shown.

Table 5.6 Number of labelled atoms incorporated in the major EI fragments of compounds **8** and **9** shown in Figure 5.6

Compound 9[a]	**m/z 179**	**m/z 178**	**m/z 150**	**m/z 109**
[U-$^{13}C_5$,^{15}N]proline	5	5	5	0
[^{13}C-1]-proline	0	0	0	0

Compound 8[a]	**m/z 161**	**m/z 146**	**m/z 132**	**m/z 118**
[U-$^{13}C_5$,^{15}N]proline	5	5	5	5
[^{13}C-1]proline	0	0	0	0

[a] generated through pyrolysis of proline/HMF model systems

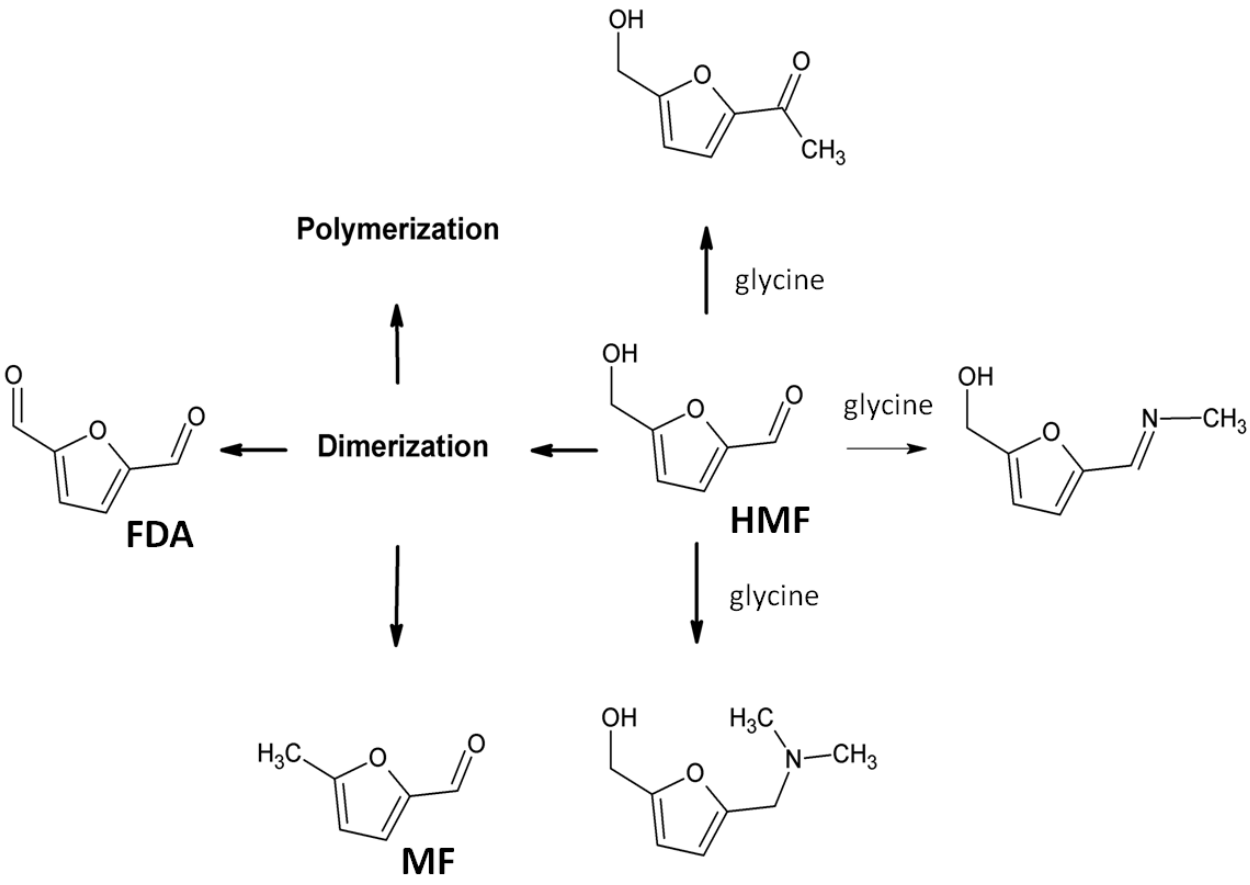
5.5 Conclusions

Although some of the HMF formed in food is prone to polymerization however, as indicated in Chapter 5 some may also undergo amino acid specific reactions. The initially formed Schiff base adducts may partially undergo decarboxylation through oxazolidin-5-one and form two isomeric reversible Schiff bases. Unlike the Schiff bases resulting from primary amines or primary amino acids, those resulting from secondary amino acids such as proline or secondary amines such as piperidine can further undergo vinylogous Amadori rearrangement resulting in covalently bonded N-substituted 5-(aminomethyl)furan-2-carbaldehyde derivatives. Lysine can exhibit both reversible and covalent adduct formation depending on the formation of either Nε-pent-4-ene-1-amine or piperidine in the reaction mixture.

CONNECTING PARAGRAPH

Chapter 5 demonstrated the different mechanistic pathways in which HMF underwent chemical transformations in the presence of lysine and its degradation products specifically Nε-pent-4-ene-1-amine and piperidine as well with other amino acids. Chapter 6 examines the thermal fate of HMF, with and without amino acids, including its polymerization, thermal decomposition and chain elongation reactions. Chapter 6 was published in the Journal of Agricultural and Food Chemistry. (Nikolov, P.Y., Yaylayan, V.A. (2011). Thermal decomposition of 5-(hydroxymethyl)-2-furaldehyde (HMF) and its further transformations in the presence of glycine. *Journal of Agricultural and Food Chemistry*, 59, 10104-10113.)

CHAPTER 6: THERMAL DECOMPOSITION OF 5-(HYDROXYMETHYL)-2-FURALDEHYDE (HMF) AND ITS FURTHER TRANSFORMATIONS IN THE PRESENCE OF GLYCINE



6.1 Abstract

Thermal decomposition of HMF has been so far studied indirectly through carbohydrate degradation reactions assuming HMF as the main product. Such studies however do not necessarily generate relevant information on HMF decomposition since many other products are generated simultaneously. Direct thermal decomposition using different concentrations of HMF in silica gel was studied using pyrolysis-GC/MS. Undiluted HMF generated four peaks corresponding to 5-methylfurfural, 2,5-furandicarboxaldehdye, HMF and a major unknown peak at retention time of 20.73 min. The diluted HMF in silica gel (15 fold) generated only the first three peaks. The generation of the unknown peak was dependant on the concentration of HMF indicating the possibility of having a dimeric structure, furthermore when HMF was generated from [U-$^{13}C_6$]glucose in the reaction mixture the highest mass in the spectrum of the unknown peak showed the incorporation of eleven carbon atoms from the glucose. Thermal decomposition studies of HMF have also indicated that in the absence of amino acids it can mainly dimerize and the initially formed dimer can degrade to generate 5-methylfurfural and 2,5-furandicarboxaldehyde. On the other hand, thermal degradation of HMF in the presence of glycine generated Schiff base adducts of HMF, 5-methylfurfural, and 2,5-furandicarboxaldehdye in addition to 2-acetyl-5-methylfuran and a newly discovered adduct 5-[(dimethylamino)methyl]-2-furanmethanol.

6.2 Introduction

Most of the studies on the thermal decomposition products of 5-(hydroxymethyl)-2-furaldehyde (HMF) have been performed utilizing different carbohydrates (Kroh, 1994; Ait-Ameur et al., 2007; Kuster, 1990) as precursors instead of HMF itself. Such studies

have indicated the decomposition of HMF into levulinic acid, formic acid, formaldehyde, 2,5-furandialdehyde (FDA) and 5-methylfurfural (MF). Under autoclaving conditions, Durham et al., (1982) also observed the formation of 5-hydroxymethyl-furoic acid and furan-2,5-dicarboxylic acid from glucose solutions and attributed their presence to the formation of HMF. On the other hand, Chambel et al., (1998) utilizing HMF as a precursor, observed its complete decomposition when heated alone at 210^{o}C and formation of four decomposition products using liquid chromatography, however, they identified the structure of only one of the four products as a symmetric ether formed through dehydration of two HMF molecules giving rise to 5,5'-oxy-dimethylene-*bis*(2-furaldehyde). The same dimer was also identified earlier by Popoff and Theander (1976) in slightly aqueous fructose and glucose solutions. Relative to aliphatic aldehydes, HMF is less volatile and chemically more stable and able to accumulate and persist longer in food to undergo polymerization. Its concentrations have been shown to vary over time experiencing continuous upward and downward fluxes especially during storage (Ferrer et al., 2005). Such changes over time can indicate the occurrence of polymerization-depolymerization, degradation (Kroh, 1994) or other reactions with N-nucleophiles such as amino acids. The ability of HMF to undergo reversible and covalent bond formation with primary and secondary amino acids respectively (Nikolov & Yaylayan, 2011a) and with human hemoglobin has been also demonstrated (Davies et al., 2009). Although HMF is one of the most abundant furfural derivatives in food however, its polymerization mechanism and its chemical interaction with amino acids have not been explored in detail. One of the drawbacks of using carbohydrate degradation to study HMF decomposition is that such studies do not necessarily generate relevant information on HMF decomposition

since many other products are formed simultaneously. The aim of this investigation was to explore direct degradation of HMF in the presence and absence of amino acids.

6.3 Materials and methods

All chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. [U-$^{13}C_6$]glucose, [^{13}C-1]glucose, [^{13}C-6]glucose, [^{13}C-1]glycine, [^{13}C-2]glycine, [^{15}N]glycine, were and purchased from CIL (Andover, MA), and 2,5-furandicarboxaldehyde (98%, TRC, Toronto, Canada) were purchased as indicated. The ^{13}C- and ^{1}H-NMR spectra were acquired in CD_3OD on a Varian VNMRS 500 MHz spectrometer. Infrared spectra were recorded on a Bruker Alpha-P spectrometer (Bruker Optic GmbH, Ettlingen, Germany) equipped with a deuterated triglycine sulphate (DTGS) detector, a temperature controlled single bounce diamond Attenuated Total Reflectance (ATR) crystal and a pressure application device for solid samples.

6.3.1 Pyrolysis-GC/MS

Analyses were conducted using a Varian CP-3800 GC coupled with a Saturn 2000 Ion Trap Mass Spectrometer (Varian, Walnut Creek, USA). The pyrolysis unit included a CDS Pyroprobe 2000 and a CDS 1500 valved interface (CDS Analytical, Oxford, USA) installed onto the GC injection port. Between 0.5-1.5 milligram of a sample mixture (see Table 6.1) was packed inside a quartz tubes (0.3mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed for 20 seconds at a temperature of 250°C. The sample separation was carried out on a DB-5MS (5% diphenyl, 95% dimethyl polysiloxane) capillary column with dimensions of 50 m length by 0.2 mm internal diameter and 0.33 μm film thickness (J&W Scientific, ON, Canada), using helium as the

Table 6.1 Composition of model systems analyzed by Py-GC/MS[a]

Target ions/product	Ratio	Model system
m/z 111		[U-$^{13}C_6$]glucose
15.452 min	1:7	HMF + [U-$^{13}C_6$]glucose
	1:1	[U-$^{13}C_6$]glucose + methylamine.HCl
		5-Methylfurfural
m/z 124		[U-$^{13}C_6$]glucose
17.820 min	1:7	HMF + [U-$^{13}C_6$]glucose
	1:1	[U-$^{13}C_6$]glucose + methylamine.HCl
		2,5-Furandicarboxaldehyde
m/z 126	1:15	HMF + silica gel
20.577min		[U-$^{13}C_6$]glucose
	1:7	HMF + [U-$^{13}C_6$]glucose
m/z 189	1:7	HMF + [U-$^{13}C_6$]glucose
20.705min	1:7	HMF + [^{13}C-1 or 6]glucose
	1:1	[U-$^{13}C_6$]glucose + methylamine.HCl
	2:1:1	[U-$^{13}C_6$]glucose + methylamine.HCl + glycine
Schiff base adducts		
m/z 123	3 : 1	HMF + [^{13}C-1 or 2]glycine
16.568min	3 : 1	HMF + [^{15}N]glycine
	3: 1	HMF + glycine
m/z 137	2 : 1 : 1	[U-$^{13}C_6$]glucose+ methylamine-HCl + glycine
20.125min	1 : 1	[U-$^{13}C_6$]glucose + methylamine-HCl
	2 : 1 : 1	Glucose + methylamine.HCl + glycine
m/z 139	1 : 1	Glucose + methylamine.HCl
21.401min	1 : 1	HMF + methylamine.HCl
		{[(5-methylfuran-2-yl)methylidene]amino}acetic
m/z 150		acid sodium salt (**7**)
21.864min		2,2'-{furan-2,5-diylbis [methylylidenenitrilo]}
		diacetic acid disodium salt (**9**)
Chain elongation adducts		
m/z 124	1:3	MF[b]/HMF + [^{13}C-1 or 2]glycine
16.907min	1:3	MF[b]/HMF + [^{15}N]glycine
	1:3	MF[b]/HMF+glycine
m/z 140		2-Acetyl-5-methylfuran[a] (**12**)
21.746min		
N-methylation of glycine		
m/z 155	1:3	HMF + [^{13}C-1 or 2]glycine
20.674min	1:3	HMF + [^{15}N]glycine
	1:3	HMF + glycine
m/z 153	1:3	HMF + sarcosine
20.674min	1:3:3	HMF + glycine + paraformaldehyde
	1:1.5:1.5	HMF + glycine + sarcosine
		5-[(dimethylamino)methyl]- 2 –furanmethanol
		hydrochloride (**16**)

[a] MF= 5-methylfurfural; FDA = 2,5-furandicarboxyaldehyde, [b]only used for 124 m/z adduct

carrier gas. The GC column flow rate was regulated by an electronic flow controller (EFC) and set at a pressure pulse of 70psi for the first 4 minutes and later maintained with a constant flow of 1.5 mL/minute for the remainder of the run. The GC oven temperature was set at -5°C for 5 minutes using CO_2 as the cryogenic cooling source. The temperature was increased to 50°C at a rate of 50°C/minute and then to 270°C at a rate of 8°C/minute, and kept at 270°C for 5 minutes. The samples were detected by using an ion-trap mass spectrometer with a scan range of 20-650m/z. The MS transfer-line temperature was set at 250°C, manifold temperature was set at 50°C, and the ion-trap temperature was set at 175°C. The ionization voltage of 70 eV was used, and EMV was set at 1700 V. Compound identification was performed using AMDIS (ver 2.65) and NIST Standard Reference Databases (data version 05 and software ver 2.0d) to compare the target compounds with the existing mass spectral libraries or by injecting commercially available standards. The reported percent label incorporation values (corrected for natural abundance and for percent enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%. The reported percent peak areas in Tables 2 & 4 are the averages of duplicate analyses with percent standard deviation of less than 10%.

6.3.2 Synthesis of {[(5-methylfuran-2-yl)methylidene]amino}acetic acid sodium salt (7)

The 5-methylfurfural (124 mg) and sodium glycinate (97 mg) was intimately mixed at room temperature in a mortar without a solvent until a homogenous yellow solid was produced (15 min). ^{1}H NMR: δ 2.35 (s, 3H, H-6), 4.16(s, 2H, H-2'), 6.18(d, 1H, H-3), 6.80(d, 1H, H-4), 7.96(s, H-1). ^{13}C NMR: δ 176.3 (C-1'), 155.8(C-5), 152.2(C-1), 149.9(C-2), 116.9(C-4), 108.0(C-3), 63.5(C-2'), 12.3(C-6). FTIR (solid): 3116 cm^{-1} (Ar-

H stretch), 2925 and 2889 cm^{-1} (-CH_3 and -CH_2- stretch), 1644 cm^{-1} (C=N stretch), 1583 cm^{-1} (COO^- anti-symmetrical stretch), 1391 cm^{-1} (COO^- symmetrical stretch). MS *m/z* (% abundance): 39(12.7), 42(14.5), 50(15.2), 51(16.3), 53(26.8), 67(14.6), 79(11.1), 80(11.6), 81(11.7), 95(100), 107(6.8), 122(79.9), 123(97.4), 124(14.8).

6.3.3 Synthesis of 2,2'-{furan-2,5-diylbis[methylylidenenitrilo]}diacetic acid disodium salt (9)

Furan-2,5-dicarboxaldehyde (124 mg) and sodium glycinate (97 mg) was intimately mixed at room temperature in a mortar without a solvent until a homogenous yellow solid was produced (15 min). ^{1}H NMR: δ 4.25(d, 4H, H-2',2"), 7.01(s, 2H, H-3,4), 8.122(t, 2H, H-1,6). ^{13}C NMR: δ 175.9(C-1',1"), 152.6(C-2,5), 151.7(C-1,6), 116.8(C-3,4), 64.0(C-2',2"). FTIR (solid): 3140 and 3107 cm^{-1} (Ar-H stretch), 2901 cm^{-1} (-CH_2- stretch), 1641 cm^{-1} (C=N stretch), 1582 cm^{-1} (COO^- anti-symmetrical stretch), 1392 cm^{-1} (COO^- symmetrical stretch). MS *m/z* (% abundance): 42(24.4), 51(10.3), 80(15.7), 81(11.3), 94(15.6), 108(30.1), 122 (10.5), 123(67.5), 149(39.9), 150(100), 151(27.5).

6.4 Results and discussion

Different furfural derivatives, such as HMF can serve as building blocks for the construction of polymeric structures (Figure 6.1) or can be transformed into amino acid adducts (Nikolov & Yaylayan, 2011a) through carbonyl-amine reactions (Figure 6.2) during the Maillard reaction. Due to the complexity of the resulting polymers, structural elucidation often relies on the identification of the subunits released during degradation of isolated polymers (Hardt & Baltes, 1989; Tehrani et al., 2002) or identification of the structure of the smaller building blocks such as dimers or oligomers having the potential for polymerization (Hayase et al., 1999a).

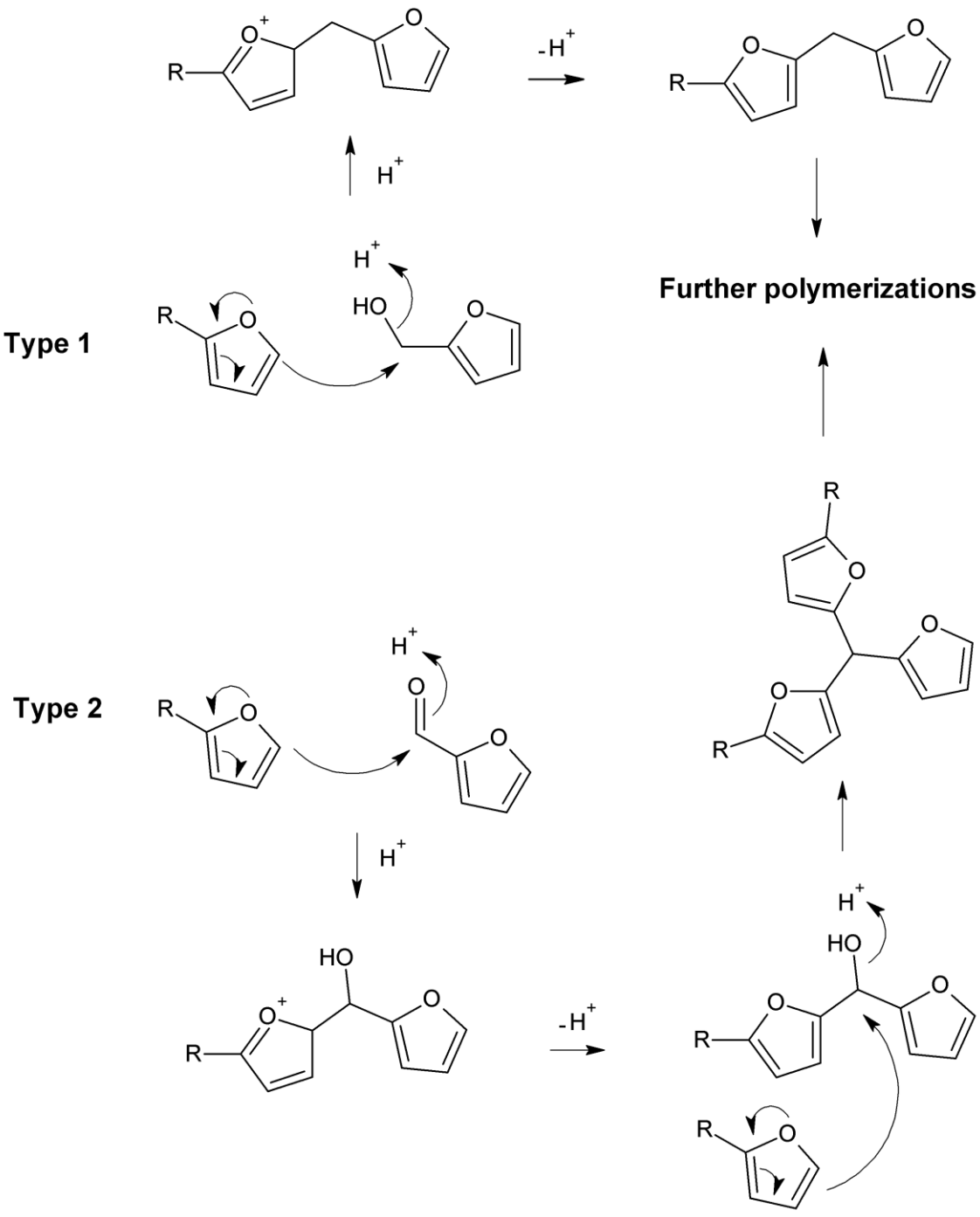


Figure 6.1 Type 1 and Type 2 polymerization reactions proposed by Tressl and colleagues (1998a, 1998b)

The applicability of the concept of "polymer generating subunits" as indicators of the structure of polymers has been demonstrated by the detection of dimers such as Blue-M1 which polymerizes readily to form a yellow polymer (Hayase et al., 1999b). Furan or pyrrole derivatives possessing a hydroxymethyl group as a side chain can undergo Type 1 polymerization through electrophilic aromatic substitution to generate a linear polymer (Tressl et al., 1998a), on the other hand, if they possess carbonyl groups as side chains they can undergo Type 2 polymerization (Tressl et al., 1998b) through electrophilic aromatic addition to generate a branched polymer (Figure 6.1). Linear oligomers of up to twelve N-methylpyrrole subunits were detected, while some branched oligomers were found to contain greater than thirty pyrrolyl-2-furylmethyl units.

