Mechanism of Formation of Thermally Generated Potential Toxicants in Food Related Model Systems

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ABSTRACT

The detailed mechanism of formation of selected Maillard-induced toxicants generated in model systems containing ^{13}C- and ^{15}N-labeled precursors, were investigated using isotope labeling and Py-GC/MS based techniques. Investigation of different sources of acrylamide (AA) formation in model systems have indicated the presence of two pathways of acrylamide generation; the main pathway specifically involves asparagine to directly produce acrylamide after a sugar-assisted decarboxylation step that passes through a 5-oxazolidinone intermediate and the second, non-specific pathway involves the initial formation of acrylic acid from different sources and its subsequent interaction with ammonia and/or amines to produce acrylamide or its N-alkylated derivatives. Furthermore, to identify the relative importance of AA precursors, the decarboxylated Amadori product (AP ARP) and the corresponding Schiff base were synthesized and their relative abilities to generate AA under dry and wet heating conditions were studied. Under both conditions, the Schiff base had the highest intrinsic ability to be converted into AA. To gain further insight into the decarboxylation step, the amino acid/sugar reactions were also analyzed by FTIR to monitor the formation of the 5-oxazolidinone intermediate known to exhibit a peak in the range of 1770-1810 cm^{-1}. Spectroscopic studies clearly indicated the formation of an intense peak in the indicated range. Similar to acrylamide, mechanism of furan formation was also studied using ^{13}C-labeled sugars and amino acids. These studies have indicated that furan can be formed through aldol condensation of acetaldehyde and glycolaldehyde and these precursors can be formed from certain amino acids, monosaccharides and ascorbic acid. Moreover, using specifically ^{13}C-labeled sucrose at C-1 of the fructose moiety, hydroxymethylfurfural (HMF) formation was studied at different temperatures. Under dry pyrolytic conditions and at temperatures above 250^{o}C, 90% of the HMF originated from fructose moiety and only 10% originated from glucose. Alternatively, when sucrose was refluxed in acidic methanol at 65^{o}C, 100% of HMF was generated from the glucose moiety. Based on the data generated, a mechanism of HMF formation from sucrose was proposed. According to this proposal sucrose degrades into glucose and a very reactive fructofuranosyl cation. In dry systems this cation can be effectively converted directly into HMF.

RÉSUMÉ

Le mécanisme détaillé de formation de certaines composantes toxiques générées par des précurseurs contenant des isotopes ^{13}C et ^{15}N, fut étudié en utilisant des techniques basées sur les principes d'incorporation d'isotopes lourds avec la pyrolyse couplée à la CG/SM (Py-CG/SM). L'investigation de différentes sources d'acrylamide a indiqué la présence de deux routes de formation; la route principale implique spécifiquement l'asparagine qui produit l'acrylamide directement suite à une décarboxylation par l'entremise des sucres passant par un intermédiaire 5-oxazolidinone, tandis qu'une seconde route non-spécifique implique une formation initiale d'acide acrylique de différentes sources suivie d'une interaction avec une molécule d'ammoniac et/ou d'amines afin de produire l'acrylamide ou ses dérivés. De plus, afin d'identifier l'importance relative des précurseurs de l'acrylamide, le produit Amadori décarboxylé (AP ARP) ainsi que la base Schiff correspondante furent synthétisés et leur habilité relative à générer l'acrylamide après un traitement thermique en milieu sec et aqueux fut étudié. Dans les deux cas, la base Schiff a démontré une habilité de conversion en acrylamide plus importante. Afin d'obtenir un aperçu approfondi de l'étape de décarboxylation, les réactions entre les sucres et les acides aminés furent aussi analysées par l'IR-TF surveillant ainsi la formation de l'oxazolidinone, une composante intermédiaire reconnue par la présence d'un pic entre 1770-1810 cm^{-1}. Les études spectroscopiques démontrent clairement la formation d'un pic intense dans cet écart. Similaire à l'acrylamide, les mécanismes de formation du furane furent aussi étudiés par l'entremise des sucres et acides aminés contenant des isotopes lourds. Ces études ont indiqué que le furane peut être formé par une condensation aldol de l'acétaldéhyde et le glycoaldéhyde et ses précurseurs, par certains acides aminés, des monosaccharides et l'acide ascorbique. De plus, la formation de l'hydroxymethyl furfural (HMF) fut étudiée à plusieurs températures à l'aide du sucrose-^{13}C isotopiquement marqué au C-1 de la molécule de fructose. Lors de conditions pyrolytiques excédant 250^{o}C, 90% du HMF provenait de la fraction fructose et seulement 10% de la fraction glucose. Par contre, lorsque le sucrose fut bouilli au reflux dans le méthanol acide à 65^{o}C, 100% de l'HMF fut généré par la fraction du glucose. Selon les résultats obtenus, un mécanisme décrivant la formation de l'HMF fut proposé. Selon cette proposition, le sucrose se dégraderait de façon à libérer une molécule de glucose et un cation fructofuranosyl très réactif. En milieu sec, ce cation peut être effectivement et directement converti en HMF.

STATEMENT FROM THE THESIS OFFICE

In accordance with the regulations of the Faculty of Graduate and Postdoctoral Studies 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 preceeding and following each manuscript are mandatory.

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When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the publishers and submit these to the Graduate and Postdoctoral Studies Office with the final deposition.

CONTRIBUTION OF AUTHORS

This thesis is presented in manuscript format, and consists of nine chapters. A general opening in Chapter 1 introduces the concept of thermally generated food toxicants and the importance of their study. It also acts as a basic framework to the proposed research, clearly displaying its rationale, objectives and significance. Chapter 2 provides a general background on the thermally generated toxicants studied and the concepts behind the analytical methods utilized in providing mechanistic evidence for their formation. Parts of sections 2.2 to 2.6 have been published as part of a book chapter. Chapters 3, 4, 5 and 6, 7 and 8 constitute the main body of the thesis. Chapters 3 to 6 are drawn based on published manuscripts. Chapters 7 and 8 have been accepted for publication. Connecting paragraphs provide logical bridges between the different manuscripts and chapters. Chapter IX establishes a closing of the topic leaving an opening for futur research. This dissertation is in accordance with the 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. Dr. Varoujan A. Yaylayan, 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. The manuscripts titled “Origin and mechanistic pathways of formation of the parent furan: a food toxicant”, “Further insight into thermal and pH induced generation of acrylamide from glucose/asparagine model systems”, “Isotope Labeling Studies on the Formation of 5-(Hydroxymethyl)-2-furaldehyde (HMF) from Sucrose by Pyrolysis-GC/MS” were co-authored with Dr. Yaylayan, and the present author was responsible for experimental work, discussion of experimental concepts and interpretation of results. In “Mechanistic Pathways of Formation of Acrylamide from Different Amino Acid” and “The Role of Creatine in the Generation of N-Methylacrylamide – A New Toxicant in Cooked Meat”, the contribution of Dr. O’Brien and Dr. Wnorowski as co-authors was in providing advisory input and participation in scientific discussion pertaining to the manuscript. Dr. Yaylayan was the first author of the following manuscripts : “The Role of Creatine in the Generation of N-Methylacrylamide – A New Toxicant in Cooked Meat”, “Vinylogous Amadori rearrangement: Implications in food and biological systems” and “Mechanistic Pathways of Formation of Acrylamide from Different Amino Acids”, and the present author’s contribution as a co-author in the manuscript preparation was to perform all experimental procedures, discuss the interpretation of results and critically evaluate the articles prior to publishing.

PUBLICATIONS

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CONFERENCE PRESENTATIONS

Perez-Locas C., Yaylayan, V.A. and Wnorowski, A. Pathways of Formation of Acrylamide from Amino Acids Other Than Asparagine. Poster Presentation, Annual International Conference of Institute of Food Technologists, Las Vegas, NV, July 2004.

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LIST OF SCHEMES

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AA	Acrylamide
AEC	Anion Exchange Chromatography
AGE	Advanced Glycation Endproducts
AP	Aminopropionamide
ARP	Amadori Rearrangement Product
AP ARP	Amino Propionamide Amadori Rearrangement Product
DG	Deoxyglucosone
FTIR	Fourier Transform Infrared Spectroscopy
HA	Heterocyclic Amine
Hb	Hemoglobin
HMF	5-(Hydroxymethyl)furfural, 5-Hydroxymethyl-2-furaldehyde
HNE	4-Hydroxy-2-nonenal
MRP	Maillard Reaction Product
PAD	Pulsed Amperometric Detection
PAH	Polycyclic Aromatic Hydrocarbons
Py-GC/MS	Pyrolysis Gas Chromatography/Mass Spectrometry
vAP	Vinylogous Amadori Product
vARP	Vinylogous Amadori Rearrangement Product

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Thermal processing of food is known to generate potentially toxic byproducts such as mutagens and carcinogens in addition to desirable aromas, colors and flavor active compounds. Heterocyclic amines (HA) were one of the first groups of carcinogens isolated and identified in food following observations of mutagenic activity in cooked meat and fish (Nagao et al., 1977; Sugimura et al., 1977). These were found to require creatine for their formation. A number of compounds in this group have been labeled as type 2A and 2B level carcinogens by the International Agency for Research on Cancer (IARC, 1993). High temperature barbecuing, roasting, broiling and frying have all been shown to produce small amounts of potent mutagens and carcinogens in a variety of foodstuffs. In 2002, the discovery of acrylamide (type 2A carcinogen) in fried potato products (Tareke et al., 2002) caused an intense worldwide investigation on the origin and mechanism of its formation. This was followed in 2003 by the discovery of furan in canned and jarred products. Numerous other thermally-generated compounds can be found in the list of daily ingested foods including several polycyclic aromatic hydrocarbons (PAH), chloropropanols and 5-hydroxymethylfurfural (HMF). For the most part, precursors include common components that make up the food such as proteins, amino acids and sugars. Studies on the toxicology and formation of thermally generated food toxicants are ongoing and much controversy remains surrounding their actual impact and the risk factors associated with cancer and other diseases. A number of attempts in understanding the origin and the formation of so-called “process-induced carcinogens” have been undertaken. Many precursors have been identified and mechanisms proposed. However such studies are often very general, scarce or lack conclusive information. There is a concerted effort by the regulatory agencies in Europe and North America to understand detailed mechanisms of thermally-generated toxicants and to propose steps to mitigate their formation in food.

1.2 Rationale and Objectives of the Proposed Research

To date research on thermally generated toxicants in food has been focused on the optimization of analytical parameters for sensitive quantitation in foodstuff and approaches directed at their mitigation in food, with less attention directed towards the

systematic understanding of their mechanism of formation. One of the challenges facing the food industry today is to minimize these toxicants without adversely affecting the positive attributes of thermal processing. To achieve this objective it is essential to have a detailed understanding of the mechanism of formation of these toxicants in processed foods.

The overall objective of the proposed research is therefore to elucidate the detailed mechanism of formation of selected Maillard-derived toxicants in food related model systems containing ^{13}C- and ^{15}N-labeled precursors. Model reactions performed with isotopically enriched starting materials generate most accurate information regarding the origin and fate of different precursors**.** A convenient method of introducing ^{13}C- and ^{15}N-labeled atoms into Maillard reaction products is through carrying out the reactions with specifically labeled sugars and amino acids using Py-GC/MS as an integrated reaction, separation and identification system. This technique requires sub-milligram quantities of the expensive labeled precursors, thus reducing drastically the cost of such experiments. In addition, these experiments could be carried out in a short period of time (30-50 min). One of the advantages of using labeled reactants is that all the atoms of a reaction product can be traced back to their origin in the starting material. This fact, not only facilitates elucidation of their mechanism of formation, but also the assignment of their mass spectral fragmentation patterns. The specific objectives of the proposal are **(1)** To develop pyrolytic micro-scale reactions able to generate known toxicants such as acrylamide, furan, HMF and other potentially toxic derivatives **(2)** Using the above approach, to perform a series of micro-scale model reactions using ^{13}C- and ^{15}N-labeled amino acids and ^{13}C-labeled D-glucose or other commercially available sugars. **(3)** Calculate percent incorporation of each label in all the identified toxicants **(4)** Use the information to propose detailed mechanism of their formation. **(5)** Study the factors (temperature, ratio, type of precursor) that promote or hinder their formation. The information gained through such studies will introduce elements of control to minimize their formation in food systems.

1.3 The Experimental Approach

The classical approach to mechanistic studies usually involves reaction mixtures containing precursors of the compound in question under reflux. The products formed must then be extracted from the solvent, purified and identified in search of intermediates or other compounds formed providing insights as to the formation of the chemical of interest. Analysis can be performed using a variety of instruments including GC/MS, HPLC or LC/MS. Specific information can be gained with the use of isotopically labeled precursors (Wnorowski and Yaylayan, 2000). However, the use of solvents, large quantities of expensive labeled precursors, time-consuming manipulations and loss of a great number of components that were not isolated has lead some researchers to adopt an alternate approach using pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS) as an integrated reaction, separation and identification system (Keyhani and Yaylayan, 1996, Yaylayan, 1999) where milligram quantities of reactants can be used as solvent-free model systems, heated in the pyrolysis interface and simultaneously separated by GC and identified by MS. Although solvents play a critical role in specific cases, they often hinder the efficiency of the reaction and extraction allows only selective separation. Consequently solvent-free reactions are considered to be more efficient models (Loupy et al., 1998). A food matrix is a highly complex system that is exceedingly difficult to analyze with regards to formation of micro-components such as mutagens and carcinogens. Model systems therefore are considered simplification of the real matrix in which only the precursors responsible for the formation of a compound in question are reacted thus significantly reducing the number of components formed and avoiding interference of other components present in the matrix (Murkovic, 2004). Within a food system, precursors of the carcinogen in question migrate through the food matrix in order to eventually collide and react. This statistically unlikely process explains in part their low overall yield, typically found in ppb or ppm. Using model systems in conjunction with Pyrolysis-GC/MS allows for a simpler and more efficient analytical approach. The direct reaction of the precursors in question limits interferences from other components in the food. It eliminates reactions of the precursors with other food components and minimizes the distance between reactants thus maximizing overall yield. Therefore the amount of starting material required is small thus maximizing heat transfer resulting in a

significant decrease in time (Yaylayan, 1999). As mentioned above, pyrolysis will be used as a micro-reactor in generating thermally toxic compounds of interest. The pyrolysis interface is coupled to a gas chromatograph (GC) for volatile separation followed by a mass spectrometer (MS) for identification and observation of label incorporation. Milligram quantities of precursors are simply placed inside a quartz tube and plugged with glass wool. The tube is positioned in the center of the pyrolyser probe surrounded by metal coils. The probe is inserted into the pyrolysis interface and the coils are heated at the desired temperature for the desired period of time (typically 250 or 350ºC/20s) leading to thermal reaction and degradation of the sample with release of volatile reaction products. Once generated, they are directly carried into the GC column and separated by various adsorption-desorption mechanisms as a function of their polarity, boiling point, molecular mass and volatility (Wampler, 1999). Molecules exiting the GC are then directed into the mass spectrometer and are broken apart by electron impact into highly reproducible characteristic fragments. The fragmentation pattern can then be matched to that of a library standard of compounds allowing for structure identification. In the case of isotope label incorporation, much information can be gained from fragment distribution of the label.

1.4 Significance of the Proposed Research

The application of isotope labeling and pyrolysis-GC/MS based techniques to the elucidation of mechanism of formation of Maillard-derived food toxicants will certainly enhance our ability to discern desirable chemical reactions from undesirable processes which is considered as a major objective of the food industry today (Yaylayan and Stadler, 2005). This approach not only can provide a comprehensive picture of chemical processes occurring during a reaction, but also can furnish the missing clues needed to distinguish between pathways leading to aroma formation from those that lead to potential toxicants. In addition it has the ability to introduce labeled atoms from precursors to the target carcinogens in a fast and cost-effective manner such that it may greatly accelerate the pace of elucidation of their formation pathways and perhaps help identify new potential carcinogens.

CHAPTER 2

LITERATURE REVIEW

2.1 General Introduction: Thermally Induced Chemical Reactions in Foods

Thermal food processing drives its focus onto three main targets: enzyme inactivation, microbial destruction, as well as the development of desirable sensory characteristics. From this perspective, heat treatment of food is clearly beneficial. However, safety remains a primary objective within the food industry. Conversely, palatability is also a crucial aspect to the success of food products and cannot be neglected (Studer et al., 2004).

As depicted in figure 2.1, various types of reactions are catalyzed in food when it is heated. The extent, variety and balance of reactions and chemicals generated will vary as a function of several factors. The development of aromas, colors and flavors generated as a means of controlling or improving sensory outcome have been extensively studied. Despite years of research much remains unknown as to the mechanisms of formation of specific compounds that impart characteristic organoleptic properties to foods. However, as recent evidence clearly indicates that ideal conditions producing desirable aromas will also generate potential food toxicants (Sugimura et al. 2002). Color and flavor development in foods is commonly classified based on enzymatic and non-enzymatic reactions. Enzymatic browning occurs at lower temperatures and is most often considered an undesirable process. Non-enzymatic browning on the other hand forms colored polymers and flavor compounds via a series of heat catalyzed chemical reactions. Non-enzymatic reactions in food can be divided into four categories: sugar-amino acids reaction, ascorbic acid degradation, thermal oxidation of lipids and sugar-sugar interactions (Nursten, 2005, Wedzicha et al., 1991). Despite their contribution to the sensory properties of thermally processed foods, they are also associated with generation of toxicants. A brief review of these non-enzymatic browning reactions will follow.

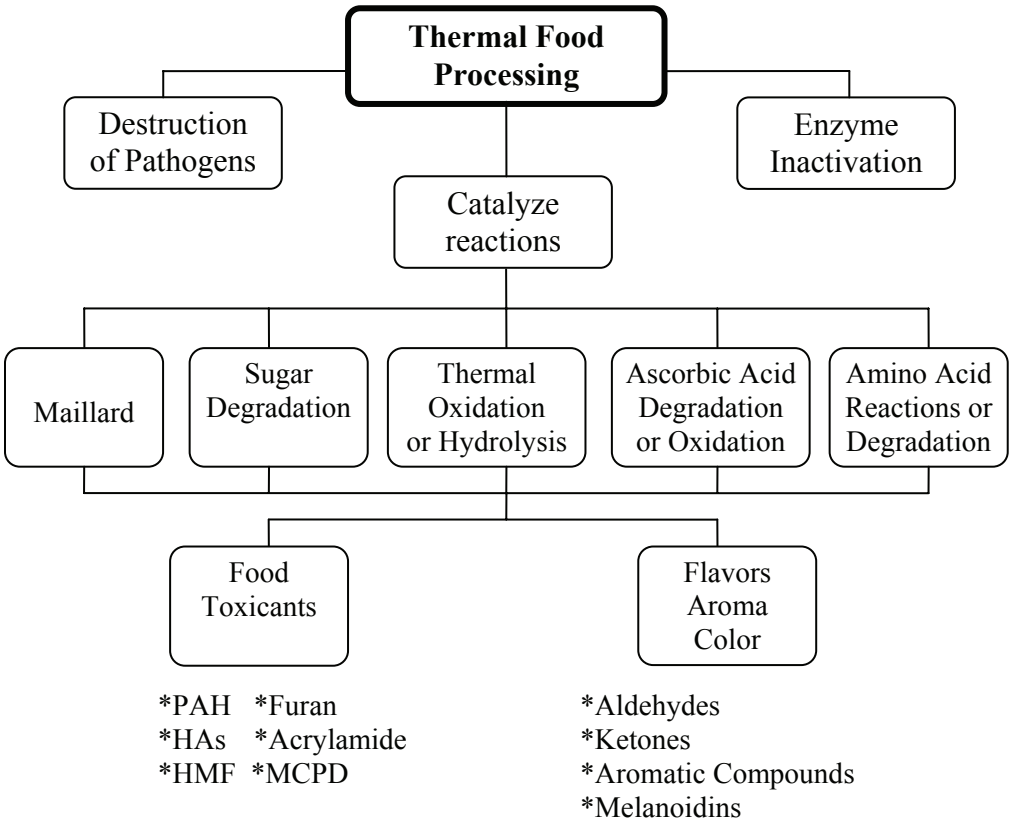


Figure 2.1 Overview of the Implication of Thermal Food Processing

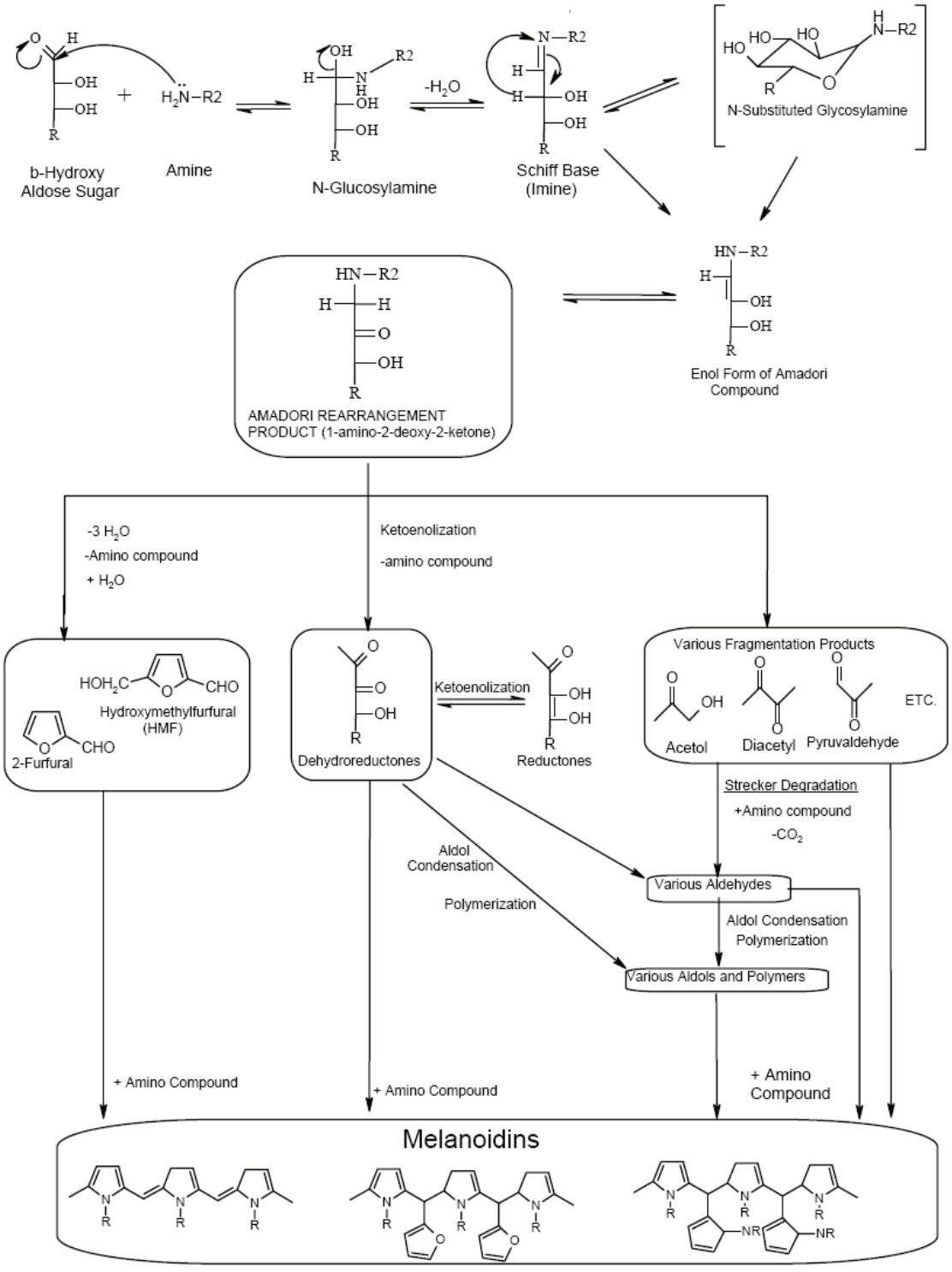
2.1.1 Thermal Interaction of Sugar-Amino Acids: The Maillard Reaction

In the early 20th Century, French chemist Louis Camille Maillard was the first scientist to study and describe resulting changes of thermal reactions between amino acids and sugars, also noting variations in chemical and aroma formation as a function of the amino acid involved (Maillard, 1912). This field of research is now known as the Maillard reaction. The chemistry supporting Louis Maillards observations on the browning reaction, however, was first described by Hodge only in 1953 (Hodge, 1953). Although more recent and detailed reviews regarding the intricacy of the reaction have been presented, J.E. Hodges review remains the pillar of Maillard chemistry research.

Despite the complexity of this reaction and the cascade of intermediates and pathways it entails, the classical Maillard reaction can be subdivided into three main categories:

initial, advanced and final. The initial reaction involves a nucleophilic attack by an amino group onto the β-hydroxy-carbonyl moiety forming an aldosylamine intermediate. Immediate dehydration yields a Schiff base in equilibrium with its less reactive and more stable cyclized form, N-glycosylamine. The Schiff base is then converted into its corresponding Amadori (if *aldo* precursor) or Heyn's (in the case of a *keto* precursor) product (see Scheme 2.1). Under aqueous conditions, the Amadori/Heyns compound is the first stable intermediate formed although, under dry conditions, isolation of the salt form of the Schiff base can be achieved (Mauron, 1981). Following Amadori/Heyns compound formation, thermal degradation in the advanced phase follows yielding highly reactive dicarbonyls, α-amino carbonyls, strecker aldehydes and hydroxy carbonyls that not only impart flavors and aromas to food, but react further, generating a plethora of products including heterocyclic compounds, providing additional characteristic organoleptic properties. The complexity remains clear as a series of reactions take place leading to decarboxylation, deamination, cyclization, dehydration, β-elimination, aldol condensations and other obscure side reactions resulting in the formation of a seemingly infinite network of compounds. More specifically however, the advanced stage can be subcategorized into three major pathways. One is simple dehydration of the Amadori leading to formation of reductones and dehydroreductones. Another pathway involves hydrolytic cleavage of the Amadori, releasing a variety of short chain compounds such as diacetyl and pyruvaldehyde capable for further undergoing Strecker degradation reactions alone or in the presence of amino compounds. Amadori degradation can occur via a third pathway by which it is dehydrated and further reacted with amino compounds and water commonly forming furan type molecules such as hydromethylfurfural and 2-furaldehyde (Nursten, 2005; Mauron, 1981). The final stage is mainly characterized by the polymerization of diverse Maillard intermediates leading to the formation of high molecular weight melanoidins. These nitrogen-containing polymeric and co-polymeric chains are responsible for the observed brown pigments in thermally processed foods. Despite several speculations and propositions, their detailed pathways of formation remain somewhat obscure (Yaylayan, 1997; Nursten, 2005). A general scheme of the Maillard reaction is presented in Scheme 2.1.

Great importance is attributed to the understanding of the series of events brought about by the Maillard reaction. Partly due to its wide spread, the Maillard reaction encompasses both positive and negative avenues. Desirable characteristics include the formation of pleasant flavors, aromas, colors and Maillard reaction product (MRP) antioxidants (Hollnagel and Kroh, 1998). Sensory and visual properties occurring when roasting, baking and frying clearly illustrate the benefits of the science. However, a darker side of the Maillard reaction has come into focus. Despite its attractive features, the Maillard reaction is also a predecessor of undesirable browning and loss of nutritional value of amino acids in foods, formation of advanced glycation endproducts (AGE) leading to protein cross-linking, DNA damage, ageing and complications associated with diabetes (Stitt, 2001, Horvat and Jakas, 2004). AGEs form through a nucleophilic attack of the amino groups of the protein side chains onto the carbonyl of carbohydrates. Following a classical Maillard-like pathway, the initial glycation leads to an irreversible modification of the molecule, thus termed AGE. In biological systems, many of these glycation adducts have the ability to covalently crosslink proteins, inhibiting their function, rendering them insoluble and over time contributing to the complications associated with diabetes and ageing such as cataract formation, atherosclerosis, stiffening of the joints, hypertension, DNA damage, among many others. Although various research groups are currently studying Maillard reaction, however, they remain a long way from a detailed understanding of the entire process (Sugimura 1986, Knasmüller et al., 2004).



Scheme 2.1- Summary of the Maillard Reaction

2.1.2 Thermal Oxidation of Lipids

Lipid oxidation is an important reaction in food responsible for a number of chemical changes including development of rancidity and other off-flavors as well as the formation of food toxicants as a result of thermal degradation at elevated temperatures of frying. Despite their beneficial contributions to health such as the lowering of LDL and raising of HDL levels, polyunsaturated fatty acids (PUFAs) are highly prone to degradation due to the presence of a greater number of double bonds which are particularly susceptible to oxidation (Watkins and German, 1998; Tang et al., 2002). Lipid oxidation is commonly depicted as a free-radical chain reaction broken down into three major steps: initiation, propagation and termination. The initiation phase refers to initial free radical formation via abstraction of a hydrogen ion from an unsaturated carbon of a fatty acid chain followed by addition of oxygen onto the newly formed radical site. Once oxidation is initiated, propagation of the free radical chain reaction follows, each free radical participating in abstraction of other ions thus creating additional radicals, until collision with another free radical terminates the reaction (Eriksson, 1982). As a result, a variety of short chain fatty acids, aldehydes, hydroxyl keto acids, keto acids and hydroperoxides are generated and contribute to off-flavor development. The general reaction is depicted in Scheme 2.2. Moreover, carbonyl containing lipid oxidation products can further participate in Maillard type reaction in the presence of amino groups consequently contributing to browning (Nursten, 2005).

Initiation Reaction: $RH + O_2 \rightarrow R^{\bullet} + {}^{\bullet}OH$

Propagation Reaction: $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$
$ROO^{\bullet} + RH \rightarrow R^{\bullet} + ROOH$
$ROOH \rightarrow RO^{\bullet} + {}^{\bullet}OH$

Termination Reaction: $R^{\bullet} + R^{\bullet} \rightarrow RR$
$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR$
$RO^{\bullet} + R\cdot \rightarrow ROR$
$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2$
$2RO^{\bullet} + 2ROO^{\bullet} \rightarrow 2ROOR + O_2$

Scheme 2.2 General Schematic of Lipid Oxidation Reaction

Prior to the free radical mechanism however, minimum amount of energy is required to trigger the oxidation reaction. Instigation of oxidation can therefore occur via reduction of the energy requirement by metals, high energy oxygen, enzymes or by addition of the necessary energy in the form of heat, light or oxidizing enzymes (Brewer, 1998). Clearly, thermal stress assists in initiating the oxidation process directly or indirectly and acts as a catalyst in its propagation reaction (Muik et al., 2005). In meats, for example, heat induced protein denaturation enables the release of oxygen and iron from hemoglobin and myoglobin units, both atoms serving as catalysts in fat oxidation (Kanner, 1994). Heating of lipids also promotes isomerization of the double bond converting cis bonds into trans (Frankel, 1998). Trans fats are well-known for their toxicity in human biological systems.

Recently, lipid oxidation products have been found to behave similarly to carbohydrates in foods and in the body. Their carbonyl or epoxy moieties have the ability to modify amine-containing molecules, including proteins and aminophospholipids, via reactions analogous to those occurring in the Maillard reaction such as Strecker degradation (Hidalgo and Zamora 2004; Hidalgo and Zamora, 2005). Moreover, they have also been shown to contribute to the burden of protein crosslinking via the formation of advanced lipoxidation endproducts (ALE) with intermediates and molecules comparable or identical to those of sugar promoted AGEs. An extensive review on the similarities of AGE and ALE has been written by Hidalgo and Zamora (2005). In addition to the formation of trans double bonds, peroxides and free radicals, thermal degradation of lipids is also responsible for generation of various cytotoxic hydroxy alkenals (Tang et al., 2002) known for their involvement in ALE generation. Their formation, characteristics and implications will be explored in a later chapter.

2.1.3 Thermal Degradation of Sugars: Caramelization

Caramelization is a thermal oxidation process by which sugars interact, undergoing dehydration, degradation and fragmentation reactions ultimately leading to the formation of volatiles and brown colored compounds (Davis, 1995). Despite involvement and generation of several intermediates and products common to those formed during the Maillard reaction, the pathways to caramelization do not involve any nitrogen containing

compounds (Nursten, 1980). Moreover, melting of the sugars prior to dehydration and initiation of caramelization is an essential step. The process therefore requires temperatures above the melting point of the sugars (typically above 80ºC) in question (Feather, 1982). Conversely, Maillard reaction has been shown to take place even at body temperature. Caramelization is characterized by a series of complex reactions. An initial isomerization rearrangement of aldose sugars into ketose form is followed by a succession of dehydration (β-elimination), fragmentation (dicarboxylic cleaving, retro-aldol), aldol condensation, polymerization and radical reactions (Nursten, 1980, Eskin, 1990). Among the numerous intermediates formed over the course of the reaction, highly reactive α-dicarbonyls such as glyoxal, methylglyoxal and diacetyl are of significant importance in promoting further reactions. Formation of a network of volatile and pigmented compounds resulting from the thermally catalyzed chemical reactions such as various furans, furanones, pyrones and carbocyclics give rise to the characteristic caramel flavor associated with caramelization (Eskin, 1990). Examples of such compounds are depicted in figure 2.2. Alternatively, toxicity derived from sugar-sugar interactions has been seen from compounds such as various polycyclic aromatic hydrocarbons (PAH) as well as 5-Hydroxymethyl-2-furaldehyde (HMF) and the parent furan.

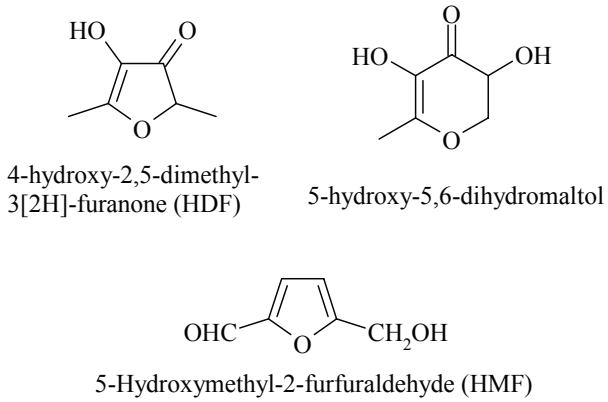
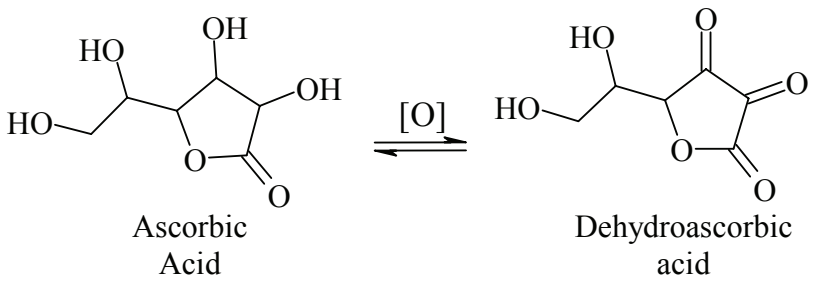


Figure 2.2 Examples of Caramel-related Compounds Generated via Sugar Interactions

2.1.4 Thermal Degradation of Ascorbic Acid

Ascorbic acid is well known for its role as a natural antioxidant, interfering with enzymatic browning, scavenging free radicals and molecular oxygen (Niki, 1991). It also has the ability to reduce orthoquinones back to their phenolic state in various fruits,

vegetables and other plant material. Enzymatic conversion of phenolics to orthoquinones and their subsequent interactions commonly generate a variety of dark pigments. Reversal of the initial enzymatic oxidation by ascorbic acid consequently prevents such further reactions (Mathew and Parpia, 1971). Ironically however, thermal degradation of ascorbic acid is associated with non-enzymatic browning. It has been shown to induce browning of catechins in wine, although the mechanism is poorly understood (Bradshaw et al., 2001). Moreover, ascorbic acid oxidation results in formation of dehydroascorbic acid as depicted in figure 5, leading to the generation of highly reactive furfurals and osones as well a series of pigmented polymers. Furfurals are known to further react producing longer chain brown polymers. Furthermore, interactions between ascorbic or dehydroascorbic acid and amino acids readily occur in thermally treated foods again forming various colored compounds via Maillard-type reactions (Nursten, 2005). Ascorbic acid degradation promptly takes place under thermal stress.



Scheme 2.3 Interconversion between Ascorbic acid and Dehydroascorbic acid

Recently, thermal degradation of ascorbic acid has been shown to produce high levels of furan, a known toxicant. Dehydroascorbic acid also produces furan (Health Canada). The relative efficiency however varies as a function of oxidative and non-oxidative conditions.

2.2 Thermally Generated Toxicants in Food

Maillard reaction has recently been linked to the formation of various carcinogens and mutagens in a variety of thermally processed foods. Acrylamide, furan and heterocyclic amines are among those toxicants.

2.2.1 Acrylamide

Acrylamide is a water-soluble compound known to be present in tap water, cigarette smoke, laboratories and several industries (IARC, 1994; Schumacher et al., 1977). In 2002, much controversy arose from the discovery of acrylamide in a variety of thermally processed foods. Although heat treatment of staple foods was clearly involved, little was known about formation and exact precursors. Despite having been classified in 1994 by the IARC as a type IIA probable human carcinogen, researchers were unaware of its presence in food. For decades, industrial synthesis of large quantities of this chemical served multiple functions, primarily revolving around its conversion to polyacrylamide, a multipurpose, non toxic impermeable solid matrix. This harmless polymeric form of acrylamide is widely used in cosmetics, as a grouting agent in the building and repairing of water tunnels, sewers and fountains, and used as a water flocculating agent for waste water treatment as well as for solid support in protein or nucleic acid separation (PAGE). (IARC, 1994; Friedman, 2003). Due to its suspected neurotoxicity and carcinogenicity, working with acrylamide involves several precautionary measures. Moreover, numerous regulations, such as implementation of boundaries for maximal acceptable trace amounts in drinking water by the EPA and the WHO, were believed to keep population safe from potentially toxic daily exposure. Serious concern came about when measured acrylamide levels in foods were observed to far exceed the regulatory tolerable level intake allowed in drinking water (Tareke et al. 2002). Consequently, research on this carcinogen has been on the priority list of governmental and industrial organizations to determine the actual risk associated with its consumption, its mechanism of action in the body, methods of detection and finally its formation mechanism in foods and methods of prevention.

2.2.1.1 Toxicology

Acrylamide is toxic to humans on various levels via inhalation, dermal absorption and ingestion. Rodent studies have proven high level exposure to be neurotoxic and even deadly (Schaumburg et al., 1974, Friedman et al., 1995, Friedman, 2003). However, the low levels of food-derived acrylamide are clearly not expected to produce such an acute response. Daily exposure to acrylamide hovers around 0.5ug/kg body wgt/day (WHO, 2002), far below the threshold of acute toxicity. Therefore, concern lies mainly upon the possible mutagenic or carcinogenic effects of low dose, long term exposure. Despite numerous studies, results and conclusions remain contradictory and lack of information with respect to human cancer risk from population studies leave much room for debate. It remains highly questionable whether or not the chemical taken in from food actually poses a risk to human health (Granath and Tornqvist, 2003). Much data has been published pertaining to cancer risk assessment of acrylamide. Evidence to date including a few large scale comprehensive human case-control studies has failed to show any association between acrylamide intake and increased incidence of various types of cancers (Pelucchi et al., 2006) but is still not excluded as a risk factor (Skog, 2007). However, regardless of the debate, maintaining carcinogen formation as low as possible remains a target. Consequently, in response to high demand, analytical companies are even providing pre-prepared packages for the monitoring of acrylamide levels in foods (Walz and Trinh, 2005). Levels of acrylamide encountered in food range anywhere between 0 and 3.5mg per kilogram. Due to the various possible routes of exposure including smoke, food and water, hemoglobin (Hb) adducts of acrylamide and of its reactive epoxide metabolite glycidamide, are often used to assess human exposure to acrylamide. Acrylamide and glycidamide are electrophilic chemicals with their α,ß-unsaturated amide groups. They therefore have the capacity of reacting in vivo and in vitro with nucleophilic compounds, forming adducts with the sulfydryl or α-amino group of proteins such as Hb. Hb adducts (formed with acrylamide or glycidamide attaching to the α-NH_2 group of the N-terminal of valine of Hb) are used as biomarkers as they are distributed in the blood and can be measured by gas chromatography/mass spectrometry (GC/MS) and correlated to the levels of exposure. One of the advantage of using Hb adducts is that they provide a time averaged estimate of exposure (Perez et al., 1999, Törnqvist and Ehrenberg., 2001).

