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PY/GC/MS INVESTIGATION OF THE MECHANISM OF MAILLARD REACTION USING ISOTOPICALLY ENRICHED AMINO-ACIDS AND D-GLUCOSES.

by

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A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Suggested Short Title

Elucidation of Mechanisms of Maillard Reaction

ABSTRACT

Pyrolysis/GC/MS was utilized as an integrated reaction, separation and identification system to study the thermal degradation products of non-volatile Maillard flavor precursors. Model systems of L-phenylalanine (aromatic amino acid), glycine and L-alanine (aliphatic amino acids), L-serine (β-hydroxy amino acids) and L-methionine (sulphur containing amino acid) were investigated. Quartz tube pyrolysis of D-glucose/amino acid or dicarbonyl/amino acid mixtures shortened the analysis time (from hours to minutes) and eliminated the need for extraction since the volatiles generated from the precursors are directly transferred into the GC column. In addition, the amount of reactants required to perform the experiments was in the order of few milligrams. This property facilitated mechanistic studies with expensive isotopically labeled reactants and with hard to obtain synthetic intermediates such as Amadori compounds.

Phenylalanine Amadori product and different model systems containing phenylalanine and different reducing sugars were studied. Ribbon pyrolysis was used to study the effect of temperature (150, 200, 250 °C) on the efficiency of formation of initial pyrolysis products from phenylalanine and Amadori phenylalanine. Quartz tube pyrolysis was used at 250 °C to enhance the secondary reactions. These studies revealed the formation of pyridine and naphthalene derivatives such as 3,5-diphenylpyridine, 1(2)-naphthaleneamine, N-methyl-1(2)-aminonaphthalene, 1-aminoanthracene, 2'-phenyl-pyrrolo[4,5-A]dihydronaphthalene, 1(2)-(N-phenethyl)naphthaleneamine and 1(2)-(N-phenethyl-N-methyl)naphthaleneamine. The precursors for pyridine and naphthalene derivatives were verified by GC/MS identification of the target compounds in the reaction mixtures of the postulated precursors.

Model studies using D-[^{13}C]glucoses and a series of dicarbonyl compounds with labeled [^{15}N/^{13}C]glycines and [^{15}N/^{13}C]alanines identified a new chemical transformation of α-dicarbonyls, that lead to the addition of alkyl groups from the amino acid to the α-dicarbonyl compounds, instead of the amino group as in the case of the Strecker type interaction between the two reactants. Thus, glyoxal and pyruvaldehyde can be transformed into pyruvaldehyde and 2,3-butanedione respectively, by glycine and 2-

ketobutanal and 2,3-pentanedione respectively, by L-alanine. The labeled glycine model studies indicated that methyl substituted pyrazines and pyrazinones formed in the model systems, have a common intermediate. Two pathways of pyrazinone formation were distinguished based on the labeling experiments, one involving the reaction of three moles of glycine and the other the interaction of the dipeptide glycylglycine with an α-dicarbonyl compound.

A major product of the reaction of D-glucose with excess glycine was detected by Py/GC/MS analysis and subsequently synthesized and isolated using focused microwave irradiation at atmospheric pressure conditions. Spectroscopic analysis by NMR, FTIR, MS and UV in conjunction with labeling studies have indicated the unknown compound to be 5-hydroxy-1,3-dimethyl-2[1H]-quinoxalinone. The labeling studies indicated the incorporation of ten carbon atoms (six from sugar, one C-1 atom of glycine, and three C-2 atoms of glycine) and two nitrogens. In addition other derivatives of quinoxalinone formed from D-glucose/glycine and D-glucose/L-alanine mixtures were also identified based on similar amino acid incorporation patterns and mass spectroscopic analysis.

L-Serine was found to be a unique amino acid generating in the absence of sugar a variety of heterocyclic compounds. Under pyrolytic conditions L-serine can be viewed as a potential mixture of glycine, alanine, serine, formaldehyde and dicarbonyl compounds.

Model studies with L-methionine provided evidence that methional (Strecker aldehyde) generated under Quartz tube pyrolysis undergoes secondary reactions with amino compounds generating 1,3-thiazines or 3-substituted pyridine in a similar fashion to that of L-phenylalanine systems where 3-substituted pyridines were also identified.

RESUME

La pyrolyse couplée avec la CG/SM fut utilisée en tant que système complexe comprenant la création, la séparation et l'identification des produits provenant de la dégradation thermique des composés non volatiles de la réaction de Maillard. Des systèmes modéles avec la L-phénylalanine (un acide aminé aromatique), la glycine et la L-alanine (des acides aminés aliphatiques), la L-sérine (un acide aminé β-hydroxylé) et la L-méthionine (un acide aminé contenant du soufre) furent utilisés. L'utilisation du tube de pyrolyse en quartz contenant des mélanges D-glucose/acide aminé et dicarbonyles/acide aminé réduisa le temps d'analyse de quelques heures à quelques minutes et élimina la nécessité de faire une extraction vu que les composés volatiles créés par le systéme étaient directement transférés dans la colonne du CG. Autre avantage, la quantité de réactifs nécessaires à la realisation de ces experiences était de l'ordre de quelques milligrammes. Cela facilitait l'étude du mécanisme réactionnel avec des réactifs radioactifs trés chers et avec des intermédiares réactionnels trés difficiles à obtenir tels que les composés d'Amadori.

Les composés d'Amadori contenant de la phénylalanine ainsi que les différents systémes contenant de la phénylalanine et des sucres réducteurs fûrents étudiés. Un ruban à pyrolyse fut utilisé pour étudier l'effet de la température (150, 200 et 250°C) sur la formation du produit initial de pyrolyse à partir de la phénylalanine et d'Amadori-phénylalanine. Des tubes de pyrolyse en quartz furent utilisés à 250°C pour favoriser les réactions secondaires. Ces études révelèrent que la formation de composés dérivant de pyridine et de naphtaléne tels que 3,5-diphénylpyridine, 1(2)- naphthaléneamine, N-méthyl-1(2)-aminonaphthalène, 1-aminoanthracène, 2'-phényl-pyrrolo-4,5-A]dihydronaphthalène, 1(2)-(N-phénetyl)naphthalèneamine et 1(2)-(N-phénetyl-N-méthyl)naphthalèneamine. Les précurseurs de la pyridine et du naphthalène furent verifiés par l'identification en CG/SM des produits cibles à partir d'un milieu réactionnel contenant les supposes précurseurs.

Des études de modéles utilisant du D-[^{13}C]-glucose et une série de composés carbonyles en présence de [^{15}N/^{13}C]glycine et [^{15}N/^{13}C]alanine permit d'identifier une

nouvelle transformation chimique des α-carbonyles qui conduisa à l'adition des groupes alkyl des acides aminés sur les composés α-carbonyl au lieu de réagir avec le groupe amine comme c'est le cas dans la dégradation de Strecker. Ainsi, le glyoxal et le pyruvaldéhyde pouvaient être transformés en pyruvaldéhyde et en 2,3-butadione respectivement par la glycine et en 2-cétobutanal et 2,3-pentadione respectivement par la L-alanine. Le systéme modéle utilisant la glycine isotope lourde montra que les pyrasines méthylées et les pyrasinones formées avaient les mêmes intermediaires. Deux voies de formation de la pyrasinone fûrent obtenues comptes tenu des résultats obtenus avec les études en présence de produits radioactifs, une premiére impliquant une réaction avec 3 molécules de glycine et une autre impliquant la réaction du dipeptide glycylglycine avec un composé α-carbonyle.

Le principal produit de la réaction du D-glucose en présence d'un exces de glycine fut détecté par pyrolyse/CG/SM puis synthétisé et isolé et utilisant l'irridiation par microonde à pression atmospherique. Des analyses spectrométriques par RMN, IRTF, MS et UV en conjonction avec des études avec des isotopes ont montrés que le composé inconnu était du 5-hydroxy-1,3-diméthyl-2[1H]-quinoxalinone. Les études avec des isotopes montrèrent l'incorporation des atomes de carbone (six du sucre, un C-1 de la glycine et trois C de la glycine) et des 2 atomes d'azote. De plus, d'autrés dérivés de quinoxalinone formes à partir des mélanges D-glucose/glycine et D-glucose/alanine fûrent également identifiés grâce à des incorporation similaires d'acides aminés et des analyses spectrométriques.

La L-sérine montrat que c'était le seul acide aminé capable de générer une grande variété de composés hétérocycliques en absence de sucres. Sous des conditions de pyrolyse, L-sérine peut paraitre comme un mélange de glycine, alanine, sérine, formaldéhyde de composés dicarbonyles.

Des études modéles avec la L-méthionine ont pu mettre en évidence que le méthional (un aldéhyde de Strecker) génèré en tude de pyrolyse en quartz subis des réactions secondaires avec des composés amines produisant des 1,3-thiazines ou des pyridines substituées en 3 de manières similaire aux systémes contenant de la L-phénylalanine où des pyridines substituées en 3 fûrent également identifiées.

FOREWORD

This thesis is presented in the traditional form, although, the contents of Chapters 4 and 5 have been presented in several publications.

While the work reported in this thesis is the responsibility of the candidate, it was supervised by Dr. V.A. Yaylayan, Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University.

PUBLICATIONS

Keyhani, A. and Yaylayan V.A. Pyrolysis/GC/MS analysis of N-(1-deoxy-D-fructose-1-yl)-L-phenylalanine: identification of novel pyridine and naphthalene derivatives. *J. Agric. Food Chem.* 1996, 44, 223-229.

Keyhani, A. and Yaylayan, V.A. Elucidation of the mechanism of pyrazinone formation in glycine model systems using labeled sugars and amino acids. *J. Agric. Food Chem.* 1996, 44, 2511-2516.

Keyhani, A. and Yaylayan, V.A. Glycine specific novel Maillard Reaction Products: 5-hydroxy-1,3-dimethyl-2-[1H]-Quinoxalinone and related compounds. *J. Agric. Food Chem.* 97, 45, *in press.*

Yaylayan V.A. and **Keyhani, A.** PY/GC/MS analysis of Amadori compounds. In *Contribution of low and non-volatile materials to the flavor of foods*. W. Pickenhagen, A. M. Spanier and C-T. Ho (eds.). Allured Publishing Company. 1996, 13-26.

Yaylayan, V.A.; **Keyhani, A.**; Paré, J.; Bélanger, J. Microwave-assisted synthesis and spectroscopic characterization of 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone. *Spectroscopy*, 1996, 13, 697-702.

Contributions To Original Knowledge

A: Analytical Techniques

1) Application of Py/GC/MS as an integrated reaction/separation/identification system to study the mechanism of formation of Maillard reaction products.

2) Development of Quartz Tube Py/GC/MS analytical system using enriched precursors to determine mechanistic pathways of the Maillard reaction of specific Maillard reaction products.

B: Mechanistic Studies

1) Identification of mechanistic routes to the formation of novel naphthalene and pyridine derivatives arising from L-phenylalanine model systems.

2) Demonstration, through utilization of enriched precursors that amino acids (glycine, L-alanine and L-serine) are involved in carbon chain elongation of smaller carbon fragments arising from sugar degradation. This reaction route involves the elongation of α-dicarbonyls by the C-2 atom of glycine, C-2 and C-3 atoms of alanine, and C-2 and C-3 atoms of serine resulting in an α-dicarbonyl with increased chain length.

3) Alkylpyrazinones, prior to this investigation had been classified as peptide specific Maillard reaction products. Evidence was provided from glycine model systems that they mainly form through the interaction of glycine with Amadori product.

4) Identification of 5-Hydroxy-1,3-dimethyl-2[1H]-quinoxalinone as a novel Maillard reaction product arising from glycine and Amadori glycine. This compound was synthesized and extracted from a D-glucose/glycine mixture using microwave assisted synthesis and extraction.

5) First comprehensive studies of D-glucose/glycine and D-glucose/L-alanine model systems utilizing completely [^{13}C] labeled carbons of glucoses and amino acids at every position and [^{15}N] amino acids.

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. V. A. Yaylayan for his support, guidance and encouragement throughout this study.