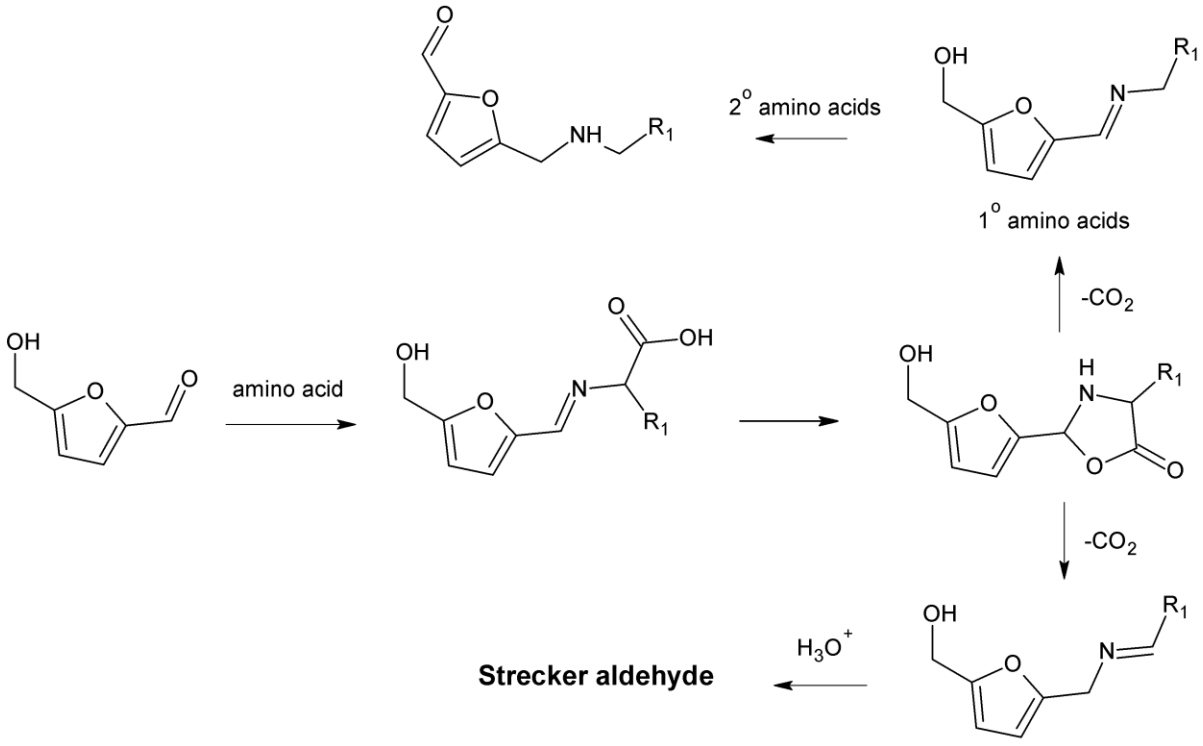


Figure 6.2 HMF reactions with amino acids according to Nikolov & Yaylayan , (2011a)

One strategy to postulate possible molecular structures of polymeric material based on the mechanisms depicted in Figure 6.1 is to identify initially formed dimeric or oligomeric structures capable of polymerization. To confirm the ability of pyrolysis to generate such dimeric subunits, furfuryl alcohol for example was pyrolyzed and structures **1**, **2** and **3** shown in Figure 6.3 were identified through NIST library searches. Structures **1** to **3** are known to be fragments of larger polymeric material. Pyrolysis of HMF on the other hand, generated two dimeric structures one minor (structure **4**) and the other major (structure **5**)**.** The structure **4** was identified through NIST library search and it can be envisaged to be formed through Type 1 mechanism involving HMF and 2-methylfuran, however, structure **5** was a major peak comprising almost 23 % of the total area of all the peaks generated from HMF degradation (Table 6.2).

Table 6.2 Percent distribution of HMF and its degradation products [a]

HMF/silica	**MF [b] (15.32 min)**	**FDA [c] (17.8 min)**	**HMF [d] (20.55 min)**	**HMF dimer [e] (20.73 min)**
1:0	13.3	18.7	8.5	22.6
1:10	3.4	29.9	6.78	10.2
1:15	1.5	29.6	3.2	0

[a] Pyrolysis at 250^{o}C for 20 s; [b] 5-methylfurfural; [c] 2,5-furandicarboxaldehyde; [d] 5-(hydroxymethyl)-2-furaldehyde; [e] m/z (%) **39** (11.5), **81** (0.23), **109** (22.4), **126** (36.4%), **127** (100), **189** (22.3) see also Figure 4 (structure **5**).

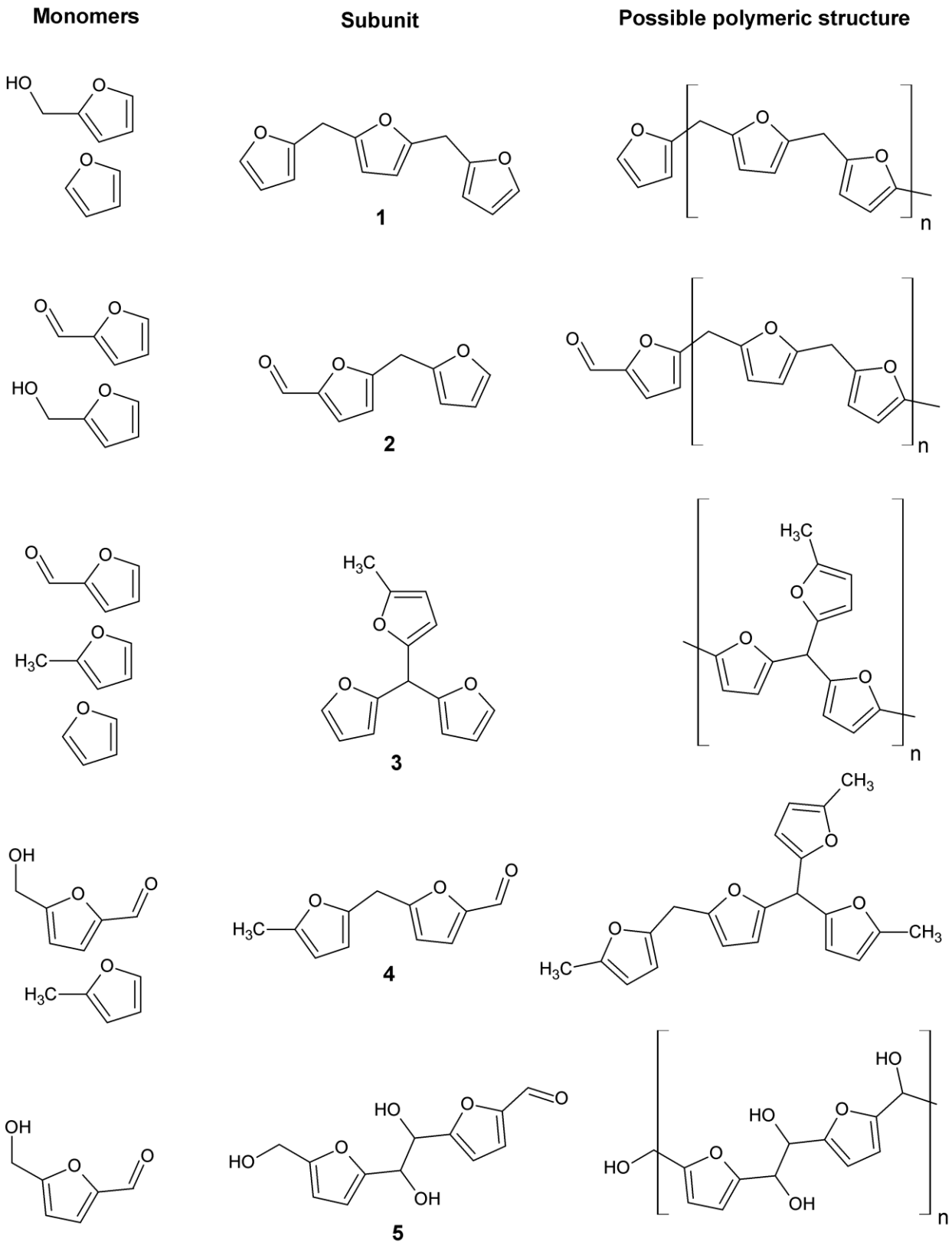


Figure 6.3 Characteristic polymeric subunits showing the monomers and possible polymeric structures

6.4.1 Thermal degradation of HMF

To study the thermal degradation of HMF and its potential to generate polymeric subunits, different concentrations of HMF in silica gel were prepared and pyrolyzed under the same conditions**.** Undiluted HMF generated four peaks (Table 6.2) corresponding to 5-methylfurfural, 2,5-furandicarboxaldehdye, HMF and an unknown peak at retention time of 20.73 min. On the other hand, the diluted HMF in silica gel (15 fold) generated only the first three peaks (Table 6.2). The generation of the unknown peak was dependant on the concentration of HMF indicating the possibility of having oligomeric structure, furthermore when HMF was generated from [U-$^{13}C_6$]glucose in the reaction mixture the highest mass in the spectrum of the unknown peak showed the incorporation of eleven carbon atoms from the glucose (Table 6.3).

Table 6.3 Number of glucose carbon atom incorporation into the mass spectral[a] fragments of the proposed HMF dimer (**5**, t_R 20.734 min)

Fragment ion	**m/z 189**	**m/z 127**	**m/z 109**	**m/z 97**	**m/z 81**
[$^{13}U_6$]glucose	11	6	6	5	5
[^{13}C-1]glucose	1	1	1	0	0
[^{13}C-6]glucose	2	1	1	1	1

[a] See Figure 6.4

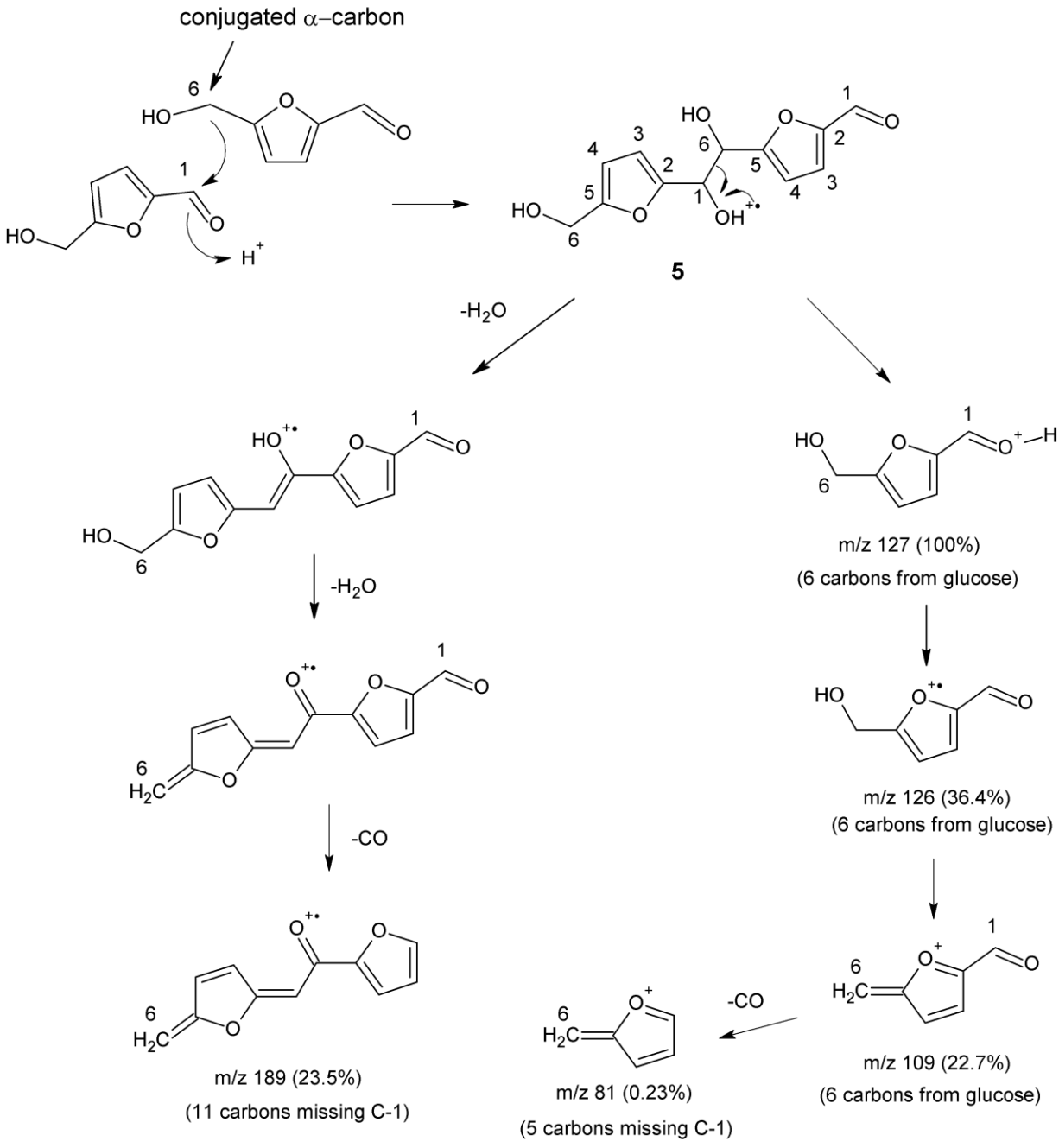


Figure 6.4 Formation of proposed HMF dimer (**5**) through vinylogous aldol addition of two HMF molecules and its mass spectral fragmentations indicating the incorporation of glucose carbon atoms.

Since HMF cannot undergo electrophilic substitution or addition reactions to form Type 1 or Type 2 polymers depicted in Figure 6.1 due to the presence of substituents at positions 2 and 5 of the furan ring, vinylogous aldol addition could be considered a possible mechanism that can generate the dimeric structure **5** that is not only consistent with the observed mass spectral fragmentation pattern but also with the label incorporation in these fragments (see Figure 6.4). HMF has been shown to undergo other vinylogous reactions such as Amadori rearrangement with secondary amino acids or amines (Nikolov & Yaylayan, 2011a) and more importantly a structure based on an identical HMF dimer was recently also identified as a building block for a melanoidin generated from glucose (Guan et al., 2011). The highest mass spectral ion at m/z 189 generated from the proposed dimeric structure shown in Figure 6.4 can be easily rationalized to be formed by the loss of two water molecules followed by the loss of a CO molecule from the parent ion, furthermore the ion at m/z 189 exhibited double incorporation of all the glucose carbon atoms with the exception of C-1 (lost as CO) (see Table 6.3) consistent with the mechanism and the structure shown in Figure 6.4. This fact also confirms the formation of a C-1 to C-6 bond during dimerization which can be achieved only through aldol type interaction. The proposed dimeric structure **5** although can be volatilized under the experimental conditions however being a reactive molecule cannot be isolated from the reaction mixtures due to its ability to undergo Type 1 polymerization at the hydroxymethyl terminal or Type 2 polymerizations at the carbonyl terminal of the molecule or continue polymerization with other HMF molecules through a series of vinylogous aldol condensations (Guan et al., 2011). As a result, very complex and non-homogenous polymers can be generated. In addition, formation of MF and FDA in the

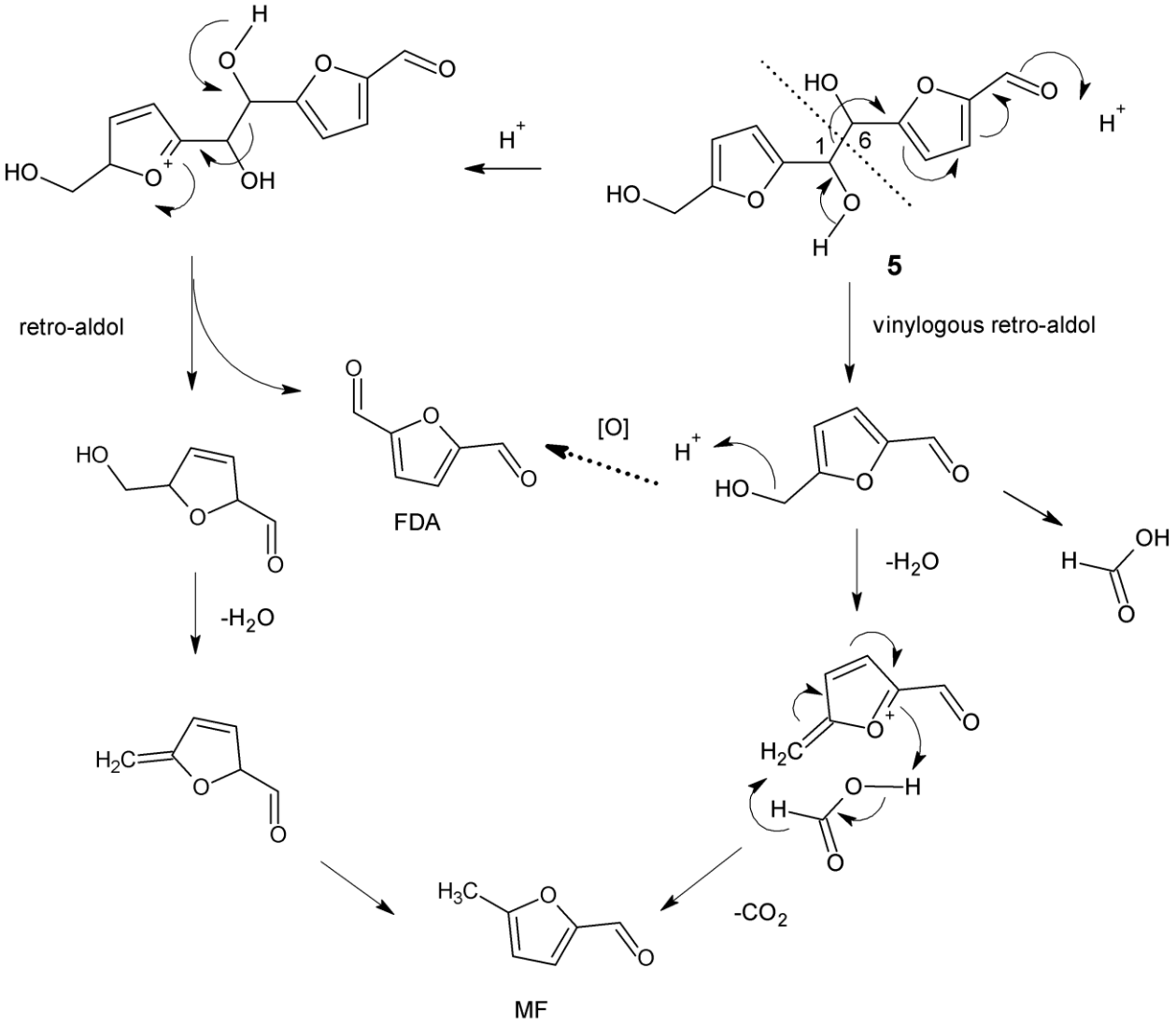


Figure 6.5 Retro-aldol reaction of protonated HMF dimer and formation of 5-methylfurfural (MF) and 2,5-furandicarboxaldehyde (FDA).

thermal degradation mixtures of HMF (see Table 6.2) which has been speculated to be formed from HMF (Sanders et al., 2003; Ait-Ameur et al., 2008) can be rationalized through the proposed degradation mechanism of the proposed dimer shown in Figure 6.5. Carbon-carbon bond cleavage of the diol group can occur through two pathways: a vinylogous retro-aldol, which can generate HMF, and a retro-aldol reaction after a protonation step that can generate FDA and 5-MF without the need for an oxidation and reduction steps required for the direct conversion from HMF. FDA and MF were identified by comparison of their retention times and their mass spectra to that of commercial standards and through NIST library matches in addition to isotope labelling data.