2.2.1.2 Analytical Methodology

Hydrophilic character of acrylamide allows for a simple water or methanol extraction from the food (Rosen and Hellenas, 2002; Tateo and Bononi, 2003). A saline solution can also be used to avoid emulsification during the sample pretreatment, water-acetone solution or a pressurized liquid extraction. Depending on the food matrix, a deffating step with an organic solvent or a deproteinating step could be necessary to remove interfering components (Delatour et al., 2004). An internal standard is also added to allow for a more reliable quantification. This standard is typically an isotopically labeled acrylamide. Further cleanup using combinations of solid phase cartridges or mixed mode cation exchange (MCX) or hydrophylic lipophilic balance (HLB) are widely used. Numerous analytical procedures have been published (Wenzyl et al., 2007) over the years focusing on maximizing extraction efficiency with removal of interferants. These methodologies also vary as a function of the type of food matrix analyzed. A thorough review has been presented by Zhang and coworker (2005). Analysis of acrylamide is generally achieved by one of two main methodologies: GC/MS or LC/MS-MS. In the case of gas chromatography, derivatization of the analyte is required in order to enhance volatility, selectivity, sensitivity and retention time. Bromination has been the derivatization methodology of choice allowing for good sensitivity although silylation has also proven itself valuable when combined with headspace SPME analysis. It is important to note however that derivatization is not necessarily essential and some groups have chosen to eliminate this lengthy procedure using a more polar GC phase, although it does not afford as low a limit of detection as the derivatization techniques (Wenzyl et al., 2006).

LC-MS/MS is analytically advantageous as it does not require any derivatization step prior to analysis yet retains selectivity. The use of a modified dC18 reverse phase minimizes retention problems whereas ion exchange chromatography reduces interferences. Both approaches have been reported as being adequate. Tandem mass spectrometry is the most common detection mode due to its high selectivity. Mass spectrometry can be used instead; however derivatization with 2-mercaptobenzoic acid is necessary for optimal confirmation (Jezussek and Schieberle, 2003).

2.2.1.3 Sources and Formation

The presence of acrylamide in processed foods is virtually universal and can be found in a wide range of products. As depicted in Table 2.1, potato products contain a high concentration of acrylamide. However, due to their generally infrequent consumption relative to other food commodities, potato products are not the primary contributors to the total dietary intake of acrylamide. On the other hand, despite low levels of acrylamide found in coffee (~1.14μg per cup, Guenther et al., 2007) and bread products, they have the potential of being principal sources of dietary acrylamide, with coffee contributing up to 39% of the total intake (Svensson et al., 2003). Alternately, meats and fish are known to have very limited amounts of acrylamide and are usually considered as negligible sources. As demonstrated in Table 2.1, accurately judging the dietary contribution of acrylamide remains challenging as broad concentration ranges of acrylamide found in similar foods can heavily impact dietary assessments. Clearly, this is a result of susceptibility of acrylamide to processing conditions and the composition of the food matrix. Despite identification of a variety of acrylamide precursors, asparagine is considered as the main source requiring however the presence of a carbonyl compound to yield significant levels of the toxicant in foods. Other amino acids have also been shown to produce acrylamide. Such amino acids include methionine, cysteine, serine, β-alanine and carnosine (Stadler et al., 2002; Yaylayan et al., 2005). Moreover, the fatty acid oxidation product acrolein commonly found in oils produces acrylamide when reacted with ammonia. However, it has not been shown to be of major importance in the frying of model food systems (Mestdagh et al., 2004).

2.2.1.4 Labeling Studies

Various isotope labeling studies in combination with mass spectrometry have clearly demonstrated that the carbon backbone of acrylamide is derived from asparagine. In addition, these studies have also confirmed the involvement of the amide nitrogen of asparagine with nearly 100% isotope label incorporation in the acrylamide structure. However, studies also show the indisputable need for a source of carbonyl, such as reducing sugars, although no label incorporation from ^{13}C-labeled sugars was observed from its co-pyrolysis with asparagine (Stadler et al., 2002). Such information strongly

confirms these carbonyls as catalyzers of the reaction, without being part of the final molecule. At this point, however, little evidence was available as to the mechanistic role of carbonyls in the formation of acrylamide.

Table 2.1 Acrylamide in Foods

Food Product	**Acrylamide (µg/kg)** [1]
Roasted Almonds	260
Roasted Asparagus	143
Boiled Potatoes	48
Potato Chips	170-3700
French Fries	200-12000
Gingerbread	90-1660
Bakery Products	70-430
Meat	30-64
Fish	30-39
Roast Coffee	45-374
Chocolate	15-102

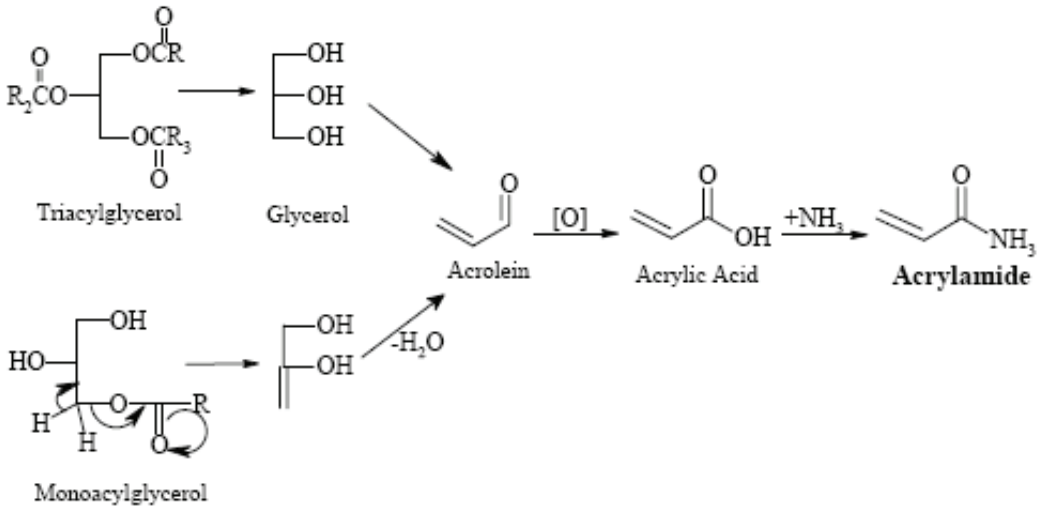
[1] Friedman et al., 2003; Andrzejewski et al., 2007; Pardo et al., 2007

2.2.1.5 Proposed Mechanisms of Formation

From the first mechanism proposed, the Maillard reaction was the main target (Mottram et al., 2002), however, early investigations clearly indicated that the mechanism of formation of acrylamide strayed somewhat from the classical Maillard reaction. The low yield resulting from the pyrolysis of Amadori compound as opposed to the high yield generated by its Schiff base precursor (or N-glycosylasparagine, the cyclized Schiff form) strongly suggested that acrylamide formation pathway occurs prior to Amadori formation (Stadler et al., 2002). Moreover, pyrolysis of Amadori intermediate revealed the difficulty of its decarboxylation. However, pyrolysis of N-glycosylasparagine yielded significantly higher levels of acrylamide than asparagine-glucose models, clearly indicating involvement of Maillard reaction. Subsequently, use of dicarbonyl moieties as

opposed to α-hydroxy carbonyls also leads to acrylamide formation (Stadler et al., 2002). Lack of α-hydroxy group blocks the Maillard reaction at the Schiff stage as it is unable to go further into Heyns/Amadori as would the classical Maillard pathway. Therefore, had acrylamide formation been entirely dependant on Maillard reaction, a dicarbonyl would not have resulted in its formation. Taking these premises into consideration, two general pathways were proposed describing the formation of acrylamide from asparagine amino acid. Zyzak and coworkers (2003) presented a mechanism involving direct decarboxylation of the Schiff with subsequent Amadori rearrangement leading directly to acrylamide or indirectly via hydrolysis of the imine yielding 3-aminopropanamide. Conversely, Yaylayan and coauthors (2003) proposed an alternate pathway where the decarboxylated Amadori product of asparagine with reducing sugars was the key precursor of acrylamide. Furthermore, data revealed decarboxylated Amadori product can be formed under mild conditions through the intramolecular cyclization of the initial Schiff base and formation of oxazolidin-5-one intermediate that can decarboxylate and produce acrylamide. The sugar-assisted decarboxylation pathway, depicted in Scheme 3.1 (Chapter 3) has also been supported by Stadler et al. (2004).

Several other precursors have been studied and shown to generate acrylamide, however general consensus targets asparagine as the main precursor of acrylamide in food. Evidence of this was demonstrated when adding asparaginase enzyme to model potato food system, resulting in a 99% drop in acrylamide formation upon heating as opposed to the asparagine-free model (Zyzak et al., 2003). Mechanisms of acrylamide formation from other amino acids or acrolein derived from fatty acid oxidation have been proposed but are considered as being relatively insignificant. Acrolein was suspected to react with ammonia resulting in acrylamide (Yasuhara et al., 2003). In a food system, free ammonia is commonly found as a result of amino acid deamination. However, due to its volatility, it tends to dissipate rapidly into the environment probably having little time to react with the acrolein in question thus explaining its minor contribution. An overview of the proposed mechanisms is depicted in Scheme 2.4.



Scheme 2.4 Proposed Mechanism of Acrylamide Formation from Lipids (Adapted from Gertz and Klostermann, 2002)

2.2.1.6 Minimizing Acrylamide

Since the discovery of acrylamide's impact on our food supply a number of investigations have been conducted targeting practical means of reduction. A key factor that has been repeatedly shown to influence acrylamide levels is temperature. Microwaving and boiling generates minimal amounts whereas harsher treatments such as frying and baking lead to high levels (Matthaus et al., 2004). Particularly in the case of potatoes, soaking them in water prior to baking or frying significantly reduces acrylamide levels as it leaches away its precursors on the surface. Further reduction has also been observed via addition of salt, citric acid or amino acids (glycine, lysine, cysteine) to the soaking solution. In some instances however, a balance must be created such as to avoid a corresponding inhibition of flavor and color development (Pedreschi et al., 2004; Ishihara et al., 2005 and 2006; Kim et al., 2005; Kolek et al., 2006; Low et al., 2006). Addition of amino acids such as glycine to dough in the making of snacks also has a negative impact on acrylamide formation (Kim et al., 2005). In response to the concerns and the scientific efforts targeting practical means of acrylamide minimization, the European Confederation of the Food and Drink Industries (CIAA) in collaboration with the Heatox project, developed a frequently updated database known as the "Toolbox".

This “Toolbox” is based on a compilation of scientific publications and advice thus providing practical guidelines for reducing acrylamide formation as a function of the food product in question and is specifically intended for industrial food application (CIAA, 2007). Among the suggestions, addition of asparaginase enzyme to some products has been recommended for keeping acrylamide levels low.

2.2.2 Furan

Furan has been known to occur in foods since the 1960s. Maga (1979) had previously reported its formation from various carbohydrates and speculated that they were the main precursors of furan in food, but lack of urgency at the time had limited further research. However, recent awareness of the widespread occurrence of furan in canned and jarred products raised questions as to its overall toxicity, precursors and formation in foods (FDA, 2003). Furan is a volatile aromatic heterocyclic organic compound exhibiting a boiling point of 31.4ºC. Therefore, once generated under thermal processing conditions, it is subsequently released into the atmosphere, unless processing occurs in an enclosed environment. Understandably, in the case of in-can (or jar) processing, the furan formed remains in the vicinity and is consequently consumed with the product.

2.2.2.1 Toxicology

Although acrylamide is listed by the IARC as a type II B possible human carcinogen (IARC, 1995) based on a series of animal studies indicating formation of malignant tumors, there is still much room for debate as to its contribution to long term cancer development in humans. Nevertheless, it has also been listed by the Department of Health and Human Services (DHHS) list of carcinogens as “reasonably anticipated to be a human pathogen”. Among the many effects, chromosomal aberration, DNA damage and hepatotoxicity have been observed in mice as a result of ingested furan (NTP, 1993). Metabolic activation of furan into one of its reactive metabolite cis-2-butene-1,4-dial is suspected to occur via cytochrome P450 and is probably responsible for some of furans cancer inducing properties (Chen et al., 1995). Lack of human epidemiological studies on the risk invoked by furan exposure prevents any further speculations, although in this case no data indicates a difference in the pathways to tumor development between rodent

and humans. Several toxicological studies are still underway helping to adequately assess the risk posed by furan.

2.2.2.2 Analytical Methodology

Volatility of furan makes it a rather ideal compound to analyze and does not require complex extraction and purification steps. Liquids can usually be analyzed as is and solids require addition of water. Common procedure consists of sealing the homogenized food sample with Furan-d_4 as internal standard in a headspace vial with an SPME fiber (Caboxen-PDMS). The fiber is then simply inserted into the GC-MS injection port where the adsorbed furan can be desorbed into a DB-5 or a non polar PLOT column. Mass spectrometry is then used for sensitive and selective identification (Becalski et al., 2005; Goldmann et al., 2005). Several reviews on furans were recently published (Yaylayan, 2006; Crew and Castle, 2007). In addition, a single issue of the journal of *Food Additives and Contaminants* was dedicated to furan and acrylamide in food (2007, Supplement 1, Volume 24).

2.2.2.3 Sources and Precursors

Furan is found in most in-pack processed food. Some examples are depicted in Table 2.2. A more extensive list is provided on the FDA website (FDA, 2003). In addition to food, sources of furan also include cigarette smoke, coal and wood smoke. It is an important intermediate in the synthesis of numerous chemicals including thiophene and tetrahydrofuran as well as in the production of pharmaceuticals and pesticides (NTP, 1993; IARC, 1995; HSDB, 2001). Maga (1979) had provided evidence of furan formation from carbohydrates. In addition to this knowledge, Health Canada (2004) was able to produce very high furan levels from thermal degradation of ascorbic acid, dehydroascorbic acid and thiamine. Amino acids alone were also found to produce trace amounts although sugar-amino acid mixtures proved to be far more efficient suggesting the involvement of the Maillard reaction. Moreover, furan was generated from thermal oxidation of polyunsaturated fatty acids. This knowledge, however, did not provide much insight as to the formation pathways of the parent furan and the FDA was requesting mechanistic information.

Table 2.2 Furan in Foods

Food Product	**Furan (ppb)** [1]
Baby Foods Applesauce	3.2-5.3
Baby Foods Sweet Potato	64.7-93.1
Baby Foods Vegetables	51-112
Coffee	<2-84.2
Jams and Jellies	1.6-37.4
Canned Beans	23.3-68.1
Fruit and Vegetable Juices	1-23.5

[1]FDA, 2003

2.2.3 Hydroxymethyl-2-furaldehyde (HMF)

Another possible toxicant, 5-hydroxymethylfurfural (HMF) originates during the thermal degradation of sugars and carbohydrates. It is known to occur at very high concentrations (exceeding 1g/kg) in carbohydrate-rich foods. Common food sources include coffee, honey, breads and cookies, caramels, heat treated milk, dried fruits as well as fruit juices and is formed as a result of thermal processing, drying or storage. In some cases it has been found in levels exceeding 1g/kg of food and up to 6.2g/kg in coffee (Schultheiss et al., 1999; Janzowski et al., 2000; Murkovic and Pichler, 2006). Despite early reports of the potential of HMF for mutagenic, genotoxic and even carcinogenic behaviour (Schoental et al, 1971; Zhang et al., 1993; Surh et al., 1994) as well as the toxicity exhibited by its metabolite 5-SMF (5-Sulfooxymethylfurfural) lack of credible evidence linking its consumption in foods to an actual increased risk for disease resulted in the establishment of maximum levels on the basis of thermal index instead of toxicity (Ameur et al., 2006; Rufian-Henares et al., 2006; Gokmen et al., 2007). Over the years, many correlations have been established between HMF and the degree of thermal treatment and it is often recognized as an essential quality parameter (AIJN, 1996; OJEC, 2002). Thus, HMF has gained much success as a chemical index in ensuring adequate heat processing and storage conditions for fruit juices, honey, cereal products, cookies and jams (Cortes et al., 2007; Berg and van Boekel, 1994; Morales et al., 1997). Recent evidence has however demonstrated potential for its toxicity.

2.2.3.1 Toxicology

Limited toxicological studies on animals and humans have hindered the evaluation of risks factors associated with consumption of HMF. Janzowski's group (2000) provided evidence for weak mutagenicity and cytotoxicity arising from HMF levels potentially comparable to those found in certain foods. Earlier estimates on daily HMF intake showed up to 2.5mg/kg body weight from dietary sources (Ulbricht et al., 1984). However, in light of more recent studies, Janzowski (2000) proposed that a more realistic assessement is an average intake of about 30-60 mg per person (0.5-1g/kg wgt/day) and overall concluded that HMF posed an insignificant health risk. However the results are controversial as some animal studies have shown dose-related tumor development (Schoental et al., 1971; Zhang et al., 1993; Surh et al., 1994) whereas others have not (Miyakawa et al., 1991). The response also varied according to the region studied. Sommer et al. (2003) showed HMF mutagenicty to *S. typhimurium* TA100 derived-strain while Glatt and coworkers (2005) demonstrated the induction of sister chromatid exchange in V79-hCYP2E1-hSULT1A1 cells by HMF as an indication for genotoxicity. Moreover, Svendsen et al., (2007) studied the potential impact of HMF and its proposed metabolite, 5-SMF (5-Sulfooxymethylfurfural), on tumerogenesis in mice revealing an increase in adenomas and colon cancer precursors particularly in the case of 5-SMF. The increased response of 5-SMF has also been previously observed by Glatt et al. (2005). Unfortunately, lack of long term carcinogenicity studies and human epidemiological studies limit the knowledge pertaining to risk posed by HMF consumption. However, assessments appear to indicate the potential for toxicity thus justifying the importance of additional toxicological studies.

2.2.3.2 Analytical Methodology

Analytical procedures for HMF determination vary slightly as a function of the food matrix and the separation tools. The HPLC methods typically involves water or methanol extraction, precipitation with clarifying agents such as Carrez solutions or TCA if necessary, filtration and direct injection into HPLC with UV or diode array detection. Wavelengths for detection appear to vary among authors between 280 and 285nm (Ramirez-Jimenez et al., 2003; Ameur et al., 2006; Murkovic and Pichler, 2006; Ameur

et al., 2007). Some more complex matrices such as coffee can require an SPE purification prior to injection to remove interfering compounds (Murkovic and Pichler, 2006). A GC-MS methodology was also proposed and validated by Teixido and coworkers (2006). Briefly, the sample is extracted with acidified water and passed through an SPE cartridge followed by concentration and derivatization. The authors tested a variety of cartridges evaluating ENV+ as giving the best response and recovery. Among the derivatizing agents tested, N,O-Bis(Trimethylsilyl) trifluoroacetamide (BSTFA) was found to provide a good response within a short time frame. The procedure is slightly more labour intensive however provides a significantly lower level of detection. Moreover it was shown to work on many types of sample including solids, liquids and semi liquids (Teixido et al., 2006).

2.2.3.3 Sources, Precursors and Formation

The widespread occurrence of HMF in food stems from widespread presence of sugars. HMF formation has long been understood to be derived from the cyclization and dehydration of 3-deoxyglucosone, a known intermediate of Maillard and caramelization reactions. Its generation is highly temperature dependant. Moreover, as the pH strongly influences the relative rate of the Maillard reaction (favored under low pH 5-6) and caramelization (favored at higher pH) it consequently impacts the pathway and the extent of HMF formation. Daily intake estimated between 30 and 150 mg/day in humans (Janzowski et al., 2006) can be attributed to a variety of popular products such as coffee (300-1900mg/kg), dried fruit (1-2200mg/kg), balsamic vinegar (up to 3400mg/kg), ketchups (0.8-189.8mg/kg), jams (4.5-159.6mg/kg) among many others (Murkovic and Pichler, 2006; Cocchi et al., 2007; Vorlova et al., 2006).

2.2.4 The 4-Hydroxy-2-Alkenals

The 4-hydroxy-2-alkenals as a group not only can be formed from sugar and amino acid degradation products but also from lipid oxidation. Oxidative stress in general has been associated with a number of degenerative diseases via intermediacy of a variety of lipid peroxidation products (Horton and Fairhurst, 1987). Polyunsaturated fatty acids (PUFA) whether part of the body or of the diet constitute important targets of oxidation due to

their numerous and reactive sites of unsaturation. As a result, more reactive hydroperoxides are formed and broken down to yield a variety of carbonyls. Among these, 4-hydroxy-2-alkenals encompass a group of highly cytotoxic α,β-unsaturated aldehydes formed from the β-cleavage of these PUFA generated hydroperoxides (Carini et al., 2004). Two highly toxic hydroxyalkenals, 4-hydroxy-2-nonenal (4-HNE) and 4-hydroxy-2-hexenal (4-HHE), are major aldehydes formed as a result of ω-6 and ω-3 PUFA peroxidation respectively (Esterbauer et al., 1991).

2.2.4.1 Toxicology

The detrimental effects of the hydroxyalkenals are associated with their ability to modify biological amino acids, proteins, peptides, nucleic acid bases as well as aminophospholipids (Gao et al., 2006). As shown in Figure 2.3, their reactivity stems mainly from two sites, allowing them to easily react with nucleophilic centers via Micheal Addition (onto the double bond) and/or Schiff base formation (onto the carbonyl) resulting in protein crosslinking (Cohn et al., 1996, Carini et al., 2004). These crosslinks disrupt normal cellular functions exerting detrimental biological effects, and contributing to neurodegenerative and cardiovascular diseases. Numerous investigations on the metabolism and biotransformation of hydroxyalkenals to assess the implications of endogeneous hydroxyalkenal formation have been conducted. Metabolism of HNE, as a representative species of the hydroxyalkenal group, is primarily mediated via conjugation with glutathione, although enzymatic reduction or oxidation of the aldehyde can occur as well (Awasthi et al, 2003). A thorough review of the metabolic pathways of hydroxyalkenals and the biotransformation of HNE published by Alary and coworkers (2003) concludes that the cytotoxicity of these species is limited by an efficient metabolism. However, although inevitably produced in vivo from phospholipids and membrane lipids, the levels are significantly enhanced during the onset of diseases involving an increase in oxidative stress, such as Diabetes and Alzheimers (Umberto-Dianzani, 2003; Zarkovic et al., 2003). A number of detailed reviews on the impact of hydroxyalkenals are available (Umberto-Diaz, 2003; Zarkovic, 2003; Hidalgo and Zamora, 2005). Recently, in addition to its own toxicity, cyclization of 4-hydroxy-2-

butenal has also been suggested to produce the toxic furan in foods (Perez-Locas and Yaylayan, 2004).

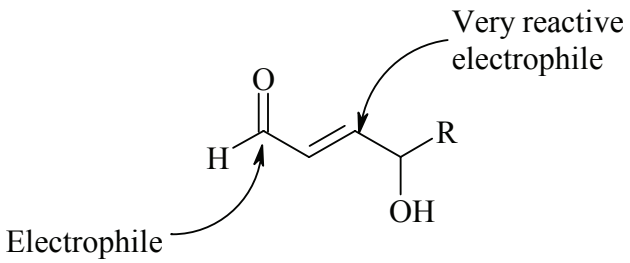


Figure 2.3 General Structure for 4-Hydroxy-2-Alkenals

2.2.4.2 Origins and Reactions of 4-Hydroxyalkenals

The 4-hydroxyalkenals can be produced as a result of food processing or storage but are also found and formed in tissues of living organisms. These molecules possess three reactive sites including an aldehyde, a double bond and a hydroxyl group, they are able to undergo a number of competing reactions, such as Schiff base formation, Micheal adduct formation, oxidation and internal hemiacetal formation. A review discussing the many reactions undergone by HNE, a hydroxyalkenal, has been published (Shaur, 2003). Similar to dicarbonyls, 4-hydroxyalkenals have the ability to decarboxylate and hydrolyse amino acids via Strecker degradation reaction (Hidalgo et al., 2005). Moreover, they exhibit their toxic potential in their ability to react with nucleophilic, leading to chemical modification of proteins and the formation of ALE.

2.3 Methodologies Employed in the Study of Maillard-type Chemical Reactions

A number of methodologies have been developed for the analysis of reaction products emanating from sugar and amino acid interaction in foods and for elucidation of their corresponding mechanistic pathways. The experimental approach generally involves reacting *m*mol quantities (~0.1-1g material) of the precursors in water or in a phosphate buffer under autoclave or reflux conditions. Once the reaction is complete, pH adjustment and multiple extractions with selected solvents such as ether, diethyl ether follow according to the properties of the compounds of interest. Final analysis is generally achieved from HPLC/MS or GC/MS for mechanistic information, whereas NMR spectroscopy can be concomitantly used for structural elucidation (Tressl et al., 1985;

1993; Hoffman and Schieberle, 1998; Frank et al, 2002). Drying, concentration and redissolution typically occur prior to analysis. In some instances, further purification or fractionation is required using preparative GC, flash chromatography or other methodologies (Tressl et al., 1985; 1993; Hoffman and Schieberle, 1998; Frank et al, 2002). Final analysis is generally achieved from HPLC/MS or GC/MS for mechanistic information, whereas NMR spectroscopy can be concomitantly used for structural elucidation (Tressl et al., 1985; 1993; Hoffman and Schieberle, 1998; Frank et al, 2002). Analysis of mechanistic pathways of formation of volatile compounds such as furan can also involve fewer preparative manipulations by favoring headspace analysis of the reaction mixture prior to GC-MS analysis as is the case of furan and acetic acid (Yaylayan, 2006; Davidek, 2005).

2.3.1 Isotope Labeling

Isotopically labeled precursors are highly valuable tools in establishing the mechanism of formation in complex mixtures (Yaylayan, 1997). Essentially, this method uses a molecule that had one or several of its original atoms replaced by its isotopic counterpart. The main isotopes used are ^{13}C, ^{15}N and ^{2}H. By reacting this modified molecule under the same conditions and with the same co-reactants as was done with the original precursor, the products generated can be scanned for label incorporation through the use of mass spectrometry. An increase in the mass of the fragments generated by each compound generally provides much insight as to the location of the isotope label in the final product. Although very advantageous, certain drawbacks including cost and availability of labeled reactants which limit the depth of the mechanistic studies. Understanding that the formation of a particular compound from a precursor can occur via more than one pathway, the information gathered from the use of only one singly labeled precursor can be restrictive. Therefore, the carbon module labeling (CAMOLA) approach was developped by Schieberle's group (2002) as a less expensive alternative to using individually labeled precursors. This method is based on the use of equimolar amounts of ubiquituously labeled carbohydrates and their completely unlabeled version, thus minimizing the use of labeled material. Observation of label incorporation via mass spectrometry provides solid information on the carbons involved, and whether the

compound of interest originates from an intact portion of the carbohydrate skeleton or is a result of fragment recombination. The possibility for characterization of transient intermediates involved is an additional advantage. This approach has been subsequently employed in a number of publications (Frank et al., 2002; Schieberle, 2005; Limacher et al., 2007).

Alternatively, the investigative approach used by Yaylayan's laboratory in conjunction with Py-GC/MS allows for the use of several independently labeled ^{13}C and ^{15}N-labeled precursors as the amounts required for experimentation are minimal (<2mg). Low temperature pyrolysis of mg amounts of precursors coupled to GC/MS allows for separation and identification of reaction intermediates and products simultaneous to observation of label incorporation. This methodology has been used for confirmation of Maillard precursors and elucidation of mechanistic pathways in numerous occasions (Yaylayan and Keyhani, 1996, 2000, 2001; Yaylayan and Wnorowski, 2001). The ease of the experimentation does not sacrifice on accuracy of results as much evidence has been provided by other groups showing the similarities of the mechanisms proposed and products generated by Py-GC/MS and that produced by the classical approach. In 2005, Davidek et al., used labeled glucose to identify the atoms involved in acetic acid formation using headspace analysis and GC/MS. The label incorporation data was nearly identical to that generated from Py-GC/MS (Wnorowski and Yaylayan, 2000). Both CAMOLA and Py-GC/MS approaches allow the study of isotopomers generated from thermal degradation of carbohydrates, recombination of their fragments or reactions with other components such as amino acids.

NMR spectroscopy in combination with isotope labeling has also been used as an essential tool when characterization of new compounds was necessary. Different groups used ^{13}C-labeling in conjunction with ^{13}C-NMR in the elucidation of two previously uncharacterized bitter tasting structures, quinizoloate and homoquinizolate (Frank et al., 2002; Schieberle, 2005). They observed "en bloc" joint transfer of several ^{13}C atoms from labeled pentoses. Recent studies conducted by Robert et al. (2005) used ^{17}O-NMR as a novel methodology for studying the mechanistic pathways involved in sugar

fragmentation by observing the exchange of ^{17}O from labeled water with oxygen-containing functional groups in dicarbonyls.

2.3.2 Ion Chromatography with Pulsed Amperometric Detection

Anion Exchange Chromatography (AEC) is a method commonly used for separation of anionic molecules as a function of their charge. The retention mechanism involves reversible adsorption of the anionic (or polar) species onto the cationic groups covalently bonded to the stationary phase. In order to enhance the anionic character of the acidic groups on the molecules of interest (hydroxyl groups of carbohydrates, sulfhydryl groups of proteins…), a basic mobile phase is required. The pH of the mobile phase buffer will therefore play a crucial role on the strength of the ionic interaction affecting the degree of separation. Proteins, endotoxins, nucleic acids and carbohydrates are common examples of solutes that can be separated or purified by AEC.

Pulsed amperometric detection (PAD) on the other hand is a type of electrochemical detection based on the electrical output of oxidation-reduction reactions at an electrode surface. More specifically, it operates on a series of multistep potential-time waveforms that are essential in preventing the inactivation of electrode. Under constant potential (DC), a number of species have the ability to stabilize the formed free radical as a result of their aromatic or vinylogous nature through resonance. However, many other species (including carbohydrates) cannot stabilize within the molecule and end up doing so by reacting irreversibly with the electrode itself. Accumulation of these carbonatious species results in “fouling” of the electrode, detected by a decrease in signal. The introduction of anodic and cathodic polarizations for desorbing carbonatious species and reactivation of the electrode has virtually eliminated this problem and is hence known as PAD, a useful detection tool for analysis of alcohols. The complexity of this form of detection lies mainly in establishing adequate waveform parameters (time and potential) for the species analyzed. Poor settings can easily lead to poor detection and electrode fouling. Papers focusing on optimization of the PAD according to the molecules of interest have been published, mainly regarding carbohydrates (Lacourse and Johnson, 1993). Other species that have the ability to be oxidized or reduced can also be detected by PAD. In some

instances, however, the surface oxide formation occurs simultaneously with the detection resulting in poor linearity. This is the case of amine-containing molecules. Quantitation therefore requires a different mode, known as Integrated Pulsed Amperometric Detection (IPAD) (Johnson and LaCourse, 1990). A thorough overview of these modes and descriptive overview of liquid chromatography with PAD is provided in a book by LaCourse (1997).

Coupling of AEC with PAD is of great value as the basic pH required for both processes simplifies the methodology. In the case of carbohydrate analysis, only one solvent system can be used eliminating the need for post column addition of NaOH. It also eliminates the need for derivatization, as in the case of GC or HPLC based approaches (Tang et al. 2007). The high pH is ideal for ionization of the acidic hydroxyl groups thus allowing for selective interaction with the cationic stationary phase. Detection with PAD subsequently follows by measuring the electrochemical current emitted from the oxidation of the carbohydrate at the gold electrode. The anodic and cathodic polarizations of the electrode will act to clean and regenerate the electrode from any free radical accumulation thus preventing fouling of the electrode. This approach is often being used in carbohydrate analysis (Cataldi et al., 2000; Tang et al., 2007).

2.3.3 Pyrolysis-GC/MS

Analytical pyrolysis is generally defined as the use of thermal energy in an inert atmosphere resulting in the rupture of chemical bonds. Consequently, the induced fragmentation leads to the formation of volatile and semi-volatile components (Irwin, 1982; Uden, 1993). Pyrolysis is commonly coupled to gas chromatography where it is used for both sample preparation and introduction, converting solid or liquid samples into volatile and semi-volatile components that are then directed by the carrier gas into the GC in analyzable form (Wampler, 1999). Furthermore, the type of pyrolyzer used can vary according to the goal as well as the state of the sample. For example, liquid samples are generally associated with ribbon probe pyrolysis where the sample is injected onto the probe. On the other hand, quartz tube pyrolysis is valuable for solid samples which are introduced inside a quartz tube. This mode promotes bimolecular interactions as the solid

sample migrates and fragments within the tube leading to primary and secondary reaction products prior to being directed into the GC column as opposed to the alternate ribbon probe in which volatiles are immediately swept away by the carrier gas not allowing further molecular interactions (Irwin, 1982, Yaylayan, 1999). Due to the nature of the experiments, quartz tube pyrolysis is the method of choice. Typically, pyrolysis temperatures ranging between 500ºC and 800ºC are applied for various purposes. Numerous applications of this methodology commonly include large scale removal of toxic volatiles from waste, as well as smaller scale studies on polymer breakdown or compound identification studies based on their fragmentation pattern. Forensics, polymers, adhesives and microorganisms are some of the areas using analytical pyrolysis (Wampler, 1999). With regards to obtaining mechanistic information pertaining to specific molecules, another, less explored application of pyrolysis is also being used. Instead of compound fragmentation and identification, quartz tube pyrolysis is employed as a micro-reactor in which precursors of thermally generated compounds of interest are being pyrolysed to instigate their formation (Yaylayan and Keyhani, 1996 and 2001; Yaylayan et al., 2003). In this case, interactions among the precursors and their intermediates are the primary focus as opposed to rapid degradation; lower temperatures ranging between 250ºC-350ºC are applied for these types of investigations.

Py-GC/MS has its place in providing valuable mechanistic information pertaining to the formation of food micro-components. Huyghues-Despointes and coworkers (1994, 1996) reacted D-glucose with proline under pyrolytic conditions and observed several similar Maillard compounds that had been previously generated under aqueous conditions by Tressl and coauthors (1993). Comparative studies between classical aqueous GC/MS and Py-GC/MS by Wnorowski and Yaylayan (2000) using glucose-glycine mixtures clearly demonstrated the relevance of pyrolysis to aqueous reaction systems. They showed that although pyrolysis leads to formation of a greater variety of compounds, most products formed in aqueous media were also formed from pyrolysis. Moreover, the use of isotope enriched precursors proved that identical components formed in both systems had matching label distribution patterns and therefore identical mechanisms of formation. Other experiments conducted by Yaylayan and Keyhani (2000) also showed consistent

patterns of label incorporation from thermally generated glucose-derived compounds using Py-GC/MS with those of Tressl et al. (1993, 1995) using classical aqueous conditions coupled with GC/MS. Therefore, Py-GC/MS in combination with isotope enriched precursors will be effective tools for mechanism elucidation of thermally generated toxic compounds.

CONNECTING PARAGRAPH

The author as a summer research assistant contributed to the study aimed at understanding the mechanism of formation of acrylamide from the amino acid asparagine (see Yaylayan, V.A., Wnorowski, A. and Perez-Locas, C. **2003**. Why Asparagine Needs Carbohydrates to Generated Acrylamide. Journal of Agricultural and Food Chemistry, 51, pp.1753-1757). In this chapter the role of other amino acids in the generation of acrylamide is investigated. Chapter 3 was published in *Advances in Experimental Medicine and Biology* (see Yaylayan, V.A., Perez-Locas, C., Wnorowski, A. and O'Brien, J. **2005**. Mechanistic Pathways of Formation of Acrylamide from Different Amino Acids. Editors: Friedman, M. and Mottram, D. In: Chemistry and Safety of Foods, Advances in Experimental Medicine and Biology 561. Springer, New York, USA, pp.191-203)

CHAPTER 3

MECHANISTIC PATHWAYS OF FORMATION OF ACRYLAMIDE FROM DIFFERENT AMINO ACIDS

3.1 Introduction

Preliminary studies (Stadler et al., 2002; Mottram et al., 2002) that followed the initial discovery of acrylamide in cooked food have lead not only to the unambiguous identification of asparagine as the main amino acid precursor of acrylamide, but also confirmed the origin of its carbon atoms and the amide nitrogen through labeling studies. Although thermal decarboxylation and deamination reactions (Yaylayan et al., 2003) of asparagine alone, in principle, can produce acrylamide, the presence of sugars was necessary to effect the conversion of asparagine into acrylamide. Subsequent studies (Becalski et al., 2003; Zyzak et al, 2003) have indicated that any carbonyl containing moiety can perform a similar transformation and that asparagine alone prefers to undergo intramolecular cyclization and form an imide rather than decarboxylate to form acrylamide. Studies related to the detailed mechanism (Yaylayan et al. 2003) of this transformation in model systems have indicated that decarboxylated Amadori product of asparagine is the key precursor of acrylamide. Furthermore, the decarboxylated Amadori product was shown to be formed under relatively mild conditions through the intramolecular cyclization of the initial Schiff base and formation of oxazolidin-5-one intermediate (Manini et al., 2001) and subsequent generation of a stable azomethine ylide which is prone to undergo an irreversible 1,2-prototropic shift (Grigg, 1989) to produce decarboxylated Schiff base and eventually decarboxylated Amadori product. Similar conclusions, using model food systems, were drawn by Zyzak et al. (2003) depicting direct decarboxylation of the Schiff base, but without invoking, oxazolidin-5-one as an intermediate. As part of our investigation of other sources of acrylamide in food and using Py-GC/MS as in integrated reaction, separation and identification system (Yaylayan, 1999) we have studied, in addition to selected α-amino acids, β-alanine and the dipeptide carnosine (N-β-alanyl-L-histidine) as potential sources of acrylamide.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

All reagents, chemicals and $^{15}NH_4Cl$ were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. The labeled [^{13}C-4]aspartic acid was purchased from Cambridge Isotope Laboratories (Andover, MA).

3.2.2 Pyrolysis-GC/MS analysis A Hewlett-Packard GC with Mass selective detector (5890 GC/5971B MSD) interfaced to a CDS pyroprobe 2000 unit was used for the Py-GC/MS analysis. One mg samples of pure reactants was introduced inside a quartz tube (0.3mm thickness), plugged with quartz wool, and inserted inside the coil probe with a total heating time of 20s. The column was a fused silica DB-5 column (50m length x 0.2mm i.d. x 0.33 μm film thickness; J&W Scientific). The pyroprobe interface temperature was set at 250°C. Capillary direct MS interface temperature was 280°C; ion source temperature was 180°C. The ionization voltage was 70 eV, and the electron multiplier was 2471V. All samples were injected in splitless mode. Three methods of analysis were used.

3.2.2.1 Method 1 had a delayed pulse of 65 psi followed by constant flow of 0.775mL/min, and a septum purge of 2 mL/min. The initial temperature of the column was set at 40°C for 2 minutes and was increased to 100°C at a rate of 30°C/min, immediately the temperature was further increased to 250°C at a rate of 8°C/min and kept at 250°C for 5 min.