I wish to acknowledge the professors, staff and students of the Department of Food Science and Agricultural Chemistry of McGill. It has been a privilege to know them and learn from them during my tenure at McGill.

Special thanks to my husband, Khashayar, for his constant love, support and encouragement.

The funding for this research was through the Natural Sciences and Engineering Research Council of Canada (NSERC).

Table Of Content

List of Schemes

List of Tables

List of Figures

OBJECTIVES

The purpose of this study was (1) to employ Py/GC/MS as a means to simultaneously generate and identify ^{13}C or ^{15}N labeled Maillard reaction products of selectively enriched sugar/amino acid mixtures or from Amadori products, (2) to utilize the data obtained in investigating the origin of reactive intermediates formed and their stable end products (3) to propose reaction mechanisms for their formation consistent with labeling experiments.

CHAPTER 1

LITERATURE REVIEW

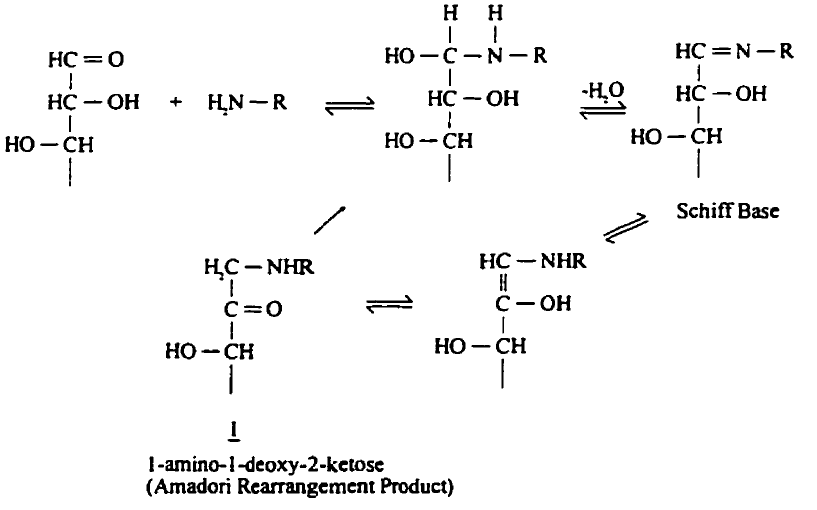
1.1 Introduction

The term Maillard reaction, which involves the interaction between carbonyls and amines, was coined in honor of its discoverer Louis Camille Maillard (1912). The study of the Maillard reaction, due to its complexity, is one of the most challenging areas of food chemistry. The reaction is initiated by the formation of a Schiff base adduct between the carbonyl and amine moieties, the eneaminol thus formed rearranges to a more stable ketoamine or Amadori product (1) as shown in Figure 1.1. Enolization, dehydration, cyclization, fragmentation and oxidation reactions eventually can lead to reactive intermediates that ultimately form stable and semi-stable end products. The introduction of fire as an aid to food preparation, a million and a half years ago linked the Maillard reaction intimately to food chemistry. Although the Maillard reaction is not the only way to impart flavor to food, however, it plays a key role in thermal generation of aromas. In thermally processed food, the Maillard reaction is responsible for changes in the flavor, color, nutritive value, and possible formation of mutagenic compounds. With the increased availability of advanced analytical techniques, studies of the origin of characteristic food flavors formed through the Maillard reaction has seen considerable progress in recent years. Improving the flavor attributes of microwavable and low calorie products and development of savory flavors by the control of the Maillard reaction are applications of basic research in this area. In addition, the Maillard reaction has been shown to occur in biological systems and has been linked with the severity of diabetic complications and the aging process.

The literature pertaining to the Maillard Reaction is extensive and its evolution can be monitored through a series of review articles (i.e. Hodge, 1953; Reynolds, 1963; Feeney and Whitaker, 1982; Ledl and Schleicher, 1990; Yaylayan and Huyghues-Despointes, 1994), symposium series (i.e. Fujimaki et al., 1986; Baynes and Monnier, 1988; Parliment et al., 1989; Finot et al., 1990; McGorrin et al., 1994; Labuza et al., 1994) and book (Ikan, 1996) which have been published in the last half century.

Unlike a classical reaction, the Maillard reaction does not result in one or few well defined products but proceeds through complex reaction pathways resulting in a large

number of products. To deal with all aspects of the Maillard reaction would be a monumentous task. The following will be a review of the basic concepts, and the evolution of methodologies to better elucidate the reaction.



Scheme 1.1. Formation mechanism of the Amadori Rearrangement Product (only acylic forms are shown)

1.2 Amadori Product

From the standpoint of carbohydrate chemistry the initial stage of the Maillard reaction has been reasonably well described by Hodge (1953). Hodge proposes that an addition reaction takes place between the amine and the open chain form of an aldose followed by water elimination producing an unstable Schiff base (Scheme 1.1). With subsequent cyclization (aldosylamine), enolization and molecular rearrangement, a 1-amino-1-deoxy-2-ketose (Amadori compound) is formed (1, Scheme 1.1). A similar sequence of reactions occurs with ketoses with the initial formation of a ketosylamine,

which undergoes rearrangement to give the corresponding 2-amino-2-deoxyaldose (Heyn's compound). Amadori compounds have been detected in heated, dried and stored foods and also in the human body (Mauron, 1981). The Amadori and Heyn's intermediates themselves do not contribute to flavor, however they are important precursors of flavor compounds.

Amadori compounds are conformationally unstable, and in solution using ^{13}C NMR spectroscopy have been demonstrated to exist in both the pyranose and furanose forms, as well as measurable amounts of open chain form (Feather and Russell, 1969; Roper et al., 1983). Compared to the parent aldose sugar, Amadori compounds are unstable (Feather and Russell, 1969), and they undergo a variety of degradation and dehydration reactions, which depend on the system's pH, temperature, water activity, and duration of heating. The difference between the rate of decomposition of an Amadori compound and the parent aldose sugar is significant. Amadori compounds, for example, when heated at pH 2.0, decompose to a large extent in a matter of hours, whereas a sugar such a D-glucose is stable at these conditions for weeks or even months. Thus, the formation of an Amadori compound provides a low energy pathway for a relatively stable sugar, such as D-glucose, to undergo a variety of degradation reactions at relatively mild conditions (Birch et al., 1984), as would be encountered during cooking and processing of foods.

Knowledge of all the degradative reactions that an Amadori compound can undergo is incomplete at present. Progress in this area has been hindered by lack of commercial availability of Amadori products and the lack of efficient synthetic methods for their preparation. The problems associated with synthesis of Amadori products lie mainly with the chemistry of reducing sugars. Reducing sugars are known to undergo mutarotation, oxidation, and acid- or base-catalyzed transformations. All of these processes interfere at some point during the synthesis. Yaylayan and Huyghues-Despointes (1994) conducted a comprehensive review of synthetic methods involving free and protected sugars. Volatile compounds arising from glucose-valine Amadori intermediates have been studied by Vernin et al. (1988). Yaylayan and Sporns (1989a) examined the diagnostic ion series produced by Amadori products using MS techniques based on electron-impact ionization as an indicator of thermal degradation pathways. To better understand the decomposition

pathways of the Amadori intermediate, electron impact mass spectrometry (EIMS) was used to obtain fragmentation schemes. The premise being that, fragmentation schemes obtained can be extrapolated to actual decomposition of Amadori products taking place in as food system at high temperatures (Yaylayan and Sporns, 1987). Yaylayan and Forage (1991) were able to separate and identify five thermal degradation products of tryptophan Amadori product using C_{18} reversed phase column and UV detection. The products were predicted to form by correlating the EIMS fragmentation data with that of thermal degradation.

The most intensely studied reactions to elucidate the mechanisms of the Maillard reaction are not those of Amadori products per se but those of amino acids and sugar. However, it is assumed that under acidic conditions the Amadori product can be considered the main precursor for the reactive intermediates in model systems. While in aqueous/basic model systems the products are formed mainly from the reactive intermediates originating from the sugar (Speck, 1958) and subsequently reacting with the amino acids.

Few studies in the literature have been aimed at determining the rate of formation or degradation of Amadori products. The kinetic models proposed for the Maillard reaction agree with a general scheme in which the reducing sugar reacts with the amine to produce a Schiff base which undergoes subsequent rearrangement to produce the Amadori product, which itself degrades to a set of reactive compounds and free amines (Vernin et al., 1992; Debrauer et al., 1991; Baisier and Labuza, 1992; Yaylayan and Forage, 1992). The kinetic analysis is complicated by the side reactions of the amino acids and sugars and the regeneration of the amino acid from the Amadori product. Huyghues-Despointes and Yaylayan (1996), investigated the kinetic analysis of formation and degradation of 1-morpholino-1-deoxy-D-fructose. The experimental conditions were designed to minimize degradation of the sugar and amino acid. The lack of carboxylic acid group in morpholine minimizes side reactions, such as decarboxylations and Strecker degradation. The kinetic data indicated that the reaction of morpholine with glucose follows a second-order kinetics and that the decomposition pathway of the Amadori compound is not only

dictated by carbon-nitrogen bond cleavage reactions but also by reactions initiated by the intact Amadori compound.

1.3 Factors affecting the Maillard reaction

The Maillard reaction is subject to control variables such as the concentration and nature of the primary reactants, pH, temperature, pressure, time, water activity, metal ions, light and inhibitors that influence the final product distribution. A large number of model studies of the Maillard reaction have been reported in the literature (Olsson et al., 1978; Hayase et al., 1985; Baltes and Bochmann, 1987; Oh et al., 1992; Bemis-Young et al., 1993; Meynier and Mottram, 1995; Tressl et al., 1995; Yu and Ho, 1995). Much of the work has been directed to understanding flavor formation in model systems in which a single amino-acid has been reacted with a reducing sugar or a sugar degradation product. In most cases, few variables were manipulated and their effects were studied.

The study of effect of the pH on pyrazine formation in equimolar D-glucose/glycine model systems heated at 120°C for 5 h indicated that increasing pH values increased the concentration and variety of pyrazines in the reaction mixture (Bemis-Young et al., 1993). To study the effect of pH on the formation of volatile compounds in meat, aqueous model systems of ribose (important as a flavor precursor in meat systems) with glycine, lysine, cysteine and methionine were adjusted to set pH levels and then heated (Meynier and Mottram, 1995). In these particular model systems the amino acid concentration was in excess of the to ribose concentration. Methionine and cysteine were chosen since sulphur containing components have been reported to be key aroma compounds in meat (Schutte, 1974; Mottram, 1991; Werkhoff et al., 1990). Even in such "simple" systems the number of products formed is very large and the interpretation of the results has been limited to identification of products, postulation of reaction mechanisms and evaluation of sensory properties. Studies have identified over 70 components from ribose/glycine reaction mixtures (Salter et al., 1988) and 120 from ribose/cysteine reaction mixtures (Farmer and Mottram, 1989). Meynier and Mottram (1995), reported that the GC analysis of the ether extracts from the various models

yielded extremely complex chromatograms, however their analysis concentrated on specific components. Qualitative and quantitative analysis (Meynier and Mottram, 1995) indicated that the Maillard reaction products were strongly affected by pH and could be classified into three groups: those which exhibited a higher concentration as pH increased (pyrazines and pyridines), those showing lower concentration at lower pH (furans and some sulphur containing compounds) and, finally those which were not affected by pH variations (some heterocyclic sulphur components). The pH of the system plays a crucial role in the Maillard reaction, since both carbonyl and amine groups have the potential to be protonated depending on the hydrogen ion concentration of the system. The relative degree of protonation of these groups is critical, because the initial step of non-enzymatic browning, the condensation of the carbonyl and amine is pH-dependent (Koehler and Odell, 1970; Tsuchida et al., 1976; Shibamoto and Bernhard, 1977; Milic and Piletic, 1984; Shu and Ho, 1988; Bemis-Young et al., 1993).