6.4.2 Thermal degradation of HMF in the presence of glycine

Glycine is known to form a Schiff base adduct with HMF (Nikolov & Yaylayan 2011a) and consequently block the carbonyl group and prevent its dimerization, similarly, glycine can react with MF and FDA formed from its degradation through similar Schiff base formation. Table 6.4 indicates that percent distribution of HMF, HMF dimer and its degradation products, changes depending on the ratio of HMF to that of glycine, increasing the ratio of glycine to that of HMF not only prevents dimerization but also reduces the intensity of MF, HMF and FDA peaks. Analysis of the new peaks that formed after the addition of glycine indicated the formation of the corresponding decarboxylated Schiff base adducts of MF, HMF and FDA (both mono and disubstituted) as shown in Figure 6.6

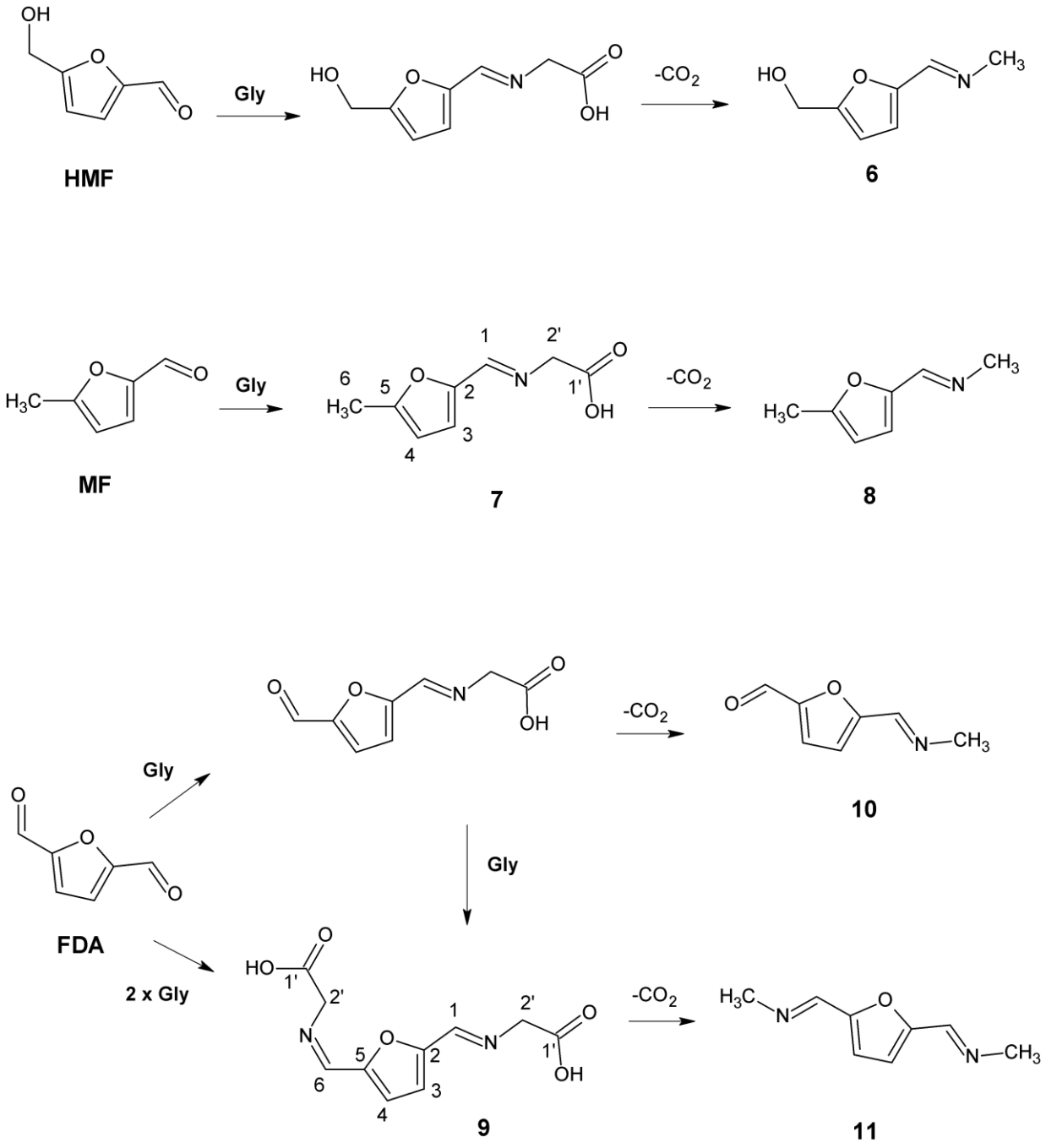


Figure 6.6 Schiff base adducts of HMF, 5-methylfurfural (MF) and 2,5-furandicarboxaldehyde (FDA) with glycine

Table 6.4 Percent distribution of HMF and its degradation products in the presence of glycine [a]

Compound	MF [b]	FDA [c]	HMF [d]	HMF dimer [e]
HMF+Glycine (3:1)	33.8	19.2	10.9	31.0
HMF+Glycine (2.5:1)	14.3	14.3	9.4	26.3
HMF+Glycine (1.5:1)	15.3	10	8.3	18.4
HMF+Glycine (1:1)	3.6	2.4	2.5	0
HMF+Glycine (1:3)	2	0.15	0	0

[a] Pyrolysis at 250 C for 20s (percentages exclude other products).; [b] 5-methylfurfural; [c] 2,5-furandicarboxaldehyde; [d] 5-(hydroxymethyl)-2-furaldehyde; [e] m/z(%) **39** (11.5), **81** (0.23), **109** (22.4), **126** (36.4%), **127** (100), **189** (22.3) see also Figure 4.

The Schiff base adduct of HMF (**6**) has been identified earlier (Nikolov & Yaylayan, 2011a), however, FDA and MF adducts (**8**, **10** and **11**) were confirmed through synthesis and pyrolysis of their corresponding glycine adducts (**7** and **9**), generating peaks with identical retention times and mass spectra. In addition, isotope labelling experiments with glycine (Table 6.5) indicated the incorporation of one nitrogen and one C-2 atom of glycine in HMF and MF Schiff base adducts, while in double substituted FDA adduct (**11**) two C-2 atoms and two nitrogen atoms were detected. *In situ* generation of labelled HMF moiety through the use of [U-$^{13}C_6$]-glucose/methylamine and [U-$^{13}C_6$]-glucose/methylamine/glycine models confirmed the presence of six carbon atoms from glucose (see Table 6.5) in each of the above adducts (**6**, **8**, **10** and **11**).

Table 6.5. Incorporation of total number of glucose and glycine labelled atoms into the Schiff base adducts from specifically labelled precursors observed in HMF/glycine model system

Compound	MF [a] (8)	HMF [b] (6)	FDA [c] (10)	FDA [d] (11)
[^{13}C-1]glycine	0	0	0	0
[^{13}C-2]glycine	1	1	1	2
[^{15}N]glycine	1	1	1	2
[$^{13}U_6$]glucose	6	6	6	6

[a] 5-methylfurfural (t_R = 16.58 min); [b] 5-(hydroxymethyl)-2-furaldehyde (t_R=20.13min); [c] 2,5-furandicarboxaldehyde single Schiff base (t_R = 21.44 min);[d] furandicarboxaldehyde double Schiff base (t_R = 21.85min)

Furthermore, glycine reaction with FDA and MF also generated compounds that eluted at the same retention times and had identical mass spectra to that of **8**, **10** and **11**. Increasing the glycine to FDA ratio from 1:1 to 3:1 resulted in a 20 fold increase of FDA-diglycine adduct relative to FDA-monoglycine adduct, indicating that FDA-diglycine is formed from the interaction of glycine with FDA-monoglycine.

6.4.3 Formation of 2-acetyl-5-methylfuran (12)

In addition to the observed Schiff base adducts of glycine with HMF, FDA and MF that showed one C-2 atom incorporation for every nitrogen atom originating from glycine, a peak incorporating only one C-2 atom from glycine without showing any incorporation of nitrogen atom was also identified. NIST library searches indicated the identity of this peak to be 2-acetyl-5-methylfuran (**12** in Figure 6.7) a known Maillard reaction product, however to the best of our knowledge its origin has not been reported yet. The structure of **12** was confirmed by comparing its retention time and mass spectrum to that generated from a commercially available standard. Since the compound incorporated only one carbon atom from glycine, the remaining six carbon atoms must have originated from HMF or MF.

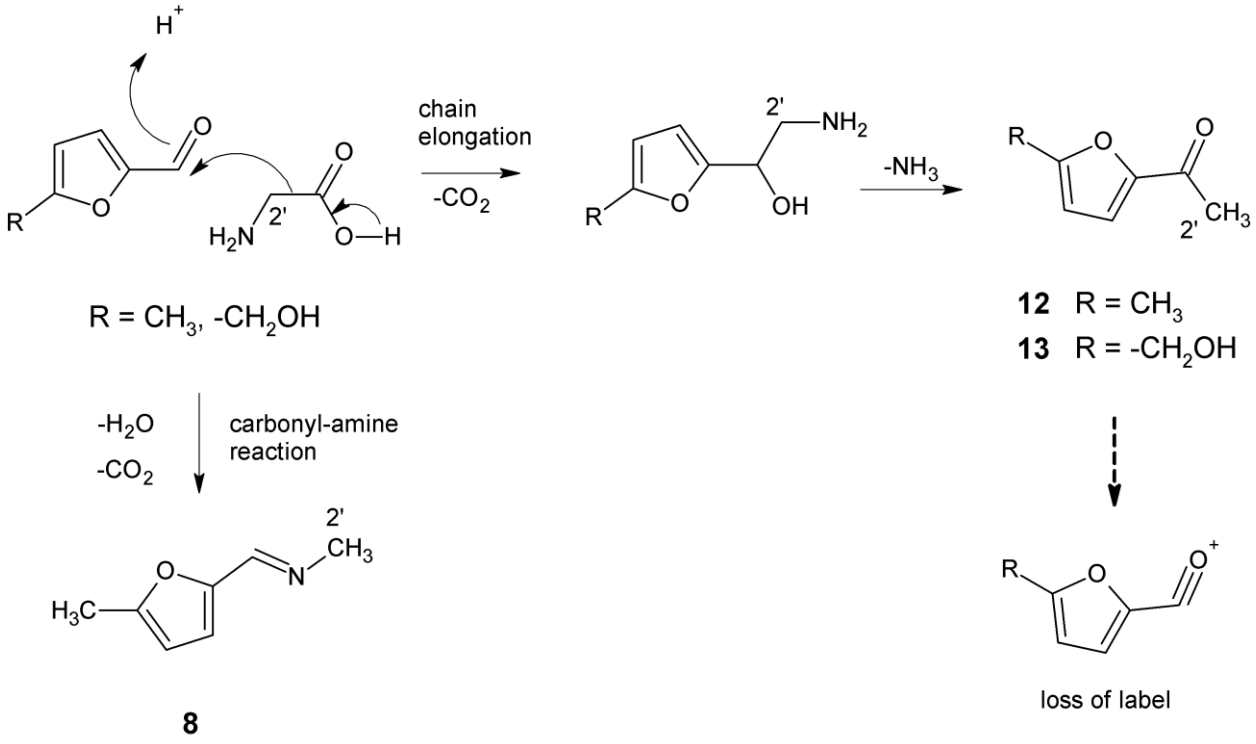


Figure 6.7 Chain elongation reactions of furfural derivatives. Dotted arrow indicates mass spectral fragmentation steps

Table 6.6 Number of glycine atom incorporation in structures **12**, **13**, **15** and **16** generated from glycine/HMF model system and shown in Figures 6.7 and 6.8.

Structure	12[a]	13[b]	15[c]	16[d]
[^{15}N]glycine	0	0	1	1
[^{13}C-1]glycine	0	0	0	0
[^{13}C-2]glycine	1	1	2[e]	2[e]

[a] 2-acethyl-5-methylfuran (mw 124 amu, t_R = 16.907 min)

[b] mw 140 amu, t_R = 21.746 min

[c] mw 153 amu, t_R = 20.728 min

[d] mw 155 amu, t_R = 20.654 min

[e] incorporation of only one C-2 is observed if excess paraformaldehyde is added to the model

Analysis of the mass spectral fragmentation pattern confirmed the location of the C-2 label from glycine as the methyl group in the acetyl side chain (Figure 6.7 and Table 6.6). This type of amino acid C-2 atom incorporation can be achieved through chain elongation mechanism (Chu & Yaylayan, 2009b) shown in Figure 6.7. HMF also undergoes similar chain elongation to produce the corresponding structure **13** that incorporates only one atom from glycine (C-2) and shows the expected molecular mass at m/z 140. However, no standards were available to confirm this proposal. When the temperature of the reaction was raised from 250°C to 350°C twenty fold increase was observed in the ion count of **12** relative to its corresponding Schiff base adduct **8** indicating chain elongation process is highly temperature dependent. The potential of furfurals to participate in chain elongation reactions has not been documented and it can provide one possible mechanism for the formation of 2-acetyl-5-methylfuran in the Maillard reaction mixtures.

6.4.4 Formation of 5-[(dimethylamino)methyl]-2-furanmethanol (16) through sarcosine

Another chromatographic peak that deviated from the expected pattern of one C-2 atom incorporation for every nitrogen atom characteristic of the Schiff base adducts, had the highest intensity in the HMF/glycine chromatogram and showed the incorporation of two C-2 atoms of glycine and only one nitrogen atom. The NIST library search indicated the possible structure of 5-[(dimethylamino)methyl]-2-furanmethanol (**16**) shown in Figure 6.8 with a high confidence.

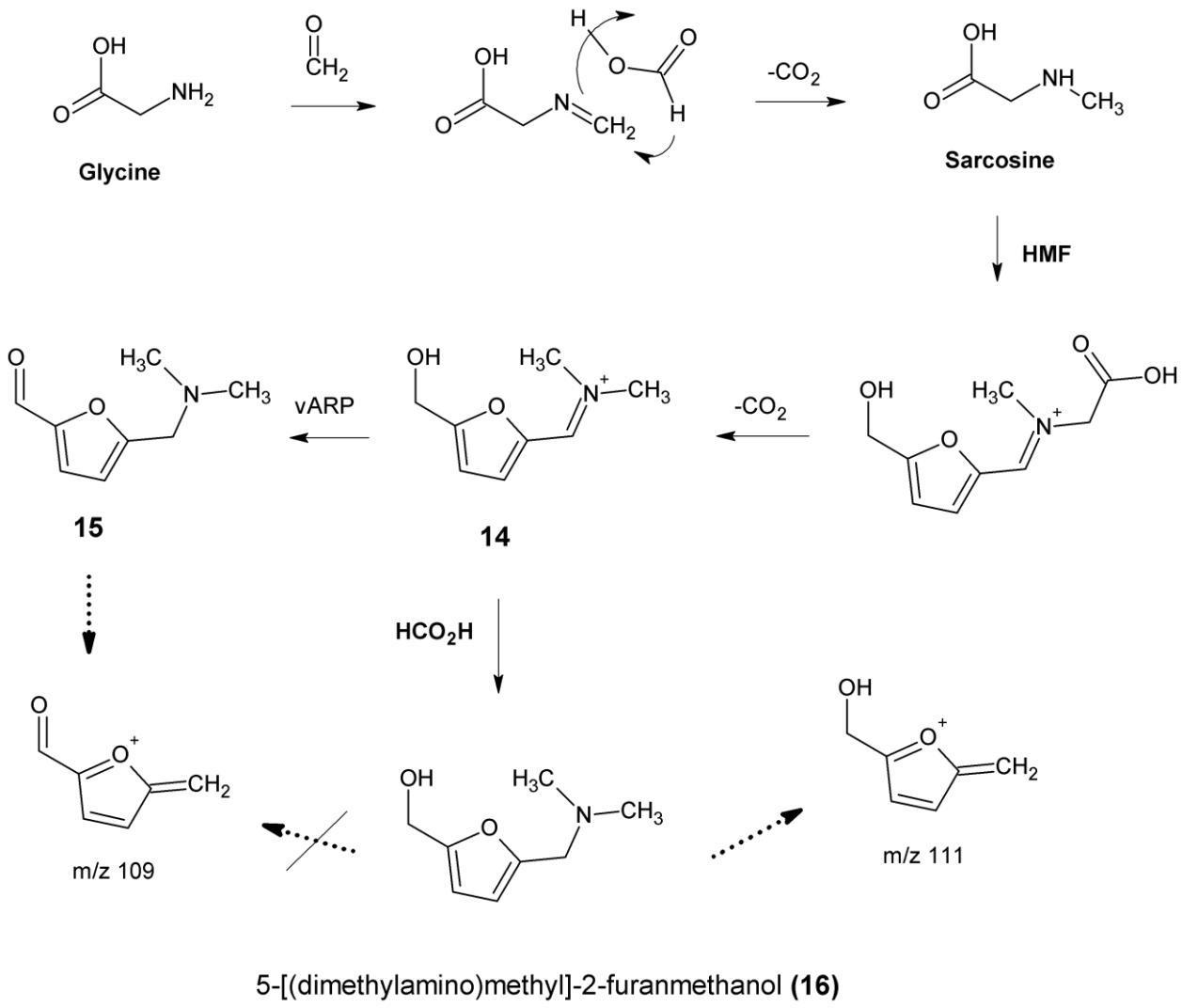


Figure 6.8 N-methylation of glycine and subsequent formation of Schiff base adduct (**14**) followed by either a reduction to from **16** or vinylogous Amadori rearrangement to form **15**. Dotted arrows indicate mass spectral fragmentation steps

Table 6.7 Number of glycine atom incorporation into the mass spectral fragments of 5-[(dimethylamino)methyl]- 2 -furanmethanol (**16**, t_R 20.65 min) in glycine/HMF model system.

m/z	**155**[a]	**138**	**124**	**111**	**94**	**83**
[^{15}N]glycine	1	1	1	0	1	0
[^{13}C-1]glycine	0	0	0	0	0	0
[^{13}C-2]glycine	2	2	2	0	1	0

[a] molecular ion

When commercially available standard was analyzed it generated a peak having the same retention time and the mass spectrum as that of the target compound confirming the proposed structure. The labelling studies (Tables 6.6 and 6.7) clearly indicated incorporation of two C-2 and one nitrogen atom from glycine, however when excess formaldehyde was added to the HMF/glycine model only one C-2 atom from glycine was found incorporated in **16** indicating formaldehyde as a possible source of the N-methyl group. The N-methylation of amino acids through reduction of Schiff base adducts of formaldehyde has already been reported (Aurelie et al., 2003) and in Figure 6.8 we propose formic acid as the reducing agent (Huyghues-Despointes & Yaylayan, 1996) originating from the degradation of HMF (Kuster, 1990) and converting the formaldehyde Schiff base adduct of glycine into sarcosine. The HMF/sarcosine model as expected also generated a peak with a matching retention time and mass fragmentation profile to that of the commercial standard. Although formaldehyde can be generated as the Strecker aldehyde of glycine, however, the reaction of HMF with glycine known to proceed with the formation of oxazolidinone intermediate (Nikolov & Yaylayan, 2011a) can also generate formaldehyde as shown in Figure 6.2. The Schiff base adduct **14,** being a secondary amine is expected to also undergo vinylogous Amadori rearrangement (Nikolov & Yaylayan, 2011a) to from structure **15**. A peak eluting at retention time of 20.73 min and corresponding to the specifications of **15** with a consistent mass spectrum (Table 6.8) was observed, as shown in Figure 6.8, the characteristic peak at m/z 109 (55.7%) was completely absent from **16** and the peak at m/z 111 (65.5%) characteristic of **16** was absent from **15**. The identity of **15** however remains to be confirmed.