3.2.2.2 Method 2 had a delayed pulse of 65 psi followed by constant flow of 1 mL/min, and a septum purge of 2 mL/min. The initial temperature of the column was set at -5°C for 2 minutes and was increased to 50°C at a rate of 30°C/min, immediately the temperature was further increased to 250°C at a rate of 8°C/min and kept at 250°C for 5 min.

3.2.2.3 Method 3 had a delayed pulse of 65 psi followed by constant flow of 1 mL/min, and a septum purge of 2 mL/min. The initial temperature of the column was set at -5°C for 2 minutes and was increased to 50°C at a rate of 8ºC/min, immediately the temperature was further increased to 100°C at a rate of 3°C/min followed by a increase to 250°C at a rate of 20 ºC/min and kept at 250°C for 5 min. The identity and purity of the chromatographic peaks were determined using NIST AMDIS version 2.1 software. The reported percent label incorporation values (corrected for natural abundance and for % enrichment) are the average of duplicate analyses and are rounded to the nearest multiple of 5%. Figure 3.2, shows examples of the three methods.

Table 3.1 Efficiency (area/mole of amino acid) of Acrylic Acid & Acrylamide Generation from 1 mg Samples of either Amino Acid or Amino Acid/Glucose (3:1) Mixtures Pyrolyzed at 350° C (data generated using method 1)

Model System	**Acrylic acid x 10^{12}**	**Acrylamide x 10^{12}**
Asparagine	not detected	trace
Asparagine/glucose	1.20 ± 0.18	5.18 ± 0.6
β-alanine	98.30 ± 0.35	14.4 ± 0.5
β-alanine/glucose	108.00 ± 7.10	13.9 ± 0.7
Carnosine	27.5 ± 6.9	12.86 ± 4.15
Carnosine/glucose	18.46 ± 1.08	5.11 ± 0.55
Aspartic acid	2.0 ± 0.08	0.18 ± 0.08
Aspartic acid/glucose	22.53 ± 2.95	0.53 ± 0.04
Cysteine	1.7 ± 0.1	trace
Cysteine/glucose	1.5 ± 0.1	trace
Serine	0.38 ± 0.01	not detected
Serine/glucose	0.68 ± 0.01	not detected

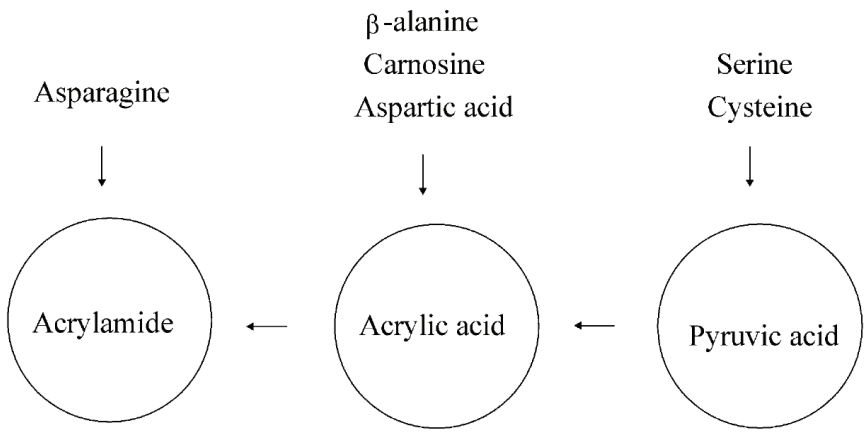


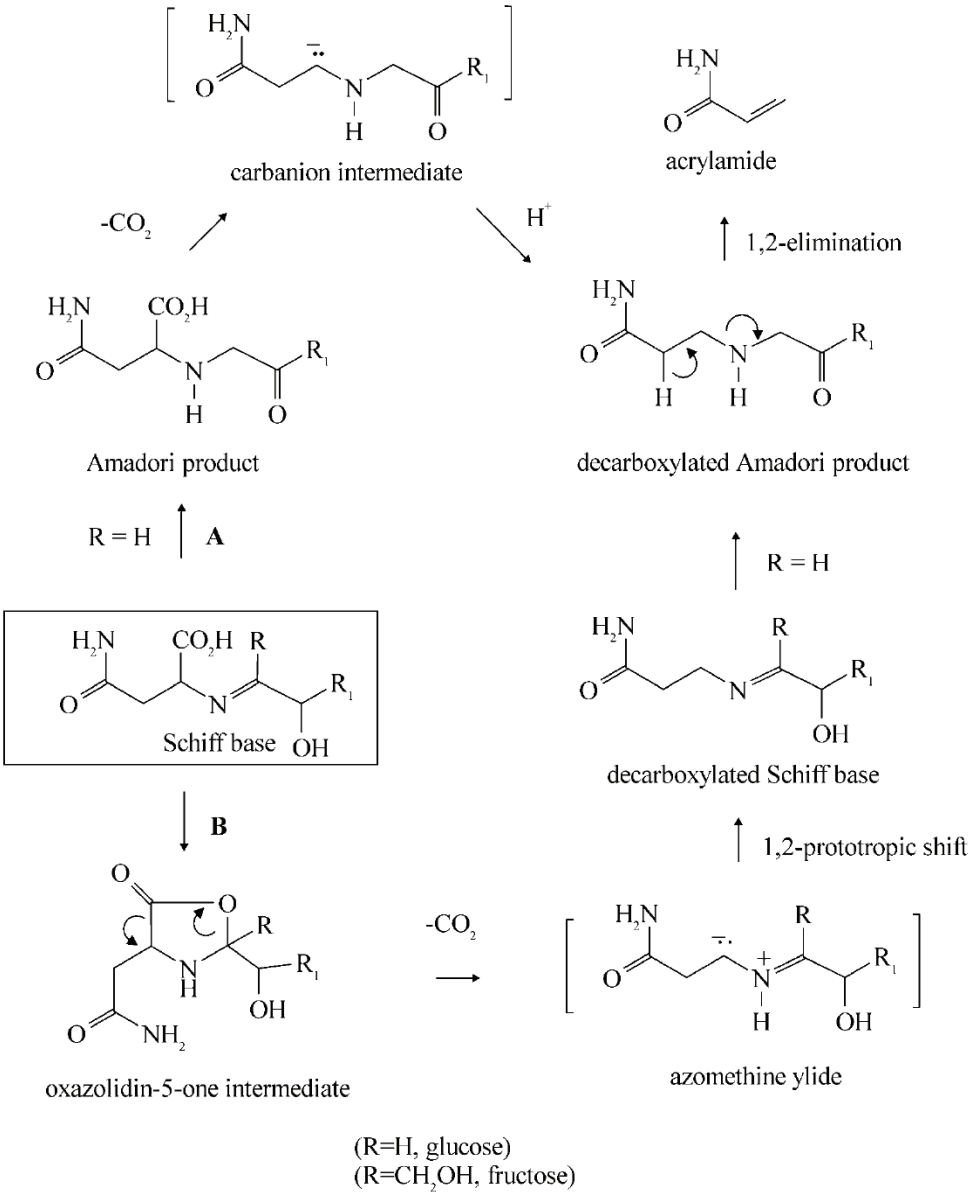
Figure 3.1 Precursors of Acrylamide

3.3 Results and Discussion

Studies with model systems containing selected amino acids and glucose (see Table 3.1) have indicated that there are two general pathways of acrylamide formation; a major pathway that generates acrylamide directly from asparagine and the second minor pathway that generates acrylamide though reaction of ammonia with acrylic acid (see Figure 3.1). Furthermore, studies have also indicated that acrylic acid itself can be generated either directly from certain amino acids or dipeptides such as carnosine, β-alanine and aspartic acid or indirectly from amino acids such as serine and cysteine, through reduction of pyruvic acid into lactic acid and its subsequent dehydration into acrylic acid.

3.3.1 Direct formation of Acrylamide from Asparagine

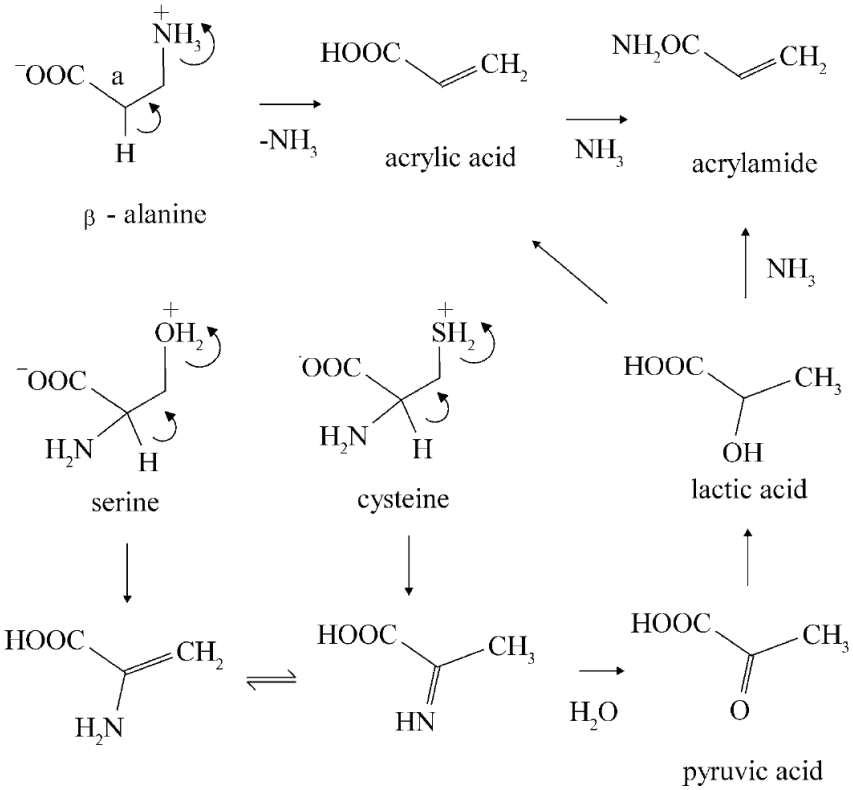
Asparagine is the only amino acid capable of directly generating acrylamide. Consequently it is considered the main source of acrylamide in food. The studies related to the detailed mechanism of this transformation have indicated that sugars and other carbonyl compounds play a specific role in the decarboxylation process of asparagine - a necessary step in the generation of acrylamide. It has been proposed (Yaylayan et al., 2003) that Schiff base intermediate formed between asparagine and the sugar provides a low energy alternative to the decarboxylation from the intact Amadori product through generation and decomposition of oxazolidin-5-one intermediate (Manini et al., 2001) leading to the formation of a relatively stable azomethine ylide (see Fig. 2). Literature data indicates the propensity of such protonated ylides to undergo irreversible 1,2-prototropic shift (Grigg et al., 1989) and produce, in this case, decarboxylated Schiff base which can easily rearrange into corresponding Amadori product. Decarboxylated Amadori products can either undergo the well known β-elimination process initiated by the sugar moiety to produce 3-aminopropanamide and 1-deoxyglucosone or undergo 1,2-elimination initiated by the amino acid moiety to directly generate acrylamide. On the other hand the decarboxylated Schiff intermediate can either hydrolyze and release 3-aminopropanamide or similarly undergo amino acid initiated 1,2-elimination to directly form acrylamide (Yaylayan and Stadler, 2004). However, their relative contribution to acrylamide formation is still under investigation.



Scheme 3.1 Mechanism of Formation of Acrylamide through Thermally-Induced Decarboxylation of intact Amadori Products (pathway A) and through Sugar-assisted Pre-Amadori Decarboxylation (pathway B)

3.3.2 Direct Formation of Acrylic Acid from β-Alanine, Carnosine and Aspartic Acid

Some amino acids can generate acrylic acid directly during their thermal decomposition. Such amino acids require the presence of ammonia to convert acrylic acid into acrylamide. One of the main sources of ammonia in food is the free amino acids. Sohn and Ho (1995) have identified asparagine, glutamine, cysteine and aspartic acid as the most efficient ammonia generating amino acids under thermal treatment.



Scheme 3.2 Proposed Mechanisms of Formation of Acrylamide from β-Alanine, Serine and Cysteine

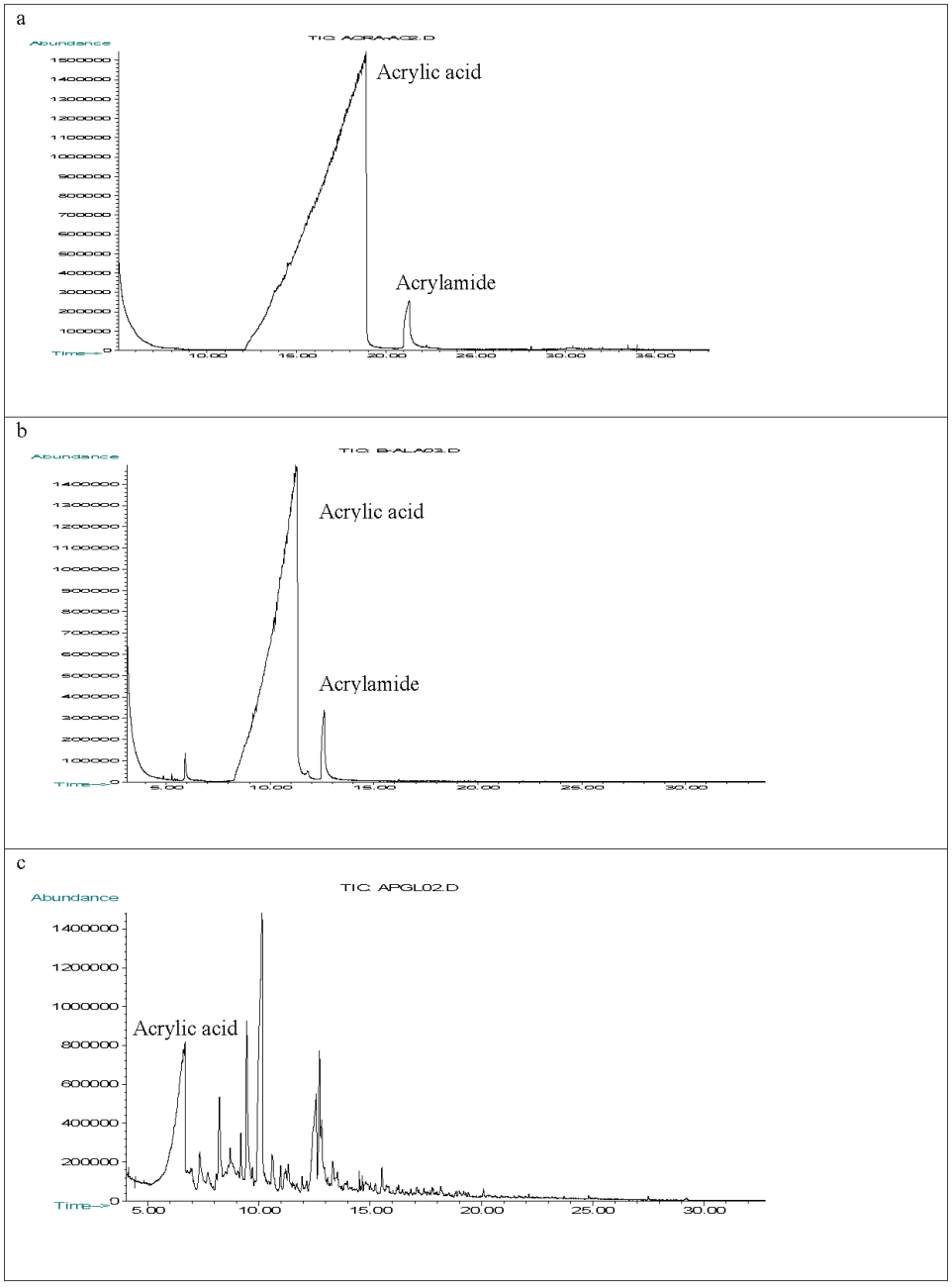
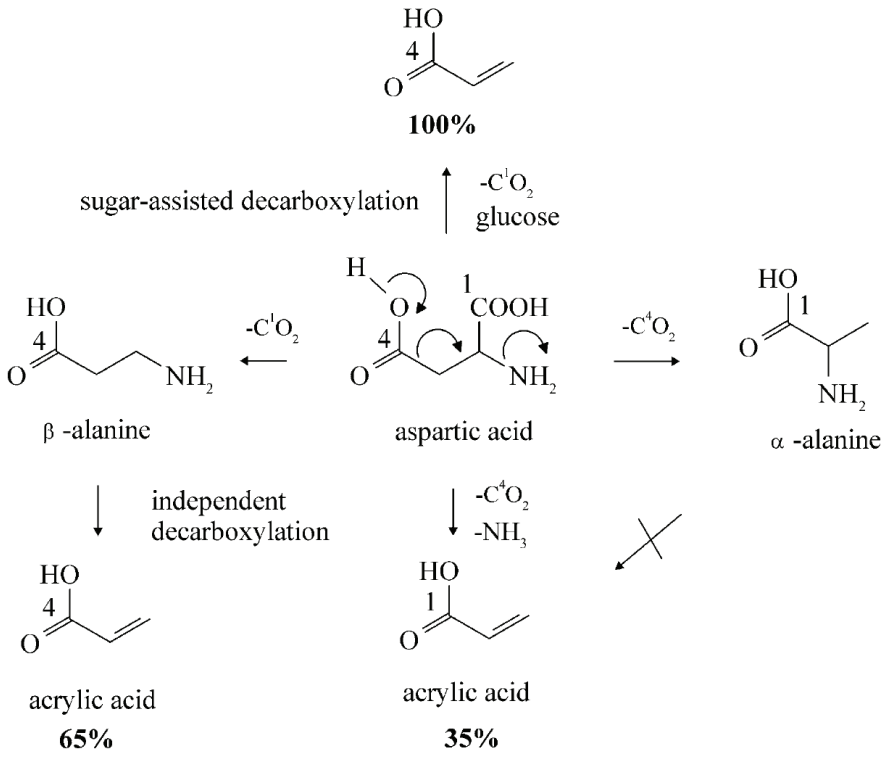


Figure 3.2 Pyrograms of Model Systems Consisting of Equimolar Mixtures of (a) Acrylic Acid/Ammonium Carbonate (using method 3) (b) β-Alanine Alone (using method 2) and (c) Aspartic acid/Glucose (using method 1)

3.3.2.1 Formation of Acrylamide from β-Alanine

The mechanism of decomposition of β-alanine to generate both reactants required for the formation of acrylamide, ammonia and acrylic acid, is shown in Scheme 3.2. Pyrolysis of β-alanine alone generated mainly acrylic acid and acrylamide, indicating deamination as a major pathway of thermal decomposition of β-alanine. The resulting acid can then interact with the available ammonia to form acrylamide (Fig. 3.2b). When (β -alanine was pyrolyzed in the presence of excess $^{15}NH_4C1$ the resulting acrylamide incorporated both the labeled (added) and unlabeled (generated from β-alanine) ammonia. Similarly, pyrolysis of commercial acrylic acid in the presence of an ammonia source (NH_4Cl, $(NH_4)_2CO_3$, etc.) also generated acrylamide (Figure 3.2a). Comparison of figures 3.2a and 3.2b indicates the efficiency of conversion of β-alanine into acrylic acid and ammonia. No significant change in the efficiency of β-alanine conversion into acrylamide was observed in the presence of glucose (see Table 3.1).



Scheme 3.3 Decarboxylation Pathways of Aspartic Acid based on Labeling Studies

3.3.2.2 Formation of Acrylamide from Aspartic Acid

Aspartic acid, on the other hand, can also form acrylic acid and subsequently acrylamide (Stadler et al., 2003; Yaylayan et al., 2004; Becalski et al, 2003) (Fig. 3.2c), but unlike β-alanine and similar to asparagine, it produces more acrylic acid in the presence of glucose (see Table 3.1). In order to identify the mechanism of acrylic acid formation from aspartic acid, [^{13}C-4]-aspartic acid was pyrolyzed alone and in the presence of glucose. According to Scheme 3.3, aspartic acid can undergo decarboxylation of either C-1 or C-4 carboxylate moieties. C-1 decarboxylation can generate β-alanine and C-4 decarboxylation can generate α-alanine as shown in Scheme 3.3. Unlike α-alanine, β-alanine is known to produce acrylic acid and consequently it was expected to observe 100% label retention in the acrylic acid mass spectrum when [^{13}C-4]-aspartic acid was pyrolyzed alone. However, analysis of the data showed the formation of 65% of labeled acrylamide and 35% unlabeled product (Figure 3.3c) indicating existence of a third pathway capable of formation of acrylamide with C-4 decarboxylation. A concerted mechanism where decarboxylation occurs simultaneously with deamination can explain the formation of unlabeled acrylic acid as shown in Scheme 3.3. Interestingly, when [^{13}C-4]-aspartic acid was pyrolyzed in the presence of glucose only 100% labeled acrylic acid was observed (Figure 3.3b), indicating preferential decarboxylation of C-1 carboxylate moiety consistent with the mechanism of sugar-assisted decarboxylation shown in Scheme 3.1. This observation, along with increased ability of aspartic acid to generate acrylamide in the presence of glucose (see Table 3.1), provides evidence for the ability of the Schiff base to provide a low energy pathway for decarboxylation of amino acids relative to decarboxylation from intact Amadori products that passes through a carbanion intermediate rather than the more stable azomethine ylide as shown in Scheme 3.1. Furthermore, similar to asparagine, reaction with sugar and formation of oxazolidine intermediate can prevent cyclization to form maleic anhydride (equivalent to succinimide in the case of asparagine) and enhance acrylic acid generation as observed.

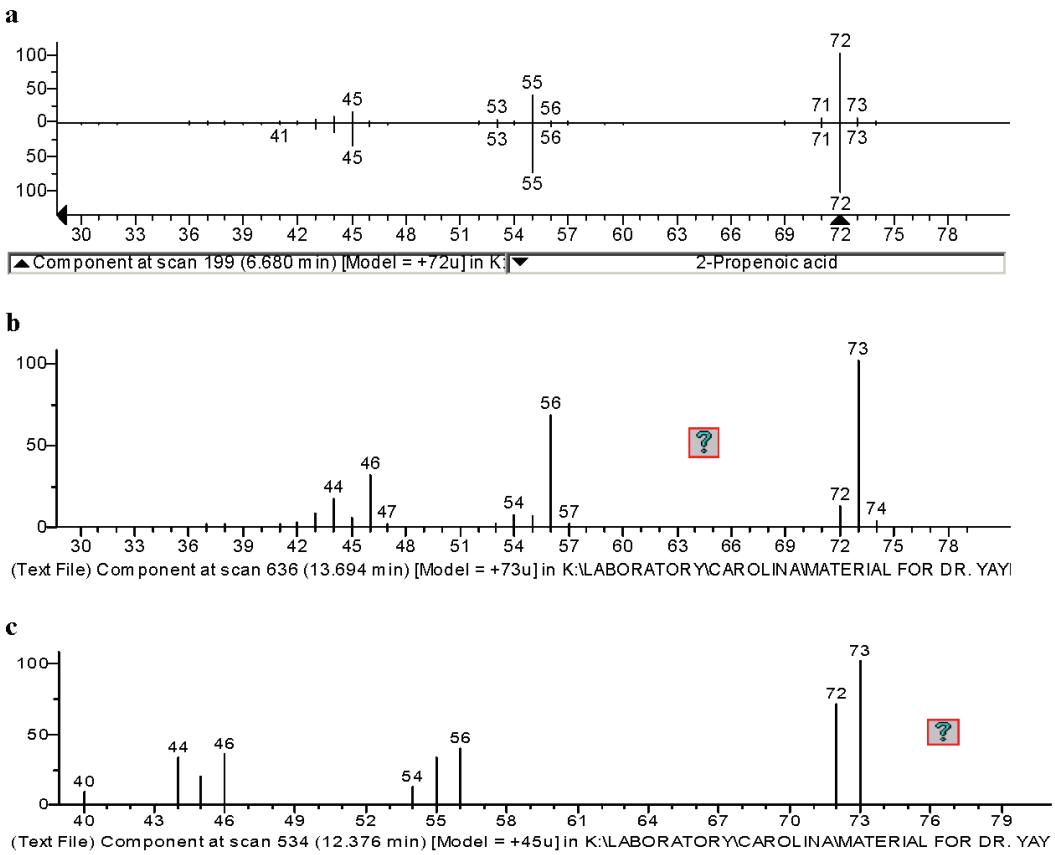
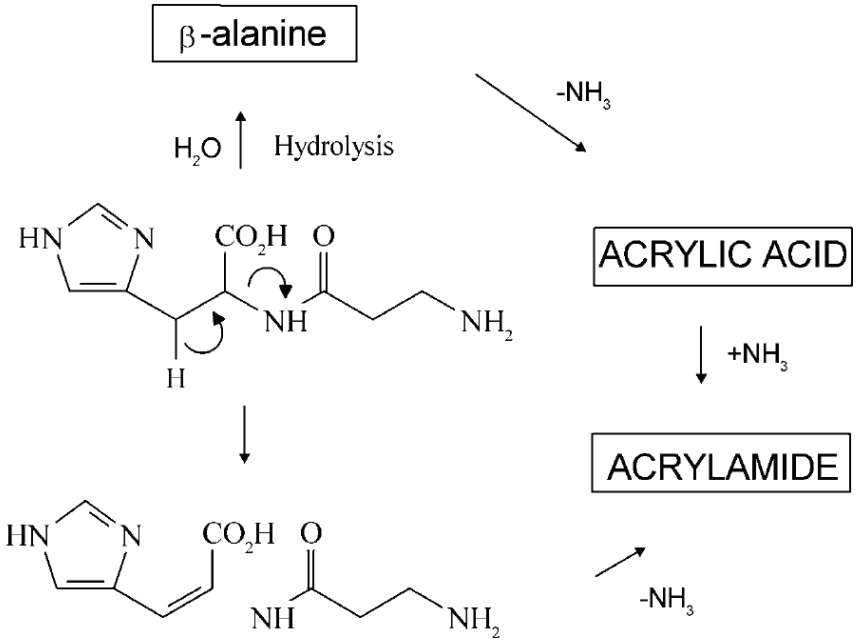


Figure 3.3 Mass Spectrum of (a) Acrylic Acid Generated from Unlabeled Aspartic Acid Compared with Authentic NIST Library Spectrum in Head to Tail Fashion. (b) Acrylic Acid Generated from [^{13}C-4]Aspartic Acid/Glucose Mixture (c) Acrylic Acid Generated from [^{13}C-4]Aspartic Acid Alone.

3.3.2.3 Formation of Acrylamide from Carnosine

The dipeptide carnosine (N-β-alanyl-L-histidine) when pyrolyzed alone produced acrylic acid and acrylamide in amounts higher than asparagine/glucose model system. However, in the presence of glucose the amounts became comparable (see Table 3.1) due to the interaction of carnosine with reducing sugars (Chen and Ho, 2002). Scheme 3.4 depicts two possible pathways of formation of acrylamide from carnosine, one through hydrolysis of the peptide bond and release of β-alanine and its subsequent deamination,

the second through release of 3-aminopropanamide and its deamination. However, the conspicuous absence of acrylamide in meat products at the scale expected to that of potatoes (Friedman, 2003) has lead us to investigate its possible fate in meat products using carnosine containing model systems. Carnosine was reacted in the presence of lysine (a reactive amino acid) and creatine (a major constituent of meat) and their effect on the amounts of acrylamide and its precursor acrylic acid was calculated. Lysine did not exert any significant effect on the formation efficiencies of acrylamide and acrylic acid. Creatine on the other hand, not only significantly reduced the acrylic acid content but also gave rise to two new potentially toxic (Hashimoto et al., 1981 & WHO, 1985) acrylamide derivatives; N-methylacrylamide and N,N-dimethylacrylamide. The decrease in acrylic acid formation can be explained by its accelerated conversion into acrylamide derivatives due to the efficient generation of ammonia and methylamines from added creatine (Yaylayan et al., 2004).



Scheme 3.4 Proposed Mechanism of Acrylamide Formation from Carnosine

3.3.3 Indirect Formation of Acrylic Acid from Serine and Cysteine

Dehydration of serine alone (see Scheme 3.2) and in the presence of sugars has been shown to generate pyruvic acid (Wnorowski and Yaylayan, 2003). Conversion of β-alanine into acrylic acid and release of acrylamide from decarboxylated Amadori product follow a similar mechanism of 1,2-elimination (see Schemes 3.1 & 3.2). Cysteine can also lose a hydrogen sulfide molecule to generate acrylic acid as shown in Scheme 3.2. Acrylic acid was detected along with pyruvic acid when serine was pyrolyzed at 350°C. This observation can be justified by proposing the reduction of pyruvic acid into lactic acid and its subsequent dehydration into acrylic acid. Model studies with lactic acid have indicated that such transformations are possible in the presence of ammonia; mixtures of lactic acid and ammonium salts produced lactamide, acrylic acid and acrylamide when pyrolyzed at 350°C.

3.4 Conclusion

Although, in theory, there are more than one amino acid that can generate acrylamide, however, the efficiency of the conversion of acrylic acid into acrylamide is limited by the availability of free ammonia in the vicinity of its production in the food matrix, in addition, this limitation is further compounded by the extreme volatility of ammonia at temperatures that are conducive to acrylamide formation. Recent studies (Stadler et al., 2003) have indicated that aspartic acid/ fructose mixtures generated acrylamide at levels 1000-fold below the levels measured for asparagines/fructose mixtures.

CONNECTING PARAGRAPH

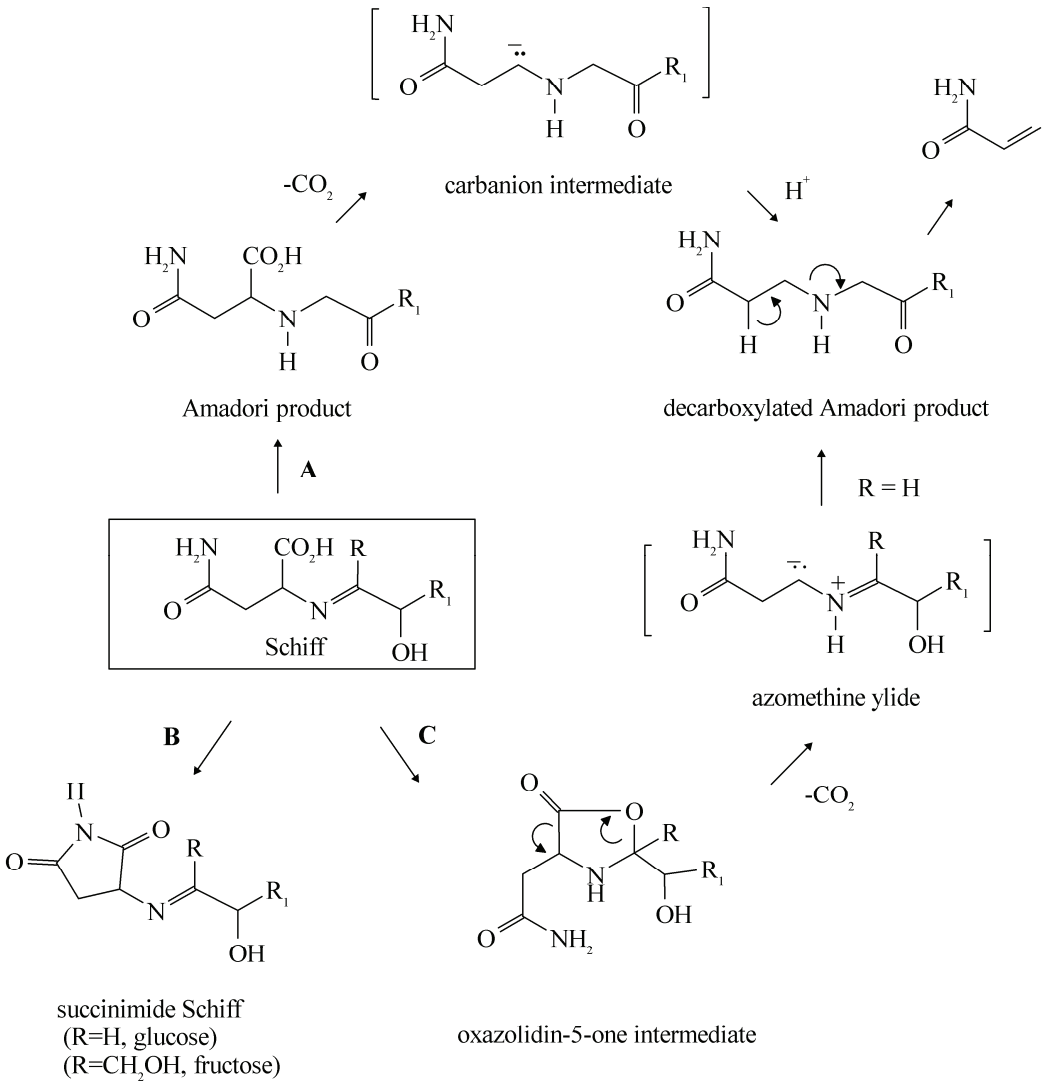
Chapter 3 provided detailed explanation on acrylamide formation from various amino acids found in foods. Carnosine was one of the amino acids studied. The unexpectedly high yields of acrylamide released from carnosine, an important amino acid in meat, led to the questioning of the reasons why meat itself has such low levels of acrylamide. Chapter 4 investigates the reaction of acrylamide with creatine and formation of two N-alkyl derivatives. Chapter 4 was published in the Journal of Agricultural and Food Chemistry. (see Yaylayan, V.A., Perez-Locas, C., Wnorowski, A. and O'Brien, J. **2004**. The Role of Creatine in the Generation of N-Methylacrylamide – A New Toxicant in Cooked Meat. Journal of Agricultural and Food Chemistry, 52, pp.5559-5565)

CHAPTER 4

THE ROLE OF CREATINE IN THE GENERATION OF N-METHYLACRYLAMIDE: A NEW TOXICANT IN COOKED MEAT

4.1 Introduction

Asparagine has emerged as the major amino acid responsible for the formation of acrylamide (Mottram et al., 2002) in food products heated at high temperatures in the presence of reducing sugars or carbonyl compounds (Stadler et al., 2002). Studies on the detailed mechanism (Yaylayan et al., 2003) of this transformation in model systems have indicated that the decarboxylated Amadori product of asparagine is the key precursor of acrylamide (Scheme 4.1). Furthermore, the decarboxylated Amadori product was shown to be formed under relatively mild conditions through the intramolecular cyclization of the initial Schiff base (Scheme 4.1, pathway C) and formation of the oxazolidinone intermediate (Manini et al., 2001). The low energy sugar-assisted decarboxylation of this intermediate makes it possible to bypass the thermally induced cyclization reaction (Scheme 4.1, pathway B), which is in competition with the high energy decarboxylation of intact Amadori product (Scheme 4.1, pathway A), and hence promote the formation of acrylamide in carbohydrate/ asparagine mixtures. Similar conclusions were drawn by other researchers (Zyzak et al., 2003; Stadler et al., 2003) using food systems and liquid chromatography/tandem mass spectrometry (LC/MS/MS) analyses. As part of our investigation of other sources of acrylamide in food and using pyrolysis (Py)-gas chromatography/mass spectrometry (GC/MS) as an integrated reaction, separation, and identification system (Yaylayan, 1999; Wnorowski and Yaylayan, 2000), we have studied, in addition to selected α-amino acids, β-alanine and the dipeptide carnosine (N-β-alanyl-L-histidine) as potential sources of acrylamide. Table 4.1 summarizes the efficiency of each amino acid alone and in the presence of glucose, to produce acrylamide and acrylic acid under pyrolytic conditions, expressed as chromatographic peak areas per mole of amino acid. According to this table, carnosine had a similar acrylamide-generating efficiency to asparagine in model systems containing reducing sugars.



Scheme 4.1 Mechanism of Formation of Acrylamide through Sugar-assisted Decarboxylation (pathway C) and Thermally-Induced Decarboxylation of Intact Amadori Product (pathway A).

Table 4.1 Efficiency (area/mole of amino acid) of Acrylic Acid & Acrylamide Generation from 1 mg samples of either Amino Acid or Amino Acid/Glucose (3:1) Mixtures Pyrolyzed at 350 ^{o}C.

Model system	**Acrylic acid x 10^{12}**	**Acrylamide x 10^{12}**
Asparagine	not detected	Trace
Asparagine/glucose	1.20 ±0.18	5.18 ±0.57
β-alanine	98.30 ±0.35	14.4 ±0.5
β-alanine/glucose	108.00 ± 7.10	13.9 ±0.7
Carnosine	27.5 ±6.9	12.86 ±4.15
Carnosine/glucose	18.46 ±1.08	5.11 ±0.55
Aspartic acid	2.0 ±0.08	0.18 ±0.08
Aspartic acid/glucose	22.53 ±2.95	0.53 ±0.04
Cysteine	1.7 ±0.1	Trace
Cysteine/glucose	1.5 ±0.1	Trace
Serine	0.38 ±0.01	not detected
Serine/glucose	0.68 ±0.01	not detected

[1] Average of duplicate analyses, using method 1

4.2 Materials and Methods

4.2.1 Chemicals and Reagents

All reagents and chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used without further purification. The labeled [^{13}C-4]aspartic acid was purchased from Cambridge Isotope Laboratories (Andover, MA), and creatine (methyl d_3) was purchased from CDN Isotopes (Pointe Claire, Quebec, Canada).

4.2.2 Py-GC/MS Analysis

A Helwett-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. One milligram samples of pure reactants or 6.8 mg of beef extract was introduced inside a quartz tube (0.3 mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed at 350°C with a total heating time of 20 s. The column was a fused silica DB-5

column (50 m length × 0.2 mm i.d. × 0.33 μm film thickness; J&W Scientific). The pyroprobe interface temperature was set at 250°C. The capillary direct MS interface temperature was 280°C; the ion source temperature was 180°C. The ionization voltage was 70 eV, and the electron multiplier was 2471 V. All injections were in the splitless mode. Three methods of analysis were optimized for specific model systems listed in Figure 4.1. Method 1 had a delayed pulse of 65 psi followed by a constant flow of 0.775 mL/min and a septum purge of 2 mL/min. Two modes of analysis were used, the scan and selected ion monitoring (SIM) modes. In the scan mode, the mass range analyzed was 33-650 amu, whereas in the SIM mode, only ions of masses 99, 98, 87, 85, 84, 72, 71, 61, 58, 55, 45, and 44 amu were monitored. The initial temperature of the column was set at 40 °C for 2 min and was increased to 100°C at a rate of 30°C/min; immediately, the temperature was further increased to 250°C at a rate of 8°C/min and kept at 250°C for 5 min. Method 2 had a delayed pulse of 65 psi followed by a constant flow of 1 mL/min and a septum purge of 2 mL/min. The mass range analyzed was 33-650 amu. The initial temperature of the column was set at -5 °C for 2 min and was increased to 50 °C at a rate of 30 °C/min; immediately, the temperature was further increased to 250°C at a rate of 8 °C/min and kept at 250 °C for 5 min. Method 3 had a delayed pulse of 65 psi followed by a constant flow of 1 mL/min and a septum purge of 2mL/min. The mass range analyzed was 33-650 amu. The initial temperature of the column was set at -5°C for 2 min and was increased to 50°C at a rate of 8°C/min; immediately, the temperature was further increased to 100°C at a rate of 3°C/min followed by a increase to 250°C at a rate of 20 °C/min and kept at 250°C for 5 min (Figure 4.1 shows examples of the three methods). The identity and purity of the chromatographic peaks were determined using NIST AMDIS version 2.1. The reported percent label incorporation values (corrected for natural abundance and for % enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

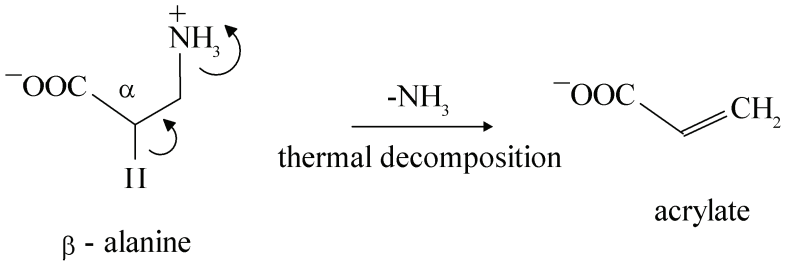
4.2.3 Extraction of N-Methylacrylamide from Beef

Beef cubes (obtained from a local store) were roasted in a domestic oven at 250°C for 30 min. A portion (28 g) of the roasted meat was homogenized and extracted by stirring for 2 h with 40% methanol/water (100 mL). After filtration, the filtrate was evaporated for 48 h under the fume hood and analyzed (after mixing 3.8 mg of the dry extract with 3 mg of

neutral alumina) with and without the addition of labeled creatine (methyl-d_3) by Py-GC/MS using method 1 in the SIM mode.