In model systems used to study the influence of pH on the Maillard reaction, it is important to maintain a constant pH during the reaction by using buffers. Investigators have demonstrated pH variations in excess of three units upon heating of unbuffered model systems containing amino acids and sugars (Whitfield et al., 1988; Wong and Bernhard, 1988). The conclusions of the majority of the pH-studies is that the Maillard reaction in general is favored by high pH, but even here interactions with other variables such as water activity (a_w) and temperature, have to be considered.

The temperature dependence of chemical reactions is often expressed as the activation energy (E_a), in the Arrhenius equation. The higher the value of E_a, the more temperature dependent is the reaction rate. Activation energy data for the Maillard reaction have been reported to span a wide range, 10-160 kJ/mole, depending on the reaction variables (Lingnert, 1990). The temperature dependence of the Maillard reaction is also influenced by the participating reactants. This was illustrated by Holmes (1970) who measured amino acid degradation when D-glucose was reacted with several amino acids for 1 hour and reported considerable differences in temperature dependence of degradation products between different amino acids. Different product profiles have been identified from the same reaction system, due to variations in temperature and time of heating (Shibimoto,

1983). Baltes et al. (1989) analyzed reaction mixtures of glucose/serine and glucose/phenylalanine which had been heated from 100-220°C in aqueous solution and on an inert material. The relative quantities of the various classes of products indicated a temperature dependence. They concluded that the reaction temperature is the most significant factor in the generation of thermal aroma.

The rate of the Maillard reaction is considered to have a maximum at some intermediate water activity (0.3-0.8). Methylpyrazine formation from non-fat dry milk was shown to increase from a_w of 0.3 to 0.75 at a constant temperature (Leahy and Reineccius, 1989). Labuza (1970), attributed the reaction rate decrease at higher a_w to the dilution of the reactants while relating the decreased reaction rate at low a_w to the increasing diffusion resistance which lowers the mobility of the reactants. It has also been shown that the water influence is highly temperature dependent (Lingert, 1990).

Many volatile flavor compounds have been identified and reported from simple model systems and the influence of specific carbohydrates or amino compounds on the composition of the volatiles has been studied. Pyrazines, pyrroles, furans and pyranones (Figure 1.1) form in the majority of sugar/amino acid model systems; however, many of the flavor compounds formed through the Maillard reaction are amino acid specific. For instance, each amino acid gives rise to one specific Strecker aldehyde (Scheme 1.2, see also Appendix A)

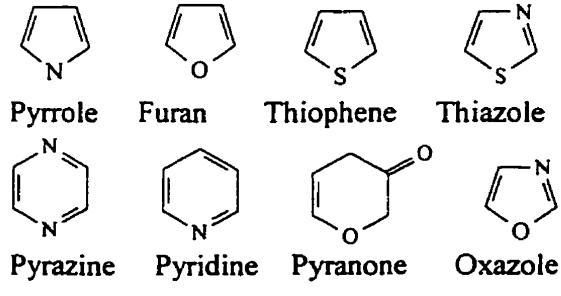
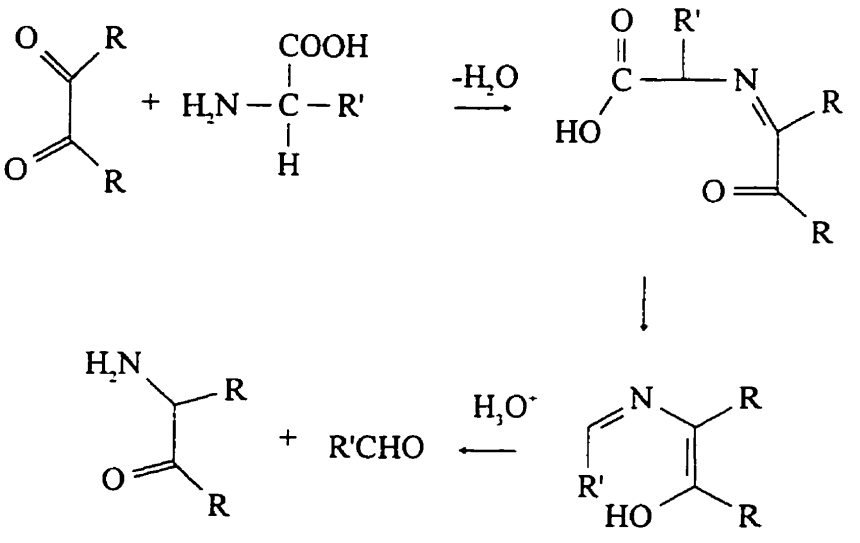


Figure 1.1. Selected heterocyclic compounds formed in Maillard model systems.



Scheme 1.2. Mechanism of Strecker Degradation of Amino Acids.

1.4 Maillard reaction products

The present thinking suggests that Amadori products (1) initially undergo enolization and dehydration to give partially dehydrated dicarbonyl sugar derivatives which serve as the precursors for the production of a variety of food flavor and aroma constituents, and possibly as intermediates that modify proteins in later stages of the reaction.

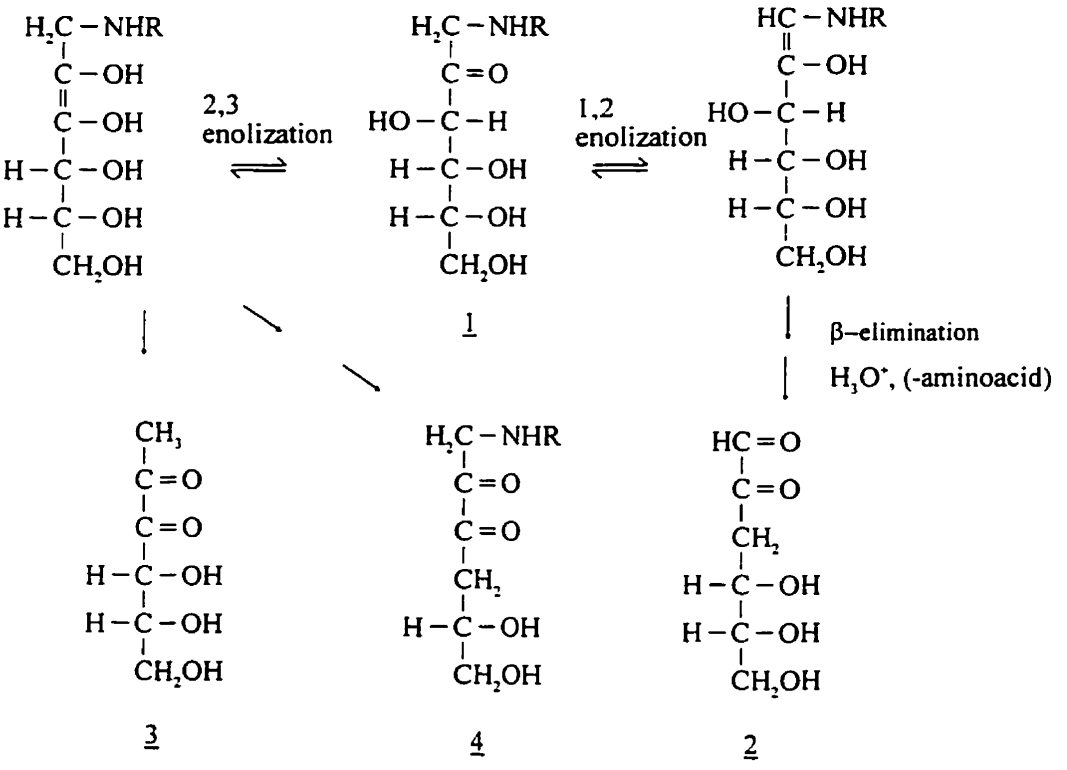
Under acidic conditions Amadori compounds can undergo 1,2-enolization followed by elimination of the amine moiety to produce 3-deoxyglucosone (2, Scheme, 1.3), a highly reactive intermediate. In acidic solution, upon dehydration it is converted to 5-(hydroxymethyl)-2-furaldehyde (HMF). At higher pH a 2,3-enolization is favored leading to the formation of 1-deoxyglucosone (3) and 1-amino-1,4-dideoxy-2,3-dicarbonyl derivative (4, Scheme 1.4). These intermediates were isolated from heated Amadori products in water at 100°C by trapping with o-phenylenediamine as their

quinoxaline derivatives (Ledl and Huber, 1990). Amadori products, deoxyglucasones and other sugar derivatives formed under Maillard reaction conditions can undergo retro-aldol reactions, especially under basic conditions, to produce C_2, C_3, C_4, and C_5 sugar fragments containing α-dicarbonyl moieties that can react more efficiently with amines than C_6 sugars. α-Dicarbonyls are generally more reactive toward nucleophiles than simple carbonyls. Generation of these fragments in sugar/amino acid or sugar/amine mixtures has been studied extensively. Morita and Takagi (1986), demonstrated by trapping α-dicarbonyl compounds with o-phenlenediamine, that D-glucose when incubated at 100°C (pH 10) for 1 hour can generate six α-dicarbonyl derivatives containing C_6, C_5, C_4 and C_3 fragments and 60% of the dicarbonyl generated was pyruvaldehyde, indicating the preference of D-glucose to undergo retro-aldol cleavage at the C3-C4 position. Glycoaldehyde and glyoxal (C_2 fragments) have been detected as Maillard fragmentation products in heated model systems of sugars and alkylamines (Hayashi and Namiki, 1980). These reactive dicarbonyls can interact with ammonia and hydrogen sulfide to produce many significant flavor compounds. The reactive dicarbonyls cause oxidative deamination and decarboxylation of α-amino acids (Ghiron et al., 1988), referred to as Strecker degradation (Scheme 1.2., see also Appendix A).

The α-aminoketones produced during Strecker degradation (Scheme 1.2) are important intermediates in the formation of several classes of heterocyclic compounds including pyrazines (Maga, 1982; Fors, 1983), oxazoles (Mottram, 1991), thiazoles (Vernin et al., 1982) and volatiles produced by further interactions such as furanthioles (Evers, 1976). These compounds contribute to the aromas of boiled or grilled meat, roasted coffee, chocolate and bread.

The Maillard browning reaction produces the brown melanoidin pigments that have given the name to the whole reaction. Models containing amino acids have an increased rate of polymer formation compared to D-glucose (Feather and Nelson, 1984). Pigment formation is the result of polymerization of the many highly reactive compounds that are formed during the advanced Maillard reaction, especially the unsaturated carbonyl compounds, heterocyclic amines, and furfural (Hodge, 1953). Not much is known about the chemistry of the formation of these polymers, except that addition and substitution

reactions play a more important role than condensation reactions (Ledl and Schliecher, 1990), so that the final stage of the Maillard reaction still remains obscure.



Scheme 1.3. Formation of deoxyglucosone intermediates. Amadori Product (1), 3-deoxyglucosone (2), 1-deoxyglucosone (3), 1-amino-1,4-dideoxyglucasone(4).

1.5 Other aspects of the Maillard reaction

While the bulk of the research on Maillard reaction products has focused on flavor and color formation there have been numerous investigations regarding other aspects of the reaction. The antimicrobial effects of the Maillard reaction products have been demonstrated in several studies (Einarsson, 1990). Temperature and pH of the reaction mixture and duration of the reaction greatly influence the formation of antimicrobial compounds (Einarsson, 1990). Maillard reaction products such as carbonyl compounds and melanoidins, the main reaction products produced during thermal processing and storage of foods have been shown to have antioxidant properties (Aeschbacher, 1990).

Under certain conditions the Maillard reaction does not impart favorable characteristics to food. Color and flavor formation have already been mentioned as the main effects of the Maillard reaction. They can produce discoloration and off-flavors. There is considerable interest in the development of compounds that inhibit Maillard reactions, particularly with regard to color development.

One of the most documented nutritional consequences of the Maillard reaction is the reduction in nutritional availability of lysine, an essential amino acid. Finot (1990), showed that the early Maillard product, deoxyketosyllysine was utilized by the laboratory rats to the extent of about 5-15% compared to lysine. Hurrell and Carpenter (1974), showed that protein-bound fructosyllysine formed in stored albumin-glucose mixtures did not serve as a lysine source. Maillard products and glycated proteins impair digestibility by directly inhibiting digestive enzymes (Pervical and Schneeman, 1971) and/or blocking access of the enzymes to peptide bonds (Friedman et al., 1981 and 1985). Polymers formed during the reaction have a molecular weight above 1000 and are considered to be chemically and biologically inert substances. However, there have been reports linking premelanoidins with the destruction of vitamins (Ford et al. 1983), and they have been reported to influence trace element metabolism (Finot and Furniss, 1986).