Table 6.8 Number of glycine atom incorporation into the mass spectral fragments of the proposed structure **15** (t_R 20.73 min) in glycine/HMF model system

m/z	**153**[a]	**124**	**109**	**81**
[^{15}N]glycine	1	1	0	0
[^{13}C-1]glycine	0	0	0	0
[^{13}C-2]glycine	2	2	0	0

[a] molecular ion

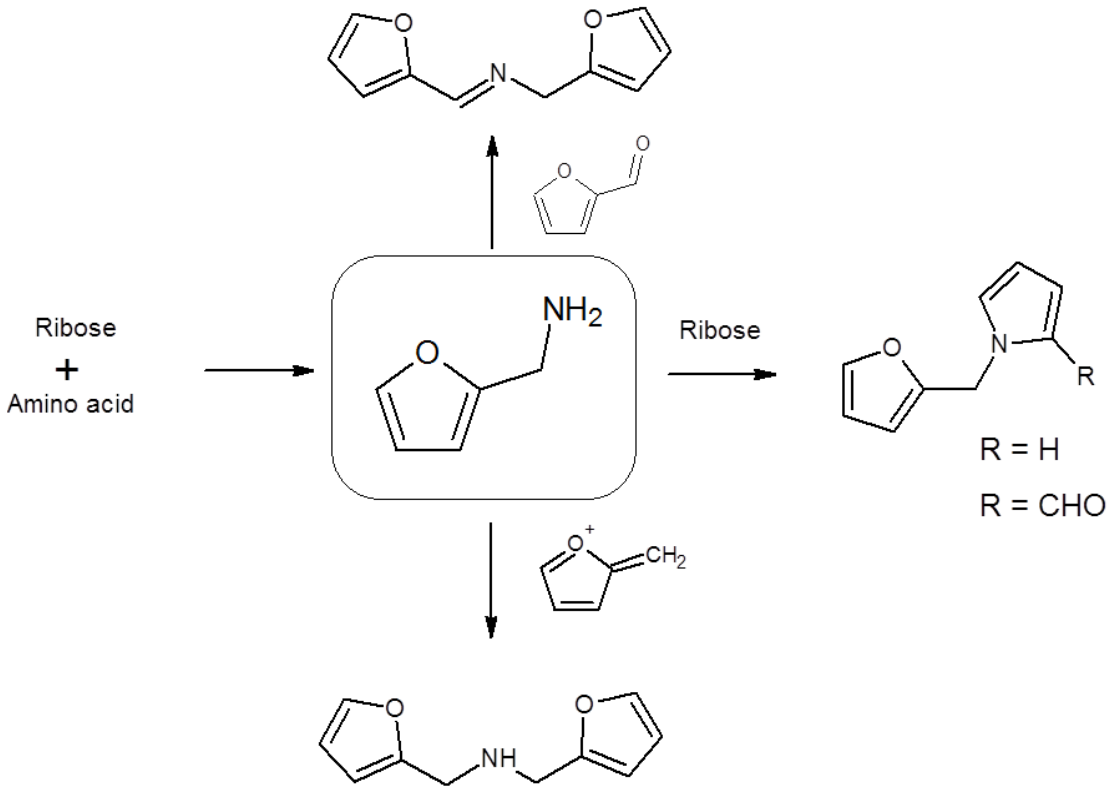
6.5 Conclusions

Thermal decomposition studies of HMF have indicated that in the absence of amino acids HMF can mainly dimerize and the initially formed dimer can degrade to generate 5-methylfurfural and 2,5-furandicarboxaldehyde. On the other hand, in the presence of amino acids such as glycine, HMF and its degradation products can form Schiff base adducts. In addition, beside undergoing carbonyl amine reactions with HMF, glycine can also perform chain elongation reactions in the presence of furfural derivatives to generate 2-acetyl furan derivatives. The abilities of HMF and glycine to generate formic acid during Maillard reaction can provide reducing agents needed to justify many of the products observed during the Maillard reaction, (Huyghues-Despointes & Yaylayan, 1996) such as the formation of sarcosine from glycine.

CONNECTING PARAGRAPH

Previous chapters have investigated the chemical behaviour of lysine in conjunction with hexoses and their thermal degradation products. Although hexoses are the most abundant monosaccharides in foodstuffs, pentoses are more reactive and have a higher contribution towards food browning. In Chapter 7, the reaction behaviour of one of the most reactive pentose sugars - ribose will be investigated with lysine. The investigation will also explore the generation of aroma compounds and analyze coffee samples for the presence of lysine/ribose reaction products. Chapter 7 was published in the Journal of Agricultural and Food Chemistry. (Nikolov P.Y., Yaylayan V. (2012) The role of ribose-specific marker furfurylamine in the formation of aroma active 1-(furan-2yl-methyl)-1H-pyrrole (or furfuryl-pyrrole) derivatives. *Journal of Agricultural and Food Chemistry*, 60, 10155-10161.)

CHAPTER 7: THE ROLE OF RIBOSE-SPECIFIC MARKER FURFURYL-AMINE IN THE FORMATION OF AROMA ACTIVE 1-(FURAN-2YL-METHYL)-1H-PYRROLE (OR FURFURYL-PYRROLE) DERIVATIVES



7.1 Abstract

Furfuryl-pyrroles possess a diverse range of organoleptic properties described as roasted, chocolaty, green, horseradish-like, mushroom-like and are detected in various foods such as coffee, chocolate, popcorn and roasted chicken. Although their origin in food was attributed to furfuryl-amine, however the latter has not been detected so far in Maillard model systems or in foods. In this chapter, furfuryl-amine was shown to be formed specifically from ribose through nitrogen atom transfer from the α-amino group of any amino acid. Such a transfer can be achieved through decarboxylation of the Schiff base adduct and isomerization followed by hydrolysis. Through the use ^{15}Nα-lysine it was revealed that only the ^{15}Nα nitrogen atom was incorporated into its structure indicating a specific role for the carboxylate moiety in the mechanism of its formation. Furthermore, isotope labelling studies have indicated that furfuryl-pyrrole derivatives can be formed by the interaction of two moles of furfuryl-amine with 3-deoxyribosone followed by dehydration and cyclization to form 1-(furan-2-yl)-N-{[1-(furan-2-ylmethyl)-1H-pyrrol-2-yl]methylidene} methanamine. After hydrolysis, this intermediate can generate furfuryl-formyl-pyrrole, furfuryl-pyrrole carboxylic acid and furfuryl-pyrrole. In this chapter, the furfuryl-amine derivatives were also detected in different coffee beans after pyrolysis and analysis by GC/MS. The potential of these compounds to form in aqueous model systems at a temperature of 120^{o}C was also demonstrated.

7.2 Introduction

Although furfuryl-amine (**1** in Figure 7.1) has not been detected in food or in model systems, however it has been postulated to be the precursor (Baltes & Knoch, 1993) of various pyrrole-containing furan derivatives (Walradt et al., 1971; Counet et al., 2002)

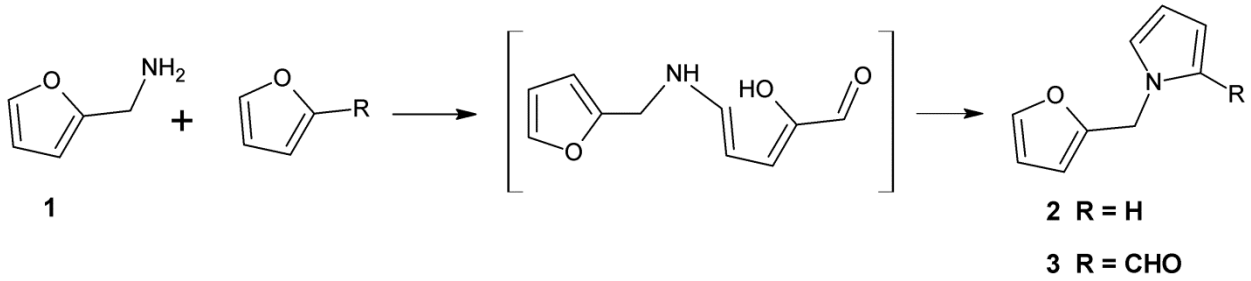


Figure 7.1. Proposed formation pathway of furfuryl-pyrrole derivatives according to Baltes & Knoch (1993)

such as furfuryl-pyrrole (**2**) shown in Figure 7.1. It has been identified in many amino acid model systems including cysteine (Mulders et al., 1973), tryptophan (Baltes & Knoch, 1993), serine, threonine (Chen & Ho, 1999) and in foods including coffee (Stoll et al., 1967), dark chocolate (Counet et al., 2002), chicory aroma (Baek & Cadwallader, 1998), popcorn (Shen & Hoseney, 1995), roasted peanuts (Walradt et al., 1971), and has been found as an aroma constituent in bread, roasted chicken, and sandalwood oil (Yannai, 2004). Furfuryl-pyrrole has been shown to possess important organoleptic properties described as roasted, chocolaty, green (Counet et al., 2002), horseradish-like, mustard seed and mushroom-like (Mosciano, 1996). Another proposed furfuryl-amine derivative furfuryl-formyl-pyrrole (**3**) has been detected in model systems including tryptophan (Baltes & Knoch, 1993), serine, glutamine and as one of the main volatile products in a ribose/threonine system (Chen & Ho, 1999). In foods, compound **3** is identified in coffee (Stoll et al., 1967), roasted peanuts (Walradt et al., 1971), popcorn (Shen & Hoseney, 1995), and chicory aroma (Baek & Cadwallader, 1998). The origin of the pyrrole moiety in furfuryl-pyrrole derivatives (**2** and **3**) has not been confirmed however, it was postulated (Rizzi, 1974) to be the result of a nucleophilic attack by the amino group of the furfuryl-amine at the carbon five position of the furan moiety (Figure

7.1). Tressl and colleagues (1986) on the other hand suggested that the source of the pyrrole moiety in furfuryl-pyrrole was not only due to the furfural but also to the presence of hydroxyproline (section 7.4.3). This hypothesis was further supported when both furfuryl-pyrrole and furfuryl-formyl-pyrrole were observed to experience 130 and 20 fold increases in peak areas respectively when dent corn was spiked with hydroxyproline (Shen & Hoseney, 1995). Due to the importance and widespread occurrence of furfuryl-pyrrole derivatives, their origin and mechanism of formation were investigated in both dry and aqueous Maillard model systems utilizing ribose, arginine and lysine as precursors. Both lysine and arginine models generated similar furfuryl-pyrrole derivatives.

7.3 Materials and methods

All chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. [^{15}Nα]lysine.2HCl, [U-$^{13}C_5$] Ribose, [^{13}C-1]ribose, were purchased from CIL (Andover, MA). Blended coffee samples were purchased from local markets.

7.3.1 Pyrolysis-GC/MS analysis

Analyses were conducted using a Varian CP-3800 GC coupled with a Saturn 2000 Ion Trap Mass Spectrometer (Varian, Walnut Creek, USA). The pyrolysis unit included a CDS Pyroprobe 2000 and a CDS 1500 valved interface (CDS Analytical, Oxford, USA) installed onto the GC injection port. Between 0.5-1.5 milligram of a sample mixture (see Table 1) were packed inside a quartz tube (0.3mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed for 20 seconds at a temperature of 250°C. The separation was carried out on a DB-5MS (5% diphenyl, 95% dimethyl polysiloxane) capillary column with dimensions of 50 m length by 0.2 mm internal diameter and 0.33

μm film thickness (J & W Scientific, ON, Canada), using helium as the carrier gas. The GC column flow rate was regulated by an electronic flow controller (EFC) and set at a pressure pulse of 70psi for the first 4 minutes and later maintained with a constant flow of 1.5 mL/minute for the remainder of the run. The GC oven temperature was set at -5°C for 5 minutes using CO_2 as the cryogenic cooling source. The temperature was increased to 50°C at a rate of 50°C/minute and then to 270°C at a rate of 8^{o}C/minute, and kept at 270°C for 5 minutes. The samples were detected by using an ion-trap mass spectrometer with a scan range of 20-650m/z. The MS transfer-line temperature was set at 250°C, manifold temperature was set at 50°C, and the ion-trap temperature was set at 175°C. The ionization voltage of 70 eV was used, and EMV was set at 1700 V. Structural identification was performed using AMDIS (ver 2.65) and NIST Standard Reference Databases (data version 05 and software ver 2.0d) and by comparison of the retention times and mass spectra to that of commercially available standards in addition to isotope labelling data. The reported percent label incorporation values (corrected for natural abundance and for percent enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

7.3.2 Sample preparation

The dihydrochloride salts of the commercially available isotopically labelled lysines were unreactive when pyrolyzed as such, however, mixing the salts with an equimolar amounts of unlabelled free lysine resulted in increased reactivity when pyrolyzed. Consequently, equimolar amounts of unlabelled DL-lysine and specifically labelled DL-lysine.2HCl were

Table 7.1 Composition of Model Systems Analyzed by Py-GC/MS[a]

Compound / target ion	Models
Furfuryl-amine (**1**) m/z 97	ribose + arginineHCl ribose + lysineHCl ribose + ornithineHCl ribose + citrulline ribose + furfuryl-amine
Furfuryl-pyrrole (**2**) m/z 147	ribose + arginineHCl ribose + arginineHCl (aqueous) ribose + lysineHCl ribose + ornithineHCl ribose + citrulline ribose + hydroxyproline ribose + furfuryl-amine furfural + arginineHCl furfural + hydroxyproline glucose + arginineHCl+furfuryl-amine 1-furfuryl-pyrrole
Furfuryl-formyl-pyrrole, (**3**) m/z 175	ribose + arginineHCl ribose + arginineHCl (aqueous) ribose + lysineHCl ribose + ornithineHCl ribose + citrulline ribose + furfuryl-amine glucose + arginineHCl+furfuryl-amine
Furfuryliden-furfuryl-amine, (**4**) m/z 175	ribose + arginineHCl ribose + lysineHCl ribose + ornithineHCl ribose + citrulline ribose + furfuryl-amine furfural + arginineHCl furfural + furfuryl-amine glucose + arginineHCl+furfuryl-amine
1-(furan-2-yl)-*N*-{[1-(furan-2-ylmethyl)-1*H*-pyrrol-2-yl] methylidene}methanamine (**5**) m/z 254	ribose + arginineHCl ribose + lysineHCl ribose + ornithineHCl ribose + citrulline ribose + arginineHCl + furfuryl-amine
di-(2,2'- furfuryl)amine (**7**) m/z 177	ribose + arginineHCl ribose + lysineHCl ribose + ornithineHCl furfuryl alcohol + furfuryl-amine
1-(furan-2-yl)-N,N-bis(furan-2-ylmethyl)methanamine (**8**) m/z 257	ribose + arginineHCl ribose + lysineHCl ribose + ornithineHCl ribose + furfuryl-amine furfuryl-amine

[a] model systems containing ribose or lysine were also analyzed using their labelled counterparts ([$^{13}U_5$]ribose or [^{13}C-1]ribose and [$^{15}N\alpha$]lysine)

mixed and homogenized before mixing with equimolar amount of D-glucose. Coffee samples (1 mg) were pyrolyzed according to the above described procedure

7.3.3 Aqueous samples

Equimolar ratio of ribose and arginineHCl were dissolved in distilled water (1mL) and the sample was placed in a sealed Q-tube reactor (Q Labtech LLC) and heated at 120°C for 20min. The sample was dried and the residue was dissolved in methanol and directly injected into the above mentioned GC/MS system using the same parameters for analysis except the oven temperature was set at 41°C and the temperature was increased to 50°C at a rate of 10°C/minute and then to 270°C at a rate of 8^{o}C/minute, and kept at 270°C for 5 minutes.

7.4 Results and discussion

The postulated furfuryl-amine derivatives mentioned above have been shown to form independently of the type of amino acid used in the model systems (Mulders, 1973; Baltes & Knoch, 1993; Chen & Ho, 1999). In contrast to the lack of specificity of the precursor amino acids, Chen et al., (1999) have demonstrated that furfuryl-amine derivatives were formed exclusively from ribose containing model systems and that glucose or fructose are unable to generate such derivatives. Rizzi (1974) attributed the formation of furfuryl-amine derivatives from ribose models to its ability to form furfural, similarly, Baltes & Knoch (1993) suggested that furfuryl-amine can be formed during the hydrolysis of the imine bond of a decarboxylated amino acid-furfural adduct. On the other hand, Shibamota et al., (1979) have proposed ammonia as a potential source of nitrogen in the formation of furfuryl-amine from furfural. To investigate the mechanism

of formation of furfuryl-amine, model systems listed in Table 7.1 were analyzed and here for the first time we report the detection of furfuryl-amine **(1)** in these model systems.

7.4.1 Mechanism of formation of furfuryl-amine in ribose/amino acid model systems

So far furfuryl-amine has been assumed to be the precursor of furfuryl-pyrrole derivatives without being detected or identified in the same model systems. When ribose/arginine model system was analyzed as described under the experimental section a prominent peak was detected at the retention time of 12.796 min matching the mass spectral fragmentation pattern and the retention time of a commercial standard of furfuryl-amine (see Table 7.2) and that of the NIST library. Spiking experiments with the standard sample further confirmed the identity of the peak. The same peak was also generated when arginine was replaced with lysine and with various other amino acids (see Table 7.1), however when ribose was replaced with glucose or fructose the furfuryl-amine peak was not observed. Furthermore, isotope labelling experiments with $^{13}U_5$-ribose and $^{15}N\alpha$-lysine confirmed the presence of five carbon atoms from ribose and one Nα nitrogen atom (100% incorporation) from lysine in the furfuryl-amine structure, no Nε nitrogen was incorporated in the structure of furfuryl-amine (Table 7.2). To examine the role of furfural in the formation of furfuryl-amine, furfural was also reacted with ammonium chloride and with several amino acids, however, none of the models studied generated furfuryl-amine including glucose/ammonium chloride and ribose/ammonium chloride model systems, thus excluding furfural and ammonia as major precursors of furfuryl-amine.

Table 7.2 Number of isotopically labelled atoms incorporated in furfuryl-amine[a] (**1**) generated in ribose/lysine models

m/z	97	81	69	54	39
[$^{13}U_5$]ribose	5	5	3	3	3
[^{13}C-1]ribose	1	1	0	0	0
[^{15}Nα]lysine	1	0	1	0	0

[a] t_R = 12.796 min, standard t_R = 12.779min, mw 97
Model m/z (%) 39 (40.9), 53 (16.2), **69 (100),** 81 (56.2), 96 (45.1), 97 (39.6)
Commercial standard m/z (%) 39 (50.2), 53 (38.7), **69 (100),** 81 (21.7), 96 (57.2), 97 (78.4)
NIST m/z (%) 39 (37.0), 53 (46.2), **69 (100),** 81 (27.5), 96 (26.8), 97 (53.9)

Clearly, furfuryl-amine is formed by the interaction of any amino acid with ribose, through Nα atom transfer at the carbon one position of ribose as indicated by the isotope labelling data in Table 7.2. Since ammonia was excluded as the source of nitrogen, one mechanism that is capable of rationalizing such Nα transfer from amino acids into carbonyl compounds is shown in Figure 7.2, this proposed mechanism is based on the formation of 5-oxazolidinone intermediate followed by a decarboxylation step (Chu & Yaylayan, 2009a; Hidalgo et al., 2010) to eventually form decarboxylated and isomeric Schiff bases of which one isomer can hydrolyze to transfer the nitrogen atom at the C-1 position of ribose and release the Strecker aldehyde (See Figure 7.2). Amino groups that are not located at the α-position of the carboxylic acid are not able to undergo such transformation due to their inability to form 5-oxazolidinone intermediates. This fact can rationalize the observation that only Nα atom was transferred into furfuryl-amine structure from lysine. As shown in Figure 7.2 the initial intermediate can undergo dehydration and cyclization reactions leading to furfuryl-amine.

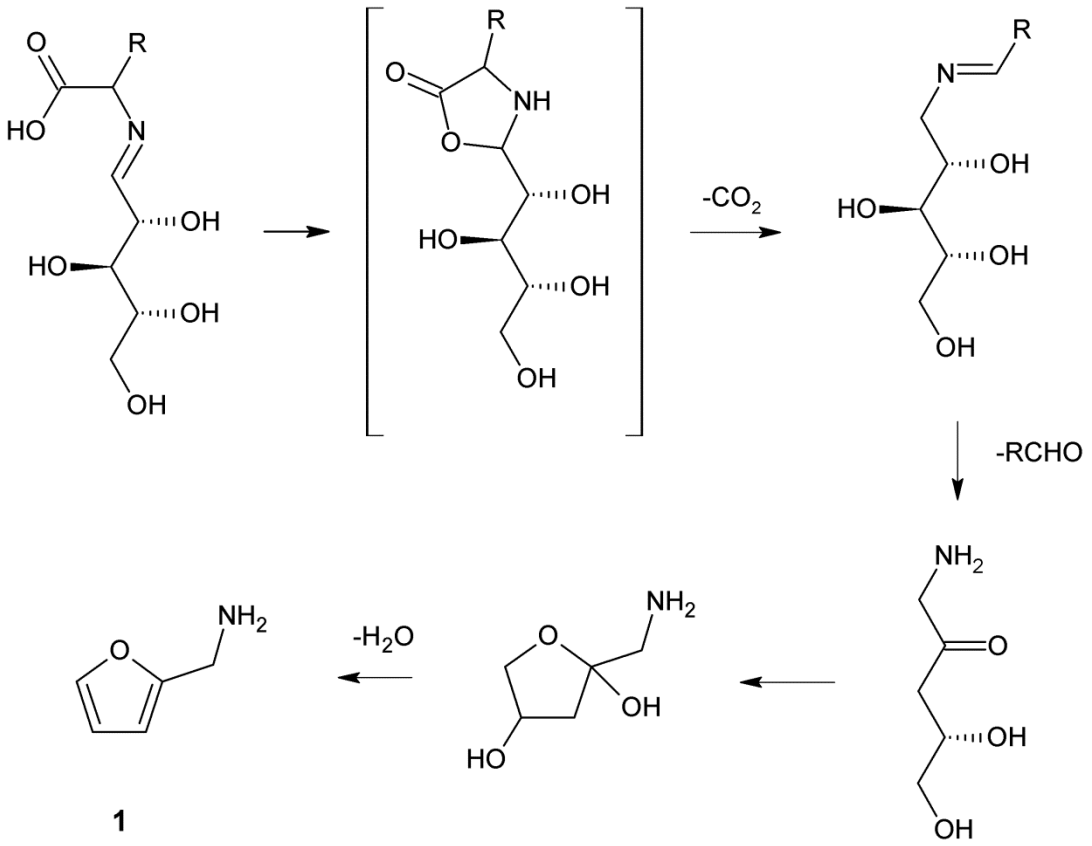


Figure 7.2 Proposed mechanism of formation of furfuryl-amine (**1**) from Schiff base adducts of ribose with any α-amino acid

7.4.2 Proposed mechanism of formation of furfuryl-pyrrole (FP) and other derivatives of furfuryl-amine

As speculated in the literature, furfuryl-amine derivatives furfuryl-pyrrole and furfuryl-formyl-pyrrole (FFP) were also detected in the same model systems studied where furfuryl-amine was observed. Furfuryl-pyrrole (**2**) eluted at the retention time of 19.8 min and its identity was confirmed by matching the mass spectral fragmentation pattern and the retention time of a commercial standard (see Table 7.3) and through the NIST library searchers. Spiking experiments with the standard sample further confirmed the identity of

Table 7.3 Number of isotopically labelled atoms incorporated in furfuryl-pyrrole[a] (**2**) generated in ribose/lysine-HCl models

m/z	147	81	53	39
[$^{13}U_5$]ribose	9	5	4	3
[^{13}C-1]ribose	1	1	1	0
[^{15}Nα]lysine	1	0	0	0

[a] t_R = 19.813, standard t_R =19.792, mw 147
Model m/z (%) 39 (9.40), 53 (33.6), **81(100),** 147 (77.2)
Commercial standard m/z (%) 39 (10.7), 53 (33.8), **81(100),** 147 (83.9)
NIST m/z (%) 39 (9.0), 53 (38.3), **81(100)**, 147 (47.0)

the peak. Isotope labelling studies have indicated that all the nine carbon atoms of FP originated from ribose with the loss of one carbon atom from C-1 position and only one nitrogen atom was originated from Nα of lysine as in the case of furfuryl-amine (Table 7.3). Furthermore, reacting ribose with a 50:50 mixture of [^{15}Nα]lysine and ammonium chloride generated the furfuryl-pyrrole peak with an 100% ^{15}Nα-lysine incorporation thus ruling out ammonia as a source of nitrogen. Interestingly, a model system consisting of only ribose and furfuryl-amine also generated furfuryl-pyrrole without the need of an amino acid, indicating furfuryl-pyrrole can result through the interaction of ribose with furfuryl-amine. A proposed mechanism consistent with the above observations is shown in Figure 7.3 which postulates double Schiff base formation between 3-deoxyribosone and two moles of furfuryl-amine. Intramolecular cyclization of this intermediate followed by dehydration can generate furfuryl-pyrrole derivative **5.** Hydrolysis of **5** can form furfury-formyl-pyrrole (**3**) which in turn after oxidation into carboxylic acid functionality can undergo decarboxylation with the loss of C-1 atom from ribose to form furfuryl-pyrrole (**2**) as confirmed above through isotope labelling experiments.