4.3 Results and Discussion

Studies on model systems containing selected amino acids and glucose (see Table 4.1) have indicated that there are in general two pathways of acrylamide generation from amino acids. The main pathway specifically involves asparagine (Scheme 4.1, pathway C) to directly produce acrylamide after a sugar-assisted decarboxylation step (Yaylayan et al., 2003). The second, nonspecific pathway involves the initial formation of acrylic acid from different sources and its subsequent interaction with ammonia to produce acrylamide. Aspartic acid, β-alanine, and carnosine follow the acrylic acid pathway.



Scheme 4.2 Proposed Mechanism of Deamination of β-Alanine

4.3.1 Formation of Acrylamide from β-Alanine.

The mechanism of decomposition of β-alanine generates both reactants required for the formation of acrylamide; ammonia and acrylic acid are shown in Scheme 4.2. The pyrolysis of β-alanine alone generated mainly acrylic acid and acrylamide, indicating deamination as a major pathway of thermal decomposition of β-alanine. The resulting acid can then interact with the available ammonia to form acrylamide (Figure 4.1b). When β-alanine was pyrolyzed in the presence of excess $^{15}NH_4Cl$, the resulting acrylamide incorporated both the labeled (added) and the unlabeled (generated from β-alanine) ammonia. Similarly, pyrolysis of commercial acrylic acid in the presence of an ammonia source [NH_4Cl, $(NH_4)_2CO_3$, etc.] also generated acrylamide (Figure 4.1a). A comparison of Figure 4.1a,b indicates the efficiency of conversion of β-alanine into acrylic acid and ammonia. No significant change in the efficiency of β-alanine conversion into acrylamide was observed in the presence of glucose (see Table 4.1).

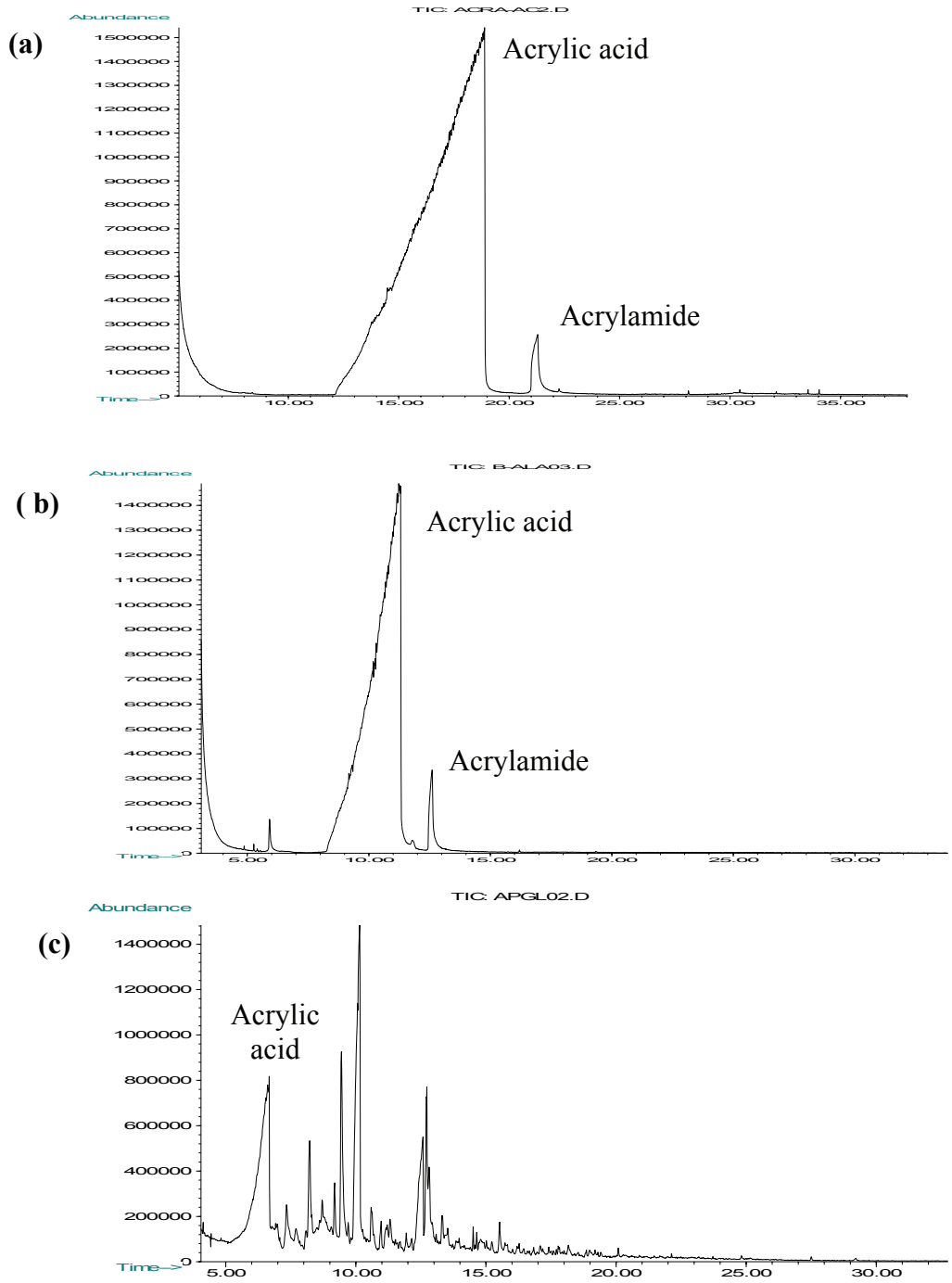
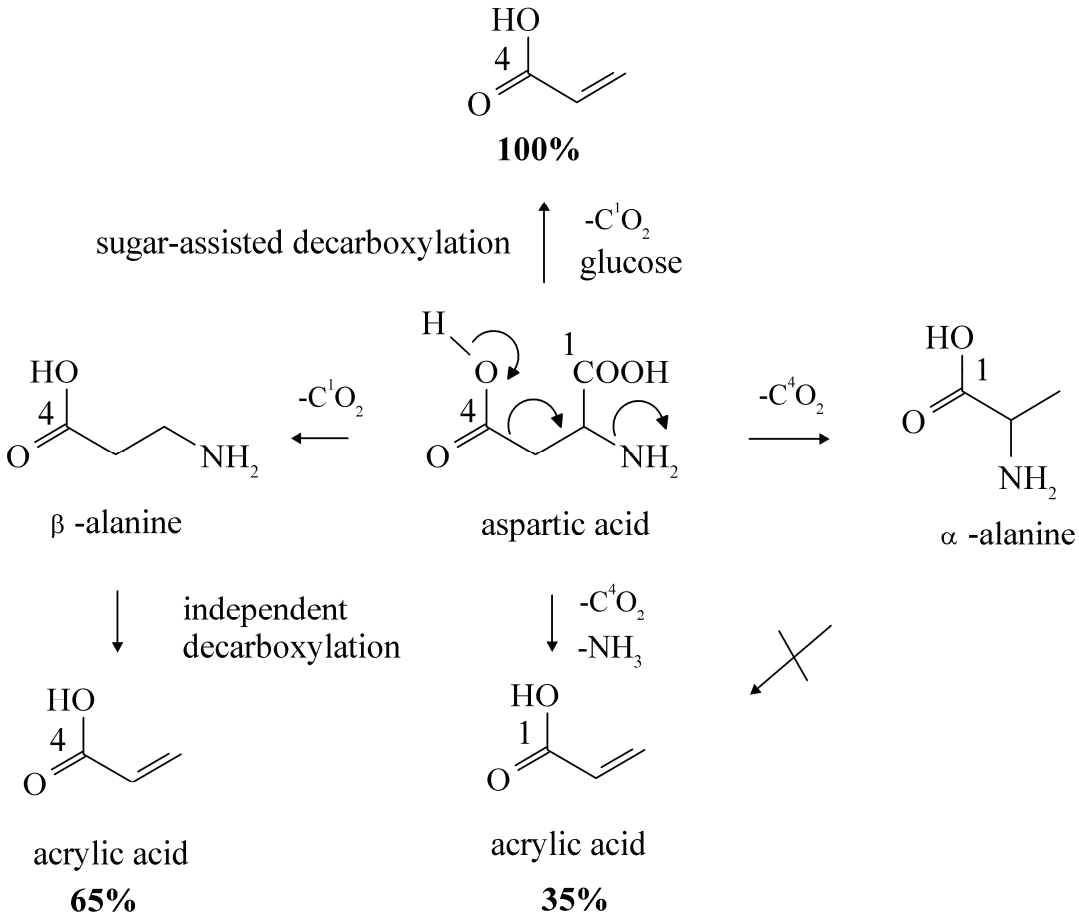


Figure 4.1 Pyrograms of Model Systems Consisting of Equimolar Mixtures of (a) Acrylic Acid/Ammonium Carbonate (using method 3) (b) β-Alanine Alone (using method 2) and (c) Aspartic Acid/Glucose (using method 1).

4.3.1 Formation of Acrylamide from Aspartic Acid.

Aspartic acid on the other hand can also form acrylic acid and subsequently acrylamide (Figure 4.1c), but unlike β-alanine and similar to asparagine, aspartic acid produces more acrylamide in the presence of glucose (Table 4.1). To identify the mechanism of acrylic acid formation from aspartic acid, [^{13}C-4]aspartic acid was pyrolyzed alone and in the presence of glucose. According to Scheme 4.3, aspartic acid can undergo decarboxylation of either C-1 or C-4 carboxylate moieties. C-1 decarboxylation can generate β-alanine, and C-4 decarboxylation can generate α-alanine as shown in Scheme 4.3. Unlike α-alanine, β-alanine is known to produce acrylic acid; consequently, it was expected to observe 100% label retention in the acrylic acid mass spectrum when [^{13}C-4]aspartic acid was pyrolyzed alone. However, analysis of the data showed the formation of 65% of labeled acrylamide and 35% unlabeled product (Table 4.2) indicating the existence of a third pathway capable of formation of acrylic acid with C-4 decarboxylation. A concerted mechanism where decarboxylation occurs simultaneously with deamination can explain the formation of unlabeled acrylic acid as shown in Scheme 4.3. Interestingly, when [^{13}C-4] aspartic acid was pyrolyzed in the presence of glucose, only 100% labeled acrylic acid was observed (see Table 4.2), indicating the preferential decarboxylation of the C-1 carboxylate moiety consistent with the mechanism of sugar-assisted decarboxylation shown in Scheme 4.1. This observation along with an increased ability to generate acrylamide in the presence of glucose (see Table 4.1) provide conclusive evidence for the ability of the Schiff base to provide a low energy pathway for decarboxylation of amino acids relative to decarboxylation from an intact Amadori product that passes through a carbanion intermediate rather than the more stable azomethine ylide as shown in Scheme 4.1. Furthermore, similar to asparagine, reaction with sugar and formation of the oxazolidinone intermediate can prevent cyclization to form maleic anhydride (equivalent to succinimide in the case of asparagine) and enhance acrylic acid generation as shown in Table 4.1.



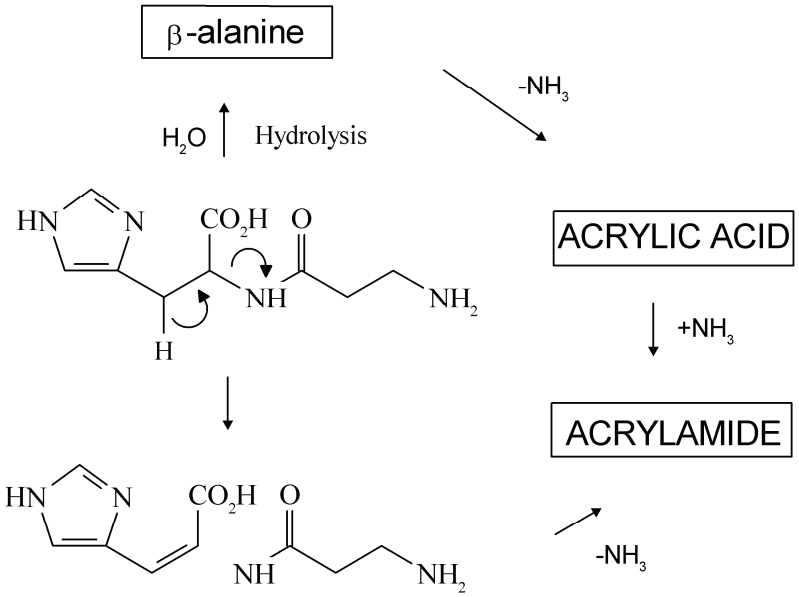
Scheme 4.3 Decarboxylation Pathways of Aspartic Acid

Table 4.2 Percent Label Distribution in Acrylic Acid Generated from Different Model Systems.

Model system	**M (m/z 72)**	**M+1 (m/z 73)**
Aspartic acid	100	0
[^{13}C-4]Aspartic acid	35	65
[^{13}C-4]Aspartic acid/Glucose	0	100

4.3.3 Formation of Acrylamide from Carnosine.

The dipeptide carnosine (N-β-alanyl-L-histidine) when pyrolyzed alone produced acrylic acid and acrylamide in amounts higher than the asparagine/glucose model system. However, in the presence of glucose, the amounts became comparable (see Table 4.1) due to the interaction of carnosine with reducing sugars (Chen and Ho, 2002). Scheme 4.4 depicts two possible pathways of formation of acrylamide from carnosine, one through hydrolysis of the peptide bond and release of β-alanine and its subsequent deamination and the second through elimination of β-alanine amide and its deamination. The conspicuous absence of acrylamide in meat products (Friedman, 2003) at the scale expected to that of potatoes has led us to investigate its possible fate in meat products using carnosine-containing model systems. Carnosine was reacted in the presence of lysine (a reactive amino acid) and creatine (a major constituent of meat), and their effects on the amounts of acrylamide and its precursor acrylic acid were calculated as shown in Table 4.3. Lysine did not exert any significant effect on the formation efficiencies of acrylamide and acrylic acid. Creatine,



Scheme 4.4 Different Pathways of Acrylamide Formation from Carnosine

on the other hand, not only significantly reduced the acrylic acid content but also gave rise to two new acrylamide derivatives: N-methylacrylamide and N,N-dimethylacrylamide. Both acrylamide derivatives appear to exhibit similar toxicological profiles to acrylamide, a known animal carcinogen and neurotoxin (WHO, 1985; Hashimoto et al., 1981). The decrease in acrylic acid formation can be explained by its accelerated conversion into acrylamide derivatives due to the efficient generation of methylamines from added creatine (Scheme 4.5).

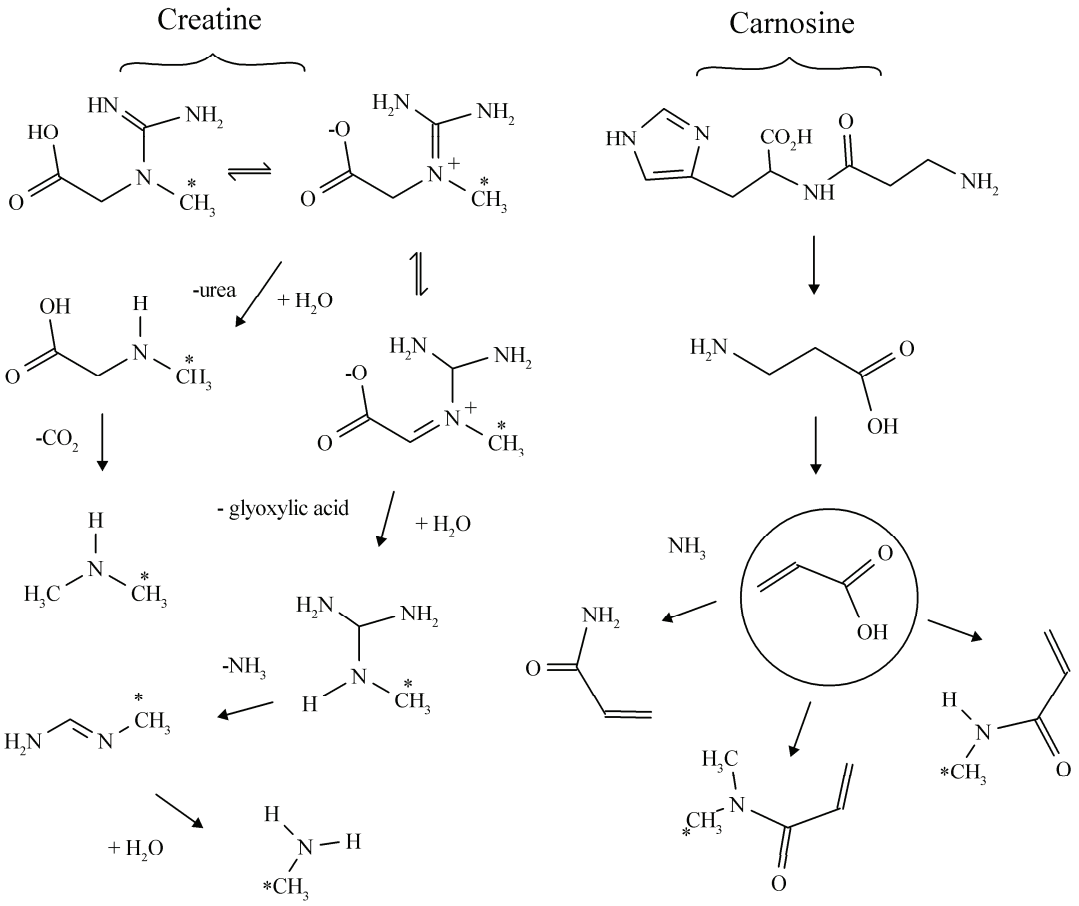
Table 4.3 Efficiency (peak area/mole of Carnosine) of Acrylic Acid & Acrylamide Generation from 1 mg Samples of Carnosine Alone and in the presence of Creatine or Lysine Pyrolyzed at 350 ^{o}C**.**

Model system	**Acrylic acid x 10^{12}**	**Acrylamide (AA) x 10^{12}**	**N-methyl-AA x 10^{12}**	**N,N-dimethyl-AA x 10^{5}**
Carnosine alone	13.8 ± 4.6	4.31 ± 1.3	not detected	not detected
Carnosine + Creatine	5.50 ± 0.6	6.42 ± 0.10	7.05 ± 0.10	6.6 ± 0.10
Carnosine + Lysine	16.9 ± 5.5	6.16 ± 2.2	not detected	not detected

[1] Average of duplicate analyses, using method 2.

4.3.4 Mechanism of Formation of N-Methylated Acrylamides in the Carnosine/Creatine Model System.

To identify the detailed mechanism of formation of N-methylated derivatives of acrylamide in the carnosine/creatine model system, carnosine was reacted with labeled creatine (methyl-d_3) under the same conditions in the presence and absence of excess labeled $^{15}NH_4Cl$ and label incorporation patterns were calculated for Nmethylacrylamide (see Table 4.4). According to these data, N-methylacrylamide incorporated 100% the N-methyl group of creatine and N,N-dimethyl acrylamide incorporated 100% one labeled N-methyl group and one unlabeled methyl group; both acrylamide derivatives did not incorporate any nitrogen atoms from the free ammonium chloride, indicating formation of methyl and dimethylamines directly from creatine. Figures 4.2a,b shows the mass



Scheme 4.5 Proposed Mechanism of Generation of Methyl and Dimethylamines from Creatine and Formation of N-Methylated Acrylamides

spectra of N-methylacrylamide generated from labeled and unlabeled model systems. The label incorporation patterns can be explained by proposing a reaction between acrylic acid (generated from carnosine) and methylamine or dimethylamine, both generated from creatine (see Scheme 4.5). The consumption of creatine by human subjects also significantly increased their urine levels of methylamine, indicating the existence of a metabolic equivalent of this transformation in human biochemistry (Mitchell and Zang, 2001). The proposed pathway of generation of methyl and dimethylamines from creatine is shown in Scheme 4.5. According to this figure, creatine in its imminium zwitterionic form can undergo hydrolysis to release urea and N-methylglycine, which after

decarboxylation can generate dimethylamine. Alternatively, the zwitterionic form can undergo isomerization followed by hydrolysis to generate glyoxylic acid and N-methyl-triaminomethane. This unstable intermediate can undergo deamination followed by hydrolysis to produce methylamine as shown in Scheme 4.5. Alternatively, it can undergo hydrolysis to produce N-methylformamide, which after oxidation and decarboxylation steps can also generate methylamine. This proposed pathway was verified by reacting creatine with acrylic acid and acrylamide separately. The reaction of acrylic acid with creatine generated acrylamide, N-methylacrylamide (major product), and N,N-dimethylacrylamide, whereas the acrylamide reaction with creatine generated none of the methylated products, confirming the proposed pathway.

Table 4.4 Percent Label Distribution in N-Substituted Acrylic Acids Generated from Isotopically Enriched Model Systems

Model System	Compound	M	M+1	M+3	M+4
Carnosine/Creatine (methyl-d3)	MA[1]	0	0	100	0
Carnosine/Creatine (methyl-d3)	DMA[2]	0	0	100	0
Carnosine/Creatine/$15NH_4Cl$	MA	100	0	0	0
Carnosine/Creatine/$15NH_4Cl$	DMA	100	0	0	0

[1]MA = N-methylacrylamide; [2]DMA = N,N-dimethylacrylamide

4.3.5 Detection of N-Methylacrylamide in Heated Beef Samples.

Py-GC/SIM-MS analysis of extracts prepared from heated beef at 250°C have indicated the presence of trace amounts of acrylic acid and acrylamide but have exhibited a significant intensity of the peak identified as N-methylacrylamide (see Figures 4.2c and 4.3). When the same extract was also analyzed after the addition of excess creatine-d_3, the peak identified as N-methylacrylamide showed 60% label incorporation. When sodium acrylate was pyrolyzed in the presence of methylamine hydrochloride, a peak was generated at the same retention time as the proposed N-methylacrylamide peak and had an identical mass spectrum. In addition, the following acrylamide derivatives N,N-dimethylacrylamide, N,N′-methylene-bis-2-propenamide, and tetrahydrofurfuryl acrylate were also detected in trace amounts.

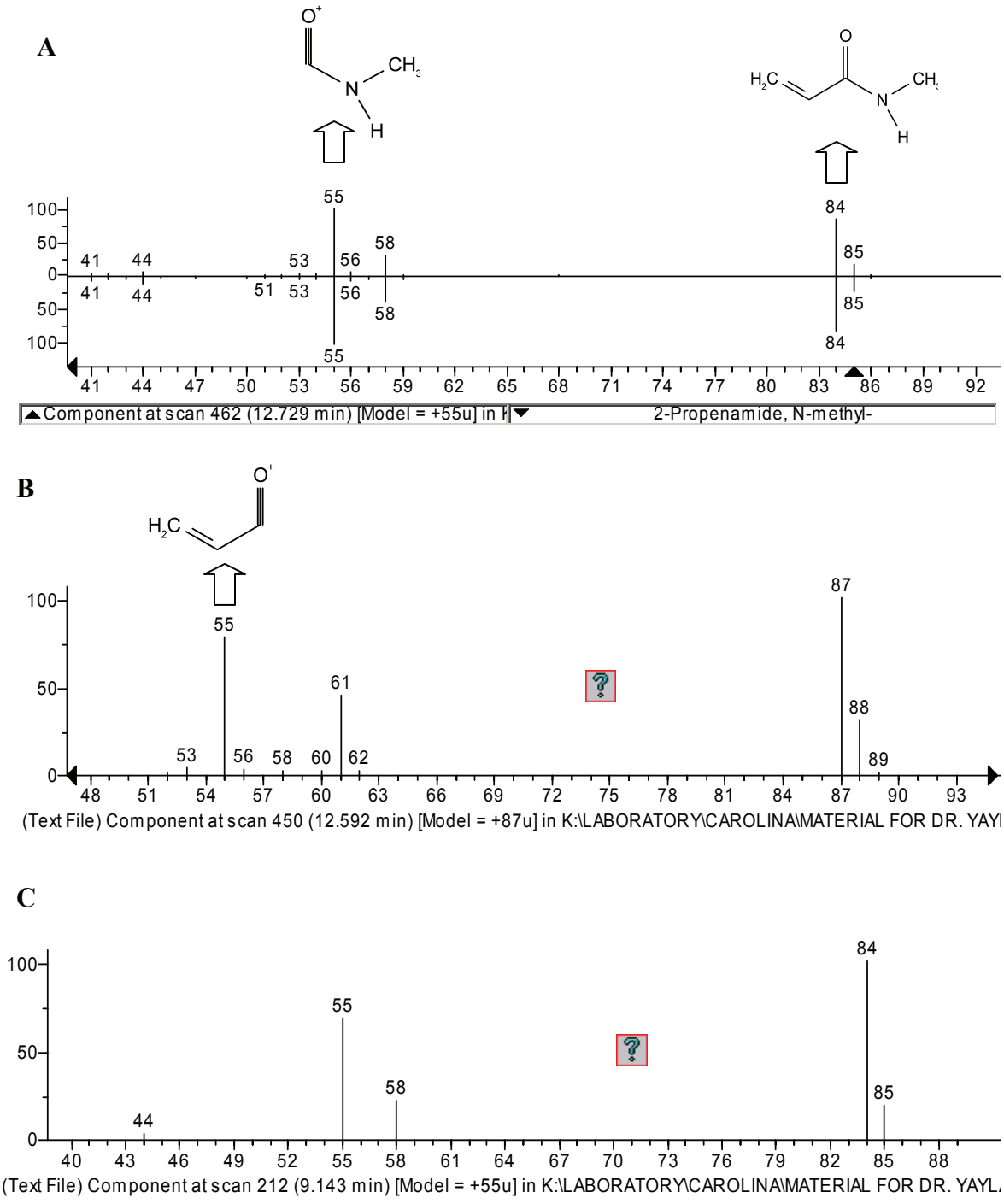


Figure 4.2 Mass Spectrum of (a) N-methylacrylamide generated from Unlabeled Creatine/Carnosine Model System compared with Authentic NIST Library Spectrum in Head to Tail Fashion. (b) N-Methylacrylamide Generated from Creatine Methyl-d_3/Carnosine Mixture. (c) N-Methylacrylamide Generated from Meat Extract.

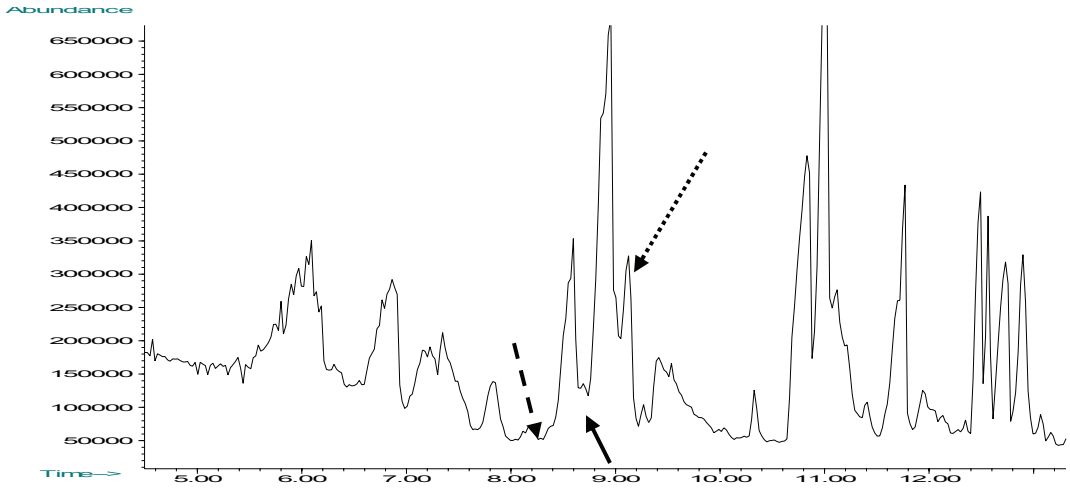


Figure 4.3 Partial Pyrogram of Meat Extract.

Dotted arrow (t = 9.14 min) indicates N-methylacrylamide peak (mass spectrum shown in Figure 4.2c), dashed arrow indicates acrylic acid peak and the block arrow indicates acrylamide peak

4.4 Conclusion

In conclusion, considering the relatively high detection limit of the method employed in measuring acrylamide derivatives in meat samples, the results obtained have provided enough evidence to speculate that levels of N-methylacrylamide in cooked meat could be as high as acrylamide levels in potato products. Further studies are needed to quantify the levels of N-methylacrylamide in different meat-related consumer products to assess the risk factors associated with its consumption.

CONNECTING PARAGRAPH

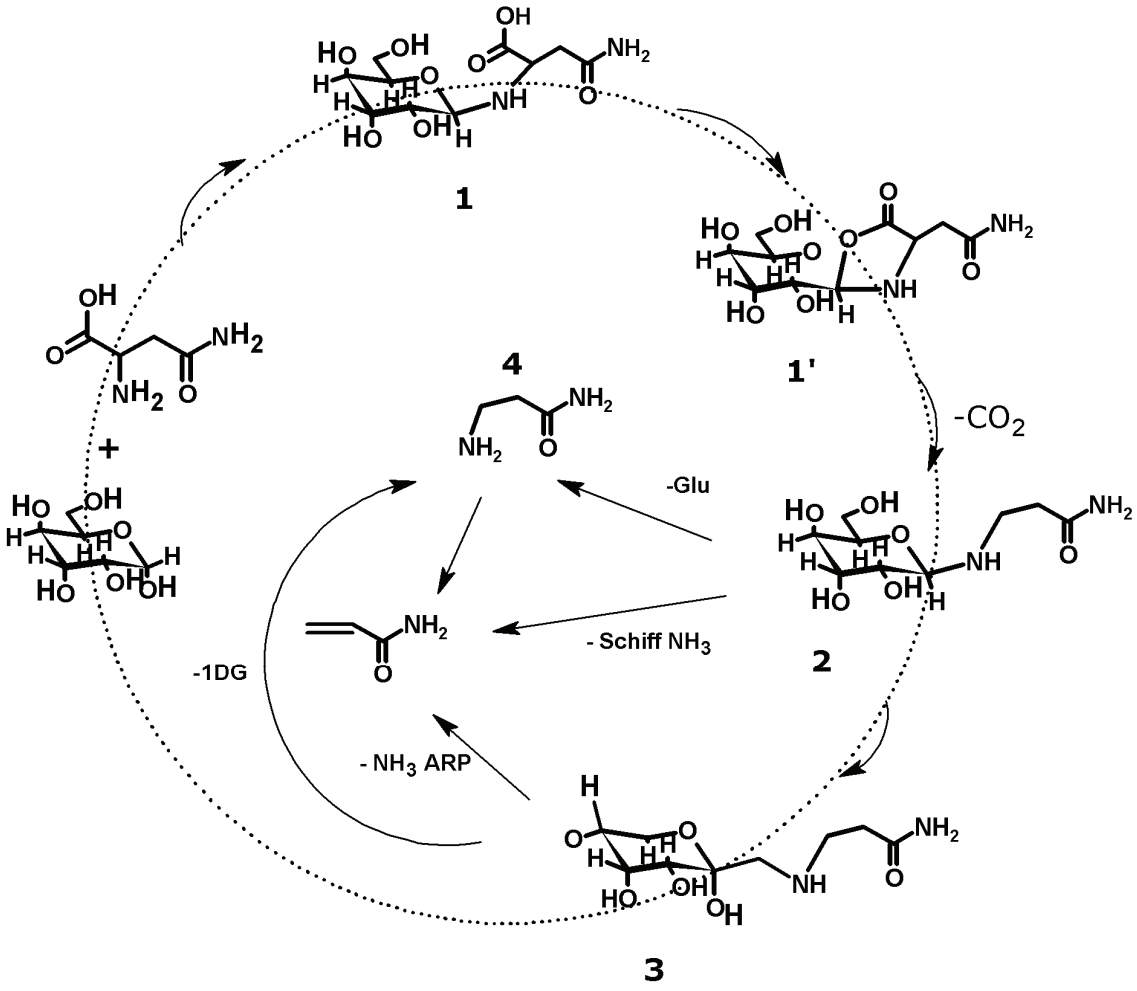
Chapters 3 & 4 were focused on acrylamide formation from various amino acids including the dipeptide carnosine. Chapter 5 takes an in depth look at the detailed mechanism of acrylamide formation specifically the relative importance of various intermediates including the oxazolidin-5-one, providing additional evidence for its formation. The content of chaper 5 will appear in the Journal of Agricultural and Food Chemistry (see Perez-Locas, C. and Yaylayan, V.A. **2008**. Further Insight into Thermal and pH Induced Generation of Acrylamide from Glucose/Asparagine Model Systems. Journal of Agricultural and Food Chemistry. Accepted for publication)

CHAPTER 5

FURTHER INSIGHT INTO THERMAL AND PH INDUCED GENERATION OF ACRYLAMIDE FROM GLUCOSE/ASPARAGINE MODEL SYSTEMS

5.1 Introduction

Although the general features of the mechanism of formation of acrylamide from asparagine and reducing sugars have been confirmed through different studies (Stadler et al., 2002; Granvogl et al., 2006; Stadler et al., 2004; Yaylayan et al., 2003; Zyzak et al., 2003), however, the detailed knowledge regarding the relative importance of the different precursors of acrylamide such as the decarboxylated glycosyl amine, its corresponding Amadori product and 3-aminopropionamide or the mechanism of decarboxylation of the asparagine are still lacking (Scheme 5.1). It is well documented that in a glucose/asparagine system, the glucos-1-yl-asparagine **(1)** undergoes decarboxylation prior to its rearrangement into Amadori product, to generate N-(D-glucos-1-yl)-3'-aminopropionamide **(2)**, this intermediate in turn can undergo Amadori rearrangement to produce N-(1-deoxy-D-fructos-1-yl)-3'-aminopropionamide **(3)**. Both aminopropionamide intermediates **(2 & 3)** are capable of directly generating acrylamide or through formation of free 3-aminopropionamide **(4).** The relative importance of these three possible routes to acrylamide is not well understood. This fact constitutes an important hindrance towards a complete understanding of the mechanistic details of acrylamide formation. In addition, no direct evidence has been presented for the mechanism of decarboxylation of glucos-1-yl-asparagine **(1)** through 5-oxazolidinone intermediate **1'** (Yaylayan et al., 2003). Decarboxylation through such intermediate can generates azomethine ylides (Tsuge et al., 1987) whose stability can determine the ease of decarboxylation and hence the ease of acrylamide formation and consequently, different carbonyl sources or sugars can provide azomethine ylides with differing stabilities and hence different abilities to generate acrylamide. In this study, for the first time, we provide evidence for the formation of 5-oxazolidinone intermediate in amino acid/carbohydrate systems. In addition, the relative abilities of the precursors **2, 3 & 4** shown in Figure 5.1 to generate acrylamide was studied under dry and wet conditions to evaluate their contribution to the total acrylamide formation. Calibration and retention time data is presented in Appendix A.



Scheme 5.1 Proposed Reactive Intermediates Involved in the Generation of Acrylamide
Glu: glucose; ARP: Amadori Rearrangement Product; 1DG: 1-deoxy-glucosone

5.2 Materials and Methods

5.2.1 Reagents and Chemicals

All reagents, chemicals and deuterated NMR solvent (CH_3OD) were purchased from Aldrich Chemical company (Milwaukee, WI) and used without further purification. The 3-amino-propionamide hydrochloride salt was purchased from Chem Impex International (Wood Dale, IL, USA). The [^{13}C-1] and [^{15}N] labeled phenylalanines were purchased from Cambridge Isotope Laboratories (Andover, MA). ^{13}C-NMR spectra were acquired on a 500 MHz Varian Unity spectrometer.

5.2.2 Synthesis of N-(D-glucos-1-yl)-3'-aminopropionamide (2).

A modified procedure for synthesis of N-glycosides of amino acids as reported by Stadler et al., (2004) was followed. The 3-amino-propionamide hydrochloride (0.2g) was dissolved in methanol (8mL) and excess KOH (0.12 g) was added and the solution was stirred for 10 min and filtered to remove the precipitated KCl. Slight excess of glucose (0.32g) was then added to the filtrate and heated in an open beaker at 110^{o}C for 30 min to help remove the water, replenishing evaporated methanol when needed. The solution was transferred into a round bottomed flask and refluxed for another 30 min. The methanol was evaporated in vacu and the resulting oil was analyzed by NMR, FTIR, Py-GC/MS and by ion chromatography which indicated a purity of > 80% the residue being unreacted excess glucose. Py-GC/MS generated mainly acrylamide as a major volatile product (85% of the total area). FTIR (cm^{-1}) 3300 s (OH); 2939m, 2834m (alkyl); 1665 s (amide I); 1612 m (amide II), 1077s 1013 s (sugar C-OH). ^{13}C NMR (500 MHz, CH_3OD): δ 175.4 ($CONH_2$, $C_{1'}$); 34.2 (CH_2CONH_2, $C_{2'}$); 40.6 (NH_1CH_2, $C_{3'}$); 89.1 (C_1); 69.2 (C_2); 72.1 (C_3); 76.2 (C_4); 75.4 (C_5); 60.3 (C_6).

5.2.3 Synthesis of N-(1-deoxy-D-fructos-1-yl)-3'-aminopropionamide (3).

The 3-amino-propionamide hydrochloride (0.2g) was dissolved in methanol (8mL) and KOH (0.03g) was added to adjust the pH to around 6 and the solution was stirred for 10 min and filtered to remove any precipitated KCl. Slight excess glucose (0.30g) was added to the filtrate and heated in an open beaker at 110^{o}C for 30 min to help remove the water, replenishing evaporated methanol when needed. The solution was transferred into a round bottomed flask and refluxed for another 40 min. The methanol was evaporated and the resulting oil was analyzed by NMR, FTIR, MS, Py-GC/MS and ion chromatography which indicated a purity of >85% residue being mainly excess glucose. Py-GC/MS generated mainly acrylamide as a major volatile product (75%). FTIR (cm^{-1}) 3313 s (OH); 2929m, 2838m (alkyl); 1728w (keto sugar); 1669 s (amide I); 1618 m (amide II), 1073 s 1015 s (sugar C-OH). ^{13}C NMR (500 MHz, CH_3OD)): δ 172.3 ($CONH_2$, $C_{1'}$); 34.4 (CH_2CONH_2, $C_{2'}$); 29.4 (NH_1CH_2, $C_{3'}$); 52.9 (C_1); 95.5 (C_2); 69.1 (C_3); 70.4 (C_4); 71.2 (C_5); 62.5 (C_6).