Since the late 1970's, extensive mutagenicity testing on the products of the Maillard browning model systems have been conducted. The three major test systems as categorized by Anderson (1988), are gene mutations, chromosomal damage and DNA

damage/repair. Maillard products and reaction mixtures have been subjected to these tests, the most popular being the Ames test. Much of the information compiled are not comparable and in part contradictory. Omura et al. (1983), observed mutagenicity from an ethanol extract of glucose/phenylalanine reaction mixture and determined HMF to be the mutagenic component, while Kong et al.(1989), found HMF to be an inhibitor of the mutagenic effect of heterocyclic compounds. Considerable evidence has been accumulated regarding the mutagenecity of several compounds such as, 2-amino-1-methylimidazo[f:b]-8-methyl-quinoline and 2-amino-1-methylimidazo[f:b]-3-methyl-quinoxoline (IQ compounds), arising from creatine and creatine phosphate. The factors speculated to be important in the formation of IQ compounds in model systems are the creatine, amino acid and sugar content, and temperature (Johansson and Jagerstad, 1993; Skog, 1993; Knize et al. 1994b).

In the last decade medical researchers have begun to appreciate the significance of the Maillard reactions in vivo and their relevence to chemical modification of proteins and other biomolecules during natural aging and in diseases such as diabetes and atheroscelerosis (Vlassara et al., 1983; Brownlee, 1988; Brownlee, 1992).

1.6 Reaction elucidation and characterization

According to Hodge (1953) "... the control of browning reactions to produce only wanted flavors and odors is an intriguing possibility. Control of browning to do mans will is the ultimate goal of browning research, but progress towards this goal can be made only as the reaction mechanisms are better understood". The great interest shown by the food industry in the Maillard reaction largely stems from a desire to produce and control characteristic flavors of roasted and baked foods. However efforts have been hindered by the variety of compounds arising under different reaction conditions and the hundreds of compounds which are involved in producing a characteristic aroma.

The Maillard reaction in a simple model system can produce compounds that are of importance to the food and flavor industry. The Maillard reaction products have diverse physical and chemical properties, and range from highly volatile aroma

compounds to the nonvolatile colored pigments known as melanoidins. Furthermore, the polarity of such compounds range from non-polar to extremely polar water soluble products. Analysis of aroma and flavor compounds from foods and model systems remains an important, yet difficult task. Research efforts on the Maillard reaction model systems were initially focused on variables affecting the rate of the reaction and identification of products. Many solution phase model studies have been conducted over a wide range of temperature (20-220°C) and reaction times (hours to weeks). While such conditions are suitable for measurements of color formation, reactant concentration and appearance of products, they do not accurately represent the conditions of a food system. The Maillard reaction, in foods such as in the roasting of coffee beans, cocoa beans, nuts, grains and meats occurs at high temperature in a low moisture environment. To more closely reflect the conditions during the heat processing of food, increasingly model studies are being conducted in high temperature-low moisture systems (Baltes and Bochmann, 1987; Oh et al., 1992; Bemis-Young et al., 1993; Meynier and Mottram, 1995; Tressl et al., 1995; Shu and Lawrence; 1995; Yu and Ho, 1995; Amrani-Hemaimi et al, 1995; Huyghues-Despointes et al., 1994). Temperatures ranging from 130-180°C were utilized to determine the volatile products generated from glucose/glycine, glucose/proline and glucose/glycine-proline dipeptide reaction mixtures (Oh et al., 1992). To determine the contribution of serine and threonine with sucrose to the aroma profile of coffee, Baltes and Bochmann (1987), analyzed model systems heated at 225°C. About 350 compounds (alkyl-, alkenyl-, acyl-substituted furans, pyrroles, pyrazines, pyridines and oxazoles) were identified from the model systems. Roasting of raw coffee under the same conditions and subsequent analysis yielded many of the same compounds.

Gas Chromatography/Mass Spectrometry (GC/MS) has been used extensively to analyze volatile aroma components of different Maillard model systems. The procedure commonly followed is illustrated in Figure 1.2, and will henceforth be referred to as the "classical approach". The reaction solution or the melt is dissolved in water, and repeatedly extracted with organic solvent. The non-polar products extracted into the organic solvent, can be analyzed by GC, provided they are sufficiently volatile, and characterized by MS. Following derivatisation (silylation, acylation), some of the polar

products have been identified by GC/MS (Yaylayan and Huyghues-Despointes, 1996). The remaining mixture, however presents great difficulties for the analyst, yet it may constitutes the major part of the reaction products. By analytical chromatographic methods (exclusion, ion exchange, adsorption, partition) fractions are obtained which yield pure substances but usually after several separation steps. Analysis of Maillard reaction mixtures by high performance liquid chromatography (HPLC) has facilitated the identification of substances and classes of compounds through the use of different detectors (UV, fluorescence, electrochemical). The increased availability of LC/MS and LC/MS/MS systems will further the knowledge of the non-volatile Maillard reaction products which may constitute a major portion of the mass balance of the Maillard reaction.

Recent efforts have been aimed at elucidating the mechanisms of reaction which predominate in Maillard reaction system. The commercial availability of sugar degradation products such as hydroxy-ketones, aldehydes, diketones and shorter chain aldoses has enabled investigators to *(i)* determine the volatile profile of a specific hexose/amino acid or hexose/dipeptide model systems, *(ii)* propose a reaction mechanism involving a reactive carbonyl fragment and then (*iii*) provide evidences to support the mechanism by production/isolation/identification of the target compound(s) from a carbonyl/amino acid model system. Since, in most cases, the variability in the product profile of these model systems is directly related to the amino acid or peptide utilized; increasingly, the investigators have concentrated on identifying amino acid or peptide specific Maillard reaction products. Tressl et al. (1985b); identified 2,3-dihydro-1H-pyrrolizines as a proline specific Maillard product, and proposed a mechanism based on the formation of this product in a equimolar 2,3-butadione/proline model.

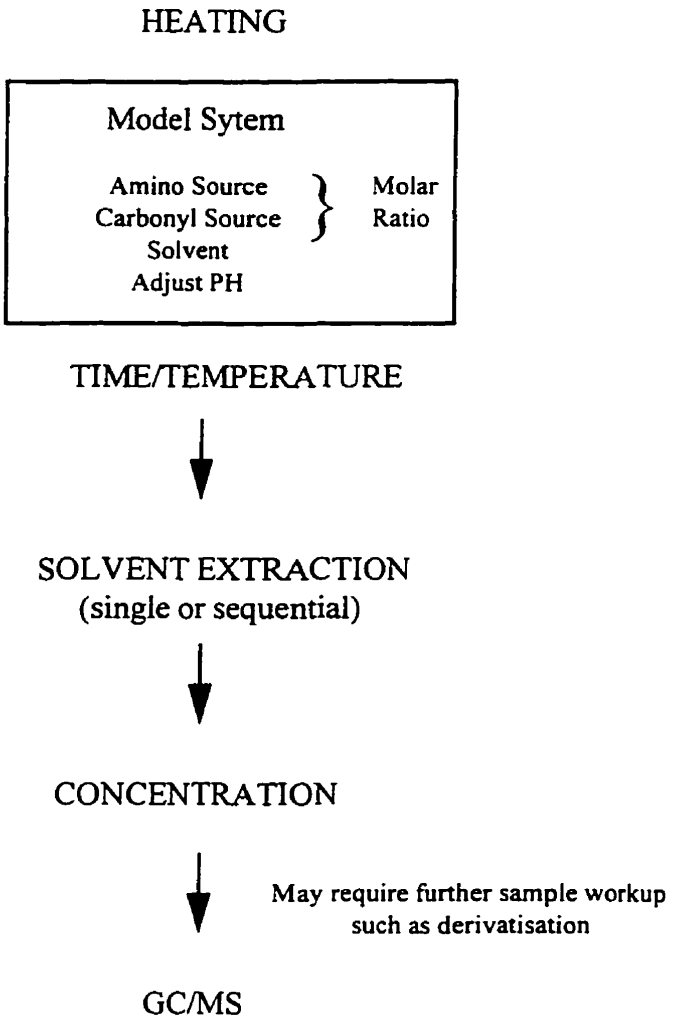
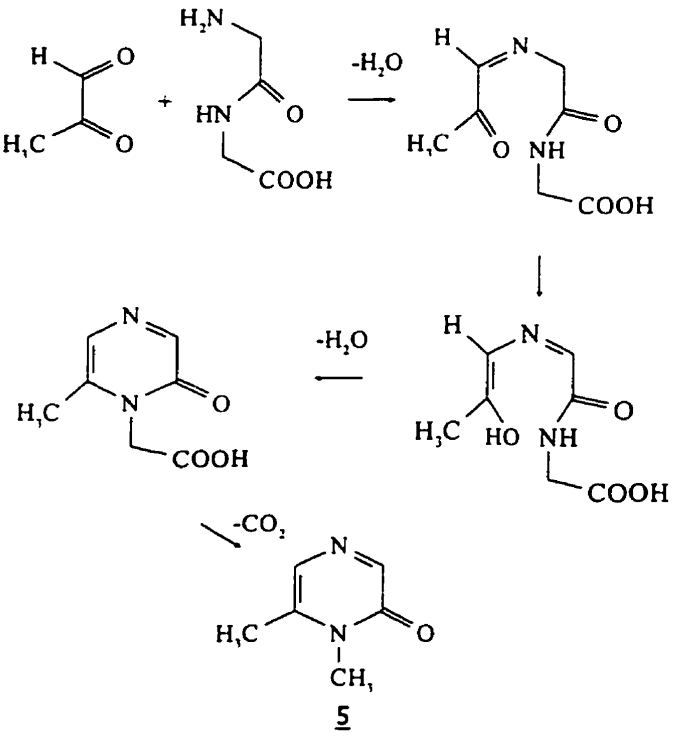


Figure 1.2. "Classical Approach" for analysis of volatiles produced during the Maillard reaction.

Novel 2(1H)-pyrazinones (1,6-dimethyl-2(1H)pyrazinone, 5) were identified in glucose/glycylglycine reaction mixtures (Oh et al., 1992). Since these compounds were not detected from glucose/glycine reaction mixtures they were classified as peptide-specific Maillard reaction products. Following detection in pyruvaldehyde/glycylglycine models a mechanism for the formation, with pyruvaldehyde as the dicarbonyl intermediate was proposed (Scheme 1.4).



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Scheme 1.4. Proposed mechanism for the formation of 1,6-dimethyl-2(1H)-pyrazinone (5) from pyruvaldehyde and diglycine(Oh et al., 1992).

The main problem encountered in identifying mechanistic pathways in the Maillard reaction is the fact that several reaction pathways may be involved in the formation of the same product. Isotope labeling experiments were introduced into studies on the Maillard reaction by Koehler et al., (1969). Nyhammar et al., (1983) and Tressl et al., (1993); demonstrated the high potential of the combined isotopic labeling and capillary GC/MS techniques for the evaluation of the course of the Maillard reaction. The isotope distribution among the detected products is calculated on the ratio of molecular mass ions M, M+1, and M+2 corrected for natural abundance of ^{13}C. Labeling positions are estimated by MS data interpretation.