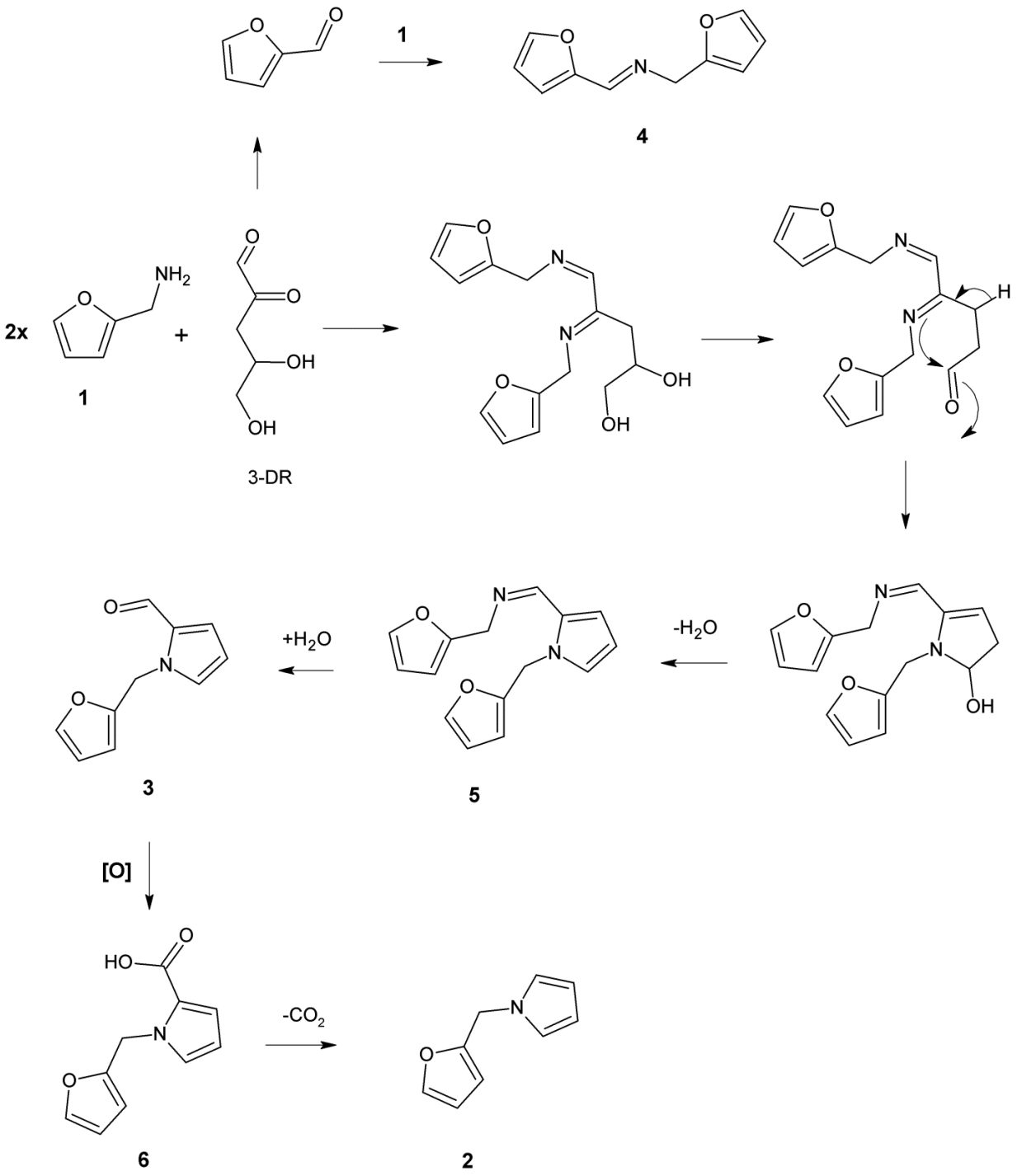


Figure 7.3 Proposed mechanism of formation of furfuryl-pyrrole (**2**) and other furfuryl-amine derivatives from the interaction of furfuryl-amine (**1**) with 3-deoxyribosone (3-DR)

The proposed intermediate **3** was observed as the highest peak in the chromatogram and at a retention time of 24.12 min (see Table 7.4) and was tentatively identified as furfuryl-formyl-pyrrole based on the matching of its reported mass spectrum (Baltes & Knoch, 1993) and based on the complementary isotope labelling data that confirmed incorporation of ten carbon atoms from ribose and only one Nα atom from lysine (see Table 7.4). Consistent with the proposed mechanism, furfuryl-formyl-pyrrole is often detected in tandem with furfuryl-pyrrole in food samples such as coffee (Stoll et al., 1967), popcorn (Shen & Hoseney, 1995), roasted peanut (Walradt et al., 1971), chicory aroma (Baek & Cadwallader, 1998), and in different models system including serine/threonine with ribose (Chen & Ho, 1999), and tryptophan (Baltes & Knoch, 1993). We were also able to detect both compounds when commercially obtained roasted coffee beans were pyrolyzed at 250^{o}C.

Table 7.4 Number of isotopically labelled atoms incorporated in furfuryl-formyl-pyrrole[a] (**3)** generated in ribose/lysine-HCl models

m/z	**175**	**147**	**81**	**53**
[$^{13}U_5$]ribose	10	9	5	4
[^{13}C-1]ribose	2	1	1	1
[^{15}Nα]lysine	1	1	0	0

[a] t_R = 24.122 min, mw 175
Model m/z (%) 39 (6.60), 53 (21.8), **81 (100),** 146 (7.6), 175 (63.5)
Literature (Baltes & Knoch, 1993) m/z (%) 39 (12.0), 53 (29.0), **81 (100),** 146 (2.0), 175 (28.0)

On the other hand, intermediate **6** was not observed in this investigation although a compound consistent with the structure of **6** has been detected and reported in ribose/tryptophan (Baltes & Knoch, 1993) model systems together with **2** and **3** further confirming the sequence of steps proposed in Figure 7.3. Furthermore, a peak at retention time of 31.442 min consistent with the proposed structure of intermediate **5** was also

detected (see Table 7.5). As expected isotope labelling data (Table 7.5) indicated the incorporation of 15 carbon atoms from ribose and two Nα atoms from lysine. In addition, spiking the ribose/arginine model system with furfuryl-amine resulted in a nearly 260 fold increase in intensity of intermediate **5**, while the peak was not detected at all in furfural/lysine or arginine models.

Table 7.5 Number of isotopically labelled atoms incorporated in the proposed structure 1-(furan-2-yl)-N-{[1-(furan-2-ylmethyl)-1H-pyrrol-2-yl]methylidene} methanamine[a] (**5)** generated in ribose/lysine-HCl models

m/z	**254**	**225**	**173**	**146**	**118**	**81**	**53**
[$^{13}U_5$]ribose	15	14	10	9	8	5	4
[^{13}C-1]ribose	3	3	2	1	1	1	1
[^{15}Nα]lysine	2	2	2	1	1	0	0

[a] t_R = 31.448 min, mw 254, m/z (%): 53 (41.4), 81 (56.4), 118 (23.5), 146 (33.1), **173 (100**), 225 (34.4), 254 (77.7).
Note: The mass spectral fragments shown and label incorporation pattern indicated in the table are consistent with the proposed structure of **5.**

In general, ribose-containing model systems are known to generate furfural as a major product which may react with furfuryl-amine and form a Schiff base adduct similar to amino acids (Nikolov & Yaylayan, 2011a, b), such a predicted product (structure **4**) was reported by Baltes & Knoch (1993) in ribose containing model systems and a peak having a retention time at 23.8 min matched its mass spectral fragmentation pattern and that of reported in the NIST library (see Table 7.6). In fact, a peak having the same retention time and mass spectral fragmentation pattern as that of **4** was generated as the major product from furfural and furfuryl-amine reaction, confirming its proposed precursors. The peak experienced an approximately 25 fold increase in intensity when ribose/arginine model was spiked with furfuryl-amine. Isotope labelling studies reported

in Table 7.6 further confirms the presence of 10 carbon atoms from ribose and one Nα from lysine.

Table 7.6 Number of isotopically labelled atoms incorporated in furfuryliden-furfuryl-amine [a] (**4)** generated in ribose/lysine-HCl models

m/z	175	147	81	53
[$^{13}U_5$]ribose	10	9	5	4
[^{13}C-1]ribose	2	2	1	1
[^{15}Nα]lysine	1	1	0	0

[a] t_R = 23.749min, mw 175
Model m/z (%) 39 (11.0), 53 (32.4), **81 (100),** 147 (19.6), 175 (24.2)
Literature (Baltes & Knoch, 1993) m/z (%) 39 (7.0), 53 (20.0), **81 (100),** 175 (17.0)
NIST m/z (%) 39 (14.6), 53 (29.8), **81 (100),** 147 (5.0), 175 (21.3)

7.4.3 Hydroxyproline-specific pathway for furfuryl-pyrrole (2)

One of the most commonly detected furfuryl-amine derivatives the furfuryl-pyrrole (**2)** has been also postulated to be formed as the result of the interaction between furfural and hydroxyproline (Tressl et al., 1986). To confirm this specific pathway, hydroxyproline reaction with ribose was investigated and indeed the model generated 600 x fold excess of furfuryl-pyrrole relative to ribose/arginine model. Although this pathway is more efficient, however it is only specific to a rare (Jones et al., 1986) amino acid in food the hydroxyproline and generates only one furfuryl-amine derivative. The proposed mechanism shown in Figure 7.4 is based on the reported reaction of furfural with secondary amines (Nikolov & Yaylayan, 2011b).

7.4.4 Other reactions of furfuryl-amine

In addition to the above mentioned furfuryl-amine derivatives other non-pyrrole containing adducts of furfuryl-amine were also identified in the same model systems studied. A peak detected at 22.98 min in a ribose/arginine model was tentatively

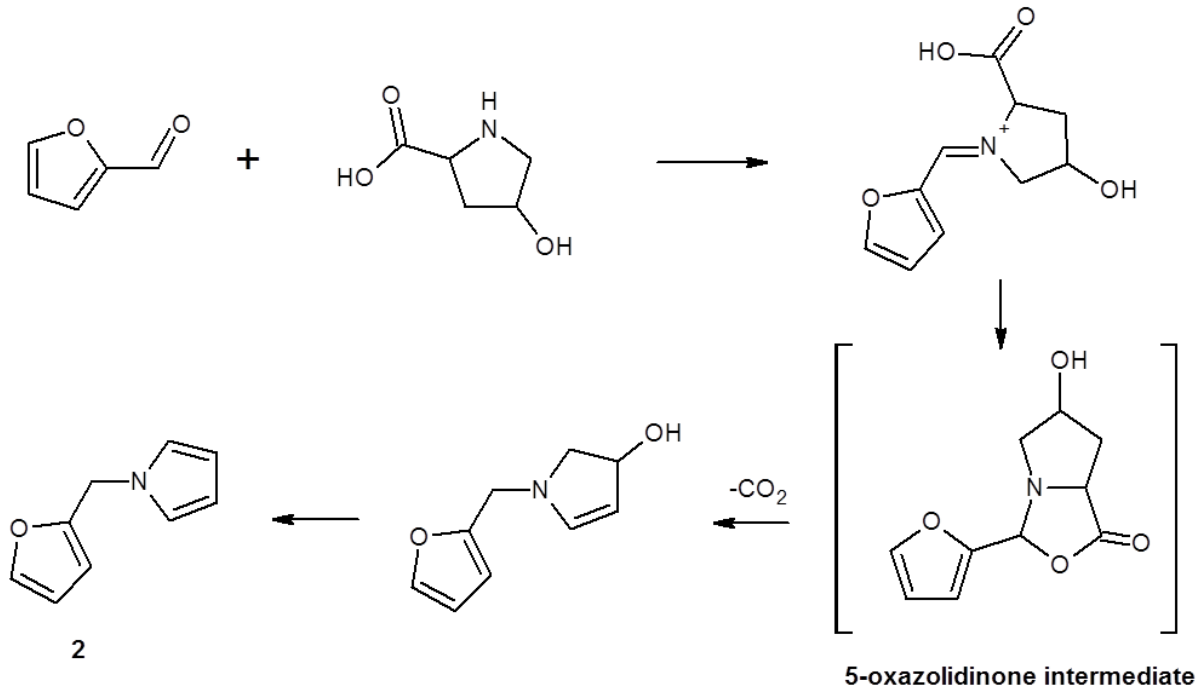


Figure 7.4 Alternate formation pathway of furfuryl-pyrrole (**2)** through hydroxyproline-specific reaction with furfural

characterized as di-(2,2'- furfuryl)amine (**7**) based on mass spectral evidence (Baltes & Knoch, 1993) and the NIST library searches in addition to the data based on isotope labelling experiments (Table 7.7). The data indicated the incorporation of 10 carbon atoms from ribose and one Nα from lysine. The same peak was also generated from furfuryl alcohol reaction with furfuryl-amine or only from furfuryl-amine, prompting us to propose 2-methylidene-2*H*-furanium ion as the key intermediate in the mechanism of formation of **7**, the furanium intermediate then can react with furfuryl amine to generate **7** (see Figure 7.5). If this assumption is correct, the resulting new amine (**7**) should similarly interact with the furanium ion to generate the trimeric derivative 1-(furan-2-yl)-N,N-bis(furan-2-ylmethyl)methanamine (**8**) shown in Figure 7.5. Indeed, a peak at the

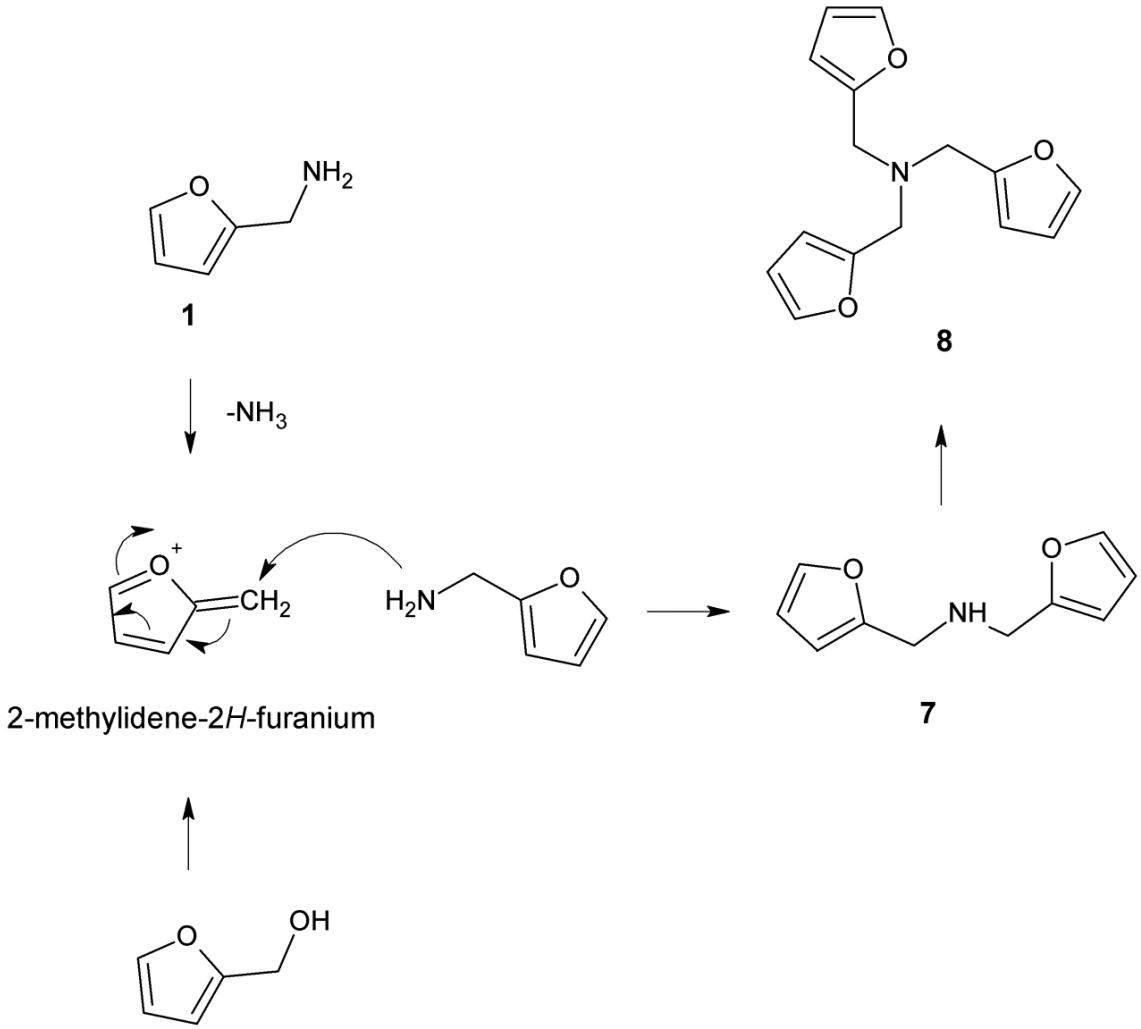


Figure 7.5 Chemical activation of furfuryl-amine and furfuryl-methanol into 2-methylidene-2H-furanium ion and formation of proposed structures **7** & **8**.

retention time of 29.211min in a ribose/lysine model system and possessing fifteen carbons atoms from ribose and one nitrogen atom from lysine was detected matching the elemental formula and the mass fragmentation profile of the proposed structure **(**see Table 7.8). As shown in Figure 7.5**,** the furanium ion can be generated from either furfuryl alcohol or furfuryl-amine. Furfuryl alcohol has been previously detected in model systems containing ribose (Meynier & Mottram, 1995) and in the present study it

Table 7.7 Number of isotopically labelled atoms incorporated in di-(2,2'- furfuryl)amine[a] (**7)** generated in ribose/lysine-HCl models

m/z	**177**	**109**	**96**	**81**	**53**
[$^{13}U_5$]ribose	10	6	5	4	4
[^{13}C-1]ribose	2	2	1	1	1
[^{15}Nα]lysine	1	1	1	0	0

[a] t_R = 22.982 min, mw 177
Model m/z (%) 39 (8.2), 53 (30.2), **81(100),** 96 (21.1), 109 (40.8), 177 (3.5)
Literature (Baltes & Knoch, 1993) m/z (%) 39 (5.0), 53 (21.0), **81(100),** 96 (25.0), 109 (27.0), 177 (3)
Note: The mass spectral fragments shown and label incorporation pattern indicated in the table are consistent with the proposed structure of **7.**

was observed at the retention time of 13.237 min confirmed through comparison of its retention time and mass spectral fragmentation pattern with that of a commercial standard in addition to the label incorporation data from a $^{13}U_5$-ribose/arginine model. Theoretically, other furfuryl alcohol derivatives such as 5-methylfurfuryl alcohol that can form from glucose should undergo similar activation and form the corresponding 5-methyl-methylidene furanium ion which can similarly interact with various amines including furfuryl-amine.