5.2.4 Pyrolytic Generation of Acrylamide (Dry Heating).

A Helwett-Packard GC with Mass selective detector (5890 GC/5971B MSD) interfaced to a CDS pyroprobe 2000 unit was used for the Py-GC/MS analysis. One mg samples of reactants were introduced inside a quartz tube (0.3mm thickness), plugged with quartz wool, and inserted inside the coil probe with a total heating time of 20s. The column was a fused silica DB-5 column (50m length x 0.2mm i.d. x 0.33 μm film thickness; J&W Scientific). The pyroprobe interface temperature was set at 250ºC. Capillary direct MS interface temperature was 280ºC; ion source temperature was 180ºC. The ionization voltage was 70 eV, and the electron multiplier was 2471 V. All injections were in splitless mode. The mass range analyzed was 33-650 amu. The initial temperature of the column was set at -5ºC for 2 minutes and was increased to 50ºC at a rate of 30ºC/min, immediately the temperature was further increased to 250ºC at a rate of 8ºC/min and kept at 250ºC for 5 min. The identity and purity of the chromatographic peaks were determined using NIST AMDIS version 2.1. The reported percent label incorporation values (corrected for natural abundance and for % enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

5.2.5 Thermal Generation of Acrylamide in the Presence of Water.

Acrylamide precursors (10mg) were mixed with water and alumina (50% w/w moisture) in a 10 mL flame sealed glass ampoule and heated in an oven at 170^{o}C for 20 min. The samples were diluted with water (100mL) and analyzed by ion chromatography as described below.

5.2.6 Quantification of Acrylamide by Ion Chromatography.

A Metrohm MIC-8 modular IC system (Herisau, Switzerland) consisting of a pulsed amperometric detector (E_1 = 0.15 v, t_1 = 400 ms, E_2 = 0.75v, t_2 = 200ms; E_3 = -0.15v, t_3 = 400ms), pump and a sample injection unit connected to Metrosep Carb1-150 anion exchange column thermostated at 31^{o}C was used for the analysis of acrylamide and its precursors. The mobile phase was 0.1N NaOH and flow rate of 1 mL/min. Calibration curve for acrylamide was constructed by injecting serially diluted solutions in the range of 1-20 ppm concentrations.

5.2.7 Generation and FTIR Analysis of 5-Oxazolidinone.

An equimolar mixture (10mg) of sugar and the amino acid was heated in toluene (2 mL, methanol and or *p*-toluene-sulfonic acid could be added to help dissolve insoluble models) for 10 min or until most reactants dissolved at 115^{o}C in an open vial. The solution was passed immediately through a glass wool and the sample (5 μL) was applied on the ATR crystal and scanned after evaporation of the solvent. Infrared spectra were recorded on a Nicolet 380 FTIR spectrometer (Thermo Electron Corporation (Madison, WI) equipped with a single bounce ATR sampling unit. A total of 64 scans at 4 cm^{-1} resolution were co-added. Processing of the FTIR data was performed using GRAMS/32 AI (ThermoGalactic). Second-order derivatization was performed using Savitsky-Golay function (30 points) to enhance closely absorbing peaks.

5.2.8 Browning Measurement by UV/VIS.

An equimolar solution of reactants (0.03M each) in dimethyl sulfoxide was stirred in the presence and absence of dimethyl fumarate (0.03M) at 80^{o}C for 30min. The solution was cooled and browning was measured by scanning between 360-830 nm using Evolution 300 scanning spectrophotometer from Thermo Electron Corporation (Madison, WI). CIE chromaticity coordinates were calculated using ACD/Labs SpecManager version 8.2 (Toronto, Canada).

5.2.9 Effect of pH on Acrylamide Generation from N-(1-deoxy-D-fructos-1-yl)-3'-aminopropionamide.

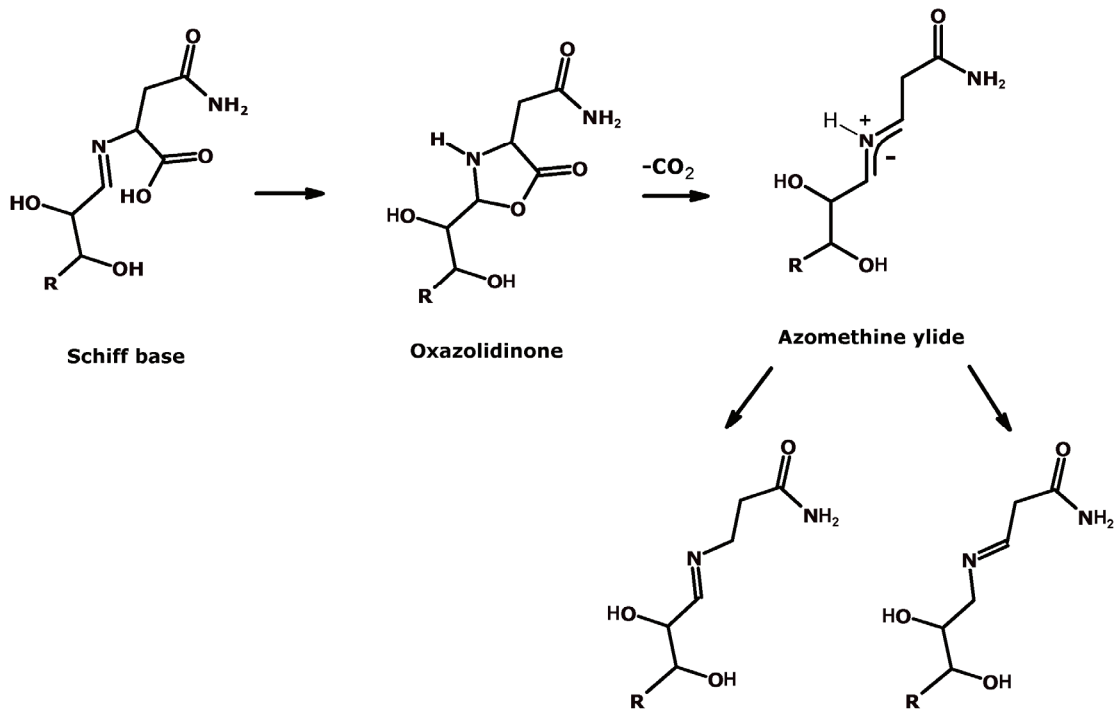
Alumina (50mg) was mixed with 3-AP ARP (20mg), glucose (30mg) and water (9 μL). The mixture was divided into four portions and heated in separate 10 mL flame sealed glass ampoules in an oven at 165^{o}C for 20 min. The two samples were diluted with water (2.5 mL) and the remaining two with equal volume of NaOH solution (pH 12) and incubated at room temperature overnight and analysed by ion chromatography as described above.

5.3 Results and Discussion

Decarboxylation of the initially formed Schiff base **(1)** of glucose with asparagine is one of the critical steps in the pathway of conversion of asparagine into acrylamide (Scheme 5.1). Although amino acids can be decarboxylated at high temperatures into their corresponding free amines, however, in the presence of aldehydes and ketones and in low moisture systems, this process is facilitated due to the formation of a relatively stable azomethine ylide (Tsuge et al., 1987) after the loss of CO_2 from 5-oxazolidinone intermediate (Scheme 5.2). In dry systems, the open-form Schiff bases are prone to undergo intramolecular cyclization to form either 5-oxazolidinone or glycosylamines, unlike in high moisture systems, where they tend to undergo Amadori rearrangement (Stadler et al., 2004); the more stable isomer. In fact, the efficiency of decarboxylation of phenylalanine for example as measured by the amount of phenethylamine produced during Py/GC-MS analysis, increased around 300 fold in the presence of phenylacetaldehyde and other carbonyl containing compounds. However, the formation of 5-oxazolidinone and subsequent generation of azomethine ylides have so far been verified only in model systems consisting of amino acids and simple aldehydes (Tsuge et al., 1987). In this study we provide evidence for their formation in amino acid/carbohydrate systems. Furthermore, in order to ascertain the relative abilities of the immediate precursors **2, 3 & 4** shown in Scheme 5.1 to generate acrylamide under dry and wet (50% moisture) conditions, the proposed intermediates were either pyrolyzed at 250^{o}C/20s or heated with moisture at 170^{o}C for 20 min as described under Materials and Methods section.

5.3.1 Spectroscopic Evidence for the Formation of 5-Oxazolidinone Intermediate in Sugar/Asparagine Model Systems.

Indirect evidence for the involvement of Schiff bases in assisting decarboxylation process was obtained earlier (Yaylayan et al., 2004) when [^{13}C-4]-aspartic acid was pyrolyzed alone and in the presence of glucose and label incorporation in the resulting decarboxylation product the acrylic acid was calculated. Analysis of the data showed the formation of 65% of labeled acrylic acid and 35% unlabeled product when [^{13}C-4]-aspartic acid was pyrolyzed alone and when [^{13}C-4]-aspartic acid was pyrolyzed in the



Scheme 5.2 Intramolecular Cyclization of the Schiff base of Asparagine into 5-Oxazolidinone Intermediate and its Subsequent Decarboxylation into Azomethine Ylide.

presence of glucose 100% labeled acrylic acid was observed (for details see Yaylayan et al., 2004), indicating preferential decarboxylation of C-1 carboxylate moiety that is able to cyclize and form 5-oxazolidinone intermediate, rather then the C-4 acid group unable to cyclize in the same fashion, consistent with the mechanism of sugar-assisted decarboxylation shown in Scheme 5.2 However, in order to provide direct evidence for the formation of 5-oxazolidinone, the amino acid/sugar reactions were analyzed by FTIR to monitor the formation of a peak in the range between 1780 to 1800 cm^{-1} where 5-oxazolidinones are known to exhibit a strong absorption band (Aurelio et al., 2003). Spectroscopic studies using glyceraldehyde/amino acid models in toluene heated at 110^{o}C clearly indicated the formation of an intense peak in the range of 1780 -1810 cm^{-1}, depending on the amino acid. The identity of the peak was verified by observing the expected 40 cm^{-1} shift when [^{13}C-1]-labeled amino acids were used. Figure 5.1 shows the

carbonyl absorption peak centered at 1778 cm^{-1} for the glyceraldehydes/asparagine model system. Furthermore, evidence for the formation of the resulting azomethine ylide was also provided using their specific ability to undergo 1,3-dipolar cycloadditions with dipolarophiles (Tsuge et al., 1987). The addition of dipolarophiles, such as dimethyl fumarate to the heated model systems has lead to a significant drop in intensity of the Maillard browning (Table 5.1), indicating the importance of the resulting imines shown in Scheme 5.2 to the generation of color.

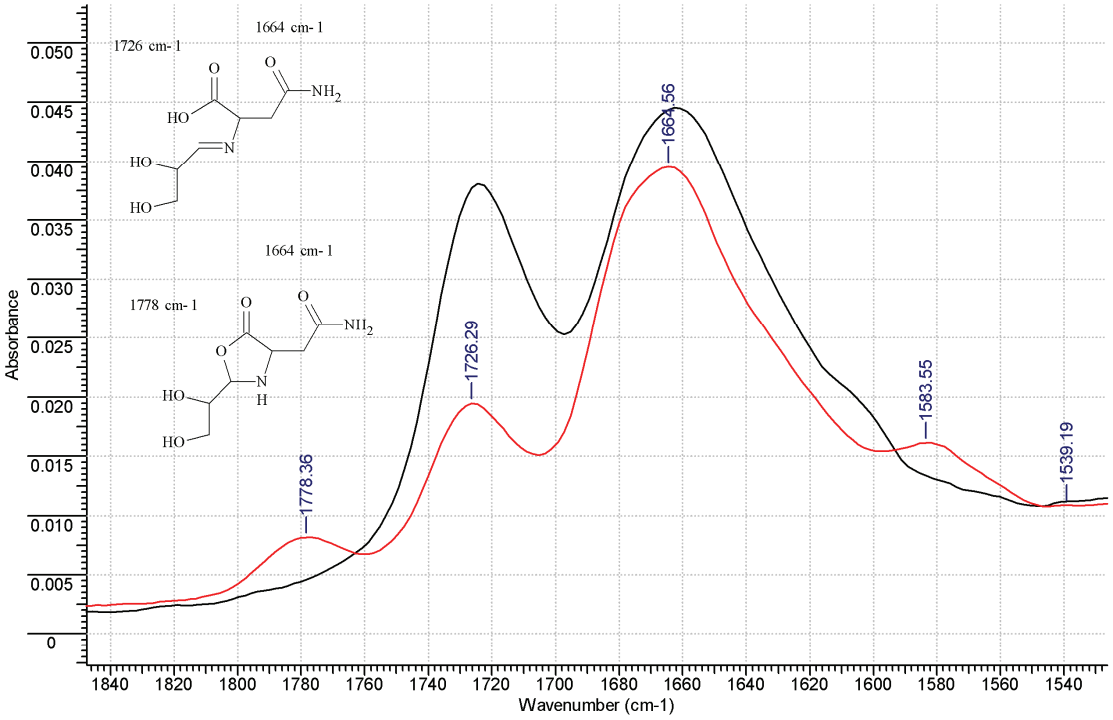


Figure 5.1 FTIR Spectrum of Asparagine and Heated Glyceraldehyde/Asparagine Mixture showing the build up of the Absorption Peak of 5-Oxazolidinone Intermediate Centered at 1778cm^{-1}.

Table 5.1 Effect of Dimethyl Fumarate on the Intensity of Absorption at 450 nm in Different Model Systems[1]

Model System	**450 nm**
Glyceraldehyde/Phenylalanine[2]	0.698
Glyceraldehyde/Phenylalanine/Dimethyl Fumarate	0.344
% difference	**-50**
Mannose/Phenylalanine	0.095
Mannose/Phenylalanine/Dimethyl Fumarate	0.074
% difference	**-22**
Control	
1-Amino-propanediol/Phenylacetaldehyde	0.060
1-amino-propanediol/Phenylacetaldehyde / Fumarate	0.063
% difference	**0**

[1] Solutions (0.03M) heated in DMSO at 80°C for 30 min. Average of two replicates with coefficient of variation <10%.

[2] heated for 8 min only due to intense color formation

5.3.2 Relative Efficiency of Different Precursors in the Generation of Acrylamide under Dry and Wet Conditions.

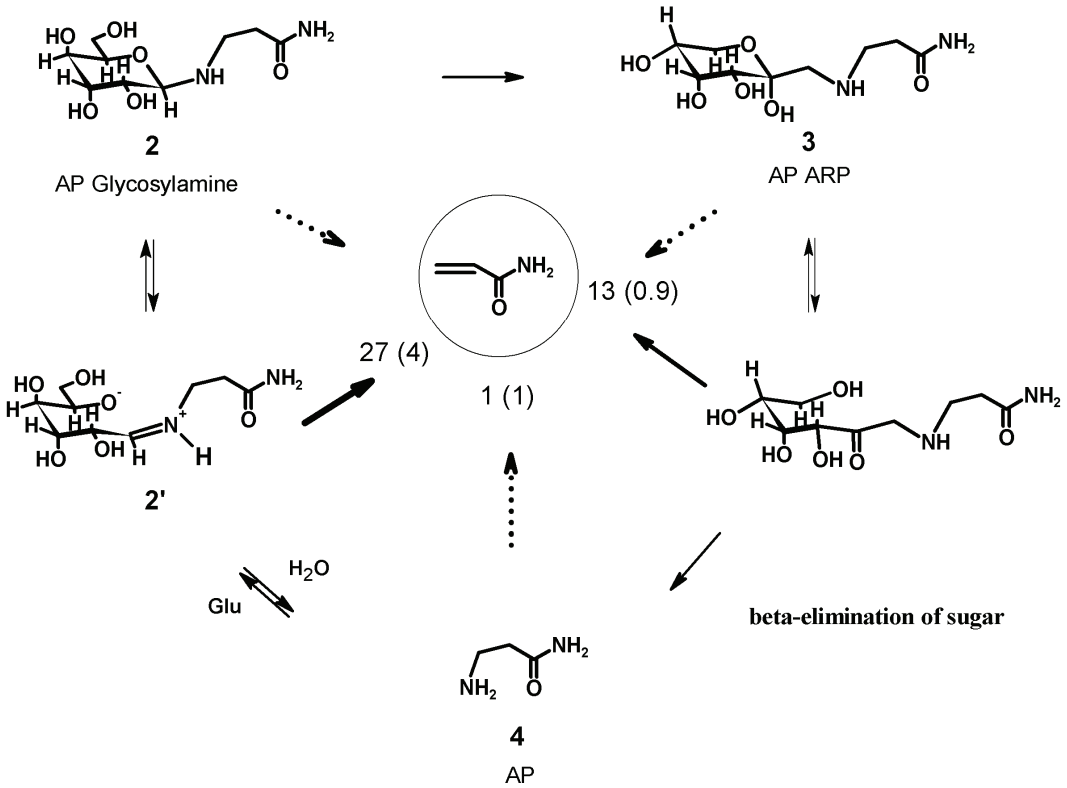
Different precursors **(2, 3 & 4)** listed in Table 5.2 and shown in Scheme 5.3 were either pyrolyzed and analyzed by GC/MS or heated in sealed glass ampoules in the presence of wet alumina and analyzed by ion chromatography as detailed under the Materials and Methods section. In both dry and wet systems, the relative amount of acrylamide was estimated from the calculated value of the area of the acrylamide peak per mole of the precursor. The relative efficiencies reported in Table 5.2 and in Scheme 5.3 are normalized values relative to the ability of the free 3-aminopropionamide (AP) to

generate acrylamide taken as unity. Inspection of Table 5.2 indicates that the most efficient precursor of acrylamide in both dry and wet systems is the N-(D-glucos-1-yl)-3'-amino-propionamide (**2**, AP Glycosylamine). In the dry system there is no significant difference between free AP **(4)** and AP ARP **(3)** in their abilities to be converted into AA, however, in the wet system there was significant increase in the ability of AP ARP **(3)** relative to AP in acrylamide generation. Furthermore, the data also indicate the importance of even small amount of water in enhancing the efficiencies of both AP Glycosylamine (6 fold increase) and AP ARP (13 fold increase) in generating AA. The role of water can be explained by its effect on ring opening and on chemical mobility in general. In addition, the common step of acrylamide formation from all three precursors is the elimination of either ammonia as in the case of AP **(4)** or amines as in the case of AP ARP **(4)** or AP glycosylamine **(3)**. However, de-amination of amines whether 1^o, 2^o or 3^o is energetically difficult process due to the basicity of the leaving groups. The common method of de-amination of amines is through their conversion into quaternary ammonium salts or quaternary imminum ions (Katritzky and EL-Mouafy, 1982), known as Hofmann elimination (Scheme 5.4). Consequently, the enhanced ability of AP Glycosylamine **(2)** to undergo de-amination can be attributed to its open form, the imminum ion (**2'** in Scheme 5.3). This form is capable of undergoing Hofmann-type elimination. The process of ring opening to form the imminium ion is greatly enhanced in the presence of moisture. A higher content of moisture of course will degrade the imminium ion and produce free AP and glucose. Similarly, increased concentration of the open form of AP ARP in the presence of moisture can increase the formation of reactive carbonyl compounds through the known degradation pathways of ARP and consequently form imminium ions similar to that of AP glycosylamine (Scheme 5.3 & 5.4). When AP or AP ARP were heated in the presence of excess sugar or vanillin (Table 5.2) increased formation of AA was observed under both dry and wet conditions, supporting the proposed mechanism of de-amination through Hofmann-type E2 mechanism after reaction with carbonyl compounds or available sugars.

Table 5.2 Relative Efficiencies (peak area/mole) of Different Precursor Systems in Acrylamide (AA) Generation under Dry and Wet Conditions

Precursor System[1]	**AA in Dry system**[2]	**AA in Wet system**[3]
AP ARP	0.9	13
AP ARP/Glucose[4]	1.2	20
AP	**1**	**1**
AP/Glucose[4]	1.6	6
AP/Vanillin[4]	2.2	n.d.
AP Glucosylamine	4	27

[1] AP = 3-aminopropionamide; ARP = Amadori Rearrangement Product
[2] As determined by Py/GC-MS (1 mg samples pyrolyzed at 250°C for 20 seconds); values represent average of two replicates with a coefficient of variation of < 10%.
[3] As determined by ion chromatography (10 mg samples diluted with alumina in 50% moisture to a total weight of 50mg, heated at 170°C for 20 min) values represent average of two replicates with a coefficient of variation of < 30%.
[4] Three fold molar excess relative to the primary precursor



Scheme 5.3 Reactive Precursors and their Relative Abilities to Generate Acrylamide in Wet and Dry (values in parathesis) Model Systems (see also Table 5.2).
Glu : Glucose; AP : 3-Aminopropionamide; ARP : Amadori Rearrangement Product

Table 5.3 Acrylamide Content in Heated N-(D-glucos-1-yl)-3'-aminopropionamide (**2**) Model System after Incubation under Acidic or Alkaline pH.

Model System[1]	Acrylamide[2] (mmol/mol)
AP ARP + excess glucose[3] Incubated overnight in water (pH 6.5)	**2.7**
AP ARP + excess glucose[3] Incubated overnight in NaOH solution (pH 12)	**11.7**

[1] Model systems (10 mg samples diluted with alumina in 50% water (w/w) to a total weight of 50 mg) were heated with alumina at 165°C for 20 min in sealed vials and incubated overnight as shown in the table.

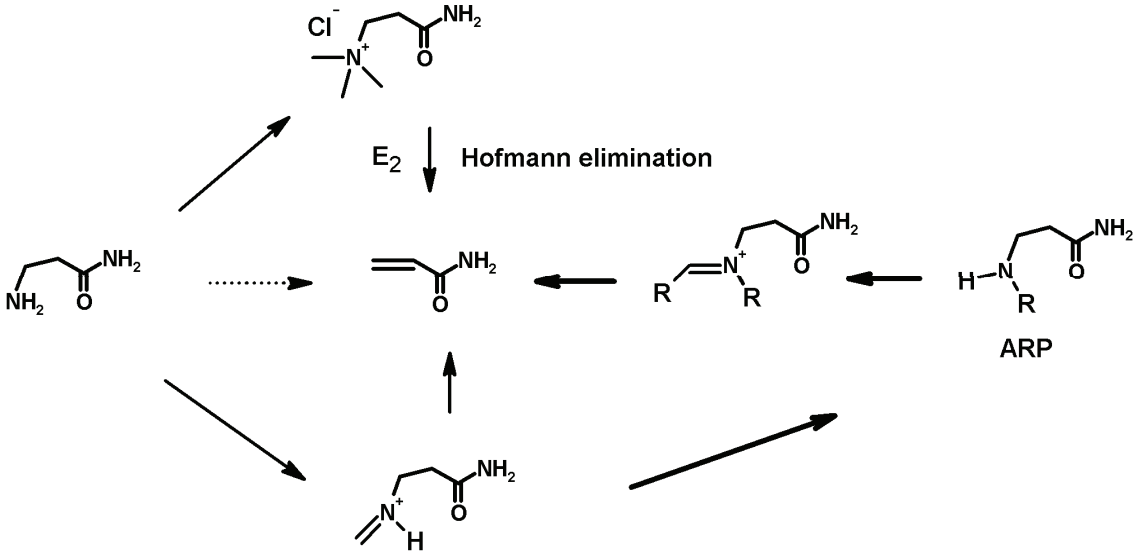
[2] based on average of two measurements with Coefficient of Variance < 15%

[3] Glucose was in three fold molar excess

AP = 3-aminopropionamide; ARP = Amadori Rearrangement Product

5.3.3 Role of Basic pH in Post-Storage Acrylamide Release.

The above proposed mechanism of base catalyzed Hofmann-type elimination shown in Scheme 5.4 can also provide a possible explanation for the observed increase in AA content of food samples if a subsequent extraction is performed under high pH conditions, indicating the presence of a water soluble, base sensitive precursor capable of releasing AA (Goldmann et al., 2006). Based on the above findings it can be assumed that among the known precursors, the AP ARP **(3)** is most likely candidate to undergo incomplete reaction and partially accumulate in food products due to its lower reactivity. During storage and as depicted in Scheme 5.4, AP ARP can react with glucose or other carbonyl compounds and be converted into a more reactive form to undergo base catalyzed Hofmann-type elimination (Katritzky and EL-Mouafy, 1982) and generate acrylamide. To test this hypothesis, AP ARP samples with excess glucose were heated with alumina at 165°C for 20 min in sealed ampoules containing 50% water (w/w) and incubated overnight; one sample was kept in distilled water and the other in NaOH solution (pH 12). As shown in Table 5.3, the sample incubated in basic solution showed more than four fold increase in AA.



Scheme 5.4 Hofmann Elimination of 3-Aminopriopionamide and of AP ARP through Successive Glycation Reactions.

R = Sugar Residue; ARP: Amadori Rearrangement Product

5.4 Conclusion

The initial interaction between asparagine and glucose can generate therefore a sequence of precursors capable of either directly generating acrylamide such as N-(D-glucos-1-yl)-3'-aminopropionamide **(2)** and N-(1-*deoxy*- D-fructos-1-yl)-3'-aminopropionamide **(3)** or through the formation of 3-aminopropionamide **(4)**. The relative importance of each of these intermediates in food systems can only be determined by their kinetic parameters. These parameters can change depending on the food matrix and moisture content. In this study only intrinsic abilities of these precursors to generate acrylamide in model systems were estimated under dry and wet conditions. Under both conditions the N-(D-glucos-1-yl)-3'-aminopropionamide (**2**, AP Glycosylamine) had the highest intrinsic ability to be converted into AA. In the dry model system the increase was almost four fold higher than AP ARP (**3**) or AP (**4**), however in the wet system, the increase was two fold higher than AP ARP but more than twenty fold higher relative to AP. Furthermore, spectroscopic studies also indicated an increased likelihood of decarboxylation through 5-oxazolidinone intermediate with decreasing moisture content of the model system.

CONNECTING PARAGRAPH

Chapters 3, 4 and 5 were mainly concerned with acrylamide formation and its precursors. Chapter 6 takes a look at the mechanism of formation of another thermally-generated food toxicant also found in carbohydrate-rich foods, known as furan. The content of chapter 6 appeared in the Journal of Agricultural and Food Chemistry (see Perez-Locas, C. and Yaylayan, V.A. **2004.** Origin and Mechanistic Pathways of Formation of the Parent Furan: A Food Toxicant. Journal of Agricultural and Food Chemistry, 52, pp.6830-6836)

CHAPTER 6

ORIGIN AND MECHANISTIC PATHWAYS OF FORMATION OF THE PARENT FURAN - A FOOD TOXICANT

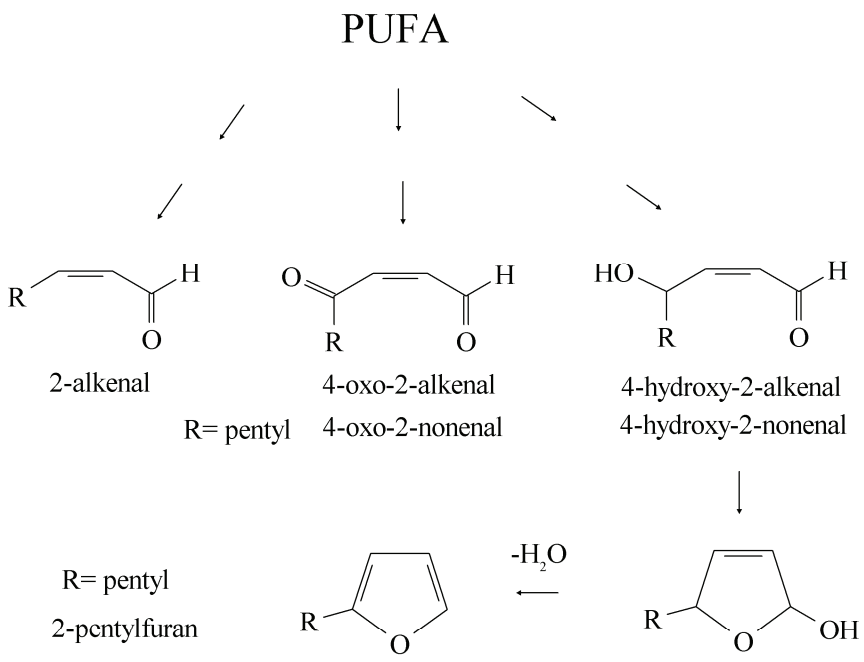
6.1 Introduction

Recently, researchers at the U.S. Food and Drug Administration (FDA, 2003) have identified the parent compound furan in a number of foods that undergo thermal treatment, especially canned and jarred foods. Furan is a volatile and colorless liquid and is classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC) (IARC,1995). Although the parent furan had previously been reported in various foods such as coffee, canned meat, baked bread, and cooked chicken (Maga, 1979; Persson and von Sydow, 1973; Stoffelsma et al., 1968), it was only recently that a more comprehensive study was performed by the FDA using larger number of food samples, which found furan levels ranging up to ~100 ppb.

According to Maga (Maga, 1979) the primary source of furans in food is thermal degradation of carbohydrates such as glucose, lactose, and fructose. According to the FDA, a variety of carbohydrate/ amino acid mixtures or protein model systems (e.g., alanine, cysteine, casein) and vitamins (ascorbic acid, dehydroascorbic acid, thiamin) have been used to generate furans in food. Furthermore, Health Canada (?) reported the formation of furan from ascorbic acid and from the oxidation of polyunsaturated fatty acids (PUFA). The latter finding, however, should not come as a surprise because a furan derivative, 5-pentylfuran, has already been detected in oxidized soybean oil (Chang et al., 1966) and is used currently as a chemical marker for rancidity. The origin of 5-pentylfuran was linked to the formation of 4-hydroxy-2- nonenal (4-HNE) by Sayre et al. (1993) when they observed the formation of the furan upon refluxing ethanolic solution of 4-HNE under acidic conditions. This observation was later confirmed by Erdelmeier et al. (1998). Recent studies found a significant correlation between 5-pentylfuran concentrations with the time of oxidation of olive oil (Vichi et al., 2003).

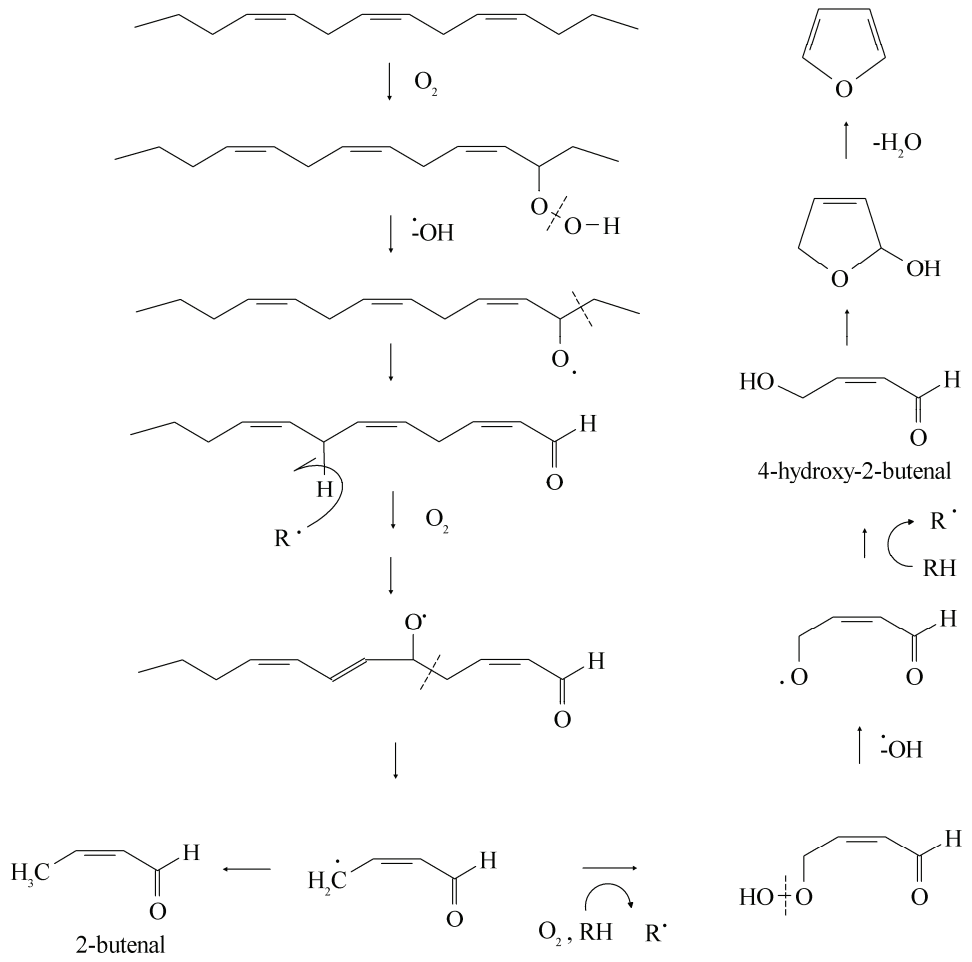
In general, the oxidative degradation of PUFAs and the formation of lipid peroxides are known to play a major role in the development of both degenerative diseases in biological systems (Xu and Sayre, 1998) and off-flavors and rancidity in food systems (Vichi et al., 2003). Lipid hydroperoxides can be formed from PUFA nonenzymatically by reactive oxygen species or enzymatically by lipoxygenases. Subsequent homolytic cleavages of

PUFA hydroperoxides, catalyzed by transition metal ions, result in the formation of 2-alkenals, 4-oxo-2-alkenals, and 4-hydroxy-2-alkenals (Xu and Sayre, 1998) (see Scheme 6.1). Highly cytotoxic 4-hydroxy-2-alkenals such as 4-HNE are capable of the modification of proteins, DNA, and low-density lipoprotein (LDL).



Scheme 6.1 Summary of Different Reactive Aldehydes formed from Oxidative Decomposition of Polyunsaturated Fatty Acids.

On the basis of the above discussion, it can be proposed that the parent furan, similar to 5-pentylfuran, could be formed from corresponding 4-hydroxy-2-butenal (Nadkarni and Sayre, 1995) through cyclization and formation of 2,5-dihydro-2-furanol and subsequent dehydration as proposed in Scheme 6.2. Ironically, this process converts the more toxic 4-hydroxy-2-alkenals into less toxic and more volatile furan derivatives. At present, there are no specific mechanisms proposed that can explain the formation of the parent furan from other sources such as carbohydrates and amino acids. In this study we provide evidence for the mechanism of formation of the parent furan from amino acids, sugars, amino acid/sugar mixtures, and ascorbic acid.



Scheme 6.2 Proposed Mechanism of Lipid Peroxidation and Subsequent Formation of Furan

6.2 Materials and Methods

6.2.1 Chemicals and Reagents

All reagents and chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. The labeled [^{13}C-1]serine (99 atom %) [^{13}C-2]serine, and [^{13}C-3]serine (99 atom %) and the labeled D-[1-^{13}3C]glucose (99 atom %), D-[2-^{13}C]glucose (99 atom %), and D-[6-^{13}C]glucose were also purchased from Aldrich Chemical Co. D-[3-^{13}C]Glucose (99 atom %), D-[4-^{13}C]glucose (99 atom %), and D-[5-^{13}C]glucose (99 atom %) were purchased from Cambridge Isotope Laboratories (Andover, MA).

6.2.2 Pyrolysis-GC-MS Analysis

6.2.2.1 Mechanistic Studies. All Py-GC/MS analyses were performed using a Hewlett-Packard 5890 series II GC fitted with a 5971B MS (Hewlett-Packard, Palo Alto, CA) and a CDS Pyroprobe 2000 interface (CDS Analytical Inc., Oxford, PA). Single compounds or binary mixtures of D-glucose/ amino acid (1:1 molar ratio, total 2 mg), except for labeling studies performed with D-glucose/L-serine (1:3 molar ratio, total 2 mg), were introduced inside a quartz tube (0.3 mm thickness), plugged with quartz wool and inserted into the coil probe. The pyroprobe was set at 250°C at a heating rate of 50°C/s with a total heating time of 20 s. The pyroprobe interface was set at 250°C. The samples were introduced in splitless mode and analyzed under a constant He flow of 1.34 mL/min, the pressure being regulated by an electronic pressure controller (Hewlett-Packard). The capillary direct MS interface temperature was 180°C; the ion source was 280°C. The ionization voltage was 70 eV and the electron multiplier, 2047 V. The MS scanned masses from *m*/*z* 17 to 500 at 1.5 scans/s; the column temperature (PLOT-Q capillary column from Hewlett-Packard, Mississagua, ON) was held at 40°C for 2 min, then increased to 100°C at a rate of 30 °C/min, and further increased to 250°C at a rate of 10°C/min and kept at 250 °C for 10 min. Compounds were tentatively identified by comparing their mass spectra with those of Wiley and NIST mass spectral databases. The identity and purity of the chromatographic peaks were determined using NIST AMDIS version 2.1 software.

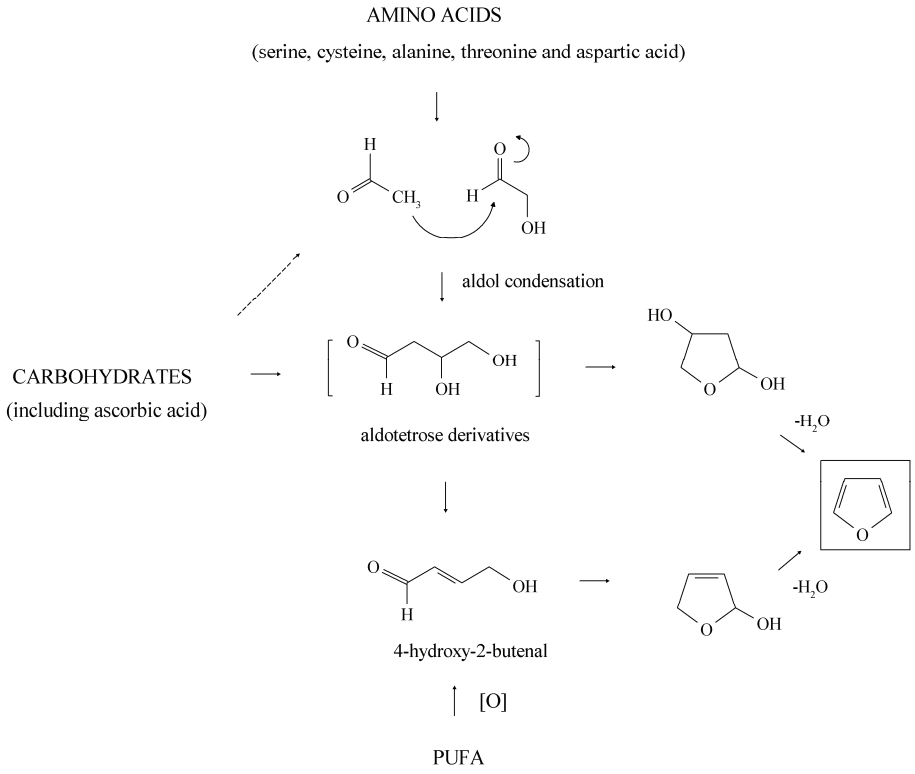
6.2.2.2 Efficiency of Furan Formation

Single compounds (7 μmol) listed in Table 6.1 were mixed with silica gel (1 mg) except ascorbic acid (reacted without silica gel) and were pyrolyzed as indicated above at 250°C unless otherwise specified. All pyrolyzed model systems contained 7 μmol of each reagent indicated except in the case of sodium formate, which was 10 μmol. The reported formation efficiency values are the average of duplicate analyses and are rounded off, with no more than 6% relative error in reproducibility.

Table 6.1 Efficiency of Furan Formation expressed as Area Count of Furan/Mole of Starting Material Generated from Different Model Systems at 250°C.