1.6.1 *Investigation of pyrrole formation from reducing sugars and amines/amino acids by labeling techniques:* The formation of pyrroles in D-glucose/amine (Jurch and Tatum, 1970; Olsson et al., 1977; Njorge et al., 1988; Beck et al., 1989) and D-glucose/amino acid mixtures (Olsson et al., 1978; Nyhammar et al., 1983; Shigematsu et al., 1971; Kato, 1967) has been studied extensively by various investigators and reaction routes and intermediates proposed. A series of labeling experiments of the Maillard reaction were conducted using [1-^{13}C]-D-glucose, [1-^{13}C]-D-arabinose, and [1-^{13}C]-D-fructose with 4-aminobutyric acid (Tressl et al., 1993c) as a Strecker inactive amino acid resembling peptide bound lysine. The observed distribution of labels supports 3-deoxyaldoketoses (2) as intermediates of 2-formylpyrroles (6 and 7, Figure 1.3) as proposed by Nyhammar et al. (1983). The lack of labeling of the acetyl moiety (Figure 1.3) disqualified the proposal by Njorge et al., (1988), that 4- and 1-deoxydiketoses were key intermediates in 2-acetylpyrrole (8 and 9) formation. Two pathways for 5-formylpyrrole (Figure 1.3) formation were demonstrated since analysis of the mass spectra indicated two isotopomers, [5-^{13}C]-5-formylpyrrole (10) and [^{13}CHO]-5-formylpyrrole (11) produced in the model systems.

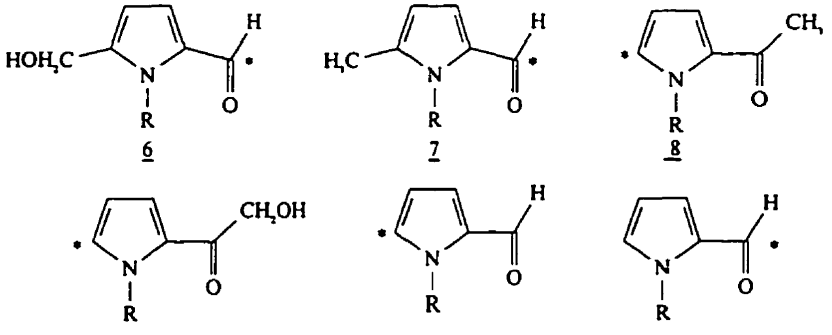
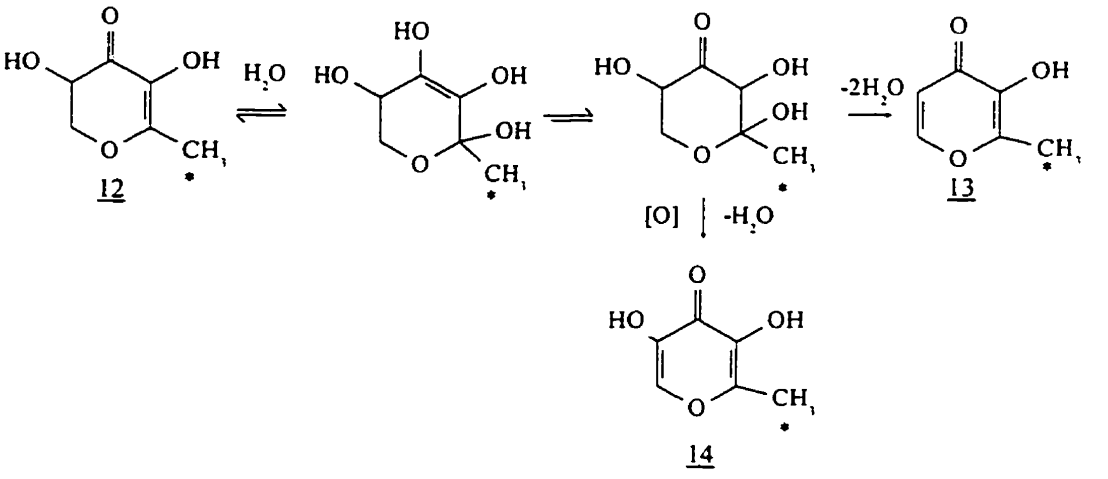


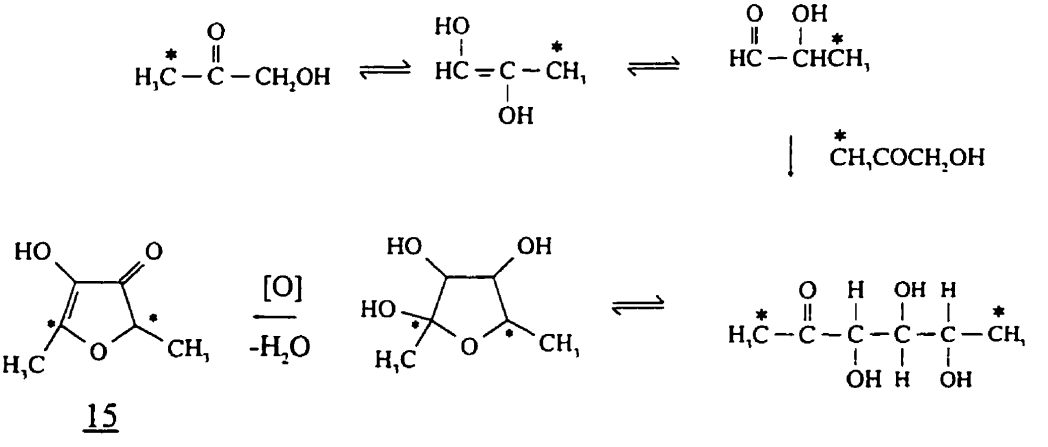
Figure 1.3. 2-formyl-5-hydroxymethylpyrrole (6), 2-formyl-5-methylpyrrole (7), 2-aectylpyrrole (8), 2-açetolpyrrole (9), $_{f}$[5-^{13}C]-5-formylpyrrole (10), [^{13}CHO]-5-formylpyrrole (11). (Tressl et al., 1993c). Asterisks indicate ^{13}C labeled carbon atoms.

The Maillard reaction of D-[1-^{13}C]glucose and D-[1-^{13}C]fructose with isoleucine was investigated (Tresssl et al., 1995). The extent and position of the isotopic labeling were used to evaluate origin, reactive intermediates, and formation pathways of pyrroles and pyridinoles. The labeling in the products correlated with proposals made in the above mentioned study (Tressl et al., 1993c)

1.6.2 *Investigation of degradation mechanisms of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-one:* Many groups have reported the detection of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-one (12) from Maillard reaction mixtures and thermally processed foods (Tatum et al., 1967; Ledl et al., 1976; Reese and Baltes, 1992; Huyghues-Despointes et al., 1994). To investigate the thermal degradation pathways of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (Scheme 1.5) in the Maillard reaction, Kim and Baltes (1996), synthesized 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (12) from D-[1-^{13}C]glucose. Thermal degradation products as detected by GC/MS included maltol (13), cyclotene, hydroxymaltol (14), 2,4-dihydroxy-2,5-dimethyl-2(H)-furanone and 2,5-dimethyl-4-hydroxy-3(2H)-furanone (15). The extent and position of the labeling of the thermal degradation products were interpreted by mass spectroscopic data and proposals were made regarding the main degradation pathways. The maltol and hydroxymaltol detected were 100% singly labeled therefore indicating one mechanistic pathway for their formation (Scheme 1.5). 1-Hydroxy-2-propanone showed two molecular masses, singly labeled and unlabeled, indicative of at least two pathways of formation. Products such as 2,5-dimethyl-4-hydroxy-3(2H)-furanone (15) were detected as singly and doubly labeled isotopomers indicating the condensation of two fragments arising from 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (Scheme 1.6). Kim and Baltes (1996), propose 2,5-dimethyl-4-hydroxy-3(2H)-furanone results from the condensation of 1-hydroxy-2-propanone (Scheme 1.6).



Scheme 1.5. Proposed degradation of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (12) to produce maltol (13) and hydroxymaltol (14) (Kim and Baltes, 1996). Asterisks indicate ^{13}C labeled carbon atoms.

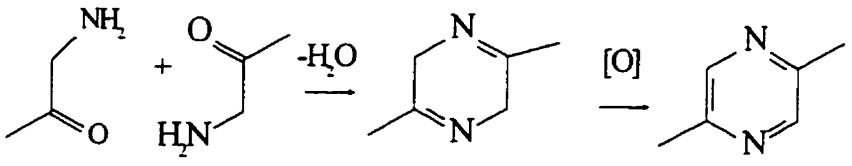


Scheme 1.6. Possible formation pathway of doubly labeled 2,5-dimethyl-4-hydroxy-3(2H)-furanone (15) from labeled 1-hydroxy-2-propanone (Kim and Baltes, 1996). Asterisks indicate ^{13}C labeled carbon atoms.

1.6.3 *Mechanisms of pyrazine formation:* Pyrazines are considered important contributors to the characteristic roast aroma. In the past 15 years in excess of 250 articles have been written on the subject. Recent investigations have centered on defining the sensory properties of various pyrazine structures and on understanding their formation pathways (Maga, 1992).

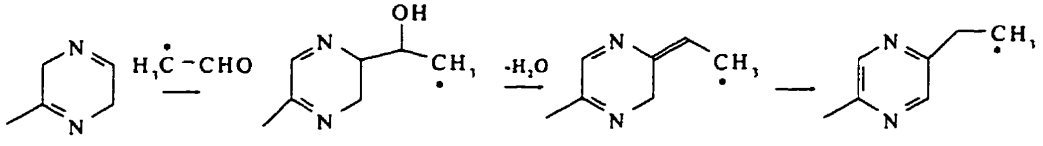
To investigate the participation of α- and ε-amino groups of lysine in pyrazine formation and the effects of pH and temperature on the relative contributions of the two amino groups of lysine to pyrazine generation, Hwang et al., (1994) utilized a α-[^{15}N]lysine. They were able to demonstrate that both α- and ε-amino groups of lysine participate in pyrazine formation. They concluded that the nitrogen atoms from the α-amino groups of lysine react more readily with dicarbonyls to form pyrazines than nitrogens from the ε-amino group.

The most direct route to pyrazine formation results from the interaction of α-dicarbonyls and amines through Strecker degradation. Koehler et al., (1969) identified sugar as the principal source of dicarbonyls and amino acids as the nitrogen source. The resulting α-amino carbonyl compounds, such as α-aminoacetone are considered to be important intermediates for the formation of dihydropyrazines (Scheme 1.7), which are then oxidized to the corresponding pyrazines (Shibamoto and Bernhard, 1977, Milic and Piletic, 1984). To account for multiple alkylations of the pyrazine ring in a basic environment, Shibomoto et al. (1979), propose the addition of Strecker aldehydes to the dihydropyrazines followed by dehydration and aromatization. Alanine is an important precursor for the aroma-relevant trialkylated pyrazines such as 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine (Cerny and Grosch, 1994). The formation of the first pyrazine is in accordance with a proposed mechanism in which acetaldehyde is added to an intermediate dimethyldihydropyrazine (Shibomoto et al., 1979; Milic and Piletic, 1984), the origin of the latter pyrazine does not fit in with the proposed mechanism.



Scheme 1.7. Reaction scheme for the formation of pyrazines from α-aminoacetones.

To investigate the contribution of amino acid carbons to pyrazine structure, mechanism of formation of alkylpyrazines in the Maillard reaction was investigated using [3-^{13}C]alanine and [2-^{13}C]glycine to determine the rate of incorporation and the position of isotopic labeling in the pyrazines formed (Amrani-Hemaimi et al., 1995). The results indicated, contrary to general belief, that alanine and glycine not only act as nitrogen donors but also contribute to the alkyl side chain of some alkyl pyrazines. They propose a reaction route which include the addition of Strecker aldehydes of L-alanine and glycine to dihydropyrazines, which are postulated as intermediates (Scheme 1.8). However, they could not account for doubly labeled pyrazines which were evident in the system. Also their proposed mechanism could not occur under the acidic conditions used in the study.



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Scheme 1.8. Proposed reaction route to [^{13}C]2-ethyl-5-methylpyrazine (16) formation in hexose/alanine systems. Asterisks indicate ^{13}C labeled carbon atoms. (from Amrani-Hemaimi et al., 1995)

The utilization of enriched starting materials has appreciably increased the current knowledge on the fundamental reaction mechanisms involved in Maillard reaction. Unfortunately comprehensive studies with enriched compounds are lacking. This is due mainly to the high cost of the enriched starting materials and the large amount of starting material required using the classical method of analysis (Appendix B).