Table 7.8 Number of isotopically labelled atoms incorporated in the proposed structure 1-(furan-2-yl)-N,N-bis(furan-2-ylmethyl)methanamine[a] (**8)** generated in ribose/lysine-HCl models

m/z	**257**	**229**	**176**	**148**	**108**	**81**	**53**
[$^{13}U_5$]ribose	15	14	10	9	6	5	4
[^{13}C-1]ribose	3	3	2	2	2	1	1
[^{15}Nα]lysine	1	1	1	1	1	0	0

[a] t_R = 29.211 min, mw 257, m/z (%) **53** (29.1), **81** (100), **108** (24.9), **148** (10.0), **176** (9.9), **229** (7.7), **257** (7.5).
Note: The mass spectral fragments shown and label incorporation pattern indicated in the table are consistent with the proposed structure of **8.**

7.4.5 Formation of furfuryl-amine derivatives in aqueous medium

Model systems mentioned so far have been studied under pyrolytic conditions in a molten state at 250^{o}C for the purpose of performing isotope labelling studies. To verify the ability of the corresponding aqueous solutions at lower temperatures to generate furfuryl-amine derivatives ribose/arginine or ribose/lysine aqueous models were heated in a sealed reactor at 120°C for 20 min and analyzed by direct injection in to GC by-passing the pyrolysis interface. This sample also generated all the derivatives of furfuryl-amine, demonstrating the ability of ribose/amino acid model systems to undergo similar pathways under aqueous conditions. Furfuryl-amine therefore can be considered an important reactive intermediate in ribose/amino acid model systems and as a characteristic marker for the presence of ribose in food products. Glucose on the other hand is unable to generate the corresponding 5-hydroxymethyl-1-furfuryl-amine structure in detectable levels due to the relative stability of the resulting glucosyl amino acid ring system versus 5-oxazolidinone, the required intermediate for its transformation into the furfuryl-amine derivative.

7.5 Conclusions

This chapter detected for the first time furfurylamine, a reactive amine that can be a marker of ribose degradation products in food. Isotopically labelled lysine and ribose data were used to propose a furfurylamine formation mechanism involving an α-amino group transfer via a 5-oxazolidinone intermediate. Furthermore, this mechanism is postulated to be applicable to all amino acids reacting with ribose. The reactivity of furfurylamine was established with the detection of two of derivatives furfuryl-pyrrole and furfuryl-formyl-pyrrole, both of which were also detected with Py-GC/MS in different coffee blends. The hydrolysis of a novel intermediate consisting of two furfurylamine and 3-DR (compound **5)** generated furfuryl-pyrrole and furfuryl-formyl-pyrrole. Furthermore, the intermediate demonstrated the potential of furfurylamine derivatives to be formed from any amino acid and not be limited to hydroxyproline models. Lastly, the ability of furfurylamine and its derivatives to be generated at mild conditions was demonstrated with the detection of the furfurylamine derivatives in aqueous models heated at 120°C for 20 minutes.

CHAPTER 8: GENERAL CONCLUSIONS AND CONTRIBUTIONS TO KNOWLEDGE

The main theme of this study was focused on the reactivity of lysine and HMF. Lysine is one of the most reactive amino acids in the Maillard reaction and HMF is one of the most abundant thermal degradation products of hexoses in food. Both reactants have a direct influence on the health of consumers. The loss of the essential amino acid lysine for example decreases the protein quality of food and some of the lysine sugar reaction products (AGE) are considered detrimental to health. On the other hand, the content of HMF is regulated in some foods such as honey due to its potential toxicity. Overall the fate of thermal degradation products of lysine and HMF is not well understood. This thesis therefore has focused on developing an in-depth understanding of the fate of many interaction products of lysine with various sugar degradation products.

Piperidine is a potentially harmful lysine-specific derivative that was demonstrated to be formed for the first time by two novel mechanisms. The first mechanism, which did not involve sugar, generated a piperidine with equal composition of either Nε or Nα atoms. In the presence of sugars it was shown that lysine, similar to asparagine and phenylalanine, undergoes a carbonyl-assisted decarboxylative deamination reaction to generate Nε-pent-4-ene-1-amine, the counterpart of acrylamide from lysine, which would cyclise into piperidine. Acrylamide is a carcinogenic compound and this novel vinylic counterpart Nε-pent-4-ene-1-amine could have a similar biological significance. Through the use of

^{15}N and ^{13}C isotopically labelled precursors, the reactivity of Nε-pent-4-ene-1-amine with methylfurfural was demonstrated and a novel adduct was characterized for the first time. Further insight into the reactivity of the lysine-specific intermediate, piperidine, was achieved through the discovery of two novel piperidine products. It was demonstrated for the first time that piperidine is chemically activated by formaldehyde. Piperidine was also displayed to generate a number of pyridine derivatives.

Furthermore, this investigation characterized successfully for the first time the interaction products of piperidine and Nε-pent-4-ene-1-amine with HMF, which is considered one of the most common sugar thermal degradation products in food. It was discovered that the type of nitrogen (1^o or 2^o) dictated the mechanistic pathway of HMF/lysine reaction products. It was shown that the primary nitrogen in Nε-pent-4-ene-1-amine/HMF adduct was stabilized through the formation of a reversible Schiff base. On the other hand the piperidine, a secondary amine, and HMF adduct underwent further stabilization through a vinylogous Amadori rearrangement to generate a covalent bond. This is the first time that HMF has been demonstrated to generate a Schiff base or vinylogous Amadori rearrangement products with amines in the absence of catalysts or solvents. Furthermore, two novel adducts of HMF and glycine or proline confirmed the ability of HMF to form reversible and covalent interactions with other primary and secondary amino acids, respectively. A novel HMF/Nε-pent-4-ene-1-amine/glucose adduct also displayed the ability of HMF to form a glycosidic bond with glucose. In the absence of amino acids HMF was demonstrated to generate a HMF dimer through a novel vinylogous aldol addition mechanism that could lead to the formation of HMF polymers. The significance

of the dimer was highlighted with the discovery of the important intermediates MF and FDA resulting from breakdown of the dimer. The reactivity of MF and FDA were demonstrated with the successful characterization of several novel adducts with lysine and glycine. In addition, HMF was also observed for the first time to participate in a chain elongation reaction and to form adducts with N-methylated amino acids.

Furfurylamine, a precursor of aroma compounds in food, was identified for first time in model systems. It was demonstrated that furfurylamine was specific to ribose model systems and could be used as a marker of the pentose sugar. Furthermore, the formation mechanism of furfurylamine, involving the transfer of the Nα group of lysine, implies that it that can be applicable to any amino acid reacting with ribose. A new formation mechanism was proposed for the furfurylamine derivatives found in food (furfuryl-pyrrole and furfuryl-formyl-pyrrole) through the discovery of a novel intermediate composed of 3-deoxy-ribosone and two furfurylamine molecules. Lastly, the furfurylamine derivatives were also found to form under aqueous conditions.

Suggestions for future research:

1. Detection of pent-ene-1-amine, piperidine and their Schiff base adducts in food and determination of their concentrations.
2. Investigating the potential biological significance of pent-ene-1-amine, piperidine and their derivatives in cell-line models.

3. Investigation of the potential role of HMF to form polymers and vinylogous Amadori products of with various secondary amines in model systems and in food.
4. To determine the impact of high temperature or long storage duration on the ratio between the native HMF and HMF reaction derivatives in model system and in food.

REFERENCES

Abdulmalik, O., Safo, M.K., Chen, Q., Yang, J., Brugnara, C., Ohene-Frempong, K., Abraham, D.J. & Asakura, T. (2005) 5-Hydroxymethyl-2-furfural modifies intracellular sickle haemoglobin and inhibits sickling of red blood cells. *British Journal of Haematology*, 128, 552-561.

Abraham, K., Gurtler, R., Berg, K., Heinemeyer, G., Lampen, A. & Appel, K.E. (2011) Toxicology and risk assessment of 5-Hydroxymethylfurfural in food. *Molecular Nutrition and Food Research*, 55, 667-678.

Abrams, A., Lowy, P.H. & Borsook, H. (1955) Preparation of 1-amino-1-deoxy-2-ketohexoses from Aldohexoses and a Amino Acids. *Journal of the American Chemical Society*, 77, 4794-4796.

Ahmed, M.U., Thorpe, S.R. & Baynes, J.W. (1986) Identification of n-epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *Journal of Biological Chemistry*, 261, 4889-4894.

Ait-Ameur, L., Mathieu, O., Lalanne, V., Trystram, G. & Birlouez-Aragon, I. (2007) Comparison of the effects of sucrose and hexose on furfural formation and browning in cookies baked at different temperatures. *Food Chemistry*, 101, 1407-1416.

Ait-Ameur, L., Rega, B., Giampaoli, P., Trystram, G. & Birlouez-Aragon, I. (2008) The fate of furfurals and other volatile markers during the baking process of a model cookie. *Food Chemistry*, 111, 758-763.

Alabed, Y. & Bucala, R. (1995) N-epsilon-carboxymethyllysine formation by direct addition of glyoxal to lysine during the Maillard reaction. *Bioorganic & Medicinal Chemistry Letters*, 5, 2161-2162.

Ames, J.M., Apriyantono, A. & Arnoldi, A. (1993) Low-molecular-weight colored compounds formed in xylose lysine model systems. *Food Chemistry*, 46, 121-127.

Ames, J.M. (1998) Applications of the Maillard reaction in the food industry. *Food Chemistry*, 62, 431-439.

Anet, E.F.L.J. (1964) 3-Deoxyglycosuloses (3-Deoxyglycosones) and the degradation of carbohydrates. *Advances in Carbohydrate Chemistry*, pp. 181-218.

Anklam, E. (1998) A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chemistry*, 63, 549-562.

Arribas-Lorenzo, G. & Morales, F.J. (2010) Estimation of dietary intake of 5-hydroxymethylfurfural and related substances from coffee to Spanish population. *Food and Chemical Toxicology*, 48, 644-649.

Ashoor, S.H. & Zent, J.B. (1987) Maillard Browning of Common Amino Acids and Sugars. *Journal of Food Science*, 49, 1206-1207.

Aurelio, L., Box, J.S., Brownlee, R.T.C., Hughes, A.B. & Sleebs, M.M. (2003) An efficient synthesis of N-methyl amino acids by way of intermediate 5-oxazolidinones. *Journal of Organic Chemistry*, 68, 2652-2667.

Bachmann, S., Meier, M. & Kanzig, A. (1997) 5-Hydroxymethyl-2-furfural (HMF) in Lebensmitteln. *Lebensmittelchemie*, 51, 49-50.

Baek, H.H. & Cadwallader, K.R. (1998) Roasted chicory aroma evaluation by gas chromatography/mass spectrometry/olfactometry. *Journal of Food Science*, 63, 234-237.

Baltes, W. (1985) Application of pyrolytic methods in food chemistry. *Journal of Analytical and Applied Pyrolysis*, 8, 533-545.

Baltes, W. & Knoch, E. (1993) Model reactions on roast aroma formation. XIII. The formation of some uncommon N-heterocyclic compounds and furans after roasting of tryptophan with reducing sugars and sugar degradation products. *Food Chemistry*, 46, 343-349.

Bengmark, S. (2007) Advanced glycation and lipoxidation end products-amplifiers of inflammation: The role of food. *Journal of Parenteral and Enteral Nutrition*, 31, 430-440.

Benzingpurdie, L.M., Ripmeester, J.A. & Ratcliffe, C.I. (1985) Effects of temperature on Maillard reaction-products. *Journal of Agricultural and Food Chemistry*, 33, 31-33.

Biemel, K.M., Buhler, H.P., Reihl, O. & Lederer, M.O. (2001) Identification and quantitative evaluation of the lysine-arginine crosslinks GODIC, MODIC, DODIC, and glucosepan in foods. *Nahrung-Food*, 45, 210-214.

Bieme, K.M., Fried, D. A. & Lederer, M.O. (2002) Identification and quantification of major maillard cross-links in human serum albumin and lens protein: Evidence for glucosepane as the dominant compound. *Journal of Biological Chemistry*, 277, 24907-24915.

Bogen, K.T. (1994) Cancer potencies of heterocyclic amines found in cooked foods. *Food and Chemical Toxicology*, 32, 505-515.

Borrelli, R.C., Mennella, C., Barba, F., Russo, M., Russo, G.L., Krome, K., Erbersdobler, H.F., Faist, V. & Fogliano, V. (2003) Characterization of coloured compounds obtained by enzymatic extraction of bakery products. *Food and Chemical Toxicology*, 41, 1367-1374.

Borrelli, R.C., Visconti, A., Mennella, C., Anese, M. & Fogliano, V. (2002) Chemical characterization and antioxidant properties of coffee melanoidins. *Journal of Agricultural and Food Chemistry*, 50, 6527-6533.

Brands, C.M.J. & Van Boekel, M.A.J.S. (2002) Kinetic modeling of reactions in heated monosaccharide-casein systems. *Journal of Agricultural and Food Chemistry*, 50, 6725-6739.

Buffo, R.A. & Cardelli-Freire, C. (2004) Coffee flavour: An overview. *Flavour and Fragrance Journal*, 19, 99-104.

Bujard, E. & Finot, P.A. (1978) Measure of available and blocked lysin in industrial milks. *Annales de la Nutrition et de l'Alimentation*, 32, 291-305.

Bunn, H.F. & Higgins, P.J. (1981) Reaction of monosaccharides with proteins: Possible evolutionary significance. *Science*, 213, 222-224.

Cabodevila, O., Hill, S., E., Armstrong, H., J., de Sousa, I. & Mitchell, J., R. (2006) Gelation Enhancement of Soy Protein Isolate using the Maillard Reaction and High Temperatures. *Journal of Food Science*, 59, 872-875.

Capuano, E. & Fogliano, V. (2011) Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism, toxicity, occurrence in food and mitigation strategies. *LWT - Food Science and Technology*, 44, 793-810.

Chambel, P., Oliveira, M.B., Andrade, P.B., Fernandes, J.O., Seabra, R.M. & Ferreira, M.A. (1998) Identification of 5,5'-oxy-dimethylene-bis(2-furaldehyde) by thermal decomposition of 5-hydroxymethyl-2-furfuraldehyde. *Food Chemistry*, 63, 473-477.

Chen, J. & Ho, C.T. (1999) Comparison of volatile generation in serine / threonine / glutamine - ribose/glucose/fructose model systems. *Journal of Agricultural and Food Chemistry*, 47, 643-647.

Chen, X.M. & Kitts, D.D. (2011) Antioxidant and anti-inflammatory activities of Maillard reaction products isolated from sugar-amino acid model systems. *Journal of Agricultural and Food Chemistry*, 59, 11294-11303.

Chevalier, F., Chobert, J.M., Popineau, Y., Nicolas, M.G. & Haertle, T. (2001a) Improvement of functional properties of β-lactoglobulin glycated through the Maillard reaction is related to the nature of the sugar. *International Dairy Journal*, 11, 145-152.

Chevalier, F., Chobert, J.M., Molle, D. & Haertle, T. (2001b) Maillard glycation of β-lactoglobulin with several sugars: Comparative study of the properties of the obtained polymers and of the substituted sites. *Lait*, 81, 651-666.

Chu, F.L. & Yaylayan, V.A. (2008) Post-Schiff base chemistry of the Maillard reaction: Mechanism of imine isomerisation. *Annals of the New York Academy of Sciences*, pp. 30-37.

Chu, F.L. & Yaylayan, V.A. (2009a) FTIR monitoring of oxazolidin-5-one formation and decomposition in a glycolaldehyde-phenylalanine model system by isotope labeling techniques. *Carbohydrate Research*, 344, 229-236.

Chu, F.L. & Yaylayan, V.A. (2009b) Isotope labeling studies on the origin of 3,4-hexanedione and 1,2-butanedione in an alanine/glucose model system. *Journal of Agricultural and Food Chemistry*, 57, 9740-9746.

Cotterill, J.V., Chaudhry, M.Q., Matthews, W. & Watkins, R.W. (2008) In silico assessment of toxicity of heat-generated food contaminants. *Food and Chemical Toxicology*, 46, 1905-1918.

Counet, C., Callemien, D., Ouwerx, C. & Collin, S. (2002) Use of gas chromatography-olfactometry to identify key odorant compounds in dark chocolate. Comparison of samples before and after conching. *Journal of Agricultural and Food Chemistry*, 50, 2385-2391.

CRC (1974) Properties of Amino Acids. In Weast, R.C. (ed) *Handbook of Chemistry and Physics*. CRC Press, Cleveland, Ohio, pp. C-741.

Creighton, T.E. (1984) Chemical nature of polypeptides *Proteins: Structures and Molecular Principles*. W. H, Freeman, New York, pp. 10-14.

Crews, C. & Castle, L. (2007) A review of the occurrence, formation and analysis of furan in heat-processed foods. *Trends in Food Science and Technology*, 18, 365-372.

Culver, C., A. & Swaisgood, H., E. (1989) Changes in the digestibility of dried casein and glucose mixtures occurring during storage at different temperatures and water activities. *Journal of Dairy Science*, 72, 2916-2920.

Daglia, M., Racchi, M., Papetti, A., Lanni, C., Govoni, S. & Gazzani, G. (2004) In vitro and ex vivo antihydroxyl radical activity of green and roasted coffee**.** *Journal of Agricultural and Food Chemistry*, 52, 1700-1704.

Davies, R., Hedebrant, U., Athanassiadis, I., Rydberg, P. & Tornqvist, M. (2009) Improved method to measure aldehyde adducts to N-terminal valine in hemoglobin

using 5-hydroxymethylfurfural and 2,5-furandialdehyde as model compounds. *Food and Chemical Toxicology*, 47, 1950-1957.

de Kok P., M.T. & Rosing, E.A.E. (1994) Reactivity of peptides in the Maillard reaction. In Parliment, T., H., Morello, M., J., McGorrin, R.J. (eds) *Thermally Generated Flavors*. American Chemical Society pp. 158-179.

Delgado-Andrade, C., Rufian-Henares, J.A. & Morales, F.J. (2007) Lysine availability is diminished in commercial fibre-enriched breakfast cereals. *Food Chemistry*, 100, 725-731.

Durham, D.G., Hung, C.T. & Taylor, R.B. (1982) Identification of some acids produced during autoclaving of D-glucose solutions using HPLC. *International Journal of Pharmaceutics*, 12, 31-40.

Dybing, E., Farmer, P.B., Andersen, M., Fennell, T.R., Lalljie, S.P.D., Muller, D.J.G., Olin, S., Petersen, B.J., Schlatter, J., Scholz, G., Scimeca, J.A., Slimani, N., Tornqvist, M., Tuijtelaars, S. & Verger, P. (2005) Human exposure and internal dose assessments of acrylamide in food. *Food and Chemical Toxicology*, 43, 365-410.

Erbersdobler, H. & Dummer, H. (1971) Analytical and physiological characterization of amino-acid damage due to heat. 3. Studies on superheated skim milk powder. *Zeitschrift für Tierphysiologie, Tierernährung und Futtermittelkunde* 28, 224-231.

Erbersdobler, H.F. & Hupe, A. (1991) Determination of lysine damage and calculation of lysine bio-availability in several processed foods. *Zeitschrift fur Ernahrungswissenschaft*, 30, 46-49.

Erbersdobler, H.F. & Somoza, V. (2007) Forty years of furosine - Forty years of using Maillard reaction products as indicators of the nutritional quality of foods. *Molecular Nutrition & Food Research*, 51, 423-430.

European Commission (2002) Join research centre: Acrylamide - Summary risk assessment report, United Kingdom.

Fay, L.B. & Brevard, H. (2005) Contribution of mass spectrometry to the study of the Maillard reaction in food. *Mass Spectrometry Reviews*, 24, 487-507.

FDA (2004) Exploratory data on furan in food. Available at http://www.cfsan.fda.gov/wdms/furandat.html.

Feather, M.S. & Nelson, D. (1984) Maillard polymers derived from D-glucose, D-fructose, 5-(hydroxymethyl)-2-furaldehyde, and glycine and methionine. *Journal of Agricultural and Food Chemistry*, 32, 1428-1432.

Feather, M.S. (1989) Sugar-derived deoxy-dicarbonyl intermediates as precursors of food flavors and aromas. In Parliment, T., H., McGorrin, R.J., Ho, C.-T. (eds) *Thermal Generation of Aromas*, Washington, pp. 209-216.

Ferrer, E., Alegria, A., Farre, R., Abellan, P., Romero, F. & Clemente, G. (2003) Evolution of available lysine and furosine contents in milk-based infant formulas throughout the shelf-life storage period. *Journal of the Science of Food and Agriculture*, 83, 465-472.

Ferrer, E., Alegria, A., Farre, R., Abellan, P. & Romero, F. (2005) High-performance liquid chromatographic determination of furfural compounds in infant formulas during full shelf-life. *Food Chemistry*, 89, 639-645.

Finkelstein, E.A., Zhen, C., Nonnemaker, J. & Todd, J.E. (2010) Impact of targeted beverage taxes on higher- and lower-income households. *Archives of Internal Medicine*, 170, 2028-2034.

Freedman, N.D., Park, Y., Abnet, C.C., Hollenbeck, A.R. & Sinha, R. (2012) Association of coffee drinking with total and cause-specific mortality. *New England Journal of Medicine*, 366, 1891-1904.

Friedman, M. (1996) Food browning and its prevention: An overview. *Journal of Agricultural and Food Chemistry*, 44, 631-653.

FSA (2005) Honey Regulations 2003. Food Standards Agency - UK.