Model System	Relative Efficiency x 10^{10} (area/mol)
L-Ascorbic Acid	140
Dehydroascorbic acid[1]	78
Dehydroascorbic acid[2]	47
D-Erythrose	32
Dehydroascorbic acid	8
D-Ribose	7
D-Sucrose	5
D-Glucose	4
D-Fructose	4
L-Serine	1
L-Cysteine	0.5
L-Threonine	0
L-Aspartic acid	0
L-Alanine	0
L-Glycine	0
D-Ribose/Serine	28
D-Sucrose/Serine	24
D-Fructose/Serine	16
D-Glucose/Cysteine	15
D-Glucose/Serine	11
D-Glucose/Alanine	10
D-Glucose/L-Aspartic acid	9
D-Glucose/formate Sodium	9
D-Glucose/L-Threonine	5
D-Glucose/Glycine	5
D-Erythrose/L-Serine	3
Glycolaldehyde[3]/L-Alanine	65
Glycolaldehyde[3]/L-Serine	9
Acetaldehyde[4]/Glycolaldehyde[3]	7
Acetaldehyde[4]/L-Serine	0.5
L-Serine/L-Alanine	0.4

[1] Pyrolysis temperature 350°C, [2] Pyrolysis temperature 300°C; [3] from glycolaldehyde dimer
[4] from acetaldehyde diethyl acetal



Scheme 6.3 Different Origins of Furan Formation

PUFA = Polyunsaturated Fatty Acid; [O] = Oxidation; Dotted arrow indicates minor pathway.

6.3 Results and Discussion

Numerous derivatives of the parent compound furan such as 5-methylfurfural, furanmethanol, 2-acetylfuran, 2-methyldihydro- 3(2*H*)-furanone, and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone have been characterized in Maillard model systems and their mechanisms of formation identified using ^{13}C-labeled sugars (Yaylayan and Keyhani, 2000; Yaylayan and Wnorowski, 2000). In these studies, the parent furan was not detected because the common gas chromatographic columns employed during such studies were more polar DB-5 type columns rather than the required less polar columns such as PLOT that are able to retain gaseous and nonpolar compounds such as the parent furan. Although we had previously analyzed serine (Yaylayan et al., 2000) and cysteine (Yaylayan et al., 2003) model systems on a PLOT-Q column and observed the formation of the parent furan, we have not reported its formation mechanism. In this study, we

investigated in detail the formation of furan from different sugars and amino acid model systems, including ascorbic acid, using Py-GC-MS. Table 6.1 summarizes the efficiency of different model systems to produce furan under identical conditions expressed as furan peak area per mole of starting material, and Scheme 6.3 summarizes the general pathways leading to furan formation starting from carbohydrates and amino acids, and including PUFA.

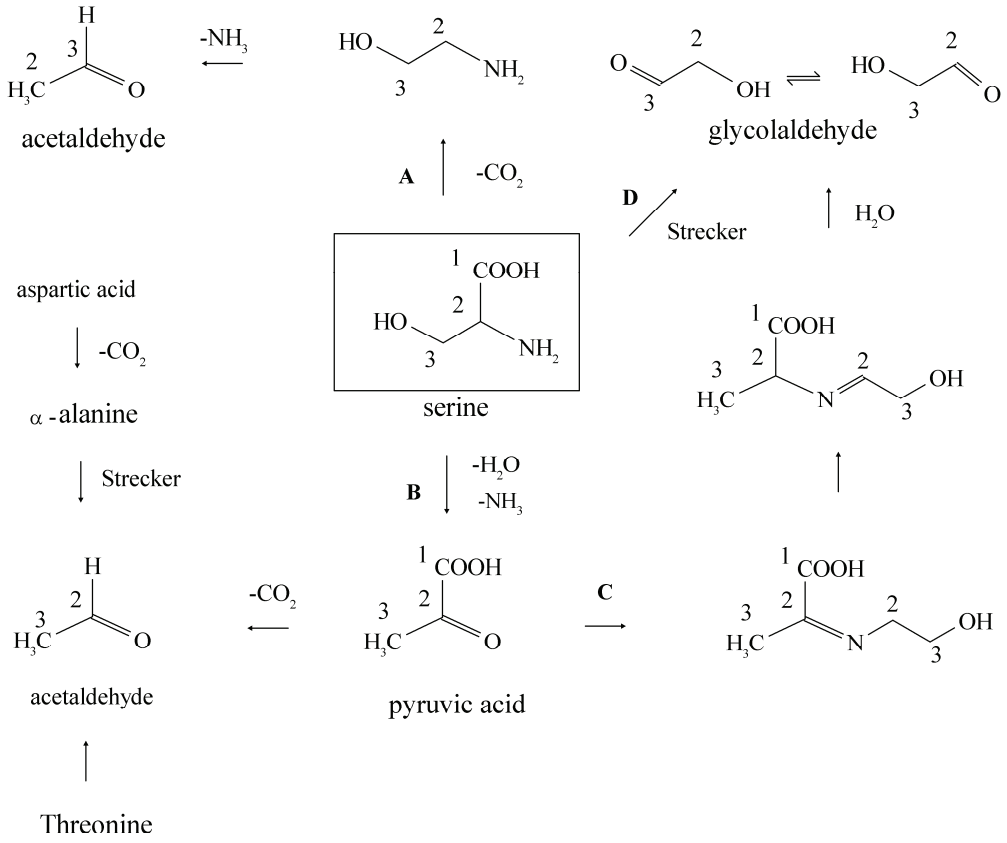
Table 6.2 Percent Label Distribution in the Parent Furan Generated from Labeled L-Serine.

Model	M	M+1	M+2	M+3	M+4
Serine	100	0	0	0	0
[1-^{13}C]Serine	100	0	0	0	0
[2-^{13}C]Serine	0	0	100	0	0
[3-^{13}C]Serine	0	0	100	0	0

6.3.1 Furan Formation through Amino Acid Degradation.

Previous studies (Yaylayan et al., 2003) using a PLOT-Q column have indicated the formation of the parent furan in model systems containing serine and cysteine, even in the absence of reducing sugars (unpublished data). To determine the mechanism of formation of furan from these amino acids, serine independently labeled at C-1, C-2, and C-3 was pyrolyzed and the label incorporation in the parent furan was calculated. The results are given in Table 6.2. According to this table, two of the four carbon atoms of furan originated from C-2 atoms of serine, and the remaining two carbon atoms originated from C-3 atoms of serine. No incorporation of the C-1 atom of serine was detected. These observations are consistent with the proposed aldol condensation mechanism (see Scheme 6.3) between an acetaldehyde and a glycolaldehyde moiety originating from serine and eventual formation of furan through an aldotetrose intermediate. Previous studies (Yaylayan and Wnorowski, 2000; Yaylayan et al., 2000; Yaylayan and Wnorowski, 2001) have also confirmed the formation of acetaldehyde and glycolaldehyde incorporating the C-2 and C-3 atoms of serine and cysteine, and their

mechanism of formation is summarized in Scheme 6.4. According to this figure, serine can decarboxylate and produce ethanolamine, which in turn can lose a molecule of ammonia and form acetaldehyde (pathway A). Alternatively, it can undergo dehydration and deamination reactions and form pyruvic acid, which in turn decarboxylates to form acetaldehyde (pathway B). Glycolaldehyde can be formed through the interaction of pyruvic acid with ethanolamine, followed by isomerization of the imine and hydrolysis (pathway C, Scheme 6.4). However, in the presence of sugars, it can also be formed through a Strecker reaction (pathway D). Similar pathways can be envisaged for cysteine.



Scheme 6.4. Mechanistic Pathways of Formation of Acetaldehyde and Glycolaldehyde (precursors of furan) from Different Amino Acids based on Labeling Studies.
Numbers indicate original amino acid carbon atom locations.

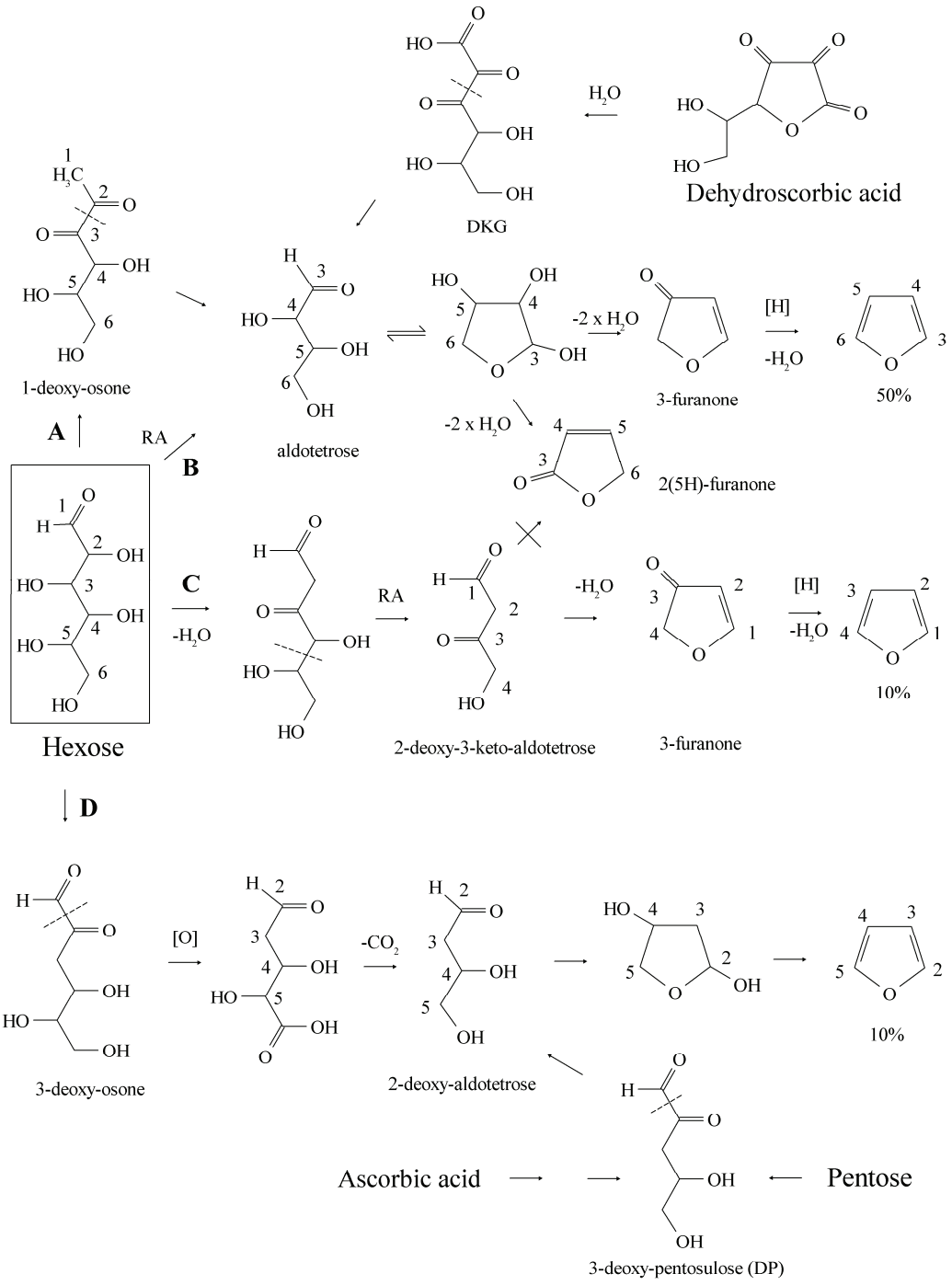
Other amino acids that can contribute to the formation of furan are aspartic acid, R-alanine, and threonine; however, these amino acids can generate only acetaldehyde and need reducing sugars to produce glycolaldehyde. The amino acid α-alanine, for example, does not produce furan alone; however, in the presence of glycolaldehyde or a glycolaldehyde source such as glucose, it can generate furan (see Table 6.1). Acetaldehyde can be generated from α-alanine through Strecker reaction, and α-alanine in turn can be generated from aspartic acid through decarboxylation reaction (see Scheme 6.4). Threonine, on the other hand, has been shown (Yaylayan and Wnorowski, 2001) to produce acetaldehyde and hence can generate furan in the presence of sugars as confirmed in Table 6.1.

Table 6.3. Percent Label Distribution in the Parent Furan Generated from Labeled L-Serine and Unlabeled D-Glucose.

Model	**M**	**M+1**	**M+2**	**M+3**	**M+4**
Serine	100	0	0	0	0
[1-^{13}C]Serine/Glucose(3:1)	100	0	0	0	0
[2-^{13}C]Serine/Glucose(3:1)	70	0	30	0	0
[3-^{13}C]Serine/Glucose(3:1)	70	0	30	0	0
[2-^{13}C]Serine/Glucose(1:3)	80	5	15	0	0
[3-^{13}C]Serine/Glucose(1:3)	80	5	15	0	0

Table 6.4 Percent Label Distribution in the Parent Furan Generated from Unlabeled L-Serine and Labeled D-Glucose (3:1 molar ratio).

Model	**M**	**M+1**	**M+2**	**M+3**	**M+4**
Serine/Glucose	100	0	0	0	0
Serine/[1-^{13}C]Glucose	90	10	0	0	0
Serine/[2-^{13}C]Glucose	80	20	0	0	0
Serine/[3-^{13}C]Glucose	30	70	0	0	0
Serine/[4-^{13}C]Glucose	30	70	0	0	0
Serine/[5-^{13}C]Glucose	40	60	0	0	0
Serine/[6-^{13}C]Glucose	50	50	0	0	0
Serine/[U-^{13}C]Glucose	30	0	0	0	70



Scheme 6.5 Mechanistic Pathways of Formation of the Parent Furan from Hexoses, Pentoses and Tetroses based on Labeling Studies.

DKG = 2,3-Diketogulonic acid; RA = Retro-aldol cleavage; [O] = Oxidation; [H] = Reduction; Numbers indicate original D-glucose carbon atom locations; Percent distribution reported takes into consideration presence of 30% unlabeled furan originating from L-serine.

6.3.2 Furan Formation through Carbohydrate Degradation

To identify possible pathways of formation of furan from carbohydrates, Py-GC-MS analysis of serine/glucose model system was used due to the availability of independently labeled serine and glucose at all of their carbon atom locations (see Tables 6.3 and 6.4). Inspection of the data in Table 6.3 indicated that in the model system where excess labeled serine was reacted with unlabeled glucose, 70% of the furan originated from glucose carbon atoms and 30% from serine carbon atoms. This conclusion was further confirmed when excess unlabeled serine was reacted with [U-^{13}C]glucose (see Table 6.4) Furthermore, inspection of Table 6.3 revealed that when glucose was in excess, 5% of the furan formed was singly labeled, indicating a minor contribution of carbohydrate degradations to the C2 + C2 aldol condensation pathway shown in Scheme 6.3. In addition, analysis of the percent label incorporation pattern (Table 6.4) has indicated that there are four pathways (A, B, C, and D in Scheme 6.5) of sugar degradation that can lead to the formation of aldotetrose derivatives that can eventually cyclize to form furan as depicted in Scheme 6.5. In the glucose/excess serine model system and as indicated above, 30% of the furan originated from serine and the remaining 70% was generated from glucose. The major pathway (50%) of glucose degradation (pathway A or B) that leads to furan formation incorporated the C3-C4-C5-C6 carbon atoms of glucose, 10% incorporated the C1-C2-C3-C4 carbon atoms of glucose (pathway C), and another 10% incorporated the C2-C3-C4-C5 carbon atoms of glucose (pathway D).

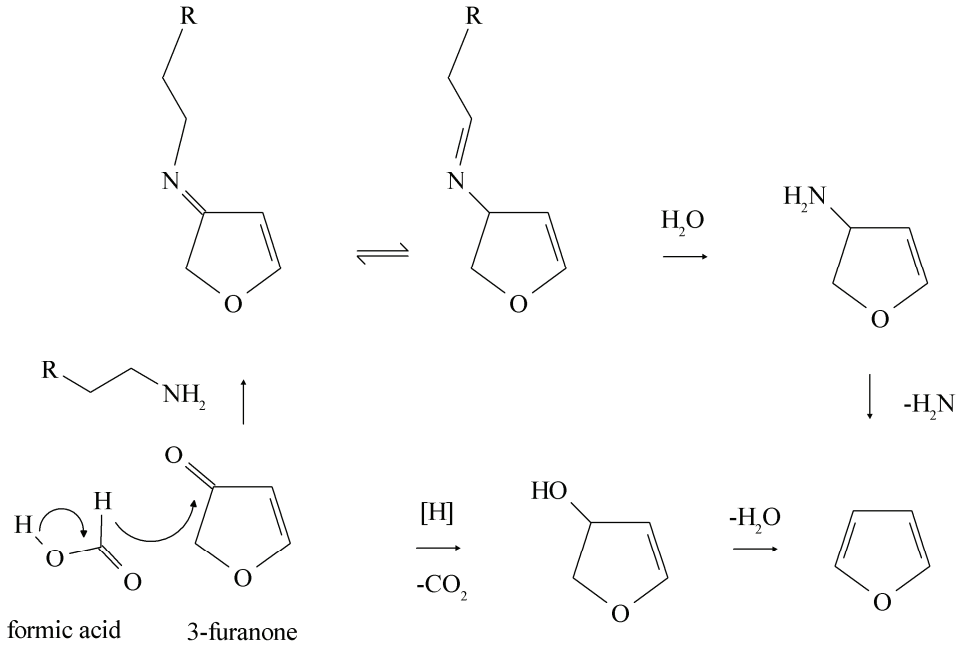
6.3.3 Proposed Pathway from Hexose Sugars

Reducing hexoses are known (Weenen, 1998) to undergo Maillard reaction in the presence of amino acids and generate reactive intermediates such as 1-deoxy- and 3-deoxyosones (pathways A and D) shown in Scheme 6.5. These intermediates are also known to be formed in the absence of amino acids (such as pathway B), however, to a lesser extent. The major pathway of furan formation (pathway A) can be initiated by the formation of 1-deoxyosone in the presence of amino acids or through a retro-aldol cleavage (pathway B). Both pathways lead to the formation of an aldotetrose sugar moiety such as erythrose. The 1-deoxyosone, however, needs to undergo α-dicarbonyl cleavage (Weenen, 1998) to produce the same intermediate. The resulting aldotetrose

moiety that contains the C3-C4-C5-C6 carbon atoms of glucose can undergo dehydration reactions and can produce both 3-furanone and 2(5*H*)-furanone. Only the former can be converted into furan through further reduction and dehydration reactions (see Scheme 6.6). Reductions in sugar/amino acid mixtures can be effected by formic acid—a major sugar degradation product (Yaylayan et al., 2003)—or through a transamination reaction as shown in Scheme 6.6. Table 6.1 also indicates that addition of sodium formate to glucose doubles the amount of furan formation, confirming the above hypothesis. The second pathway that incorporates the C1-C2- C3-C4 carbon atoms of glucose into furan can originate from glucose after a dehydration reaction (Scheme 6.5, pathway C) followed by a retro-aldol cleavage to form 2-deoxy-3-ketoaldotetrose. The latter intermediate can cyclize after a dehydration step to produce 3-furanone, similar to pathway A or B. The third pathway (Scheme 6.5, pathway D) that incorporates the C2-C3-C4-C5 carbon atoms of glucose into furan can arise through R-dicarbonyl cleavage of the 3-deoxyosone intermediate followed by oxidation of the terminal primary hydroxyl group into the carboxylic acid moiety. This intermediate can lose the C-6 carbon through decarboxylation to generate 2-deoxyaldotetrose, which can be easily converted into furan as shown in Scheme 6.5. The observation that 2(5H)-furanone incorporates only the C3-C4-C5-C6 carbon atoms of glucose (see Table 6.5) provides further evidence to the above proposed pathways.

Table 6.5 Percent Label Distribution in 2(5H)Furanone Generated from Unlabeled L-Serine and Labeled D-Glucose (3:1 molar ratio).

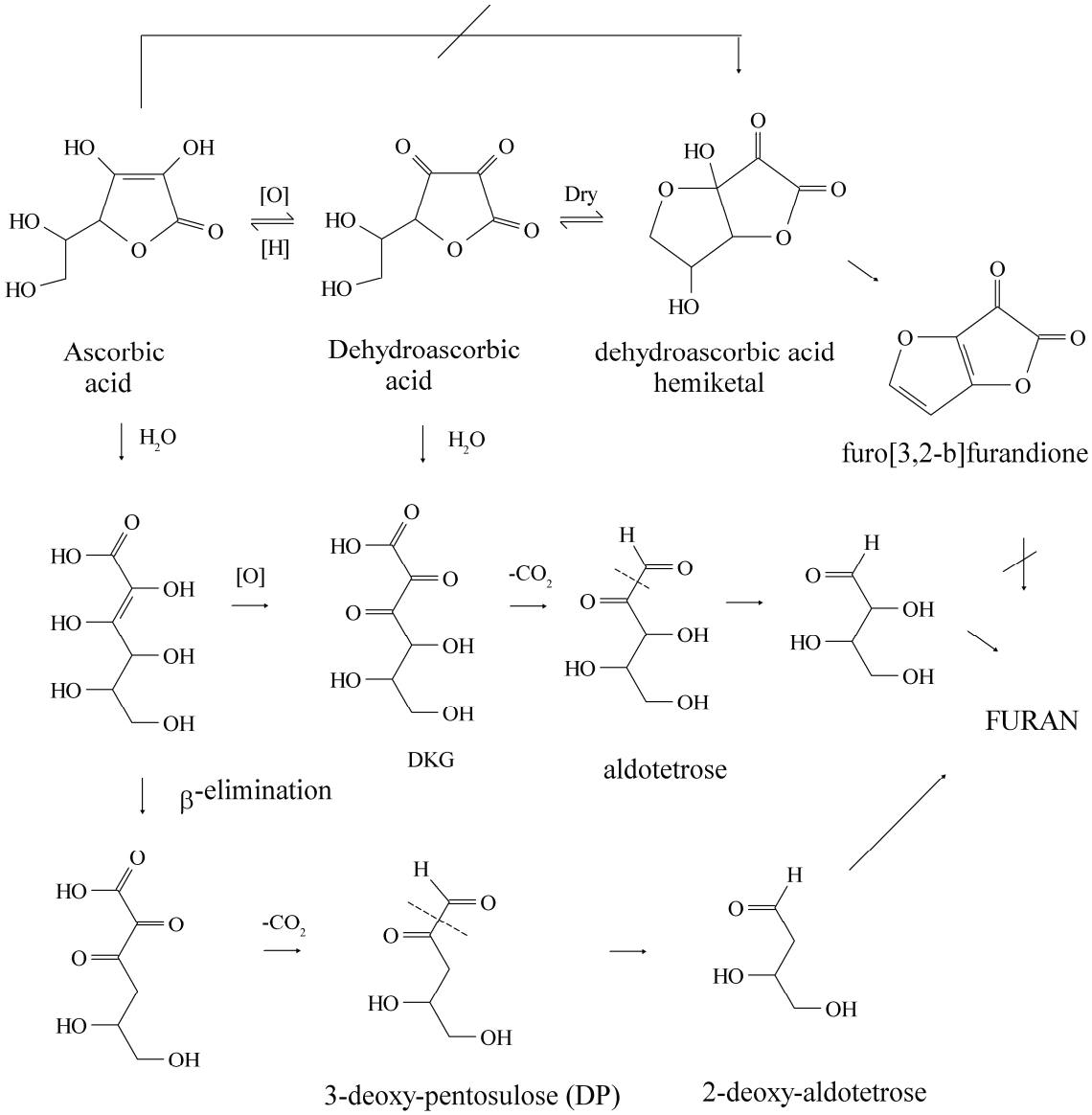
Model	**M**	**M+1**	**M+2**	**M+3**	**M+4**
Serine/Glucose	100	0	0	0	0
Serine/[1-^{13}C]Glucose	100	0	0	0	0
Serine/[2-^{13}C]Glucose	100	0	0	0	0
Serine/[3-^{13}C]Glucose	0	100	0	0	0
Serine/[4-^{13}C]Glucose	0	100	0	0	0
Serine/[5-^{13}C]Glucose	0	100	0	0	0
Serine/[6-^{13}C]Glucose	0	100	0	0	0
Serine/[U-^{13}C]Glucose	0	0	0	0	100



Scheme 6.6. Proposed Mechanism of Conversion of 3-Furanone into Furan through Reduction and Transamination.

6.3.4 Proposed Pathway from Pentose Sugars

Pentose sugars such as ribose can also generate the parent furan, but more so in the presence of amino acids (see Table 6.1). Similar to hexoses, pentoses can be converted into their 3-deoxyosone derivatives (Weenen, 1998) either through amino acid reaction or through dehydration at the C-3 hydroxyl group. The resulting intermediate can undergo α-dicarbonyl cleavage to produce 2-deoxyaldotetrose, a direct precursor of furan as shown in Scheme 6.5. The increase in the efficiency of ribose or hexose sugars to produce furan in the presence of amino acids can be directly related to their ability to catalyze the formation of deoxyosone derivatives.



Scheme 6.7. Proposed Mechanism of Oxidative and Non-oxidative Degradation of L-Ascorbic Acid into Furan.

PUFA = Polyunsaturated Fatty Acid

6.3.5 Proposed Pathway from Ascorbic Acid

Ascorbic acid is known to oxidize quickly into dehydroascorbic acid and hydrolyze in food systems (Liao and Seib, 1987; Shephard et al., 1999) into 2,3-diketogulonic acid (DKG). A pathway to furan can be envisaged from DKG through R-dicarbonyl cleavage after decarboxylation and generation of the same aldotetrose moiety as hexose sugars (see Scheme 6.5). Although, under mainly nonoxidative pyrolytic conditions, ascorbic acid cannot undergo oxidation to produce DKG, instead, it can hydrolyze and undergo *â*-elimination (Niemelä, 1987) followed by decarboxylation to produce 3-deoxypentosulose (DP) and encounter the ribose pathway of decomposition to generate furan as shown in Schemes 6.5 and 6.7. Unlike the aldotetrose intermediate, the 2-deoxyaldotetrose does not require a reduction step and directly produces the parent furan. Therefore, under nonoxidative conditions, ascorbic acid is a more efficient source of furan than dehydroascorbic acid (see Table 6.1). This order may be reversed under oxidative degradation conditions. Furthermore, under dry-heating conditions, dehydroascorbic acid can cyclize and exist mainly in its hemiketal form, thus preventing the formation of furan as shown in Schemes 6.7. Due to the unavailability of labeled ascorbic acid, we could not confirm the proposed pathways of formation of furan from ascorbic acid as shown in Schemes 6.5 and 6.7. An alternative pathway could be envisaged from aldol condensation of acetaldehyde and glycolaldehyde, similar to the pathway originating from amino acids (see Schemes 6.3). These intermediates are proposed to be formed from the thermal degradation of ascorbic acid at 180 °C (Vernin et al., 1998).

CONNECTING PARAGRAPH

Chapter 6 explored possible mechanisms of formation of the parent furan from different precursors. This chapter investigates the mechanism of formation of one of its potentially toxic derivatives 5-hydroxymethyl-2-furaldehyde (HMF) from sucrose. The content of this chapter is submitted to the Journal of Agricultural and Food Chemistry (see Perez-Locas, C., Yaylayan, V. A. Mechanism of Thermal Generation of 5-(Hydroxymethyl)-2-Furaldehyde (HMF) from ^{13}C-labeled Sucrose. Journal of Agricultural and Food Chemistry).

CHAPTER 7

ISOTOPE LABELING STUDIES ON THE FORMATION OF 5-(HYDROXYMETHYL)-2-FURALDEHYDE (HMF) FROM SUCROSE BY PYROLYSIS-GC/MS

7.1 Introduction

Similar to the widespread occurrence of acrylamide in thermally processed food, 5-(hydroxymethyl)-2-furaldehyde (HMF) is also detected in variety of food products but in relatively higher concentrations (exceeding 1g/kg). HMF is one of the major degradation products of carbohydrates that have been studied extensively as an indicator of heat damage (AIJN, 1996; OJEC, 2002). HMF has been used successfully as a chemical index in ensuring adequate heat processing or for monitoring storage conditions for fruit juices, milk, honey, cereal products, cookies and jams (Cortes et al., 2007; Berg and van Boekel, 1994; Morales et al., 1997). Formation of HMF from carbohydrates has been found to depend on many factors such as time, water activity, temperature, amount and type of catalyst and sugar used (Kuster, 1990). Ketoses generate more HMF than aldoses and the yield increases with increase in the temperature and the concentration of the acid catalyst although, it can also be formed in slightly lower yields in the absence of a catalyst (Antal et al., 1990*)*. Numerous studies have indicated that fructose is the most reactive sugar relative to sucrose and glucose, in the formation of HMF under acidic conditions. According to Lee and Nagy (1990) at 50 °C and pH of 3.5, fructose was 31.2 times faster than glucose, whereas sucrose was 18.5 times faster than glucose in the rate of HMF formation. Furthermore, rates of HMF formation from glucose and sucrose showed slight enhancement in the presence of the amino acids, whereas virtually no enhancement occurred when fructose was the substrate. Without acid catalysis and at 250 °C the conversion rate of glucose into HMF was 24% and for fructose the rate was 36% (Antal et al., 1990). However, increasing the acid concentration significantly improved the rate of HMF formation from fructose relative to glucose. At 1 mM H2SO4, 42% of fructose was converted into HMF versus 31% for glucose. Interestingly, when sucrose was heated under identical conditions, the yield of HMF per mole of fructose increased from 36% to 47% for the uncatalyzed reaction and from 42% to 53% for the acid catalyzed reaction (Antal et al., 1990). The enhanced HMF formation from sucrose per mole of fructose moiety at high temperatures can be justified by the fact that the glycosidic bond of sucrose can be easily cleaved under mild acidic conditions to produce fructofuranosyl cation, the direct precursor of HMF (see Scheme 7.1), however, it is much more difficult for the free fructose to generate the same cation (Queneau et al., 2008) under identical

conditions. Numerous studies have also indicated the formation of fructofuranosyl cation from fructose as the first step in the formation of HMF (Queneau et al., 2008; Manley-Harris and Richards, 1993; Roman-Leshkov, 2006). On the other hand, glucose cannot be converted into HMF through dehydration from cyclic forms for obvious reasons and is therefore recognized to generate HMF through cyclization of 3-deoxyglucosone (3-DG) intermediate formed from the open-ring form of glucose (see Scheme 7.1). Due to the low propensity of glucose to exists in open ring form and due to many other side reactions of 3-DG, the rate of its conversion into HMF is low compared to fructose especially at higher temperatures where fructose can directly dehydrate from its cyclic forms through the intermediacy of fructofuranosyl cation without the need to undergo thermodynamically controlled ring opening process.

Recent findings on the acute toxicity of HMF (Sommer et al., 2003; Svendsen et al., 2007; Glatt et al., 2005) and lack of evidence from isotope labeling studies confirming the above proposed mechanism of conversion of sucrose into HMF, prompted us to investigate the mechanism of HMF formation utilizing ^{13}C-labeled precursors.

7.2 Materials and Methods

7.2.1 Chemicals

All reagents, chemicals were purchased from Aldrich Chemical company (Milwaukee, WI) and used without further purification. 3-Deoxyglucosone was purchased from Toronto Research Chemicals (Ontario, Canada). The [^{13}C]glucoses were purchased from Cambridge Isotope Laboratories (Andover, MA). [1-^{13}C]fructose and [1-^{13}Cfru]sucrose were purchased from Omicron Biochemicals Inc. (Indiana, USA).

7.2.2 Pyrolysis-GC/MS analysis of HMF

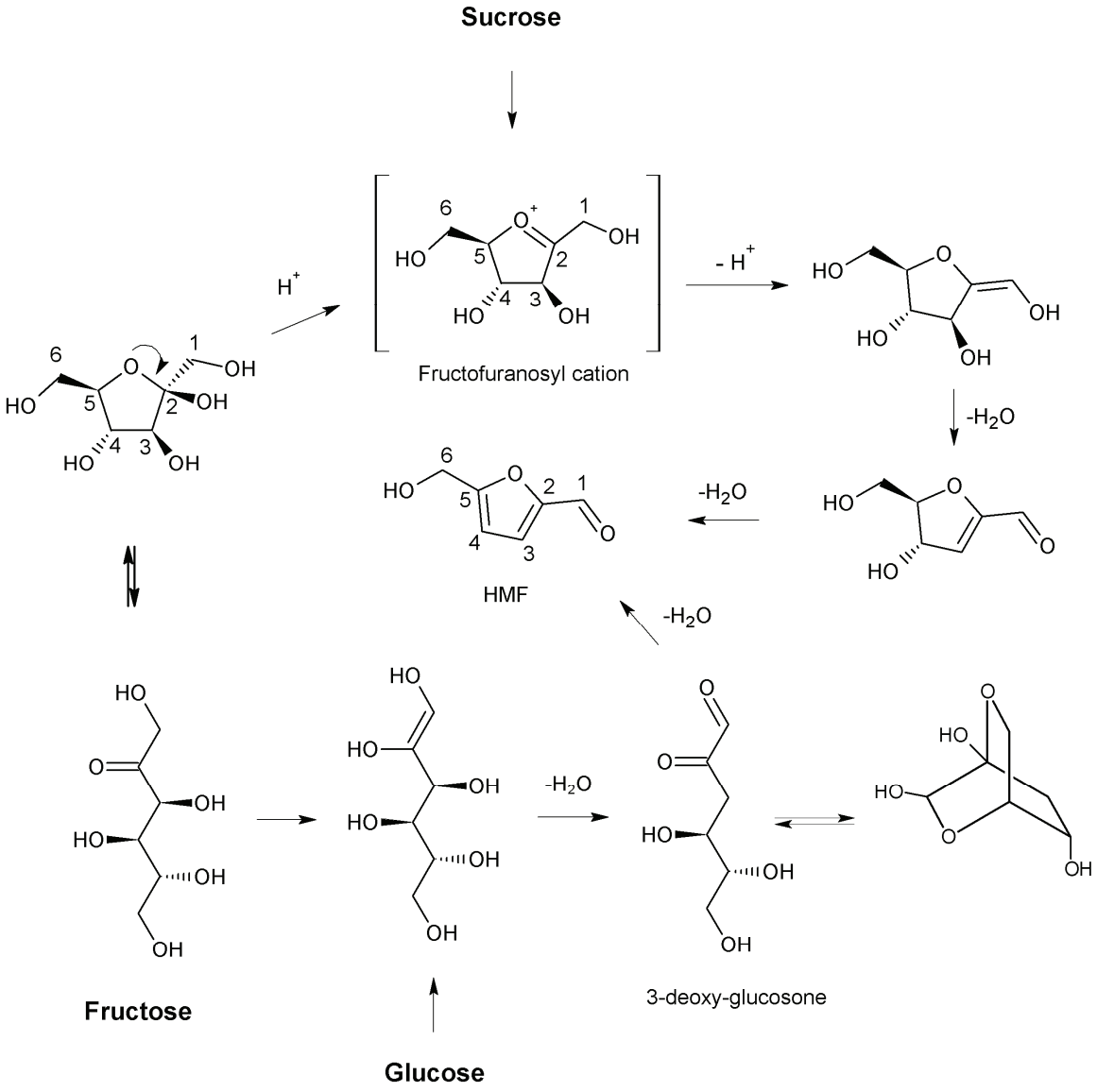
A Helwett-Packard (Palo Alto, California) GC with Mass selective detector (5890 GC/5971B MSD) interfaced to a CDS pyroprobe 2000 unit (CDS Analytical, Oxford, PA) was used for the Py-GC/MS analysis. One mg samples of reactants were mixed either with silica gel or introduced into the quartz tube (0.3 mm thickness) as is, plugged with quartz wool, and inserted inside the coil probe and pyrolyzed at indicated

temperatures with a total heating time of 20s. The column was a fused silica DB-5 column (50m length x 0.2mm i.d. x 0.33 μm film thickness; J&W Scientific). The pyroprobe interface temperature was set at 250ºC. Capillary direct MS interface temperature was 280ºC; ion source temperature was 180ºC. The ionization voltage was 70 eV, and the electron multiplier was 2471 V. All injections were in splitless mode. The mass range analyzed was 33-650 amu. The initial temperature of the column was set at 37 for 2 minutes and was increased to 100ºC at a rate of 30ºC/min, immediately the temperature was further increased to 250ºC at a rate of 8ºC/min and kept at 250ºC for 5 min. The identity and purity of the chromatographic peaks were determined using NIST AMDIS version 2.1 (http://chemdata.nist.gov/mass-spc/amdis/). The reported percent label incorporation values (corrected for natural abundance and for % enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

7.2.3 (1-^{13}Cfru)Sucrose Reaction in Methanol. The (1-^{13}Cfru)sucrose (4 mg) was refluxed in methanol (200 μL) for 10 min in the presence of *p*-toluenesulfonic acid monohydrate (3 mg). A 20 μL portion was injected into the GC/MS and analyzed using the same method as described above.

7.2.4 Detection of Levoglucosan by Ion Chromatography.

A Metrohm MIC-8 modular IC system (Herisau, Switzerland) consisting of a pulsed amperometric detector (E_1 = 0.15 v, t_1 = 400 ms, E_2 = 0.75v, t_2 = 200ms; E_3 = -0.15v, t_3 = 400ms), pump and a sample injection unit connected to Metrosep Carb1-150 anion exchange column thermostated at 31^{o}C was used for the analysis of carbohydrate residue after pyrolysis. The mobile phase was 0.1N NaOH and flow rate of 1 mL/min. The retention time of the commercial levoglucosan was 2.62 min. The residue after pyrolysis of sucrose was dissolved in distilled water and diluted before injection. One of the major peaks had a retention time of 2.62 min identical to the levoglucosan standard.



Scheme 7.1 Proposed Mechanism of HMF Formation from Glucose, Fructose, 3-Deoxyglucosone and Sucrose

7.3 Results and Discussion

7.3.1 Sucrose Degradation and Origin of HMF

Although there are some reports in the literature (Kuster, 1990) to indicate that fructose is the main moiety in sucrose that contributes to the generation of HMF, however, there is no quantitative data. According to Antal et al. (1990) at 250^{o}C and under 34.5 MPa, 30% of the uncatalyzed sucrosesolution (0.05M) can be converted into HMF in 32 seconds. This conversion rate increases to 50% under acid catalysis. Using the data provided by the Antal et al. (1990) regarding the amount of free glucose and fructose remaining after sucrose hydrolysis and the rates of HMF formation from glucose and fructose under identical conditions in addition to the amount of other products formed, we were able to estimate the amount of HMF formed from fructose moiety of sucrose to be ~84% during both uncatalyzed and acid catalyzed reactions. In order to verify this number, the ability of sucrose labeled only at C-1 of fructose moiety to generate HMF was analyzed by Py-GC/MS at various temperatures. If HMF can be produced only from glucose, no incorporation of ^{13}C-label will be observed in the parent ion of HMF at m/z 126, whereas if HMF was generated only from fructose moiety 100% ^{13}C-label incorporation should be observed in HMF and finally if both sugar moieties are responsible for HMF formation less than 100% incorporation will be observed. Moreover, percent label incorporation will indicate percent contribution of fructose to total HMF production from sucrose.

In order to extract mechanistic information from such labeling studies, knowledge of the elemental composition and the structures of the important mass spectral fragments are essential. Consequently, to gain insight into mass spectral fragmentation patterns of HMF singly labeled glucoses were pyrolyzed to generate HMF and label incorporation was analyzed (see Figures 7.1 , Scheme 7.2 and Tables 7.1 and 7.2). These figures show the structures of the relevant fragment ions identified based on the label incorporation pattern listed in Tables 7.1 and 7.2. It is important to note that similar to any aldehyde, HMF exhibits an M-1 peak due to the loss of aldehydic hydrogen. During labeling studies the percent of M-1 peak will be used to indicate unlabeled HMF arising from the glucose moiety, therefore it is important to confirm this number accurately. Table 7.1 indicates

that based on six labeled glucoses and one labeled fructose this value is 16% of the intensity of the parent ion at m/z 126. When [1-^{13}Cfru]sucrose was pyrolyzed to generate labeled HMF the corresponding M-1 peak for the labeled HMF was 26% indicating that 10% was due to the unlabeled HMF arising from glucose and 16% due to loss of aldehydic proton. Therefore, 90% of HMF generated from sucrose arises from fructose moiety during pyrolysis at high temperatures (see Table 7.1). These results are consistent with the above estimation of 84% based on literature data generated at 250^{o}C.