To further the progress in understanding the detailed mechanistic events which occur in a Maillard reaction system it is necessary to trace back the genealogy of specific products and confirm the origin of their precursors. The chromatograms obtained using the "classical approach" excludes many of the components available in the reaction pool since the solvent extraction/concentration steps are set to isolate a given class of compounds depending on the solubility characteristics of the solvent (Figure 1.2). This limitation in conjunction with the high cost per model system utilizing enriched starting materials hinders significant advances in the classification of the Maillard reaction into its elementary processes.

CHAPTER 2

PYROLYSIS/GC/MS AS AN INTEGRATED REACTION/SEPARATION/IDENTIFICATION SYSTEM TO STUDY MECHANISTIC PATHWAYS OF THE MAILLARD REACTION

2.1 Introduction

Pyrolysis is defined as thermal decomposition in the absence of air. With low molecular weight compounds this process may result in an increase in molecular weight through chemical interactions. Generally however, this process leads to molecules of lower mass due to thermal fission if polymers are used. When the heat energy applied to a molecule is greater than the energy of specific bonds, the molecule will dissociate in a predictable and reproducible manner. Pyrolysis has also been defined as the controlled thermal degradation of macromolecules in an inert atmosphere. These fragmentation products are usually more amenable to analysis and may yield qualitative and quantitative information concerning the initial material. The analytical pyrolysis approach is concerned with the characterization of the original molecule by the analysis of its pyrolysis products (pyrolyzate). This technique involves an integrated pyrolysis-analysis system which is designed to generate reproducible results, using small (often in the ng-ug range) amounts of sample.

Py/GC/MS provides a profile of thermal degradation products that are directly or indirectly related to the original composition of the sample. A typical system (Figure 2.1) consists of a pyrolyzer, a high resolution capillary GC and a detector. The sample is degraded in the pyrolyzer and the volatile pyrolyzates are swept into the high resolution GC for separation. The value of the pyrolysis GC data is dependent upon the GC detector employed. Retention time data alone from Py/GC may be unreliable in providing information on the identity of the pyrolysis fragments. Therefore the detector of choice for Py/GC has become the mass spectrometer because it can provide structural information about the pyrolysis fragments.

Py/GC/MS has the following advantages. The chromatogram can be used as a pyrolysis fingerprint. The resolution (number of compounds distinguished) is high and isomeric mixtures can also be identified and quantified. The Py/GC/MS system has become the technique of choice to identify the pyrolysis fragments and their origin. The major drawback is that fragments which are not volatile cannot be analyzed.

Small samples are often used for pyrolysis to prevent thermal gradient formation across the sample. Pyrolysis occurs mostly upstream from the injection port of the gas chromatograph. The pyrolyzates must be carried out of the pyrolysis chamber and into the GC. To compensate for the added volume and transfer effects, a high split ratio at the injector may be used, which moves the pyrolyzates through the system and into the GC quickly venting most of the analytes. To compensate for the system dead volume while permitting the analysis of very small samples a cryogenic cooling of the GC oven is used to trap the pyrolyzate at the head of the column. Upon the completion of pyrolysis the volatiles are collected onto the cooled capillary column and then revaporized at the beginning of the GC run (Figure 2.1).

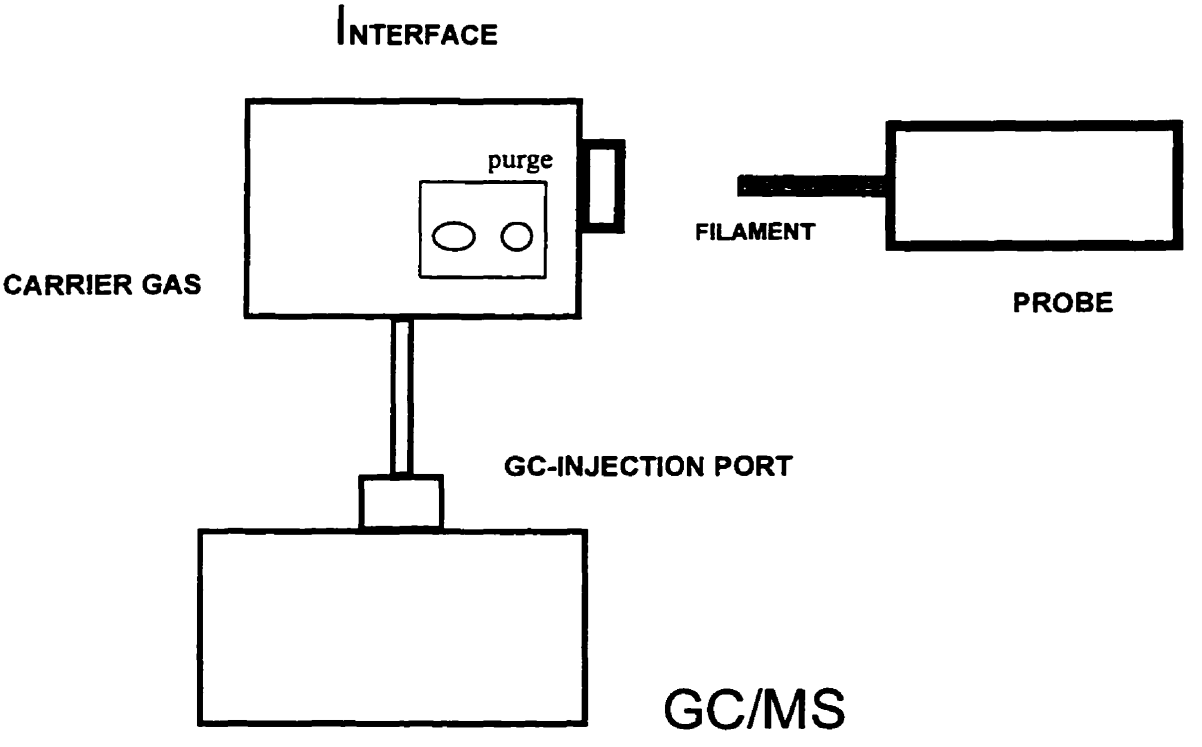

Figure 2.1. Schematic diagram of a Pyrolysis/GC/MS system

The term pyrogram (Figure 2.2), is used to describe chromatograms produced by this technique. To acquire reproducible and accurate pyrograms, the pyrolyzer and the GC must be interfaced properly to ensure efficient transfer of the pyrolysis products preventing the loss of large polar species on cold or absorbing sites. The appearance of the pyrogram will depend on the column (capillary or packed), its length and the nature and loading of the stationary phase, the nature and linear velocity of the carrier gas, the temperature of analysis and the detector response. Temperature programming enhances the detection and resolution of the products of the system.

Traditionally, pyrolysis has been used to degrade polymers at high temperatures (500-1000°C) into smaller units for identification purposes. In our laboratories the pyrolyzer has been used as a "chemical reactor" in which chemical reactions can be performed under controlled temperature and time, using few milligrams of reactants.

2.2 Pyrolysis/GC/MS (Py/GC/MS) as a tool to investigate the Maillard reaction.

The GC/MS analysis of the volatile thermal decomposition products of Maillard reaction precursors are usually carried out on an organic solvent extract of an aqueous reaction mixture (often methanol/water) refluxed for several hours (Hayase et al., 1985; Nursten and O'Reilly, 1983). To increase the reaction temperature and to prevent the loss of volatiles the model systems are placed in sealed ampoules or reaction vessels which can be exposed to higher temperatures (and pressure) in an oil bath or autoclave. This type of analysis is the favored approach in recent investigations of the Maillard reaction (Tressl et al., 1986, 1993; Oh et al., 1992; Meynier and Mottram, 1995; Bemis-Young et al., 1992; Amrani-Hemami et al., 1995; Samsudin et al., 1996, Blank and Fay 1996). Baltes and Bochmann (1987), developed an apparatus to collect the volatiles in cooling traps as they formed, thereby, collecting the volatiles as moisture condensates. While the above mentioned methods initiate the Maillard reaction and heat the reaction mixtures under various time/temperature conditions, the final reaction mixture must be extracted into an organic solvent prior to GC/MS analysis, as outlined in Figure 1.1. Thereby,

making sample preparation labor intensive and time consuming in addition to discriminating between various classes of compounds.

Initiating the Maillard reaction between amino and carbonyl compounds in the pyrolysis probe of a Py/GC/MS system can substantially reduce the analysis time (from hours to minutes) and eliminates the need for extraction since the volatiles generated from the non-volatile precursors are directly transferred into the GC column. In addition, the amount of reactants required to perform the experiments is in the order of few milligrams. This property can facilitate mechanistic studies with expensive isotopically labeled reactants or with difficult to obtain synthetic intermediates such as Amadori products.

Pyrolysis of Amadori products, coupled to GC/MS can also provide a fast and convenient method of studying the effect of temperature on the decomposition products and generating at the same time, pyrograms that can be used as fingerprints for specific Amadori products. Pyrolysis can be performed at various temperatures, with different temperature programs. Different techniques of sample introduction, such as application of the sample on a ribbon probe (platinum filament) as a solution or as a solid in a quartz tube inserted inside the coil probe, can provide valuable information regarding the differences between the initial and the advanced pyrolysis products. On the ribbon probe, the pyrolysis products are swept away as soon as they are formed by the carrier gas into the GC column, without undergoing further secondary reactions. On the other hand, the pyrolysis products produced in the quartz tube should migrate from the sample to the hotter regions of the pyrolyzer and this may initiate additional fragmentation and produce secondary products by further reactions (Irwin, 1982). Quartz tube pyrolysis of sugar/amino acid or dicarbonyl/ amino acid mixtures can be viewed as a mini reaction vessel producing in a single pyrogram a complete product profile of the possible volatiles produced upon thermal degradation and interaction of the components at a specific temperature.

Lapolla et al. (1992), applied Py/GC/MS to study the products arising from the interaction of glucose with poly-lysine to identify diagnostic markers for the advanced glycation reaction. Huyghues-Despointes et al. (1994), demonstrated that Py/GC/MS can

be used to identify primary and secondary pyrolysis products of the proline Amadori compound with minimum sample preparation and analysis time [20 seconds of total heating time (THT) and 35 min. of chromatographic run time] and that the pyrolysis of the proline Amadori compound in the quartz tube for 20 seconds at 250 ^{o}C, is comparable to autoclaving of proline/glucose mixture at 150^{o}C for 1.5 h in water (Tressl et al., 1985a, b, c).

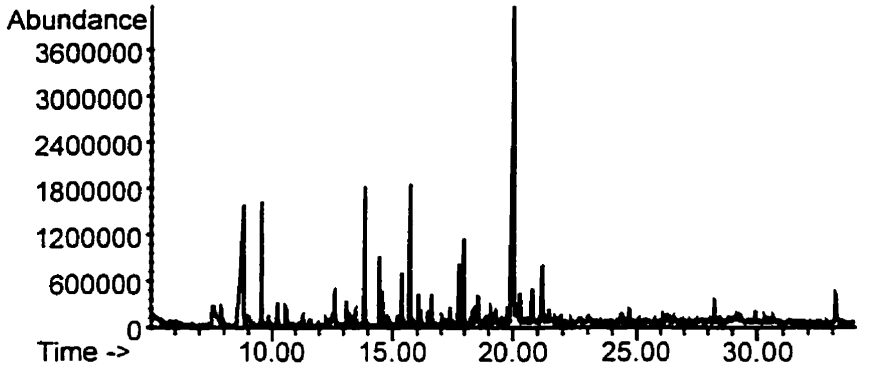

Figure 2.2. Pyrogram of Amadori glycine (250°C, 20s)

The lack of efficient analytical methods to better elucidate the Maillard reaction have hindered progress in this area. Understanding the chemical behavior of Maillard reaction systems could lead to important predictions as to the type of flavor compounds that might arise under various conditions. Huyghues-Despointes and Yaylayan (1996), investigated the retro-aldol and redox reactions of Amadori compounds utilizing Py/GC/MS in conjunction with labeled D-[^{13}C]glucoses. Utilization of enriched compounds in conjunction with Py/GC/MS permits comprehensive studies on the products formed and reaction routes followed by precursors under Maillard reaction conditions.