Fu, M.X., Requena, J.R., Jenkins, A.J., Lyons, T.J., Baynes, J.W. & Thorpe, S.R. (1996) The advanced glycation end product, N-(epsilon)(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *Journal of Biological Chemistry*, 271, 9982-9986.

Gasser, U. & Grosch, W. (1990) Primary odorants of chicken broth - A comparative study with meat broths from cow and ox. *Zeitschrift fur Lebensmittel-Untersuchung und -Forschung A*, 190, 3-8.

Ge, S.J. & Lee, T.C. (1997) Kinetic significance of the schiff base reversion in the early-stage Maillard reaction of a phenylalanine-glucose aqueous model system**.** *Journal of Agricultural and Food Chemistry*, 45, 1619-1623.

Glatt, H., Schneider, H. & Liu, Y. (2005) V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, 580, 41-52.

Glatt, H. & Sommer, Y. (2006) Health risks of 5-hydroxymethylfurfural (HMF) and related compounds. In Skog, K., Alexander, J. (eds) *Acrylamide and Other*

Hazardous Compounds in Heat-Treated Foods. Woodhead Publishing Limited, Cambridge, pp. 328-357.

Glatt, H. & Sommer, Y. (2007) Health risks of 5-hydroxymethylfurfural (HMF) and related compounds *Acrylamide and other hazardous compounds in heat-treated foods*. Woodhead Publishing in Food Science, Technology and Nutrition, pp. 328-357.

Godfrey, V.B., Chen, L.J., Griffin, R.J., Lebetkin, E.H. & Burka, L.T. (1999) Distribution and metabolism of (5-hydroxymethyl)furfural in male F344 rats and B6C3F1 mice after oral administration. *Journal of Toxicology and Environmental Health - Part A*, 57, 199-210.

Goldmann, T., Davidek, T., Gouezec, E., Blank, I., Bertholet, M.-C. & Stadler, R. (2009) Formation of styrene during the Maillard reaction is negligible. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment*, 26, 583-594.

Gothwal, P., P & Bhavadasan, M., K. (1991) Proteolytic hydrolysis of milk proteins as influenced by browning. *Indian Journal of Dairy Science*, 44, 501-509.

Granvogl, M. & Schieberle, P. (2006) Thermally generated 3-aminopropionamide as a transient intermediate in the formation of acrylamide. *Journal of Agricultural and Food Chemistry*, 54, 5933-5938.

Grosch, W. & Schieberle, P. (1997) Flavor of cereal products - A review. *Cereal Chemistry*, 74, 91-97.

Guan, Y.G., Wu, X.L., Yu, S.J. & Xu, X.B. (2011) Proposed formation mechanism, antioxidant activity and MDA-MB-231 cells survival analysis of two glucose-ammonium sulfite caramel colour melanoidins fractions. *Carbohydrate Polymers*, 86, 948-955.

Halket J.M. & Schulten H.R. (1988) Fast profiling of food by analytical pyrolysis. *Zeitschrift fur lebensmittel-untersuchung und-forschung*, 186, 201-212.

Hansen, L., P. & Millington, R., J. (1979) Blockage of protein enzymatic digestion (carboxypeptidase-B) by heat-induced sugar-lysine ractions. *Journal of Food Science*, 44, 1173 - 1177.

Hardt, R. & Baltes, W. (1989) Analysis of caramel colors - curie-point pyrolysis-high-resolution gas-chromatography mass-spectrometry and simulation of pyrolysis mass-spectrometry *Journal of Analytical and Applied Pyrolysis*, 15, 159-165.

Hartkopf, J. & Erbersdobler, H.F. (1995) Model experiments with sausage meat on the formation of n-epsilon-carboxymethyllysine. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung*, 201, 27-29.

Hayase, F., Takahashi, Y., Tominaga, S., Miura, M., Gomyo, T. & Kato, H. (1999) Identification of blue pigment formed in a D-xylose-glycine reaction system. *Bioscience Biotechnology and Biochemistry*, 63, 1512-1514.

Hayase, F., Sshirahashi, Y., Machida, T., Ito, T., Usui, T. & Watanabe, H. (2010) Chemistry of pigments as intermediate of melanoidins. In Thomas, M., C., Forbes, J. (eds) *Maillard Reaction: Interface Between Aging, Nutrition and Metabolism*. RSC, Cambridge, England.

Hayward, D., L. & J. Angyal, S. (1977) The circular dichroism of reducing sugars. Part I. A symmetry rule for the circular dichroism of reducing sugars, and the proportion of carbonyl forms in aqueous solutions thereof. *Carbohydrate Research*, 53, 13-20.

Henle, T. (2005) Protein-bound advanced glycation endproducts (AGEs) as bioactive amino acid derivatives in foods. *Amino Acids*, 29, 313-322.

Hewedy, M.M., Kiesner, C., Meissner, K., Hartkopf, J. & Erbersdobler, H.F. (1994) Effects of UHT heating of milk in an experimental plant on several indicators of heat-treatment. *Journal of Dairy Research*, 61, 305-309.

Heyns, K., Muller, G. & Paulson, H. (1967) Quantitative Untersuchungen der Reaktion von Hexosen mit Aminosauren. *Justus Liebigs Annalen der Chemie*, 703, 202-214.

Heyns, K. & Roeper, H. (1974) Gas chromatographic trace analysis of volatile nitrosamines in various types of wheat flour after application of different nitrogen fertilisers to the wheat. *IARC (International Agency for Research on Cancer) Scientific Publications*, vol 9, 166-172.

Hidalgo, F.J., Delgado, R.M., Navarro, J.L. & Zamora, R. (2010) Asparagine decarboxylation by lipid oxidation products in model systems. *Journal of Agricultural and Food Chemistry*, 58, 10512-10517.

Hodge, J.E. (1953) Dehydrated foods - chemistry of browning reactions in model systems *Journal of Agricultural and Food Chemistry*, 1, 928-943.

Hoenicke, K., Fritz, H., Gatermann, R. & Weidemann, S. (2004) Analysis of furan in different foodstuffs using gas chromatography mass spectrometry. *Czech Journal of Food Science*, 22, 357-358.

Hofmann, T. (1998) Studies on the relationship between molecular weight and the color potency of fractions obtained by thermal treatment of glucose/amino acid and

glucose/protein solutions by using ultracentrifugation and color dilution techniques. *Journal of Agricultural and Food Chemistry*, 46, 3891-3895.

Hofmann, T. (2001) On the preparation of glucose/glycine standard melanoidins and their seperation by using dialysis, ultrafiltration and gel permeation chromatography. In Ames, J.M. (ed) *Melanoidins in Food and Health*. European Communities, Luxembourg, pp. 11-21.

Hollnagel, A. & Kroh, L.W. (2002) 3-Deoxypentosulose: An a-dicarbonyl compound predominating in nonenzymatic browning of oligosaccharides in aqueous solution. *Journal of Agricultural and Food Chemistry*, 50, 1659-1664.

Hurrell, R.F. & Carpenter, K.J. (1981) The estimation of available lysine foodstuffs after Maillard reactions. *Progress in Food and Nutrition Science*, 5, 159-176.

Husoy, T., Haugen, M., Murkovic, M., Jobstl, D., Stolen, L.H., Bjellaas, T., Ronningborg, C., Glatt, H. & Alexander, J. (2008) Dietary exposure to 5-hydroxymethylfurfural from Norwegian food and correlations with urine metabolites of short-term exposure. *Food and Chemical Toxicology*, 46, 3697-3702.

Huyghues-Despointes, A., Yaylayan, V.A. & Keyhani, A. (1994) Pyrolysis/GC/MS analysis of 1-[(2'-carboxy)pyrrolidinyl]-1-deoxy-D-fructose (proline amadori compound). *Journal of Agricultural and Food Chemistry*, 42, 2519-2524.

Huyghues-Despointes, A. & Yaylayan, V.A. (1996) Retro-aldol and redox reactions of Amadori compounds: Mechanistic studies with variously labeled D-[13C]glucose. *Journal of Agricultural and Food Chemistry*, 44, 672-681.

Hwang, H.I., Hartman, T.G., Rosen, R.T., Lech, J. & Ho, C.T. (1994) Formation of pyrazines from the maillard reaction of glucose and lysine-alpha-amine-n-1. *Journal of Agricultural and Food Chemistry*, 42, 1000-1004.

Hwang, H.I., Hartman, T.G. & Ho, C.T. (1995a) Relative reactivities of amino acids in pyrazine formation. *Journal of Agricultural and Food Chemistry*, 43, 179-184.

Hwang, H.I., Hartman, T.G. & Ho, C.T. (1995b) Relative reactivities of amino-acids in the formation of pyridines, pyrroles, and oxazoles. *Journal of Agricultural and Food Chemistry*, 43, 2917-2921.

IARC (1994) Acrylamide. In IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. International Agency for Research on Cancer, Lyon, France, pp. 389-433.

IARC (1995) International Agency for Research on Cancer. Dry cleaning some chlorinated solvents and other industrial chemicals *Monographs on the evaluation of carcinogenic risks to humans*. IARC, Lyon, pp. 394-407.

Irwin, W.J. (1982) Analytical Pyrolysis. Marcel Dekker, Inc., New York, NY.

Jane, M., J. & Nursten, H. (1983) The Variety of Odors Produced in Maillard Model Systems and How They Are Influenced by Reaction Conditions. In Waller, G., R., Feather, M., S. (eds) *The Maillard Reaction in Foods and Nutrition*. American Chemical Society, pp. 141-158.

Janzowski, C., Glaab, V., Samimi, E., Schlatter, J. & Eisenbrand, G. (2000) 5-Hydroxymethylfurfural: Assessment of mutagenicity, DNA-damaging potential and reactivity towards cellular glutathione. *Food and Chemical Toxicology*, 38, 801-809.

Jenny, G. & Glomb, M.A. (2009) Degradation of glucose: reinvestigation of reactive Î±-dicarbonyl compounds. *Journal of Agricultural and Food Chemistry*, 57, 8591-8597.

Jeuring, H.J. & Kuppers, F.J. (1980) High performance liquid chromatography of furfural and hydroxymethylfurfural in spirits and honey. *Journal of the Association of Official Analytical Chemists*, 63, 1215-1218.

Jing, H. & Kitts, D.D. (2004) Antioxidant activity of sugara-lysine Maillard reaction products in cell free and cell culture systems. *Archives of Biochemistry and Biophysics*, 429, 154-163.

Jones, A.D., Homan, A.C., Favell, D.J. & Hitchcock, C.H.S. (1986) High-performance liquid chromatographic column switching method for the determination of hydroxyproline in meat and meat products. *Journal of Chromatography*, 353, 153-161.

Kerscher, R., Nurnberg, K., Voigt, J., Schieberle, P. & Grosch, W. (2000) Occurrence of 12-methyltridecanal in microorganisms and physiological samples isolated from beef. *Journal of Agricultural and Food Chemistry*, 48, 2387-2390.

Keyhani, A. & Yaylayan, V.A. (1996) Elucidation of the mechanism of pyrazinone formation in glycine model systems using labeled sugars and amino acids. *Journal of Agricultural and Food Chemistry*, 44, 2511-2516.

Kim, H.J. & Richardson, M. (1992) Determination of 5-hydroxymethylfurfural by ion-exclusion chromatography with UV detection. *Journal of Chromatography*, 593, 153-156.

Klopfer, A., Spanneberg, R. & Glomb, M.A. (2011) Formation of arginine modifications in a model system of N-α-Tert-butoxycarbonyl (Boc)-arginine with methylglyoxal. *Journal of Agricultural and Food Chemistry*, 59, 394-401.

Knecht, K.J., Feather, M.S. & Baynes, J.W. (1992) Detection of 3-deoxyfructose and 3-deoxyglucosone in human urine and plasma: Evidence for intermediate stages of the Maillard reaction in vivo. *Archives of Biochemistry and Biophysics*, 294, 130-137.

Koehler, P.E. & Odell, G.V. (1970) Factors affecting the formation of pyrazine compounds in sugar-amine reactions. *Journal of Agricultural and Food Chemistry*, 18, 895-898.

Koschinsky, T., He, C.J., Mitsuhashi, T., Bucala, R., Liu, C., Buenting, C., Heitmann, K. & Vlassara, H. (1997) Orally absorbed reactive glycation products (glycotoxins): An environmental risk factor in diabetic nephropathy. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 6474-6479.

Kroh, L.W. (1994) Caramelisation in food and beverages. *Food Chemistry*, 51, 373-379.

Kuhn, R. & Dansi, A. (1936), Ber 69: 1745.

Kuster, B.F.M. (1990) 5-Hydroxymethylfurfural (HMF). A review focussing on its manufacture. *Starch*, 42, 314-321.

Kwak, E.J. & Lim, S.I. (2004) The effect of sugar, amino acid, metal ion, and NaCl on model Maillard reaction under pH control. *Amino Acids*, 27, 85-90.

Laroque, D., Inisan, C., Berger, C., Vouland, E., Dufosse, L. & Guerard, F. (2008) Kinetic study on the Maillard reaction. Consideration of sugar reactivity. *Food Chemistry*, 111, 1032-1042.

Ledl, F. & Schleicher, E. (1990) New aspects of the maillard reaction in foods and in the human-body. *Angewandte Chemie-International Edition*, 29, 565-594.

Lee, S., H & Nagy, S. (1990) Relative reactivities of sugars in the formation of 5-hydroxymethyl furfural in sugar-catalyst model systems. *Journal of Food Processing and Preservation*, 14, 171-178.

Lee, Y.C., Shlyankevich, M., Jeong, H.K., Douglas, J.S. & Surh, Y.J. (1995) Bioactivation of 5-hydroxymethyl-2-furaldehyde to an electrophilic and mutagenic allylic sulfuric acid ester. *Biochemical and Biophysical Research Communications*, 209, 996-1002.

Lederer, M.O. & Buhler, H.P. (1999) Cross-linking of proteins by Maillard processes - Characterization and detection of a lysine-arginine cross-link derived from D-glucose. *Bioorganic & Medicinal Chemistry*, 7, 1081-1088.

Lederer, M.O. & Klaiber, R.G. (1999) Cross-linking of proteins by Maillard processes: Characterization and detection of lysine-arginine cross-links derived from glyoxal and methylglyoxal. *Bioorganic & Medicinal Chemistry*, 7, 2499-2507.

Limacher, A., Kerler, J., Davidek, T., Schmalzried, F. & Blank, I. (2008) Formation of furan and methylfuran by Maillard-type reactions in model systems and food. *Journal of Agricultural and Food Chemistry*, 56, 3639-3647.

Lindenmeier, M., Faist, V. & Hofmann, T. (2002) Structural and functional characterization of pronyl-lysine, a novel protein modification in bread crust melanoidins showing in vitro antioxidative and phase I/II enzyme modulating activity. *Journal of Agricultural and Food Chemistry*, 50, 6997-7006.

Locas, C.P. & Yaylayan, V.A. (2008) Further insight into thermally and pH-induced generation of acrylamide from glucose/asparagine model systems. *Journal of Agricultural and Food Chemistry*, 56, 6069-6074.

Maillard, L.C. (1912) Action des acides aminés sur les sucres: formation des mélanoïdines par voie méthodique. *Compte rendu de l'Académie des Sciences*, 154, 66-68.

Marko, D., Kemeny, M., Bernady, E., Habermeyer, M., Weyand, U., Meiers, S., Frank, O. & Hofmann, T. (2002) Studies on the inhibition of tumor cell growth and microtubule assembly by 3-hydroxy-4-[(E)-(2-furyl)methylidene]methyl-3-cyclopentene-1,2-dione, an intensively coloured Maillard reaction product. *Food and Chemical Toxicology*, 40, 9-18.

Marko, D., Habermeyer, M., Kemeny, M., Weyand, U., Niederberger, E., Frank, O. & Hofmann, T. (2003) Maillard reaction products modulating the growth of human tumor cells in vitro. *Chemical Research in Toxicology*, 16, 48-55.

Martins, S.I.F.S., Jongen, W.M.F. & Van Boekel, M.A.J.S. (2000) A review of Maillard reaction in food and implications to kinetic modelling. *Trends in Food Science and Technology*, 11, 364-373.

Mauron, J. (1981) The Maillard reaction in food - a critical-review from the nutritional standpoint. *Progress in Food and Nutrition Science*, 5, 5-35.

Meynier, A. & Mottram, D.S. (1995) The effect of pH on the formation of volatile compounds in meat-related model systems. *Food Chemistry*, 52, 361-366.

Miller, R., Olsson, K. & Pernemalm, P.A. (1984) Formation of aromatic-compounds from carbohydrates .IX. reaction of d-glucose and l-lysine in slightly acidic, aqueous-solution. *Acta Chemica Scandinavica Series B-Organic Chemistry and Biochemistry*, 38, 689-694.

Mills, F.D., Baker, B.G. & Hodge, J.E. (1969) Amadori compounds as nonvolatile flavor precursors in processed foods. *Journal of Agricultural and Food Chemistry*, 17, 723-727.

Monien, B.H., Frank, H., Seidel, A. & Glatt, H. (2009) Conversion of the common food constituent 5-hydroxymethylfurfural into a mutagenic and carcinogenic sulfuric acid ester in the mouse in vivo. *Chemical Research in Toxicology*, 22, 1123-1128.

Morales, F.J., Romero, C. & Jimenez-Perez, S. (1997) Chromatographic determination of bound hydroxymethylfurfural as an index of milk protein glycosylation. *Journal of Agricultural and Food Chemistry*, 45, 1570-1573.

Mosciano, G. (1996) Organoleptic characteristics of flavor materials. *Perfumer and Flavorist*, 21, 47.

Mottram, D.S. (1998) Flavour formation in meat and meat products: A review. *Food Chemistry*, 62, 415-424.

Mulders, E.J. (1973) Volatile components from the non-enzymic browning reaction of the cysteine/cystine-ribose system. *Zeitschrift fur Lebensmittel-Untersuchung und – Forschung A*, 152, 193-201.

Murata, M., Totsuka, H. & Ono, H. (2007) Browning of furfural and amino acids, and a novel yellow compound, furpipate, formed from lysine and furfural. *Bioscience Biotechnology and Biochemistry*, 71, 1717-1723.

Murkovic, M. & Pichler, N. (2006) Analysis of 5-hydroxymethylfurfual in coffee, dried fruits and urine. *Molecular Nutrition and Food Research*, 50, 842-846.

Nagao, M., Takahashi, Y., Yamanaka, H. & Sugimura, T. (1979) Mutagens in Coffee and Tea. *Mutation Research*, 68, 101-106.

Nakamura, S., Kato, A. & Kobayashi, K. (1992) Enhanced antioxidative effect of ovalbumin due to covalent binding of polysaccharides. *Journal of Agricultural and Food Chemistry*, 40, 2033-2037.

Nakamura, S., Kobayashi, K. & Kato, A. (1994) Role of positive charge of lysozyme in the excellent emulsifying properties of Maillard-type lysozyme-polysaccharide conjugate. *Journal of Agricultural and Food Chemistry*, 42, 2688-2691.

Negrean, M., Stirban, A., Horstmann, T., Hohls, B., et al (2005) Dietary advanced glycation end products (AGEs) impair acute endothelium-dependent vasodilation in patients with type 2 diabetes mellitus (T2DM). *Diabetes*, 54, A178.

Nielsen, H.K., De Weck, D. & Finot, P.A. (1985) Stability of tryptophan during food processing and storage. 1. Comparative losses of tryptophan, lysine and methionine in different model systems. *British Journal of Nutrition*, 53, 281-292.

Nikolov, P.Y. & Yaylayan, V.A. (2010a) Chemical activation of piperidine by formaldehyde and formation of lysine-specific Maillard reaction products. *Food Chemistry*, 123, 684-690.

Nikolov, P.Y. & Yaylayan, V.A. (2010b) Formation of pent-4-en-1-amine, the counterpart of acrylamide from lysine and its conversion into piperidine in lysine/glucose reaction mixtures. *Journal of Agricultural and Food Chemistry*, 58, 4456-4462.

Nikolov, P.Y. & Yaylayan, V.A. (2011a) Reversible and covalent binding of 5-(hydroxymethyl)-2-furaldehyde (HMF) with lysine and selected amino acids. *Journal of Agricultural and Food Chemistry*, 59, 6099 - 6107.

Nikolov, P.Y. & Yaylayan, V.A. (2011b) Thermal decomposition of 5-(hydroxymethyl)-2-furaldehyde (HMF) and its further transformations in the presence of glycine. *Journal of Agricultural and Food Chemistry*, 59, 10104-10113.

NTP (1993) Toxicology and carcinogenesis studies of furan (CAS No.110-00-9) in F344/N rats and B6C3Fl mice (gavage studies). NTP Technical Report No. 402. In U.S. Department of Health and Human Services, P.H.S., National Institutes of Health, Research Triangle Park, NC. (ed).

Nursten, H. (2005) *The Maillard Reaction: Chemistry, Biochemistry and Implications*. The Royal Society of Chemistry, pp 5-26, Cambridge.

Omura, H., Jahan, N., Shinohara, K. & Murakami, Y. (1983) Formation of mutagens by the Maillard reaction. In Waller, G.R., Feather, M.S. (eds) *The Maillard Reaction in Foods and Nutrition*. American Chemical Society Washington, DC, pp. 537-563.