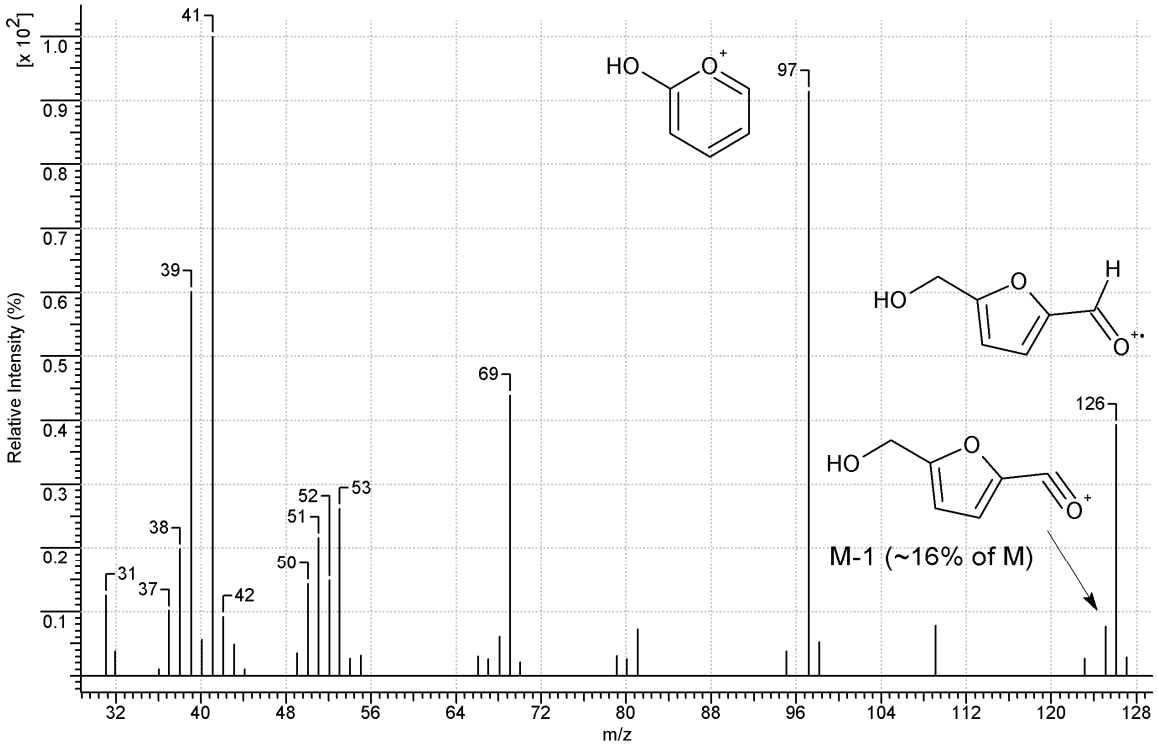
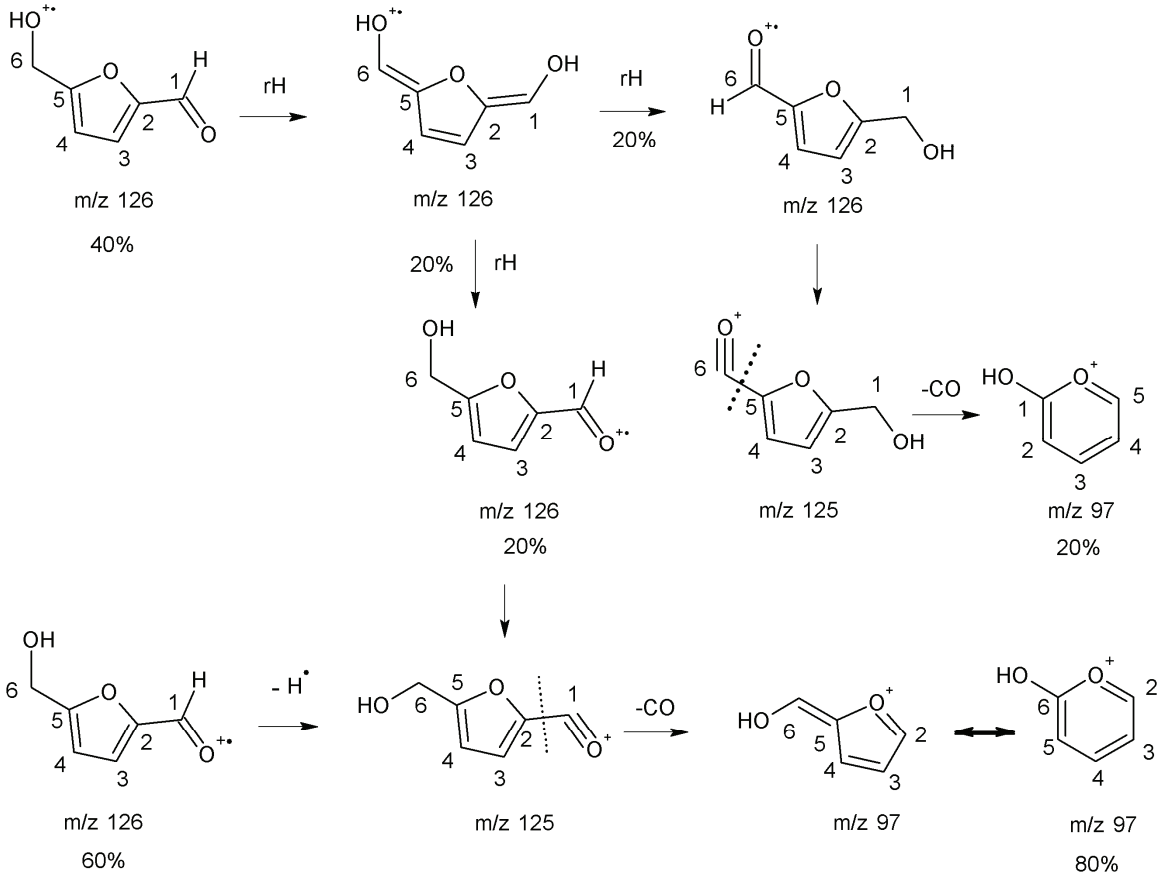


Figure 7.1 Mass spectrum of HMF and the Structure of the Major Ions

7.3.2 H-Rearrangement and Scrambling of Labels in the Molecular Ion of HMF Generated under Electron Impact (EI) Conditions.

During studies on the fragmentation patterns of HMF, inspection of ion at m/z 97 (see Figures 7.1 and Scheme 7.2) has indicated an unexpected label incorporation pattern as shown in Table 7.2. The ion at m/z 97 arises from ion at M-1 by the loss of aldehydic CO as shown in Scheme 7.2. According to Scheme 7.2, when the precursor of HMF is either

glucose-1-^{13}C or fructose-1-^{13}C, the ion at m/z 97 is expected to completely lose the label as ^{13}CO, however, both labeled sugars retained 20% of the label as shown in Table 7.2. In addition, the remaining 80% was incorporated into the C-6 as indicated from the data on pyrolysis of glucose-6-^{13}C (see Table 7.2). These observations can be explained by the formation of two molecular ions as shown in Scheme 7.2, one by the loss of electron from carbonyl oxygen (60%) and the other by the loss of electron from hydroxyl oxygen (40%). The latter can initiate a series of two hydrogen rearrangement reactions to generate two isotopomers of m/z 126 in equimolar amounts (20% each) due to the symmetrical nature of the intermediate formed after the first rearrangement. Consequently, 80% of the HMF in the mass detector will incorporate C-1 as the aldehydic carbon and 20% will incorporate C-6 as the aldehydic carbon.



Scheme 7.2 Mass Spectral Fragmentation Pattern of HMF and Formation of two Molecular Ions at m/z 126. rH = Hydrogen Rearrangement

Table 7.1. Percent Label[1] Incorporation in M+1, M and M-1 ions[2] of HMF Generated from Various Precursors[3]

Compound	M + 1 m/z 127	M m/z 126	M - 1 m/z 125
3-Deoxyglucosone	0	100	16 (% of M)
D-Glucose	0	100	15 (% of M)
D-Glucose-6-^{13}C	100	16 (% of M+1)	
D-Glucose-5-^{13}C	100	16 (% of M+1)	
D-Glucose-4-^{13}C	100	15 (% of M+1)	
D-Glucose-3-^{13}C	100	16 (% of M+1)	
D-Glucose-2-^{13}C	100	15 (% of M+1)	
D-Glucose-1-^{13}C	100	16 (% of M+1)	
D-Fructose-1-^{13}C	100	16 (% of M+1)	
[1-^{13}Cfru]sucrose[4]	**90**	**26 (% of M+1)**	
[1-^{13}Cfru]sucrose[5]	**90**	**26 (% of M+1)**	
[1-^{13}Cfru]sucrose[6]	**0**	**16 (% of M+1)**	

[1] all singly labeled and corrected for ^{13}C natural abundance.

[2] see Schemes 7.2 and 7.3

[3] values represent average of two replicates with standard deviation of not more than 5%.

[4] pyrolyzed at 250^{o}C and corrected for loss of aldehydic hydrogen

[5] pyrolyzed at 350^{o}C and corrected for loss of aldehydic hydrogen

[6] sucrose (4 mg) was refluxed in methanol for 10 min in the presence of p-toluenesulfonic acid monohydrate (3 mg)

Table 7.2 Percent Label[1] Incorporation in Fragment at m/z 97[2] of HMF Generated from Different Precursors[3]

Compound	m/z 97	m/z 98
3-Deoxyglucosone	100	0
D-Glucose	100	0
D-Glucose-6-^{13}C	20	80
D-Glucose-5-^{13}C	0	100
D-Glucose-4-^{13}C	0	100
D-Glucose-3-^{13}C	0	100
D-Glucose-2-^{13}C	0	100
D-Glucose-1-^{13}C	80	20
D-Fructose-1-^{13}C	80	20
[1-^{13}Cfru]sucrose[4]	**81.5**	**18.5**
[1-^{13}Cfru]sucrose[5]	**81.5**	**18.5**

[1] all singly labeled and corrected for ^{13}C natural abundance.
[2] see Schemes 7.2 and 7.3
[3] values represent average of two replicates with standard deviation not more than 5%.
[4] pyrolyzed at 250^{o}C
[5] pyrolyzed at 350^{o}C

7.3.3 Proposed Mechanism of Thermal Generation of HMF from Sucrose

In order to confirm the literature data (Antal et al., 1990) generated at 250^{o}C and under a pressure of 34.5 MPa regarding the relative conversion efficiency of sucrose into HMF; fructose, glucose and sucrose were pyrolyzed at 250, 300 and 350^{o}C and the areas of HMF peaks produced are reported as area per mmol of the starting sugar (see Table 7.3). The data indicated that at all temperatures studied sucrose indeed generated more HMF per mol relative to both fructose and glucose. For mechanistic considerations the efficiency of HMF formation from these sugars at 300^{o}C relative to 3-deoxyglucosone (3-DG) was also studied (see Table 7.4). According to this table and relative to 3-DG, sucrose generated 4.5 fold more HMF and fructose generated 2.4 fold more HMF, on the other hand, glucose generated only 0.16 fold relative to 3-DG. These results clearly show that 3-DG is not the main precursor of HMF in the case of fructose and sucrose otherwise it would have generated more HMF as is the case relative to glucose. These conclusions

Table 7.3 Efficiency[1] of HMF Formation at Different Temperatures from Selected Sugars

Sugar[2]	Temperature (°C)		
	250	300	350
Sucrose	1.79E+09	5.94E+09	7.49E+09
Fructose	1.07E+09	3.13E+09	3.78E+09
Glucose	7.85E+08	1.51E+09	1.78E+09
Levoglucosan	0.0	0.0	0.0

[1] expressed as chromatographic peak area of HMF/ mmol of the sugar. Values represent average of two replicates with percent standard deviation < 5%.

[2] Sugars were homogenized with Silica gel (60%) to maximize reproducibility

Table 7.4 Comparison of Relative Efficiency[1] of 3-DG in HMF Formation Relative to Glucose and Fructose at 300°C

Sugar[2]	Relative efficiency
Glucose	0.16
3-DG	1
Sucrose	4.5
Fructose	2.4

[1] Based on chromatographic peak area of HMF/ mmol of the sugar. Values represent average of two replicates with percent standard deviation < 5%.

[2] Sugars were homogenized with Silica gel (60%) to maximize reproducibility

are consistent with the above assertion (shown in Scheme 7.1) that sucrose and fructose generate HMF through fructopyranosyl cation pathway and glucose generates HMF through 3-DG pathway. Furthermore, to confirm the ability of glycosidically linked terminal fructose (as in sucrose) to generate more HMF relative to free fructose, other oligosaccharides such as raffinose and stacchiose having similar fructose linkages were also analyzed and the results are shown in Table 7.5. According to this table, both raffinose and stacchiose exhibited higher efficiency of HMF formation compared to lactose a disaccharide lacking a terminal fructose moiety as in sucrose.

Table 7.5 Comparison of Relative Efficiency[1] of HMF Formation from Different Oligosaccharides containing Terminal Fructose (except lactose) at 250°C

Sugar[2]	**Relative efficiency**
Sucrose[3]	1
Raffinose[4]	1.3
Stacchiose[5]	0.8
Lactose[6]	0.2

[1] Based on chromatographic peak area of HMF/ mmol of the sugar. Values represent average of two replicates with percent standard deviation < 5%.

[2] Sugars were homogenized with Silica gel (60%) to maximize reproducibility

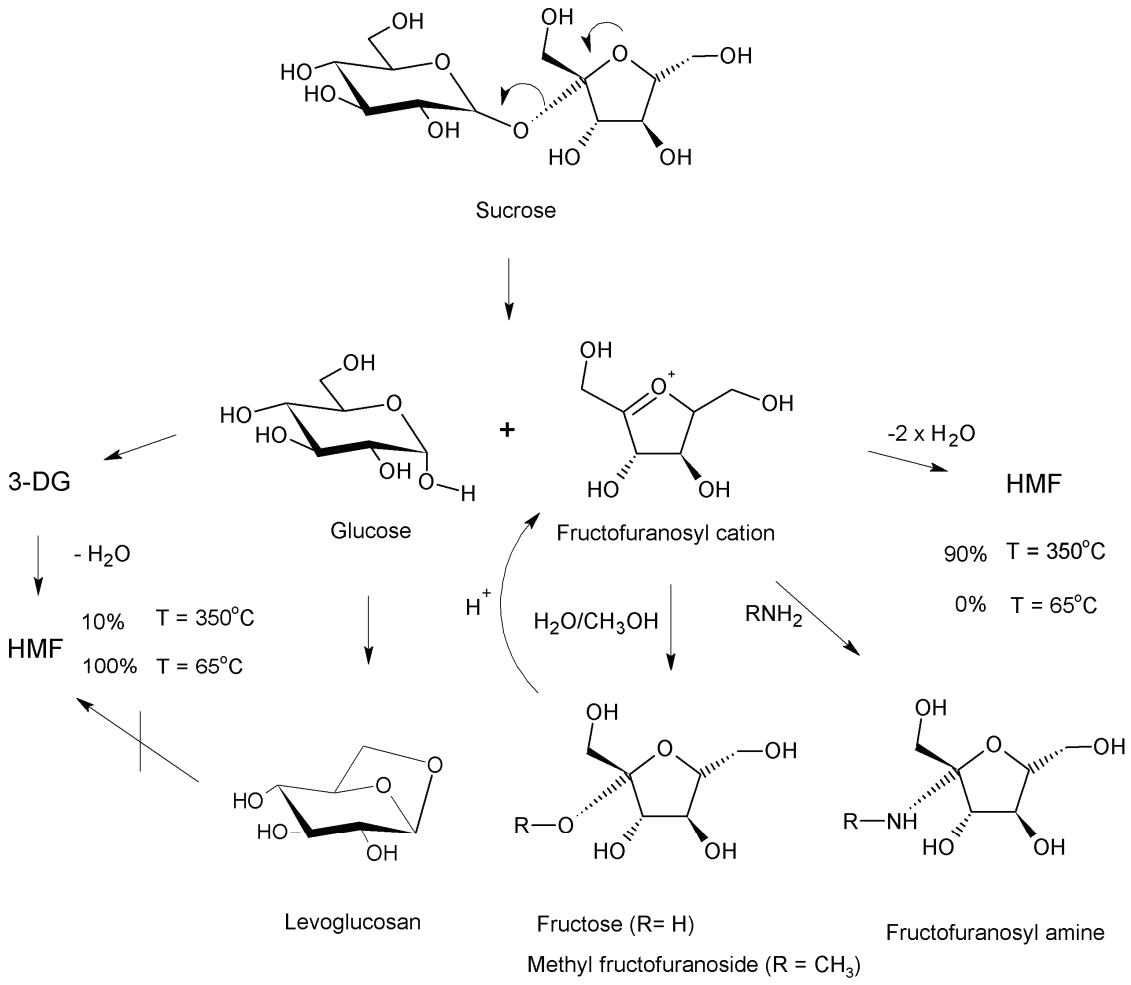
[3]A disaccharide (Glu-Fru)

[4] A trisaccharide (Gal-Glu-Fru)

[5] A tetrasaccharide (Gal-Gal-Glu-Fru). Decomposes at 250°C.

[6]As control (Gal-Glu)

Based on the above observations it can be proposed that the major pathway of sucrose decomposition is the direct formation of fructofuranosyl cation in addition to glucose and 1,6-anhydro-glucose (levoglucosan), a known degradation product of glucose and cellulose (see Scheme 7.3). To confirm the formation of levoglucosan from different sugars, the sugars were pyrolyzed at 250, 300 and 350°C and the data are reported in Table 7.6. According to this table, glucose is the most efficient precursor of levoglucosan followed by sucrose. Fructose however, did not generate any levoglucosan. Pyrolysis of levoglucosan itself indicated that it is volatile enough to be detected at high temperatures (see Table 7.6) and that it did not produce any HMF (see Table 7.3). Furthermore, the formation of levoglucosan from sucrose was also confirmed by ion chromatography, when the sucrose residue generated after pyrolysis was dissolved in water and analyzed. In addition to glucose, comparable amounts of levoglucosan were also detected using commercially available levoglucosan as a standard.



Scheme 7.3 Proposed Mechanism of Thermal Generation of Glucose, Levoglucosan, and Fructofuranosyl Cation, showing Percent Contribution of Glucose and Fructose Moieties to HMF Formation at 350 and 65°C.

Table 7.6 Efficiency[1] of Levoglucosan Formation at Different Temperatures from Selected Sugars

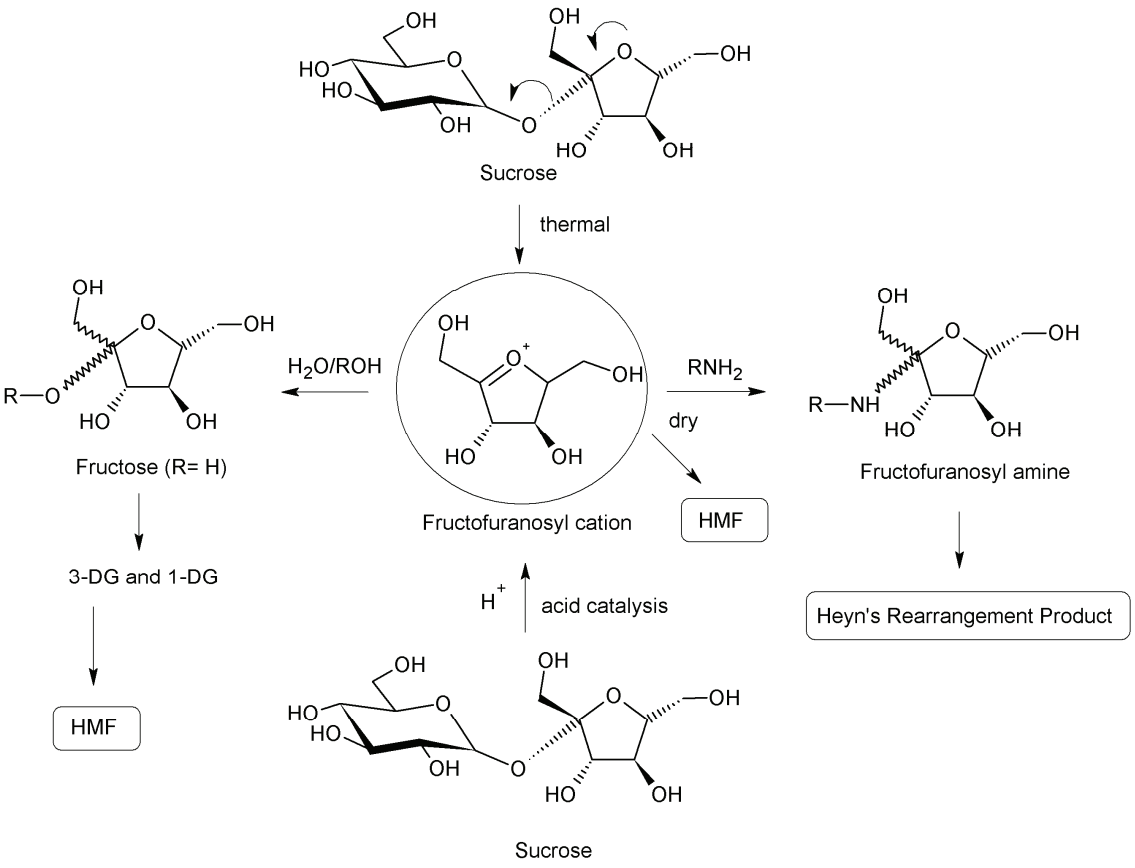
Sugar[2]	Temperature (°C)		
	250	300	350
Sucrose	0.0	5.74E+07	2.87E+08
Fructose	0.0	0.0	0.0
Glucose	2.74E+08	3.58E+08	8.35E+08
Levoglucosan	0.0	9.66E+08	1.97E+09

[1] expressed as chromatographic peak area of levoglucosan/ mmol of hexose. Values represent average of two replicates with percent standard deviation < 5%.
[2] Sugars were homogenized with Silica gel (60%) to maximize reproducibility

Finally, to confirm that HMF mainly arises from fructofuranosyl cation, [1-^{13}Cfru]sucrose was heated in refluxing methanol in the presence of *p*-toluenesulfonic acid as catalyst. According to Moody and Richards (1983) when methanol is used as solvent under acidic conditions, the fructofuranosyl cation, if formed, will immediately react with the solvent to produce methyl fructofuranoside, thus preventing the formation of HMF from the fructose moiety through the 3-DG pathway and the low temperature of refluxing methanol (65°C) will prevent formation of HMF through fructofuranosyl cation pathway. Alternatively, if sucrose was being hydrolyzed into glucose and fructose, without the formation of fructofuranosyl cation as an intermediate, then both fructose and glucose moieties can generate HMF through the less efficient 3-DG pathway as shown in Scheme 7.1. In effect, generation of HMF exclusively from the glucose moiety in the refluxing methanol solution of [1-^{13}Cfru]sucrose can be considered as evidence for the fructofuranosyl cation formation and its generation form both glucose and fructose moieties can be considered as evidence against the fructofuranosyl cation formation.

Consequently, when [1-^{13}Cfru]sucrose was refluxed in methanol for 10 min in the presence of *p*-toluenesulfonic acid monohydrate and the solution was analyzed by GC/MS, the data indicated that the low concentration of HMF detected was exclusively produced from glucose alone. This was illustrated by the lack of any label incorporation

in the HMF (see Table 7.1), thus confirming that hydrolysis of sucrose proceeds exclusively through the formation of fructofuranosyl cation. Based on the above observations, a mechanism of HMF formation from sucrose is proposed as shown in Scheme 7.3. According to this Figure, sucrose under thermal treatment and/or acid catalysis can easily cleave the glycosidic bond of the fructose moiety with the assistance of the lone pair electrons of the fructofuranosyl ring oxygen to release a free glucose and a fructofuranosyl cation as a reactive intermediate. At high temperatures and in dry systems this cation can quickly be converted into HMF, whereas in methanol and at low temperatures it can be trapped as methyl fructofuranoside and therefore only free glucose moiety can be converted into HMF through 3-deoxyglucosone pathway following ring opening and enolization steps (see Scheme 7.1). This pathway is less efficient than direct dehydrations from cyclic forms (see Table 7.4).



Scheme 7.4 Fate of Fructofuranosyl Cation under Various Conditions

7.4 Concluding Remarks

The facile formation of fructofuranosyl cation from sucrose either under acid catalysis or just thermally generated, can also explain the unusual reactivity of sucrose observed in the Maillard reaction (Karel and Labuza, 1968). The fate of this cation depends on the condition of its generation. When it is generated at high temperatures under dry conditions it can directly dehydrate into HMF or react with nucleophiles such as amino acids if present. The fructofuranosyl amine formed as a result of this interaction, can rearrange into the well known Heyns product (see Scheme 7.4). When sucrose was pyrolyzed in the presence of asparagine for example, more acrylamide was generated compared to glucose and fructose combined (Yaylayan et al., 2003). However, if the fructofuranosyl cation is generated under catalysis by dilute acid and at lower temperatures it will mainly be converted into fructose due to its fast reaction with water. As the temperature increases, it is more likely for the fructofuranosyl cation to be converted into HMF or react with nucleophiles especially in low moisture systems. The amount of HMF formed from sucrose is expected therefore to be higher than that of fructose or glucose at higher temperatures due to the more efficient conversion pathway of fructofuranosyl cation into HMF relative to less efficient 3-DG pathway that glucose and fructose follow at lower temperatures under dilute aqueous conditions.

CONNECTING PARAGRAPH

Chapters 6 and 7 provided evidence for the mechanism of formation of furan and one of its derivatives 5-hydroxymethyl-2-furaldehyde (HMF). It was concluded in chapter 6 that 4-hydroxy-alkenals were potential sources of furan in food and that they can be formed through Maillard-type reactions from non-lipid sources. In this chapter their further interaction with amino acids were explored and found to undergo an Amadori type rearrangement termed "vinylogous Amadori rearrangement" generating potentially toxic side effects. The content of this chapter appeared in the journal Molecular Nutrition and Food Research (see Yaylayan, V.A. and Perez-Locas, C. **2007**. Vinylogous Amadori Rearrangement: Implications in Food and Biological Systems. Molecular Nutrition and Food Research, 51, pp. 437-444)

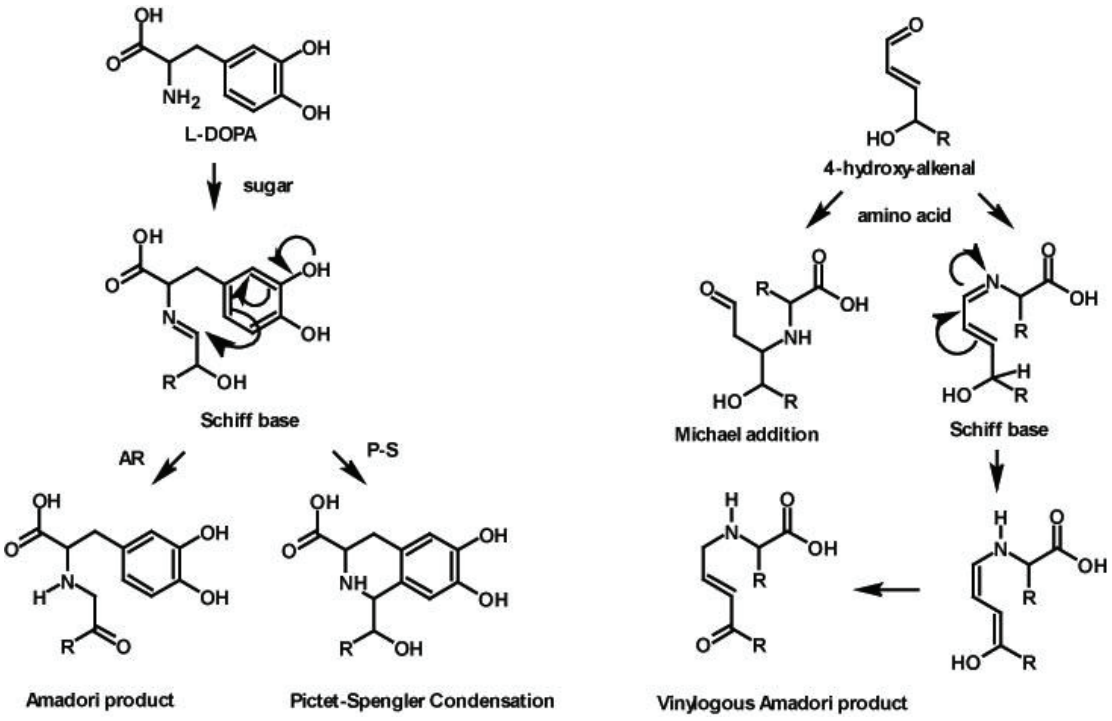
CHAPTER 8

VINYLOGOUS AMADORI REARRANGEMENT: IMPLICATIONS IN FOOD AND BIOLOGICAL SYSTEMS

8.1 Introduction

In the past two decades, nonenzymatic molecular transformations in living systems occurring outside the framework of normal metabolic processes and generating toxic by-products have gained considerable importance (Golubev, 1996; Yin and Chen, 2005). The so called "Amadori rearrangement" (AR) of reducing sugars (Yaylayan and Huyghues Despointes, 1994) is one of several such transformations that include Pictet–Spengler (P–S) condensation, Michael addition of sulfur nucleophiles to 4-hydroxy-2-alkenals – important lipid oxidation products (see Scheme 8.1), and modifications of biomolecules with oxygen centered free radicals. Interestingly, all these nonenzymatic transformations have their counterparts in food systems (Henle, 2005). AR initiates the well known Maillard reaction sequence responsible for the desirable aromas and colors in food and similarly, it is also responsible for the glycation of many biologically relevant proteins that leads to the pathogenesis of different age-related disorders (Baynes, 2003; Monnier, 2003). This rearrangement of reducing sugars can occur with any amino acid without any structural restrictions. On the other hand, P–S condensation (see Scheme 8.1), is specific to β-arylethylamines such as L-DOPA (levodopa), histamine, tyrosine, tryptamine, dopamine, tryptophan, etc. and similar to AR, is initiated by a carbonyl–amine interaction and formation of Schiff base. This reaction was discovered around the same time as the Maillard reaction. Factors that govern the competition between the two reactions were studied by Manini et al. (Manini et al.., 2005). Glycation reactions of reducing sugars through AR leads to the formation of a wide range of chemical structures including acyclic, monocyclic, fused bicyclic, and other potentially toxic compounds that can inflict irreparable damage to biological systems (Baynes, 2003). On the other hand, P–S condensations can generate mainly two moieties, tetrahydroisoquinoline and b-carboline. The drug L-DOPA, extensively used to treat Parkinson's disease, eventually induces dyskinesias in the patients after prolonged administration due to the generation of neurotoxic tetrahydroisoquinoline derivatives through P–S condensation (see Scheme 8.1). Similarly, the amino acid tryptophan can generate β-carboline derivatives both in food and biological systems (Yaylayan et al., 1990). These compounds have been demonstrated to possess antioxidant activities and to inhibit platelet aggregation, monoamine oxidase, and binding to benzodiazepine receptors. In addition to reducing

sugars, 4-hydroxy-2-alkenals are also known to undergo metabolic side-reactions with amino acids (see Scheme 8.1). They are generated through lipid oxidation (Zamaro and Hidalgo, 2005) and are considered to be toxic intermediates due to their reaction with amino acids and proteins. Although 4-hydroxy-alkenals can undergo both Michael addition and Schiff base formation with amino acids, the latter can lead to the formation of vinylogous Amadori rearrangement product (vARP). This pathway has not been characterized in detail. This report provides spectroscopic evidence for such rearrangement and explores the possible impact in food and biological systems.



Scheme 8.1. Comparison of Amadori Rearrangement (AR) with Pictet-Spengler (P-S) Condensation and Michael Addition with Vinylogous Amadori Rearrangement.

8.2 Materials and Methods

8.2.1 Chemicals and Reagents

All reagents and chemicals were purchased from Aldrich (Milwaukee, WI) and used without further purification. HNE (in ethanol) was purchased from Cayman Chemical Co. (Ann Arbor Michigan).

8.2.2 Sample preparation

All samples were dissolved in ethanol (total volume 10 μL) at a concentration of 10 mg/mL. Reaction mixtures were equimolar.

8.2.3 FTIR Analysis

Infrared spectra were recorded on a Nicolet 8210 spectrometer equipped with a temperature controlled single bounce ATR sample accessory. A total of 64 scans at 4 cm^{-1} resolution were added together. Processing of the FTIR data was performed using GRAMS/32 AI (ThermoGalactic) software. Second-order derivatization was performed using Savitsky–Golay function (30 points) to enhance closely absorbing peaks. Peak assignments were performed according to the standard procedures (Lambert et al., 1998).

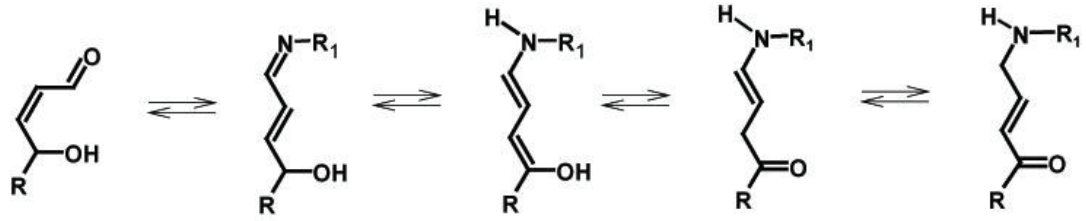
8.2.4 Temperature Studies

Sample solutions (10 μL) were placed on the ATR crystal and the solvent was allowed to evaporate before data acquisition at the specified temperature and/or time. The time of the initial mixing of the reactants was considered as time zero. Infrared spectra were recorded as described in Section 7.2.3.

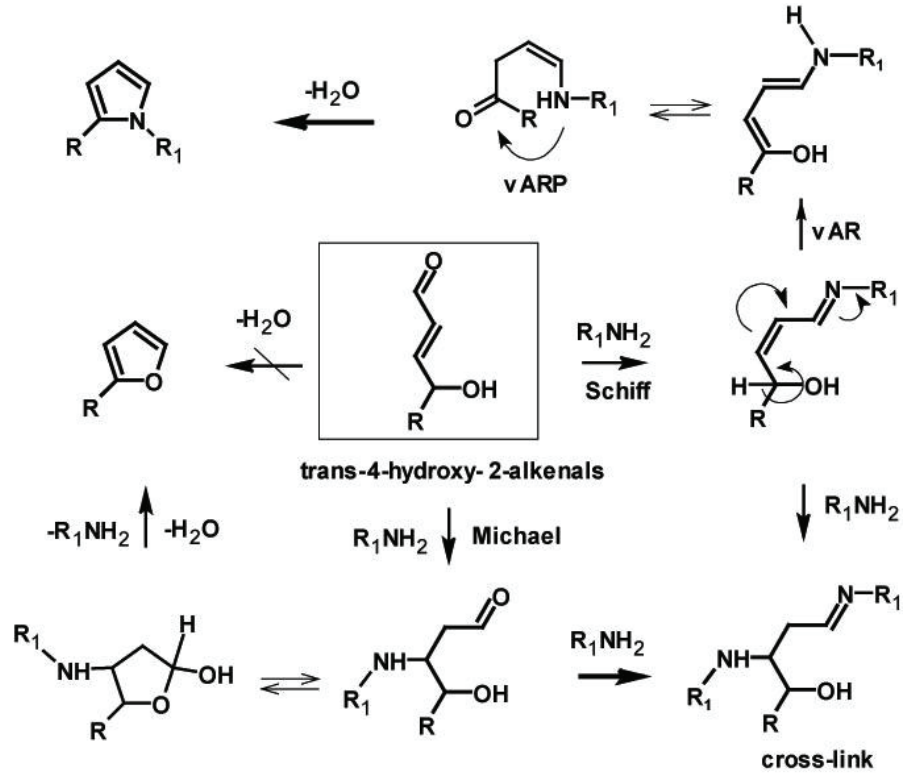
8.3 Results and Discussion

Similar to biological glycation and Maillard reactions in food, oxidative degradation of PUFAs and subsequent formation of lipid peroxides are known to play a major role both in the development of degenerative diseases in biological systems (Xu and Sayre, 1998) and off-flavors or rancidity in food systems (Vichi et al., 2003). In addition, many of the dicarbonyl intermediates produced by Maillard reaction, such as glyoxal and methylglyoxal, have also been shown to be generated from lipid oxidation (Zamora and Hidalgo, 2005). Not only the products generated by lipid oxidation and Maillard reactions are similar in structure, but some, such as 4-hydroxy-2-alkenals and 4,5-epoxy-2-alkenals can also undergo Strecker-type degradation (Hidalgo and Zamora, 2004; Hidalgo et al., 2005). These alkenals result from the subsequent homolytic cleavages of PUFA hydroperoxides, catalyzed by transition metal ions (Xu and Sayre, 1998). Similar to reducing sugars, these highly cytotoxic 4-hydroxy-2-alkenals, such as 4-hydroxy-2-

nonenal (HNE), are capable of modification of proteins, DNA, and LDL through either Michael addition and/or Schiff base formation which can undergo vinylogous AR (see Scheme 8.2).



Scheme 8.2 Vinylogous Amadori Rearrangement of 4-Hydroxy-2-Alkenal Moiety with Amino Acids.
R_1= -CH(R)COOH



Scheme 8.3. Various Reactions of 4-Hydroxy-2-Alkenals with Amino Acids.
vARP = vinylogous Amadori rearrangement product.
vAR= vinylogous Amadori rearrangement.

8.3.1 FTIR Monitoring of HNE Reaction with Amino Acid

The reactions of HNE with amino acids and proteins have been investigated extensively. The type of product formed, whether Schiff base or Michael adduct, is dependent on the reaction conditions as well as on the properties of the nucleophile (Esterbauer et al., 1991). For example, 2,4 dinitrophenylhydrazine has been shown to react exclusively via Schiff base to form 2,4-dinitrophenylhydrazone with a strong yellow color that absorbs at *k*max 360–380 nm (Benedetti et al., 1980). On the other hand, sulfur nucleophiles (glutathione, cysteine, etc) are known to react with HNE exclusively through Michael reaction (Esterbauer et al., 1991). The Michael adducts are usually in equilibrium with their corresponding acetal forms as shown in Scheme 8.3. HNE can react with different amino acids at different rates, sequentially forming first a Schiff base followed by Michael adduct or forming Michael adduct first followed by Schiff base generating identical crosslink structure (see Scheme 8.3). Although the sequence of the addition of amino acids on to HNE will make no difference in the structure of the crosslink, however, the initial Michael adduct is prone to acetal formation due to the disruption of the trans geometry that prevents cyclization and makes it much more stable than the corresponding cis isomer (Benedetti et al., 1980). The acetal can undergo dehydration and deamination to produce 2-pentylfuran, a product that has been identified mainly in food systems. On the other hand, the initial Schiff adduct can undergo vinylogous AR, followed by cyclization to produce N-substituted 2-pentypyrrole as shown in Figure 8.1. Such pyrroles were isolated from HNE and primary amine reaction mixtures and characterized by independent synthesis (Sayre et al., 1993b). Furthermore, immunochemical evidence supporting the formation of 2-pentylpyrrole on proteins exposed to HNE was also later provided by Sayre et al. (1993a). Sayre et al. (1993b) have proposed vinylogous AR as a mechanism for the formation of 2-pentylpyrroles from the interaction of HNE with amines, without naming it as such. However, Wondrak et al. (1997) characterized the reaction mechanism that leads to the formation of pyrroles as a vinylogous AR in amino acid/2-deoxy-ribose model system. Consequently, it can be concluded that, vARPs can only be isolated when the product is prevented from cyclization to form pyrroles. Hidalgo et al. (2005) inadvertently, isolated such a product (m/z 277 in Scheme 8.4) in the model system of phenylalanine and HNE.