In the following chapters Py/GC/MS analysis of model systems containing, L-phenylalanine (an aromatic amino acid); glycine and L-alanine (aliphatic amino acids), L-serine (α-hydroxy-amino acid) and L-methionine (sulfur containing amino acid), are

conducted to determine Maillard reaction products formed from these systems. Labeled D-[^{13}C]glucoses and ^{13}C/^{15}N amino acids are utilized to determine the major mechanistic routes of Maillard product formation, and to characterize novel Maillard reaction products.

CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents: All reagents, chemicals and D-[1-^{13}C]glucose (99 % enriched), D-[2-^{13}C]glucose (99 % enriched), D-[6-^{13}C]glucose (99 % enriched) and [^{15}N]glycine (98 % enriched), were purchased from Aldrich Chemical Company (Milwaukee, WI). D-[3-^{13}C]glucose (99 % enriched), D-[4-^{13}C]glucose (99 % enriched), D-[5-^{13}C]glucose (99 % enriched), [1-^{13}C]glycine (99 % enriched), [2-^{13}C]glycine (90 % enriched), [1,2-^{13}C]glycine (99 % enriched), DL-[3-^{13}C]alanine (99 % enriched) and L-[1-^{13}C]alanine (99 % enriched), were purchased from ICON Services Inc. (Summit, New Jersey). L-[^{15}N]alanine (99 % enriched) was purchased from CIL, Inc. (Andover, MA). DL-[2-^{13}C]alanine (92.1 % enriched) and L-[3-^{13}C]serine were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec). Pre-coated preparative silica gel plates were obtained from Merck (Germany).

3.2 Spectroscopic Analysis : ^{13}C and ^{1}H-NMR spectra were recorded in $CDCl_3$ at 300 MHz using TMS as internal standard on an Varian Unity Inova 300 instrument. Infrared spectra were recorded in CaF_2 IR cells with 25μ Teflon spacer, on a Nicolet 8210 Fourier-transform spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector.

3.3 Synthesis: The synthesis of Amadori glycine, Amadori phenylalanine, Amadori methionine was performed according to the method of Sosnovsky et al. (1993).

3.4 Confirmation of the precursors of pyridine and naphthalene derivatives: Equimolar concentrations of the postulated reactants were mixed with or without a solvent (ether) and heated at 130 °C for 15 h and then analyzed by GC/MS. The details of each reaction are elaborated in the text.

3.5 Pyrolysis-GC/MS Analysis: A Hewlett-Packard GC/mass selective detector (5890 GC/5971B MSD) interfaced to a CDS pyroprobe 2000 unit was used for the Py/GC/MS analysis (Appendix D). Two modes of sample introduction were used for the Py/GC/MS analysis: (a)1mg equivalent of sample dissolved in deionized water was applied to the platinum filament of the ribbon probe with a total heating time (THT) of 20s (The water was evaporated using a nitrogen stream) at 100, 150, 200, 250°C or (b) 2-3mg of solid samples was introduced into a quartz tube (0.3mm thickness), plugged with quartz wool and inserted inside the coil probe then pyrolyzed (THT of 20s at 250°C). The GC column flow rate was 0.8 ml/min. for a split ratio of 92:1 and a septum purge of 3 ml/min. The pyroprobe interface temperature was set at 250 °C. Capillary direct MS interface temperature was 180 °C; ion source temperature was 280 °C. The ionization voltage was 70 eV, and the electron multiplier was 1682 V. The mass range analyzed was 30-300 amu. The column was a fused silica DB-5 column (50m length x 0.25 mm i.d. x 25 um film thickness; Supelco, Inc.). Unless otherwise specified, the column initial temperature was held at -5 °C for 2 min. then it 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 minutes. Products that were not found in the mass spectral libraries were identified by generating them from their proposed precursors and comparing mass spectra and chromatographic retention times.

3.6 Microwave-assisted synthesis and extraction of 5-hydroxy-1,3-dimethyl-2[1H]-quinoxalinone: D-glucose (1.00 g, 0.005 moles) and glycine (1.25 g, 0.016 moles) mixture was transferred to the 250 ml quartz extraction vessel of the Soxwave 100 microwave extraction system. Two ml of water was then added. The vessel was inserted inside the extraction cavity and fitted with a condenser. The irradiation was carried out in the following sequence at full power (300 W), 2 min. on, 30s off, 2 min. on, 30s off, 2 min. on, 30s off and 2 min. on. A total of 8 min. of irradiation. At the end of the irradiation sequence a dark brown and dry slurry was obtained. The extraction step was carried out with a 40 ml of hexane using the following sequence of irradiation, 40 s on, 30 s off, 90 s on. The solvent was decanted and dried over sodium sulfate and evaporated

under vacuum. The resulting oil was further purified by thick layer chromatography on silica gel using ethyl acetate as the solvent. ν_{max} in ethylether (absorbance) 350 nm (0.68), 279 nm (2.58), 207 nm (2.47). FTIR ($CDCl_3$), 3158 cm^{-1} (Ar-OH), 2979 cm^{-1} (-CH_3), 2894 cm^{-1} (-CH_3), 1668 cm^{-1} (N-C=O), 1653 cm^{-1} (C=N), 1600, 1560, 1539 cm^{-1} (ArH), 1469, 1382 cm^{-1} (CH_3), 1095 cm^{-1} (C-O). ^{1}H-nmr ($CDCl_3$) δ: 7.49 (ArH, dd, 1H), 6.66 (ArH, dd, 1H), 6.50 (ArH, q, 1H), 2.62 (N=C-CH_3, s, 3H), 3.60 (N-CH_3, s, 3H), 2.20 (OH, broad, H/D exch). EIMS, m/z (relative intensity), 191 (12), 190 (100), 162 (29), 161 (22), 134 (20), 133 (51), 120 (6), 119 (28), 106 (7), 93 (14), 92 (22), 81 (7), 79 (5), 78 (6), 77 (11), 76 (5), 68 (5), 67 (5), 66 (9), 64 (14), 63 (5), 56 (44), 55 (5), 54 (5), 52 (7), 51 (23), 50 (9), 42 (12), 39 (14), 38 (5).

3.7. MS data interpretation of labeled compounds: Isotopic label distributions were determined by calculating the ratio of molecular ion intensities M+n, which were corrected to their natural content of ^{13}C isotopes and, if necessary, to any M-1 interferences (see Appendix C for calculation procedure). Label positions were estimated by interpretation of characteristic mass fragmentation.

CHAPTER 4

L-PHENYLALANINE MODEL SYSTEMS

4.1 Introduction

Pyrolysis coupled with gas chromatography/mass spectrometry (Py/GC/MS) has been demonstrated to be a fast and convenient technique for the analysis of Maillard reaction products arising from the Amadori intermediate, especially with amino acids that are stable enough under pyrolysis conditions to react with the sugar, rather than decompose (Huyghues-Despointes et al., 1994). Py/GC/MS analysis reduces reaction characterization to a microscale level, which permits the efficient use of isotopically enriched compounds for mechanistic studies (Huyghues-Despointes and Yaylayan, 1996). In addition, different sample introduction techniques can reveal primary and secondary pyrolysis products. For example, quartz tube pyrolysis promotes bimolecular interactions and ribbon pyrolysis produces initial degradation products (Huyghues-Despointes et al., 1994).

L-Phenylalanine and its corresponding Amadori compound (ARPP) degrade at temperatures above 150 °C (Kato et al., 1971; Westphal et al., 1988). Kato et al. (1971) identified, in the volatiles generated from heating of L-phenylalanine at 300 °C, several alkyl substituted benzene derivatives, aromatic amines, bibenzyl, stilbene, acetaldehyde and aromatic aldehydes. Papadopoulou and Ames (1994) extracted and identified two non-volatile products; N-(2-phenethyl)-3,4-diphenylpyrrole and N-(2-phenethyl)-3,4-diphenyl-3-pyrroline-2,5-dione from heated L-phenylalanine at 210 °C in paraffin oil. According to Baltes and Mevissen (1988), under roasting conditions L-phenylalanine with six fold excess of D-glucose produces 155 volatile components including furans, pyranones, pyrazines, pyrroles, aromatic compounds and in addition to parent pyridine (17), 4-methylpyridine (18) and 3-phenylpyridine (19). In a subsequent study, Kunert-Kirchhoff and Baltes (1990), identified 58 L-phenylalanine specific products from the reaction of an equimolar mixture of D-glucose and L-phenylalanine at 120, 150 and 180 °C, among them, 3-(2'-furyl)-2-phenyl-2-propenal, phenylhydroxyketones,

benzylpyrazines, phenethylpyrazines and phenethylamides in addition to 2-(5'-hydroxymethyl-2'-formylpyrrol-1'-yl)-3-phenylpropionic acid lactone. Sugimara et al. (1982), found mutagenic activity in L-phenylalanine pyrolysate and identified a pyridine derivative, 2-amino-5-phenylpyridine, arising from L-phenylalanine as mutagenic. In this chapter, we report the effect of temperature on the products arising from pyrolysis of L-phenylalanine and its Amadori product, especially naphthalene derivatives, that have not been reported in L-phenylalanine model systems. The increased utilization of Maillard reaction mixtures to impart roasted flavors to microwavable, vegetarian, and extruded foods has prompted regulatory agencies to focus their attention on the chemical composition of flavors containing Maillard reaction products (Manley, 1994).

4.2 Results and Discussion

To investigate the mechanism of formation of L-phenylalanine specific products during Maillard reaction; L-phenylalanine, Amadori L-phenylalanine (ARPP) and equimolar mixtures of D-glucose/ L-phenylalanine, D-ribose/L-phenylalanine, DL-glyceraldehyde/L-phenylalanine and Amadori L-phenylalanine in the presence of L-phenylalanine were pyrolyzed using Py/GC/MS as described under Materials and Methods. Ribbon pyrolysis was used to study the effect of temperature (150, 200, 250 °C) on the efficiency of formation of initial pyrolysis products from L-phenylalanine and Amadori L-phenylalanine. Quartz tube pyrolysis was used at 250°C to enhance the secondary reaction products. The products identified in all the model systems studied using the quartz tube at 250°C are listed in Table 4.1. Thermal degradation of L-phenylalanine alone at temperatures above 150°C leads to the formation of components with benzyl and phenyl residues as characteristic structural features (Kato et al., 1971). Py/GC/MS analysis of L-phenylalanine on the ribbon probe and in the quartz tube yields phenethylamine as the main degradation product. No thermal degradation products of L-

phenylalanine were observed below 200°C. However, ARPP degraded at 150°C and exhibited a more complex product profile. The main reaction products were phenylacetaldehyde (Strecker aldehyde), styrene, 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (12, pyranone) and three unknown compounds designated as X, Y and Z (see Figure 4.1). In addition, pyrazines were identified only in the ARPP model systems. The identification of the Strecker aldehyde and the pyrazines in the D-glucose/L-phenylalanine model system and the similarity of the pyrograms of ARPP and D-glucose/L-phenylalanine mixture, is indicative of the efficiency of Amadori rearrangement under pyrolysis conditions similar to D-glucose/proline system (Huyghues-Despointes et al., 1994). Formation of phenylacetaldehyde from L-phenylalanine has been reported at trace levels (Kato et al., 1971). Due to its acidic α-hydrogens, phenylacetaldehyde is capable of condensation reactions with other aldehydes such as glycoladehyde to form 3-phenylfuran as reported by Baltes et al., (1988) and was also detected in our model systems containing D-glucose, ribose and DL-glyceraldehyde. Labeling experiments with D-[1-^{13}C] glucose indicated that 70 % of glycoaldehyde moiety in 3-phenylfuran originates from C1-C2 of the sugar (see Table 4.2). 2-(5'-hydroxymethyl-2'-1'-yl)-3-phenylpropionic acid lactone reported by Kunert-Kirchhoff et al., (1990) was similarly observed in the model systems containing D-glucose as the carbohydrate source.