Paine III, J.B., Pithawalla, Y.B. & Naworal, J.D. (2008) Carbohydrate pyrolysis mechanisms from isotopic labeling. Part 2. The pyrolysis of d-glucose: General disconnective analysis and the formation of C1 and C2 carbonyl compounds by electrocyclic fragmentation mechanisms. *Journal of Analytical and Applied Pyrolysis*, 82, 10-41.

Parliment, T.H. (1989) Thermal Generation of Aromas. In Parliment, T., McGorrn, R., Ho, C.-T. (eds) *Thermal Generation of Aromas*. ACS Symposium Series 409, Washington, D.C., pp. 2-11.

Perez-Locas, C. & Yaylayan, V.A. (2008) Isotope labeling studies on the formation of 5-(hydroxymethyl)-2-furaldehyde (HMF) from sucrose by pyrolysis-GC/MS. *Journal of Agricultural and Food Chemistry*, 56, 6717-6723.

Pilkova, L., Pokorny, J. & Davidek, J. (1990) Browning reactions of Heyns rearrangement products. *Die Nahrung-Food*, 34.

Popoff, T. & Theander, O. (1976) Formation of aromatic compounds from carbohydrates. Part III. Reaction of D -glucose and D -fructose in slightly acidic aqueous solution. Acta Chem. Scand. 30 *(50),* 397 – 442.

Porretta, S. (1991) Nonenzymatic browning of tomato products. *Food Chemistry*, 40, 323-335.

Pripi-Nicolau, L., De Revel, G., Bertrand, A. & Maujean, A. (2000) Formation of flavor components by the reaction of amino acid and carbonyl compounds in mild conditions. *Journal of Agricultural and Food Chemistry*, 48, 3761-3766.

Rada-Mendoza, M., Olano, A. & Villamiel, M. (2002) Determination of hydroxymethylfurfural in commercial jams and in fruit-based infant foods. *Food Chemistry*, 79, 513-516.

Raghavan SK, Ho, C.T. & H, D. (1986) Identification of soy protein in meat by pyrolysis high-resolution gas-chromatography. *Journal of Chromatography* 351, 195-202

Rahbar, S., Blumenfe.O & Ranney, H.M. (1969) Studies of an unusual hemoglobin in patients with diabetes mellitus. *Biochemical and Biophysical Research Communications*, 36, 838-843.

Ramirez-Jimenez, A., Guerra-Hernandez, E. & Garcia-Villanova, B. (2000) Browning indicators in bread. *Journal of Agricultural and Food Chemistry*, 48, 4176-4181.

Ramirez-Jimenez, A., Garcia-Villanova, B. & Guerra-Hernandez, E. (2004) Effect of storage conditions and inclusion of milk on available lysine in infant cereals. *Food Chemistry*, 85, 239-244.

Reihl, O., Rothenbacher, T.M., Lederer, M.O. & Schwack, W. (2004) Carbohydrate carbonyl mobility - The key process in the formation of a-dicarbonyl intermediates. *Carbohydrate Research*, 339, 1609-1618.

Resmimi, P., Pelegrino, L. (1994) Occurance of protein-bound lysylpyrrolaldehyde in dried pasta. *Cereal Chemistry*, 71, 254-262.

Rizzi, G.P. (1974) Formation of N-alkyl-2-acylpyrroles and aliphatic aldimines in model nonenzymic browning reactions. *Journal of Agricultural and Food Chemistry*, 22, 279-282.

Rizzi, G.P. (1997) Chemical structure of colored Maillard reaction products. *Food Reviews International*, 13, 1-28.

Rosatella, A.A., Simeonov, S.P., Frade, R.F.M. & Afonso, C.A.M. (2011) 5-Hydroxymethylfurfural (HMF) as a building block platform: Biological properties, synthesis and synthetic applications. *Green Chemistry*, 13, 754-793.

Rufian-Henares, J.A., Guerra-Hernandez, E. & Garcia-Villanova, B. (2004) Pyrraline content in enteral formula processing and storage and model systems. *European Food Research and Technology*, 219, 42-47.

Sakamoto, J., Takenaka, M., Ono, H. & Murata, M. (2009) Novel yellow compounds, dilysyldipyrrolones a and b, formed from xylose and lysine by the maillard reaction. *Bioscience, Biotechnology and Biochemistry*, 73, 2065-2069.

Sanders, E.B., Goldsmith, A.I. & Seeman, J.I. (2003) A model that distinguishes the pyrolysis of D-glucose, D-fructose, and sucrose from that of cellulose. Application to the understanding of cigarette smoke formation. *Journal of Analytical and Applied Pyrolysis*, 66, 29-50.

Sandu, O., Song, K.Y., Cai, W.J., Zheng, F., Uribarri, J. & Vlassara, H. (2005) Insulin resistance and type 2 diabetes in high-fat-fed mice are linked to high glycotoxin intake. *Diabetes*, 54, 2314-2319.

Sanz, M.L., Del Castillo, M.D., Corzo, N. & Olano, A. (2003) 2-furoylmethyl amino acids and hydroxymethylfurfural as indicators of honey quality. *Journal of Agricultural and Food Chemistry*, 51, 4278-4283.

Schieberle, P. (2005) The carbon module labeling (CAMOLA) technique: A useful tool for identifying transient intermediates in the formation of Maillard-type target molecules *Annals of the New York Academy of Sciences*, pp. 236-248.

Schieberle, P. & Hofmann, T. (1998) Characterization of key odorants in dry-heated cysteine—carbohydrate mixtures: comparison with aqueous reaction systems. *Flavour Analysis*, 705, 320-330.

Schlummer, B. & Hartwig, J.F. (2002) Bronsted acid-catalyzed intramolecular hydroamination of protected alkenylamines. Synthesis of pyrrolidines and piperidines. *Organic Letters*, 4, 1471-1474.

Schmidtglenewinkel, T., Nomura, Y. & Giacobini, E. (1977) Conversion of lysine into piperidine, cadaverine, and pipecolic acid in brain and other organs of mouse. *Neurochemical Research*, 2, 619-637.

Schoental, R., Hard, G.C. & Gibbard, S. (1971) Histopathology of renal lipomatous tumors in rats treated with the "natural" products, pyrrolizidine alkaloids and, -unsaturated aldehydes. *Journal of the National Cancer Institute*, 47, 1037-1044.

Sebekova, K., Hofmann, T., Boor, P., Sebekova Jr, K., Ulicna, O., Erbersdobler, H.F., Baynes, J.W., Thorpe, S.R., Heidland, A. & Somoza, V. (2005) Renal effects of oral Maillard reaction product load in the form of bread crusts in healthy and subtotally nephrectomized rats. *Annals of the New York Academy of Sciences*, pp. 482-491.

Sebekova, K. & Somoza, V. (2007) Dietary advanced glycation endproducts (AGEs) and their health effects - PRO. *Molecular Nutrition & Food Research*, 51, 1079-1084.

Seiquer, I., Diaz-Alguacil, J., Delgado-Andrade, C., Lopez-Frias, M., Hoyos, A.M., Galdo, G. & Navarro, M.P. (2006) Diets rich in Maillard reaction products affect protein digestibility in adolescent males aged 11-14 y. *American Journal of Clinical Nutrition*, 83, 1082-1088.

Shen, G. & Hoseney, R.C. (1995) Comparisons of aroma extracts of heat-treated cereals. *LWT - Food Science and Technology*, 28, 208-212.

Shibamoto, T. & Bernhard, R.A. (1977) Investigation of pyrazine formation pathways in sugar-ammonia model systems. *Journal of Agricultural and Food Chemistry*, 25, 609-614.

Shibamoto, T., Akiyama, T., Sakaguchi, M., Enomoto, Y. & Masuda, H. (1979) A study of pyrazine formation. *Journal of Agricultural and Food Chemistry*, 27, 1027-1031.

Shinohara, K., Jahan, N. & Tanaka, M. (1983) Formation of mutagens by amino-carbonyl reactions. *Mutation Research*, 122, 279-286.

Shipp, A., Lawrence, G., Gentry, R., McDonald, T., Bartow, H., Bounds, J., Macdonald, N., Clewell, H., Allen, B. & Van Landingham, C. (2006) Acrylamide: Review of toxicity data and dose-response analyses for cancer and noncancer effects. *Critical Reviews in Toxicology*, 36, 481-608.

Shizuuchi, S. & Hayase, F. (2003) Antioxidative activity of the blue pigment formed in a D-xylose-glycine reaction system. *Bioscience, Biotechnology and Biochemistry*, 67, 54-59.

Somoza, V., Lindenmeier, M., Wenzel, E., Frank, O., Erbersdobler, H.F. & Hofmann, T. (2003) Activity-Guided Identification of a Chemopreventive Compound in Coffee Beverage Using in Vitro and in Vivo Techniques. *Journal of Agricultural and Food Chemistry*, 51, 6861-6869.

Somoza, V. (2005) Five years of research on health risks and benefits of Maillard reaction products: An update. *Molecular Nutrition and Food Research*, 49, 663-672.

Southgate, D.A., Paul, A.A., Dean, A.C. & Christie, A.A. (1978) Free sugars in foods. *Journal of Human Nutrition*, 32, 335-347.

Stadler, R.H., Verzegnassi, L., Varga, N., Grigorov, M., Studer, A., Riediker, S. & Schilter, B. (2003) Formation of vinylogous compounds in model Maillard reaction systems. *Chemical Research in Toxicology*, 16, 1242-1250.

Stadler, R.H., Robert, F., Riediker, S., Varga, N., Davidek, T., Devaud, S., Goldmann, T., Hau, J. & Blank, I. (2004) In-depth mechanistic study on the formation of acrylamide and other vinylogous compounds by the Maillard reaction. *Journal of Agricultural and Food Chemistry*, 52, 5550-5558.

Stavric, B. (1994) Biological significance of trace levels of mutagenic heterocyclic aromatic-amines in human diet - a critical-review. *Food and Chemical Toxicology*, 32, 977-994.

Stoll, M., Winter, M., Gautschi, F., Flament, I. & Willhalm, B. (1967) Research on the aromas. On the aroma of coffee. 1. *Recherches sur les aromes. Sur l'arome de cafe*, 50, 628-694.

Surh, Y.J. & Tannenbaum, S.R. (1994) Activation of the maillard reaction product 5-(hydroxymethyl)furfural to strong mutagens via allylic sulfonation and chlorination. *Chemical Research in Toxicology*, 7, 313-318.

Svensson, K., Abramsson, L., Becker, W., Glynn, A., Hellenas, K.E., Lind, Y. & Rosen, J. (2003) Dietary intake of acrylamide in Sweden. *Food and Chemical Toxicology*, 41, 1581-1586.

Tehrani, K.A., Kersiene, M., Adams, A., Venskutonis, R. & De Kimpe, N. (2002) Thermal degradation studies of glucose/glycine melanoidins. *Journal of Agricultural and Food Chemistry*, 50, 4062-4068.

Thorpe, S.R. & Baynes, J.W. (1996) Role of the maillard reaction in diabetes mellitus and diseases of aging. *Drugs and Aging*, 9, 69-77.

Totsuka, H., Tokuzen, K., Hiroshi, O.N.O. & Murata, M. (2009) A novel yellow compound and furpipate derivatives formed from furfural or 5-hydroxymethylfurfural in the presence of lysine. *Food Science and Technology Research*, 15, 45-50.

Tressl, R., Gruenewald, K.G., Kersten, E. & Rewicki, D. (1986) Formation of pyrroles and tetrahydroindolizin-6-ones as hydroxyproline-specific Maillard products from erythrose and arabinose. *Journal of Agricultural and Food Chemistry*, 34, 347-350.

Tressl, R., Helak, B., Kersten, E. & Rewicki, D. (1993a) Formation of proline- and hydroxyproline-specific maillard products from [1-13C]glucose. *Journal of Agricultural and Food Chemistry*, 41, 547-553.

Tressl, R., Kersten, E. & Rewicki, D. (1993b) Formation of 4-aminobutyric acid specific maillard products from [1-13C]-D-glucose, [1-13C]-D-arabinose, and [1-13C]-D-fructose. *Journal of Agricultural and Food Chemistry*, 41, 2278-2285.

Tressl, R., Nittka, C., Kersten, E. & Rewicki, D. (1995) Formation of isoleucine-specific Maillard products from [1-13C]-D-glucose and [1-13C]-D-fructose. *Journal of Agricultural and Food Chemistry*, 43, 1163-1169.

Tressl, R., Wondrak, G.T., Kruger, R.P. & Rewicki, D. (1998a) New melanoidin-like Maillard polymers from 2-deoxypentoses. *Journal of Agricultural and Food Chemistry*, 46, 104-110.

Tressl, R., Wondrak, G.T., Garbe, L.A., Kruger, R.P. & Rewicki, D. (1998b) Pentoses and hexoses as sources of new melanoidin-like Maillard polymers. *Journal of Agricultural and Food Chemistry*, 46, 1765-1776.

Tu, D., Xue, S., Meng, C., Espinosa-Mansilla, A., De La Pena, A.M. & Lopez, F.S. (1992) Simultaneous determination of 2-furfuraldehyde and 5-(hydroxymethyl)-2-furfuraldehyde by derivative spectrophotometry. *Journal of Agricultural and Food Chemistry*, 40, 1022-1025.

Ulbricht, R.J., Northup, S.J. & Thomas, J.A. (1984) A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions. *Fundamental and Applied Toxicology*, 4, 843-853.

Ulrich, P. & Cerami, A. (2001) Protein glycation, diabetes, and aging. *Recent Progress in Hormone Research*, 56, 1-21.

Uribarri, J., Cai, W.J., Sandu, O., Peppa, M., Goldberg, T. & Vlassara, H. (Year) Diet-derived advanced glycation end products are major contributors to the body's AGE pool and induce inflammation in healthy subjects. In: Baynes, J.W., Monnier, V.M., Ames, J.M., Thorpe, S.R. (eds). City. p. 461-466.

Usui, T., Ohguchi, M., Watanabe, H. & Hayase, F. (2008) The formation of argpyrimidine in glyceraldehyde-related glycation. *Bioscience, Biotechnology and Biochemistry*, 72, 568-571.

Van Boekel, M., A., J., S. & Rehman, Z. (1987) Determination of hydroxymethylfurfural in heated milk by high performance liquid chromatography. *Netherlands Milk and Dairy Journal*, 41, 297-306.

Van Boekel, M.A.J.S. (2006) Formation of flavour compounds in the Maillard reaction. *Biotechnology Advances*, 24, 230-233.

Van Lancker, F., Adams, A.N. & De Kimpe, N. (2010) Formation of pyrazines in maillard model systems of lysine-containing dipeptides. *Journal of Agricultural and Food Chemistry*, 58, 2470-2478.

Van Lancker, F., Adams, A. & De Kimpe, N. (2011) Chemical modifications of peptides and their impact on food properties. *Chemical Reviews*, 111, 7876-7903.

Van Lancker, F., Adams, A. & De Kimpe, N. (2012) Impact of the N-terminal amino acid on the formation of pyrazines from peptides in maillard model systems. *Journal of Agricultural and Food Chemistry*, 60, 4697-4708.

Vinale, F., Monti, S.M., Panunzi, B. & Fogliano, V. (1999) Convenient synthesis of lactuloselysine and its use for LC-MS analysis in milk-like model systems. *Journal of Agricultural and Food Chemistry*, 47, 4700-4706.

Vlassara, H., Cai, W.J., Goodman, S., Pyzik, R., Yong, A., Chen, X., Zhu, L., Neade, T., Beeri, M., Silverman, J.M., Ferrucci, L., Tansman, L., Striker, G.E. & Uribarri, J. (2009) Protection against Loss of Innate Defenses in Adulthood by Low Advanced Glycation End Products (AGE) Intake: Role of the Antinflammatory AGE Receptor-1. *Journal of Clinical Endocrinology & Metabolism*, 94, 4483-4491.

Vlassara, H. & Striker, G.E. (2011) AGE restriction in diabetes mellitus: A paradigm shift. *Nature Reviews Endocrinology*, 7, 526-539.

Walradt, J.P. (1971) Volatile components of roasted peanuts. *Journal of Agricultural and Food Chemistry*, 19, 972-979.

Ward, M.H., Sinha, R., Heineman, E.F., Rothman, N., Markin, R., Weisenburger, D.D., Correa, P. & Zahm, S.H. (1997) Risk of adenocarcinoma of the stomach and esophagus with meat cooking method and doneness preference. *International Journal of Cancer*, 71, 14-19.

Warthesen, J.J., Scanlan, R.A., Bills, D.D. & Libbey, L.M. (1975) Formation of heterocyclic N-nitrosamines from the reaction of nitrite selected primary diamines and amino acids. *Journal of Agricultural and Food Chemistry*, 23, 898-902.

Weenen, H. & Apeldoorn, W. (1996) Carbohydrate cleavage in the Maillard reaction. In A.J., T., D.S., M. (eds) *Flavour Science: Recent Developments*. The Royal Society of Chemistry, Cambridge, pp. 211-216.

Wellsknecht, K.J., Zyzak, D.V., Litchfield, J.E., Thorpe, S.R. & Baynes, J.W. (1995) Mechanism of autoxidative glycosylation - identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry*, 34, 3702-3709.

White, J.W. (1979) Spectrophotometric method for hydroxymethylfurfural in honey. *Journal of the Association of Official Analytical Chemists*, 62, 509-514.

WHO (2005) Summary report of the sixty-fourth meeting of the Joint FAO/WHO expert committee on food additive (JECFA). Rome, Italy: The ILSI Press International Life Sciences Institute, Wa, Washington, DC, pp. 1-47.

WHO (2008) Summary report of the sixty-fourth meeting of the Joint FAO/WHO expert committee on food additive (JECFA). Rome, Italy: The ILSI Press International Life Sciences Institute, Washington, DC, pp. 1-47.

Wondrak, G.T., Tressl, R. & Rewicki, D. (1997) Maillard reaction of free and nucleic acid-bound 2-deoxy-D-ribose and D-ribose with omega-amino acids. *Journal of Agricultural and Food Chemistry*, 45, 321-327.

Wnorowski, A. & Yaylayan, V.A. (2000) Influence of pyrolytic and aqueous-phase reactions on the mechanism of formation of Maillard products. *Journal of Agricultural and Food Chemistry,* 48, 3549-3554.

Wrodnigg, T., M. & Eder, B. (2001) The Amadori and Heyns Rearrangements: Landmarks in the History of Carbohydrate Chemistry or Unrecognized Synthetic Opportunities? *Topics in Current Chemistry*, 215, 115-152.

Yannai, S. (2004) 1-(2-furanylmethyl)-1H-pyrrole. In Yannai, S. (ed) *Dictionary of food compounds; additives, flavors, and ingredients*. Chapman and Hall, pp. 508.

Yaylayan, V. & Sporns, P. (1989) Intramolecular cyclizations of lysine amadori rearrangement products. *Food Chemistry,* 33, 81-91.

Yaylayan, V. & Forage, N.G. (1991) Determination of the kinetics and mechanism of decomposition of tryptophan amadori rearrangement product by RP-HPLC analysis. *Journal of Agricultural and Food Chemistry,* 39, 364-369.

Yaylayan, V.A. & Huyghues-Despointes, A. (1994) Chemistry of Amadori rearrangement products: analysis, synthesis, kinetics, reactions, and spectroscopic properties. *Critical reviews in food science and nutrition,* 34, 321-369.

Yaylayan, V.A. & Ismail, A.A. (1995) Investigation of the enolization and carbonyl group migration in reducing sugars by FTIR spectroscopy. *Carbohydrate Research,* 276, 253-265.

Yaylayan, V.A. & Keyhani, A. (1999) Origin of 2,3-pentanedione and 2,3-butanedione in D-glucose/L-alanine Maillard model systems. *Journal of Agricultural and Food Chemistry*, 47, 3280-3284.

Yaylayan, V.A., Wnorowski, A. & Locas, C.P. (2003) Why asparagine needs carbohydrates to generate acrylamide. *Journal of Agricultural and Food Chemistry,* 51, 1753-1757.

Yaylayan, V. & Wnorowski, A. (2002) The role of b-hydroxyamino acids in the Maillard reaction - transamination route to Amadori products. In Horiuchi, S., Taniguchi, N., Hayase, F., Kurata, T., Osawa, T. (eds) *Maillard reaction in food chemistry and*

medical sciences: Update for the postgenomic era. International Congress Series 1245. Elsevier Science, Amsterdam, The Netherlands, pp. 195-200.

Yaylayan, V.A. & Locas, C.P. (2007) Vinylogous Amadori rearrangement: Implications in food and biological systems. *Molecular Nutrition & Food Research*, 51, 437-444.

Yaylayan, V.A. (2009) Acrylamide formation and its impact on the mechanism of the early Maillard reaction. *Journal of Food and Nutrition Research*, 48, 1-7.

Zamora, R., Delgado, R.M. & Hidalgo, F.J. (2010) Model reactions of acrylamide with selected amino compounds. *Journal of Agricultural and Food Chemistry*, 58, 1708-1713.

Zhang, X.M., Chan, C.C., Stamp, D., Minkin, S., Archer, M.C. & Bruce, W.R. (1993) Initiation and promotion of colonic aberrant crypt foci in rats by 5-hydroxymethyl-2-furaldehyde in thermolyzed sucrose. *Carcinogenesis*, 14, 773-775.