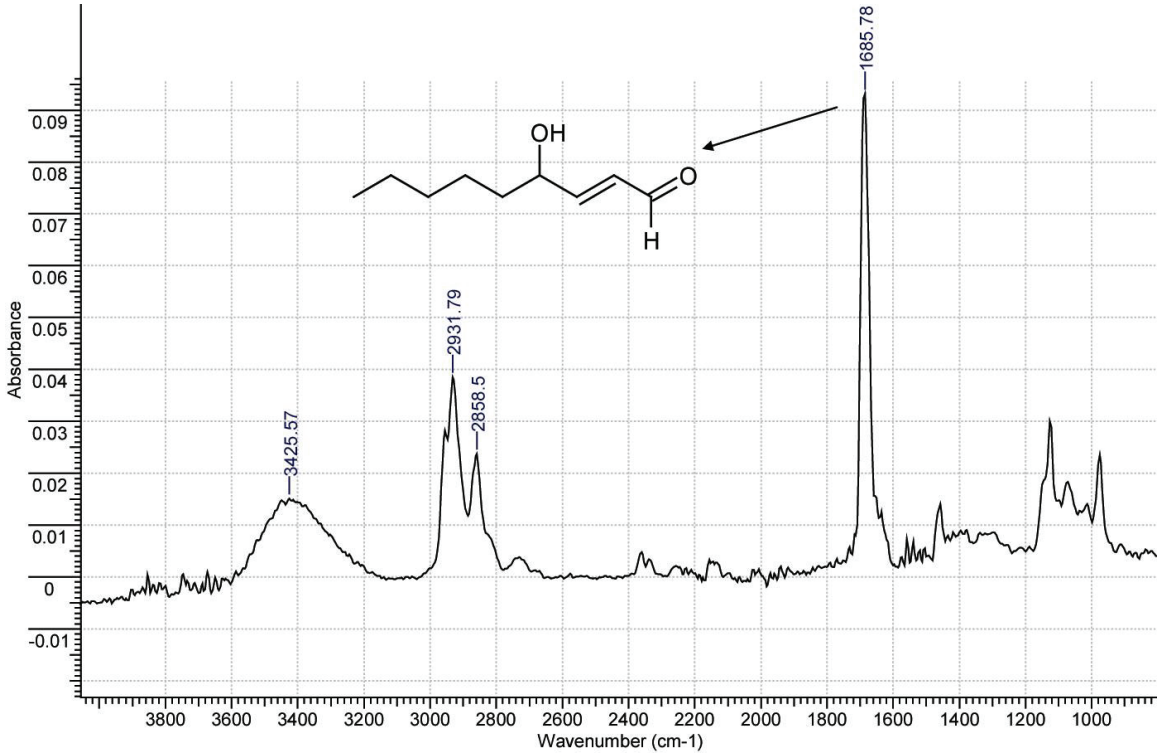
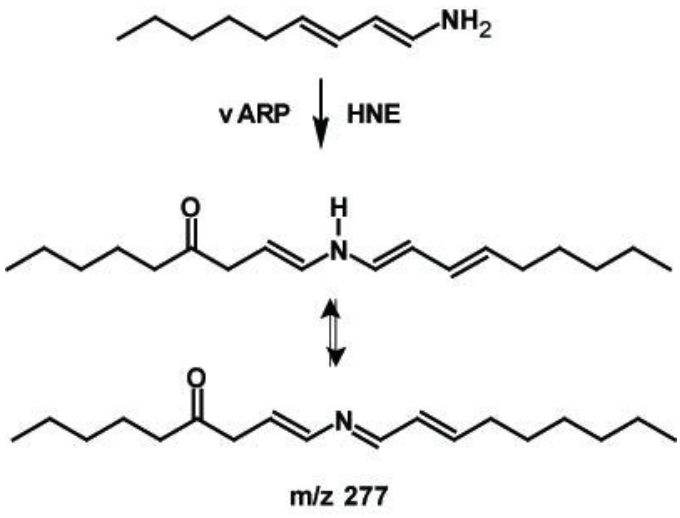


Figure 8.1. FTIR Spectrum of HNE.



Scheme 8.4. Vinylogous Amadori Rearrangement (vARP) of Nonadiene-Amine with HNE (based on Hidalgo et al., 2005)

Although the authors have provided mass spectral data consistent with our proposed structure shown in Scheme 8.4, they did not characterize the structure as a vARP. Due to the isomerization of the double bonds in the structure of the amine (a degradation product in the model system), the ability of the amine to undergo nucleophilic attack and form a pyrrole ring is considerably diminished and hence the vARP survives in the reaction mixture to be detected by GC/MS. Furthermore, they have also identified the vARP of HNE with phenylalanine itself, after its decarboxylation and cyclization into a pyrrole moiety. Again, the authors have not characterized the product as arising from a vinylogous AR. On the other hand, the reaction of proline with HNE should also produce relatively stable vARP due to the formation of a tertiary amine unable to cyclize into a pyrrole ring and this reaction can be conveniently monitored by FTIR. Figure 5 shows the FTIR spectrum of HNE and Figure 8.2 shows time dependant spectra of HNE incubated with proline at 28ºC. According to this figure, the carbonyl band disappears within 15 min at 28ºC forming the Schiff base. When temperature was increased to 50ºC, a new carbonyl band appeared at 1708 cm^{-1} consistent with the formation of vARP (see Figure 8.3).

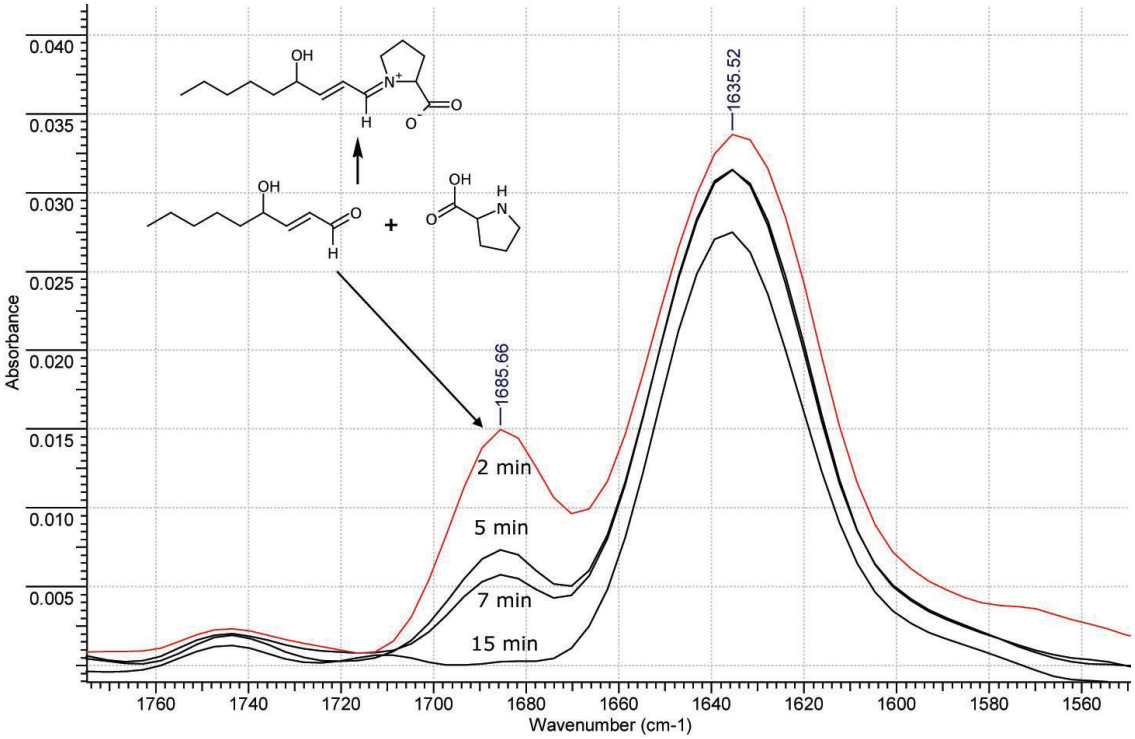


Figure 8.2. Time-Dependent Spectra of HNE Incubated with Proline at 28°C

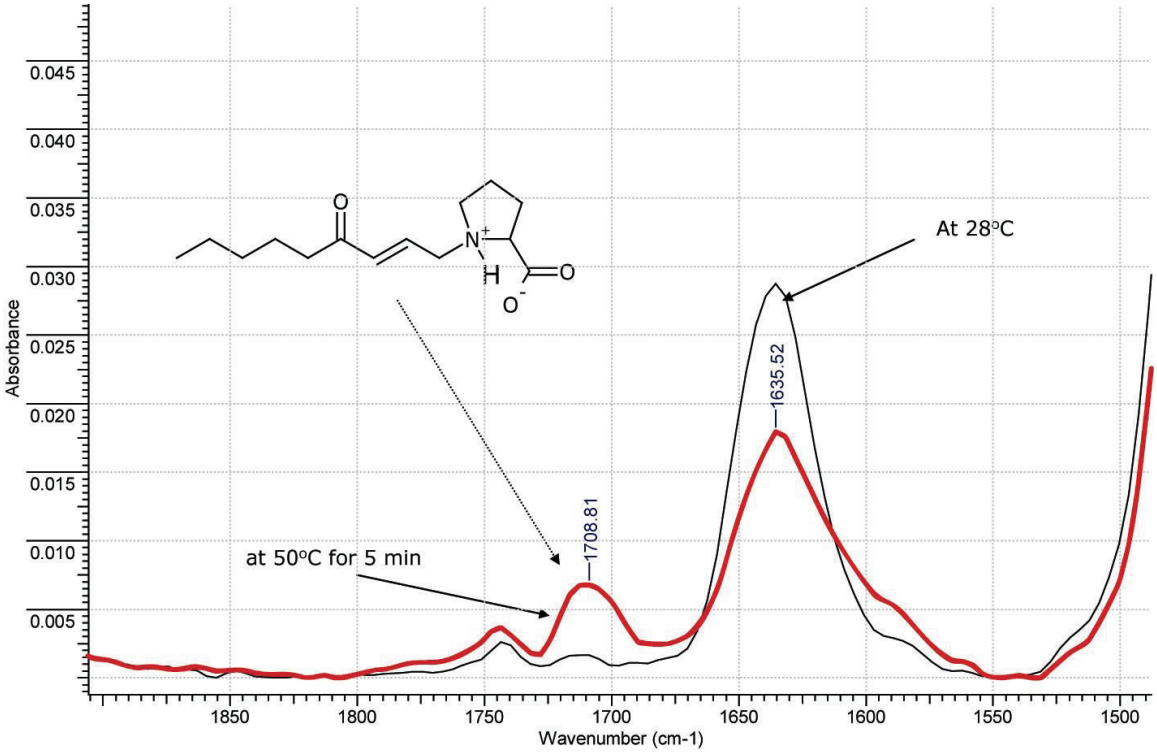
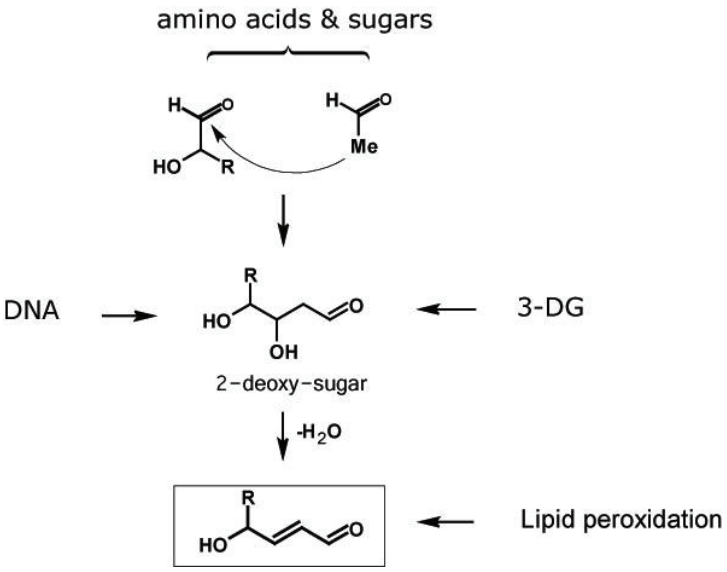


Figure 8.3 FTIR Spectrum of HNE Incubated with Proline for 5 min at 50°C

8.3.2 Precursors of 4-Hydroxy-2-Alkenal Moieties in Nonlipid Systems: 2-Deoxy-Sugars

The main precursor of 4-hydroxy-alkenals in nonlipid systems is the 2-deoxy-sugar moiety that can be formed during Maillard reaction through several pathways (Perez-Locas and Yaylayan, 2004) and eventually can be dehydrated to produce 4-hydroxy-2-alkenal (see Scheme 8.5). Hydrolysis of DNA can also provide 2-deoxyribose as a precursor. Studies performed on model systems using pyrolysis-GC/MS analysis and ^{13}C-labeled sugars and amino acids (Perez-Locas and Yaylayan, 2004) have indicated that certain amino acids such as serine and cysteine can degrade and produce acetaldehyde and glycolaldehyde that can undergo aldol condensation to produce 2-deoxy-aldotetrose moiety. Other amino acids such as aspartic acid, threonine and a-alanine can degrade and produce only acetaldehyde and thus need sugars as a source of glycolaldehyde to generate similar 2-deoxy-sugars. On the other hand, monosaccharides are also known to undergo degradation to produce both acetaldehyde and glycolaldehyde, in addition to 2-



Scheme 8.5 Origin of 4-Hydroxy-2-alkenal Moiety in Non-Lipid Systems.
3-DG = 3-Deoxy-glucosone. (2-deoxy-sugars include 2-deoxy-ribose from DNA, 2-deoxy-glucose from 3-deoxy-glucosone and 2-deoxy-tetrose from amino acid and sugar degradation)

deoxy-sugar moieties. Studies performed using ^{13}C-labeling have also revealed that glucose can degrade through the formation of 3-deoxy-glucosone (3-DG) and produce 2-deoxy-aldotetrose moiety incorporating the C2–C3–C4–C5 carbon chain of glucose. To provide evidence in support of the formation of 4-hydroxy-2-alkenal from 2-deoxy-sugars, 2-deoxy-ribose was selected as a model using Fourier transform infrared spectroscopy (FTIR) to monitor the dehydration reaction. Such transformations of an isolated aldehyde into a conjugated system can be easily detected due to a shift of the carbonyl absorption band from 1716 cm^{-1} (open form 2-deoxy-ribose) to a lower frequency such as 1686 cm^{-1} as in HNE. When 2-deoxy-ribose was incubated at different temperatures in the presence of proline or 5-amino valeric acid in methanol and the carbonyl region was monitored by FTIR, the intensity of the band centered at 1716 cm^{-1} diminished over time and new band appeared around 1672 cm^{-1}, indicating the formation of a conjugated system (see Figure 8.4). Such amino acid-catalyzed dehydration of 2-deoxy-ribose into 4-hydroxy-2-pentenal had also been observed previously by Nelsestuen (Nelsestuen, 1979). Furthermore, the second derivative spectrum of proline model system (Figure 8.5) also indicated the presence of an absorption band centered at 1708 cm^{-1} indicating the formation of a stable vARP.

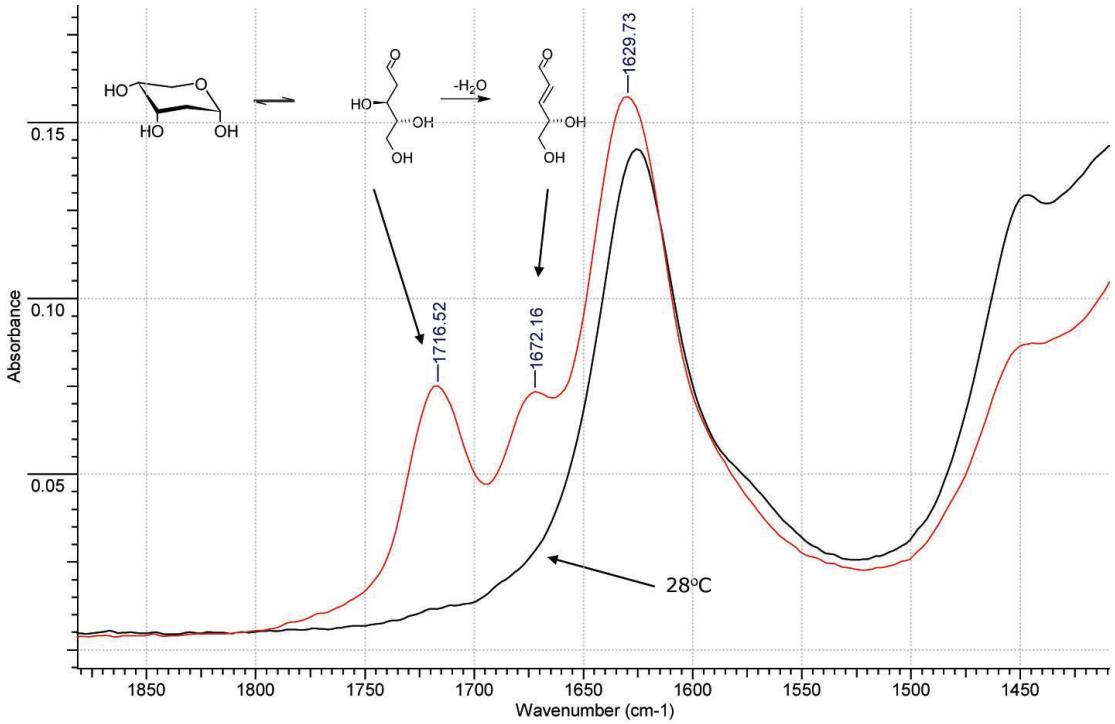


Figure 8.4 FTIR Spectrum of 2-deoxy-ribose Incubated with Proline for 15 min at 50°C

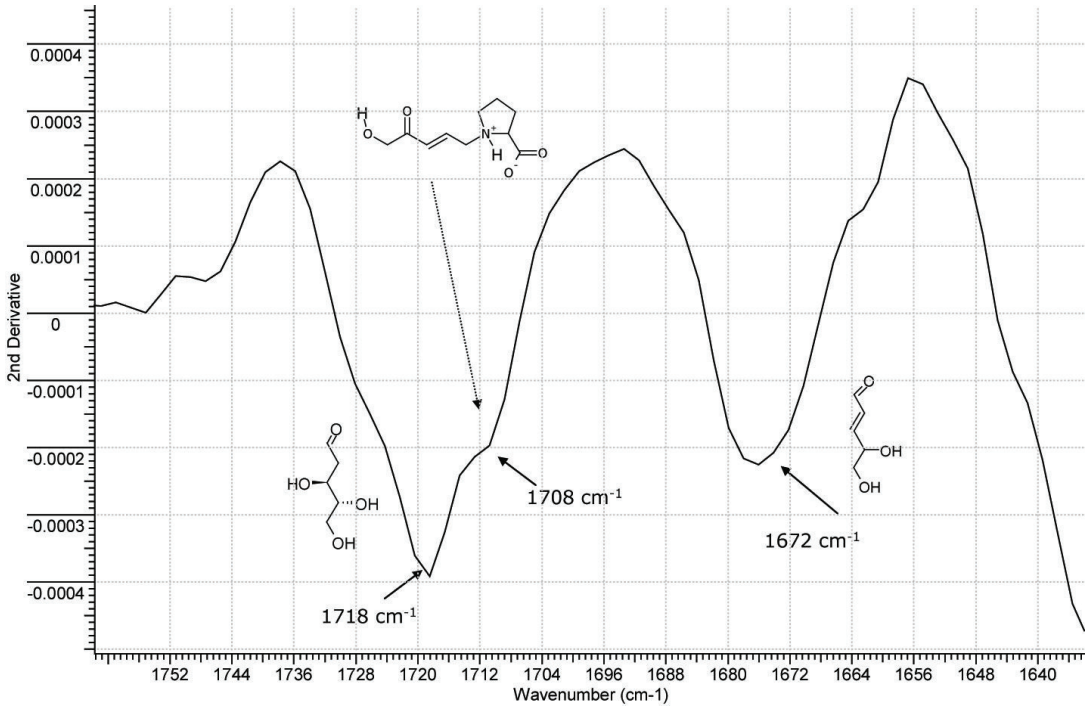


Figure 8.5 Second Derivative Spectrum of 2-deoxy-ribose Incubated with Proline for 15 min at 50°C

8.4 Conclusion

Reactive 4-hydroxy-2-alkenals can be generated not only in lipid containing systems but also through the dehydration of 2-deoxy-sugars. Most likely, the latter pathway will generate a cis/trans mixture. The cis isomers can readily cyclize into furans as recently detected in different food systems (Yaylayan, 2006). On the other hand, the trans isomer is more prone to react with different biological nucleophiles and form crosslinks or pyrrole moieties through vinylogous AR, causing the accumulation of considerable damage on important body proteins.

CHAPTER 9

GENERAL CONCLUSIONS AND SUMMARY OF CONTRIBUTIONS TO KNOWLEDGE

The process-induced toxicants will inevitably play an important role in determining the future direction of food safety policy not only by regulatory agencies but also by many food companies. Mitigation strategies to reduce many of these potential toxicants in food will rely on detailed knowledge of their formation mechanisms. This thesis has mainly focused on elucidation of the molecular mechanisms of formation of thermally generated compounds of recent concern such as acrylamide and its derivatives, furan, hydroxymethyl-2-furaldehyde (HMF) and 4-hydroxyalkenals.

Investigation of different sources of acrylamide formation in model systems consisting of amino acids and sugars have indicated the presence of two pathways of acrylamide generation; the main pathway specifically involves asparagine to directly produce acrylamide after a sugar-assisted decarboxylation step and the second, non-specific, pathway involves the initial formation of acrylic acid from different sources and its subsequent interaction with ammonia and/or amines to produce acrylamide or its N-alkylated derivatives. Aspartic acid, β-alanine and carnosine were found to follow the acrylic acid pathway. In addition, creatine was found to be a good source of methylamine in model systems and was responsible for the formation of N-methylacrylamide through the acrylic acid pathway. Labeling studies using creatine (methyl-d_3) and $^{15}NH_4Cl$ have indicated that both nitrogen and methyl group of methylamine had originated from creatine. Furthermore, analysis of cooked meat samples has also confirmed the formation of N-methylacrylamide during cooking. Labeling studies using [^{13}C-4]aspartic acid have confirmed the occurrence in this amino acid of a previously proposed sugar-assisted decarboxylation mechanism identified in asparagine/glucose model system. To gain further insight into this decarboxylation step, the amino acid/sugar reactions were analyzed by FTIR to monitor the formation of 5-oxazolidinone intermediate known to exhibit a peak in the range of 1770-1810 cm^{-1}. Spectroscopic studies clearly indicated the formation of an intense peak in the indicated range, the precise wavelength was

dependant on the amino acid and the sugar used. The identity of the peak was verified by observing a 40 cm^{-1} shift when [^{13}C-1]-labeled amino acid was used. Furthermore, to identify the relative importance of acrylamide precursors, the decarboxylated Amadori product (AP ARP) and the corresponding Schiff base were synthesized and their relative abilities to generate AA under dry and wet heating conditions were studied. Under both conditions, the N-(D-glucose-1-yl)-3'-aminopropionamide had the highest intrinsic ability to be converted into AA. In the dry model system, the increase was almost four fold higher than the corresponding AP ARP or AP, however in the wet system, the increase was two fold higher relative to AP ARP but more than twenty fold higher relative to AP.

Moreover, studies performed on model systems using pyrolysis-GC/MS analysis and ^{13}C-labeled sugars and amino acids including ascorbic acid, have indicated that certain amino acids such as serine and cysteine can degrade and produce acetaldehyde and glycolaldehyde that can undergo aldol condensation to produce furan after cyclization and dehydration steps. Other amino acids such as aspartic acid, threonine and α-alanine can degrade and produce only acetaldehyde and thus need sugars as a source of glycolaldehyde to generate furan. On the other hand, monosaccharides are also known to undergo degradation to produce both acetaldehyde and glycolaldehyde; however, ^{13}C-labeling studies have revealed that hexoses in general will mainly degrade into aldotetrose derivatives to produce the parent furan. Dehydration of aldotetroses can generate potentially toxic 4-hydroxyalkenals that was demonstrated to undergo a variant of Amadori rearrangement termed vinylogous Amadori rearrangement. This process can further diversify the toxic pathways of 4-hydroxyalkenals through their interaction with amino acid and proteins. Finally, the formation of HMF from sucrose through fructofuranosyl cation at dry and high temperatures as a distinct and more efficient mechanism from that of 3-deoxyglucosone formation from monosaccharides under aqueous conditions was demonstrated, improving our understanding of relative contribution of these sugars to HMF formation in different food products. In essence, the body of work presented in this thesis has significantly enhanced our detailed knowledge of the formation of important food hazards.

The contribution to knowledge is summarized below:

Through its different chapters, the work carried out in this thesis provided for the first time:

1. Detailed pathways for the formation of acrylamide from amino acids β-alanine, aspartic acid, serine, cysteine, and the dipeptide carnosine
2. A pathway for the formation of N-alkylated derivatives of acrylamide in meat through labeling studies and py-GC/MS.
3. Evidence for the involvement of the oxazolidin-5-one in acrylamide formation from asparagine using FTIR and isotope labeling techniques.
4. Developed a methodology with AEC-PAD capable of simultaneously detecting Maillard intermediates such as Amadori and glycosylamines from asparagine-glucose reactions as a useful tool for reaction monitoring. It also had the ability of detecting acrylamide formation over time.
5. Provided detailed mechanistic pathways describing the origin and formation of the parent furan from amino acids, sugars, amino acid/sugar mixtures, and ascorbic acid through labeling studies and py-GC/MS.
6. Provided evidence for the involvement of fructofuranosyl cation as a crucial intermediate in the formation of HMF from sucrose though labeling studies and py-GC/MS.
7. Demonstrated the ability of deoxysugars to produce 4-hydroxy-2-alkenals and provided evidence for 4-hydroxy-2-alkenals to undergo vinylogous Amadori rearrangement with nucleophiles, a previously uncharacterized pathway.

APPENDIX A

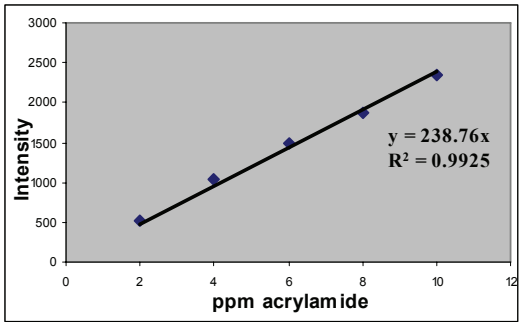
Simultaneous Determination of Acrylamide and its Precursors by Ion Chromatography with Pulsed Amperometric Detection

Among the advantages of coupling of anion exchange column with the PAD system is the use of a single solvent system, as opposed to normal or RP-HPLC which would have required a mobile phase for the column followed by post-column derivatization of the analytes with NaOH prior to PAD analysis. The system was originally set up for carbohydrate analysis, however detection voltage was slightly modified to afford an adequate sensitivity for all species.

Calibration

PAD is known to allow sensitive and quantitative evaluation of various carbohydrates (Johnson and LaCourse, 1990). Calibration of glucose showed a LOD in the low ppb range. As expected, a correlation coefficient (R^2) of 0.9992 expressed remarkable linearity over the range of 5-25 mg/L (ppm). Acrylamide was also surprisingly responsive with a LOD of 0.2 ppm. Although exhibiting more limitation in its linear range than glucose, excellent linearity was revealed between 2 and 10 ppm with an R^2 of 0.9925. Calibration of both species was undertaken using triplicate injections resulting in a RSD below 4%. Calibration curves for acrylamide and glucose are depicted in figures 1a and 1b, respectively.

a)



b)

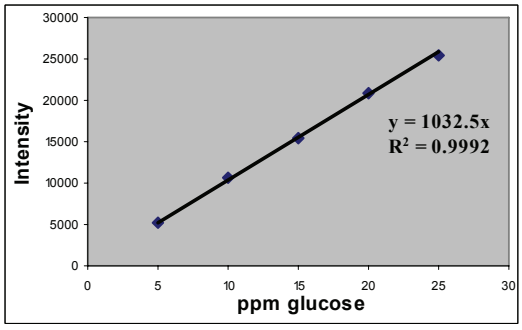
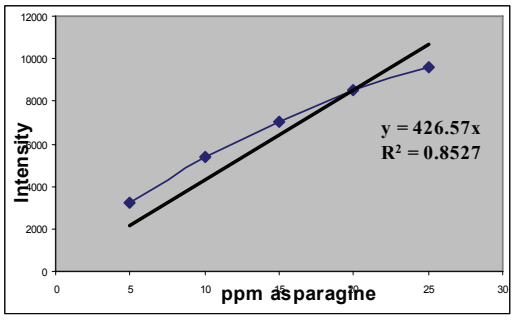


Figure 1 Calibration based on Triplicate Injections for a) Acrylamide b) Glucose

Although the system allowed for adequate detection of AP, asparagine, N-(D-glucos-1-yl)-3'-amino-propionamide, N-(1-deoxy-D-fructose-1-yl)-3'aminopropionamide and N-(D-glucos-1-yl)-asparagine, their linear ranges were rather narrow, as predicted in literature. Reproducibility was adequate with standard deviations between triplicates below 5%, however as depicted in the calibration plots for asparagine and 3-aminopropionamide in figures 2a and 2b, linearity is poor with R^2 of 0.8527 and 0.8829, respectively. Under these circumstances, quantitation of these species is not ideal, however not crucial to our objectives.

a)



b)

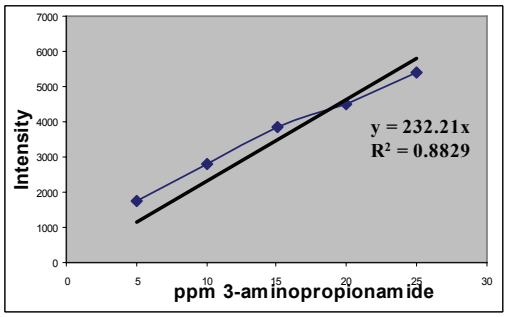


Figure 2 Calibration based on Triplicate Injections for a) Asparagine b) 3-AP

In all cases, quantitation was attempted using peak area and peak height. Peak areas consistently revealed better linearity with a lower standard deviation and was hence used. Similarly to RP-HPLC, small shifts in retention time (±0.05s) can arise as a result of slight variations in mobile phase concentration from one batch to another. However the variability was deemed insignificant and daily injection of standard solutions was an easy remedy to this issue.

Despite the cleaning and regenerating cycles of the gold electrode, characteristic to PAD, saturation can occur over time and affect the response, thus minor adjustments in calibration every few weeks may be required. A good way of evaluating consistent response is by injecting a standard solution of the analytes of interest to test for reproducibility.

Asparagine-Glucose Model System

Despite some modest limitations, this approach finds its strength in its intrinsic ability to monitor the formation and dissociation of an array of compounds having oxidation-reduction potential. This attribute is exploited for monitoring of acrylamide formation from various model systems. Its ability to separate acrylamide from its precursors is clearly depicted in figure 3.

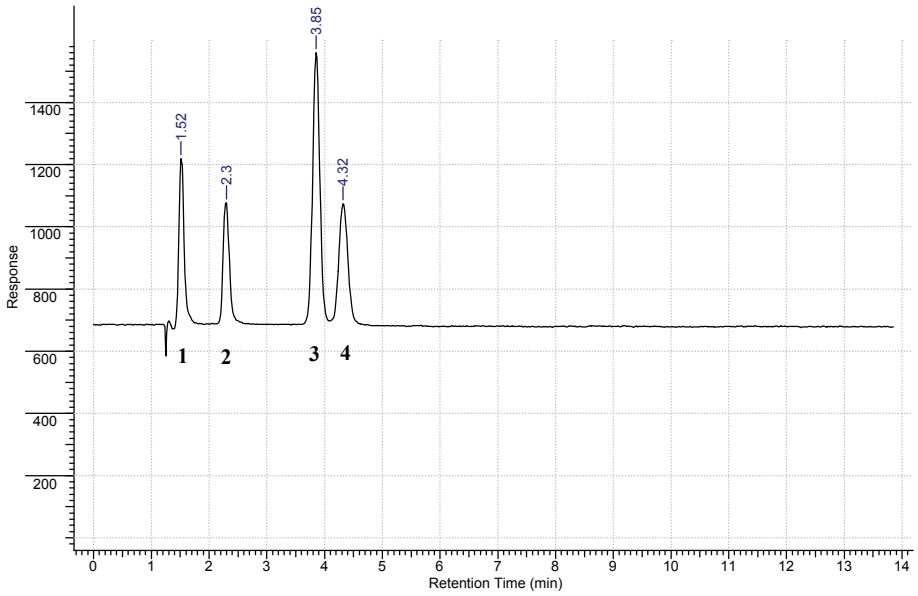


Figure 3 Ion Chromatogram depicting Simultaneous Monitoring of 3-AP (peak 1, 15ppm), Acrylamide (peak 2, 15ppm), D-Glucose (peak 3, 8.1ppm), D-Asparagine (peak 4, 15 ppm) Standards. Mobile phase: 0.1N NaOH

Moreover, as presented in figure 4 and 5, one can observe the formation of acrylamide and its intermediates over time from asparagine glucose reaction as a result of different heating conditions. Figure 4 introduces the formation of the decarboxylated asparagine glycosylamine, or AP glycosylamine ((N-D-glucos-1-yl)-3'-aminopropionamide), prior to acrylamide formation. Prolonged heating under wet conditions, reveals the formation of acrylamide and decarboxylated asparagine Amadori product N-(D-fructose-1-yl)-3'-aminopropionamide (see Figure 5). However, no claims can be made with regards to 3-aminopropionamide as its retention time is identical to that of aspartic acid, also a potential product of asparagine. Table 1 depicts the retention time values for the analytes.

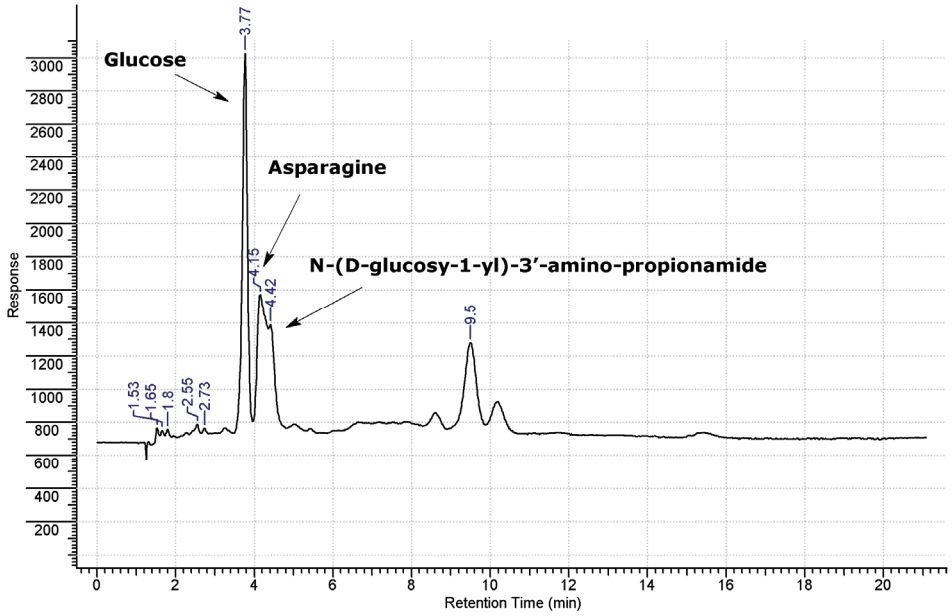


Figure 4. Asparagine/Glucose Mixture (1:1) heated in Silica at T=170ºC for 10 minutes under Dry Conditions

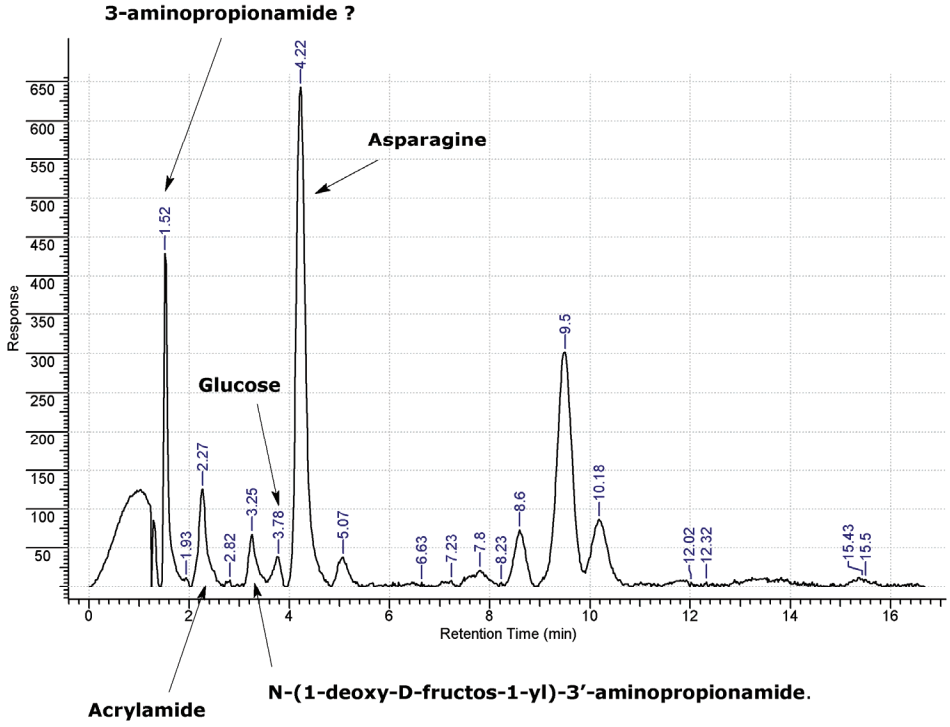


Figure 5. Asparagine/Glucose Mixture (1:1) heated in Silica containing 50% Water (w/w) at T=170ºC for 20 minutes.

Table 1 Retention Times for Acrylamide and its Precursors

Compound	Retention time (min)
Aminopropionamide (AP)	1.53
Acrylamide	2.25
N-(1-deoxy-D-fructose-1-yl) 3'aminopropionamide (APARP)	3.25
Glucose	3.78
Asparagine	4.23
N-(D-glucos-1-yl)-3'-aminopropionamide (APglyc)	4.42

Glycosylamine and Amadori Model System

In addition to the formation of acrylamide and its intermediates from asparagine, the ability to track acrylamide release from the glycosylamines and Amadori products of aminopropionamide over time offers additional insight that would otherwise be difficult to obtain. In the case of N-(D-glucos-1-yl)asparagine, a known intermediate in acrylamide formation from asparagine and sugar reaction, no peak was observed upon injection of the synthesized standard, this is likely due to poor detection of the species by the PAD system. However, heating of the intermediate released increasing amounts of acrylamide over time. Shifts in relative intensities of the various precursors as a function of heating time can be also be studied relating it to acrylamide formation and degradation although methods more responsive for their quantification would be essential. Perhaps another PAD mode would resolve the issues such as the integrated pulse amperometric detection discussed by Johnsson and La Course (1990).

CONCLUSION

Overcoming the lack of a reliable method for the study of acrylamide formation in model systems has been one of the key factors in the slow progress for gathering mechanistic information. The use of anion-exchange chromatography with pulsed amperometric detection allows for quantification of acrylamide from model systems and its correlation to sugar consumption by asparagine as well as observation of the formation and degradation of its intermediates. Further studies will be undergone for fine tuning parameters aimed at studying each individual intermediate.

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