4.2.1 Pyridine Derivatives: Pyridines as a group have exhibited mutagenic activity based on the Ames and Williams tests (Sasaki et al., 1987). Two pyridine derivatives, 3-phenylpyridine (19) and 4-(phenylmethyl)pyridine (20), were identified in the model systems based on spectral library search. 3-Phenylpyridine has also been identified by Baltes and Mevissen (1988). In addition, one of the major chromatographic peaks designated as X (molecular ion at m/z 231) in the pyrogram of ARPP (see Figure 4.1), showed close similarity to 2,6-diphenyl and 3,4-diphenylpyridine mass spectra (see Table 4.2). However, one of the common major peaks (m/z 230) differed in relative intensity

when compared with the known diphenylpyridine spectrum, indicating a different isomer and was tentatively assigned to 3,5-diphenylpyridine (21) structure.

Table 4.1. Products identified during pyrolysis of L-phenylalanine model systems, using quartz tube at 250 °C for 20 s.

Compounds	MW	Phe	ARPP	ARPP/Phe	Glu/Phe	Rib/Phe	Gly/Phe
FURANS							
2,2'-methylene-bis(5-methyl)furan	176	-	-	-	+	-	-
2- ethylfuran	96	-	+	-	-	-	-
2- furancarboxaldehyde	96	-	-	-	+	+	-
2,5 dimethylfuran	96	-	-	-	+	+	+
2-furanmethanol	98	-	+	-	+	+	-
1-(2-furanyl)ethanone	110	-	+	-	+	+	-
5-methyl-2- furancarboxaldehyde	110	-	+	-	+	-	+
3-phenylfuran	144	-	+	+	+	+	+
PYRAZINES							
3,4-dimethyl-pyrrolo[1,2-a]pyrazine	146	-	-	-	-	-	+
2,6 dimethylpyrazine	108	-	-	-	-	-	+
trimethylpyrazine	122	-	+	-	-	-	+
methyl pyrazine	94	-	+	-	+	-	-
PYRROLES							
2-methyl-1H -pyrrole	81	-	+	-	-	-	-
1-(1H-pyrrol-2-yl)-ethanone	109	-	+	-	-	-	-
1-(-2-furanylmethyl)-1H-pyrrole	147	-	-	-	-	+	-
AROMATIC							
1,1'-(1,2-ethanediyl)bis-benzene	182	+	-	-	+	-	-
1-propynyl benzene	116	+	-	-	-	-	+
benzenemethanamine	107	+	-	+	-	-	-
phenethylamine	121	+	-	+	-	-	-
benzeneacetonitrile	117	+	+	-	-	-	-
benzenepropanitrile	131	+	+	-	-	-	-
phenylacetaldehyde	120	-	+	-	+	+	-
N-(2-phenethyl)acetamide	163	-	-	-	+	+	+
benzenepropionic acid	150	-	+	+	-	+	-
styrene	104	+	+	+	+	+	-
N-(2-phenethyl)formamide	149	-	+	+	+	-	+
benzenethanol	122	-	+	+	+	+	+
trans-trans-1,4-diphenyl-1,3-butadiene	206	+	+	+	+	+	-
methyl benzene	92	+	+	+	+	+	+
ethyl benzene	106	+	+	+	+	+	+
1,1-diphenylpropene	194	+	+	+	+	+	+

Table 4.1. - Continued

Compounds	MW	Phe	ARPP	ARPP/Phe	Glu/Phe	Rib/Phe	Gly/Phe
PYRIDINE							
3-Phenylpyridine	155	+	+	+	+	+	-
4-(phenylmethyl)pyridine	169	-	+	+	+	+	-
3,5-diphenylpyridine (X)	231	+	+	+	+	+	+
NAPHTHALENE							
2'-phenyl pyrollo(4,5-A)-3,4-dihydronaphthalene	245	-	+	+	+	-	-
1(2)-naphthalenamine	143	+	+	+	+	+	-
N-methylnaphthalenamine	157	+	+	+	+	-	+
aminoanthracene	193	+	+	+	+	-	-
N-phenethyl-1(2)-naphthalenamine (Y)	247	+	+	+	+	+	+
N-phenethyl-N-methyl-1(2)-naphthalenamine (Z)	261	+	+	+	+	+	+
MISCELLANEOUS COMPOUNDS							
1,2 dimethyl-1H- Imidazole	96	-	-	-	-	-	+
lactone*	255	-	+	-	+	-	-
pyranone*	144	-	+	-	+	-	-
acetic acid	60	-	+	-	+	-	-
1-hydroxy-2 -propanone	74	-	+	-	-	+	+

** Phe L-phenylalanine, Glu D-glucose, Gly DL-glyceraldehyde, Rib D- ribose, ARPP Amadori phenylalanine*
pyranone 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one; Lactone 2-(5'-hydroxymethyl-2'-1'-yl)-3-phenylpropionic acid lactone; + present, - absent

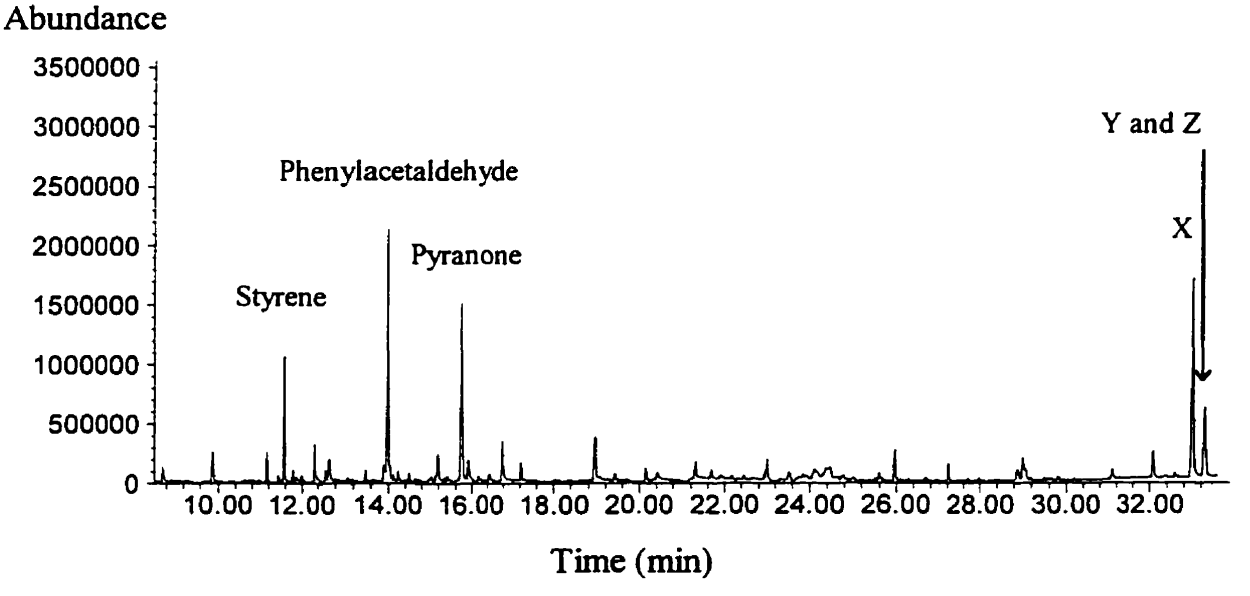

Figure 4.1. Pyrogram of L-phenylalanine Amadori product generated from the ribbon probe at 250 °C for 20 s. *Pyranone = 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one*

The two pyridine derivatives; 3-phenylpyridine (19) and 4-(phenylmethyl)pyridine (20) and the compound X were formed during pyrolysis of ARPP on the ribbon probe at 250 °C. While at 150°C only compound X was formed (Table 4.3) and it increased in intensity with increasing temperature (Table 4.4). Pyrolysis of L-phenylalanine on the ribbon probe produced the compound X at all temperatures studied whereas the other two pyridine derivatives were formed only at 250°C . These observations indicate that the precursors of compound X are readily formed from L-phenylalanine compared to the precursors of the two other pyridine derivatives.

4.2.1.1 *Proposed mechanism of formation of 3,5-diphenylpyridine (21, compound X) and 3-phenylpyridine(19):* Phenethylamine can be formed by the decarboxylation of L-phenylalanine. Phenylacetaldehyde on the other hand, can be formed through Strecker degradation, although it has been detected in trace amounts in the absence of sugars (Kato et al., 1971). These two compounds are known to react to form an imine which rearranges into enamine due to the conjugation with the benzene ring (Scheme 4.1). The corresponding benzaldehyde adduct (22, N-(phenylmethylene)phenethylamine) was detected in the heated (10 min.) methanolic solution of ARPP in 10 % water (Table 4.2). If the above enamine reacts with another mole of phenylacetaldehyde as an N-nucleophile, it eventually leads to the formation of N-(2-phenethyl)-2,4-diphenylpyrrole (Papadopoulou and Ames, 1994). However, enamines can act as C-nucleophiles and undergo condensation reactions with other carbonyls such as phenylacetaldehyde or acetaldehyde to produce 23 (Scheme 4.1). Intermediate 23 can lose a styrene molecule as shown in Scheme 4.1 to produce the triene 24 which can undergo thermally allowed electrocyclic ring closure to produce either 3,5-diphenylpyridine (21) or 3-phenylpyridine (19) after an aromatization step. To test the validity of this assumption, equimolar concentrations of phenylacetaldehyde and phenethylamine were dissolved in ether

Table 4.2. Mass Spectrometric Data From L-phenylalanine Models

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3-Phenylfuran
145(12), **144(100)**, 116(11), 115(86), 89(14), 87(3), 86(3), 63(12), 62(5)
[a]146(10), **145(100)**, 144(38), 117(10), 116(82), 115(36), 90(10), 89(10), 64(7), 63(10), 63(12), 62(5).

3,5 Diphenylpyridine (X)
232(19), **231(100)**, 230(24), 203(5), 202(12), 115(3), 102(10), 101(6), 77(4), 76(6)

3,4-Diphenylpyridine
232(19), **231(100)**, 230(70), 216(14), 202(21), 115(9), 114(9), 102(8), 101(13), 88(8), 76(7)

2,6-Diphenylpyridine
232(18), **231(100)**, 230(61), 228(6), 204(4), 203(3), 202(9), 154(6), 127(10), 116(4), 114(8), 102(20), 101(7), 77(15), 76(10)

4-(phenylmethyl)pyridine
170(13), 169(98), **168(100)**, 167(38), 166(6), 155(3), 154(18), 143(1), 142(8), 141(13), 139(9), 115(20), 63(10), 62(3),
[a]171(11), 170(84), **169(100)**, 168(53), 167(13), 166(1), 155(14), 154(7), 143(5), 142(11), 141(21), 140(8), 139(7), 115(19), 63(9), 62(4)

3-phenylpyridine
156(12), **155(100)**, 154(49), 128(11), 127(13), 102(10), 77(5), 51(7)
[a]157(4), 156(26), **155(100)**, 154(46), 128(12), 127(13), 102(11), 77(10), 51(12),

2-(5'-Hydroxymethyl-2'-formylpyrrol-1'-yl)-3-phenylpropionic acid lactone
256(11), 255(27), 211(4), 210(5), 193(2), 182(2), 180(2), 167(2), 148(5), 147(5), 131(11), 120(9), 108(12), 104(5), 103(4), 92(11), **91(100)**, 78(7), 77(7), 63(5), 51(8).

N-(phenethyl)-1(2)-naphthaleneamine (Y)
248(7), 247(31), 157(10), **156(100)**, 143(9), 128(6), 127(3), 78(9), 77(4)

N-(phenethyl)-N-methyl-1(2)-aminonaphthalene (Z)
262(7), 261(34), 171(13), **170(100)**,157(3), 155(3), 156(3), 128(7), 92(4)

N-(phenylmethylene)phenethylamine
210(1), 209(5), 208(3), 132(7), 119(9), **118(100)**, 117(6), 103(4), 92(7), 91(95), 90(6), 89(7), 78(4), 77(11), 65(11), 63(4), 51(7).

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[a] with D-[1-^{13}C]glucose

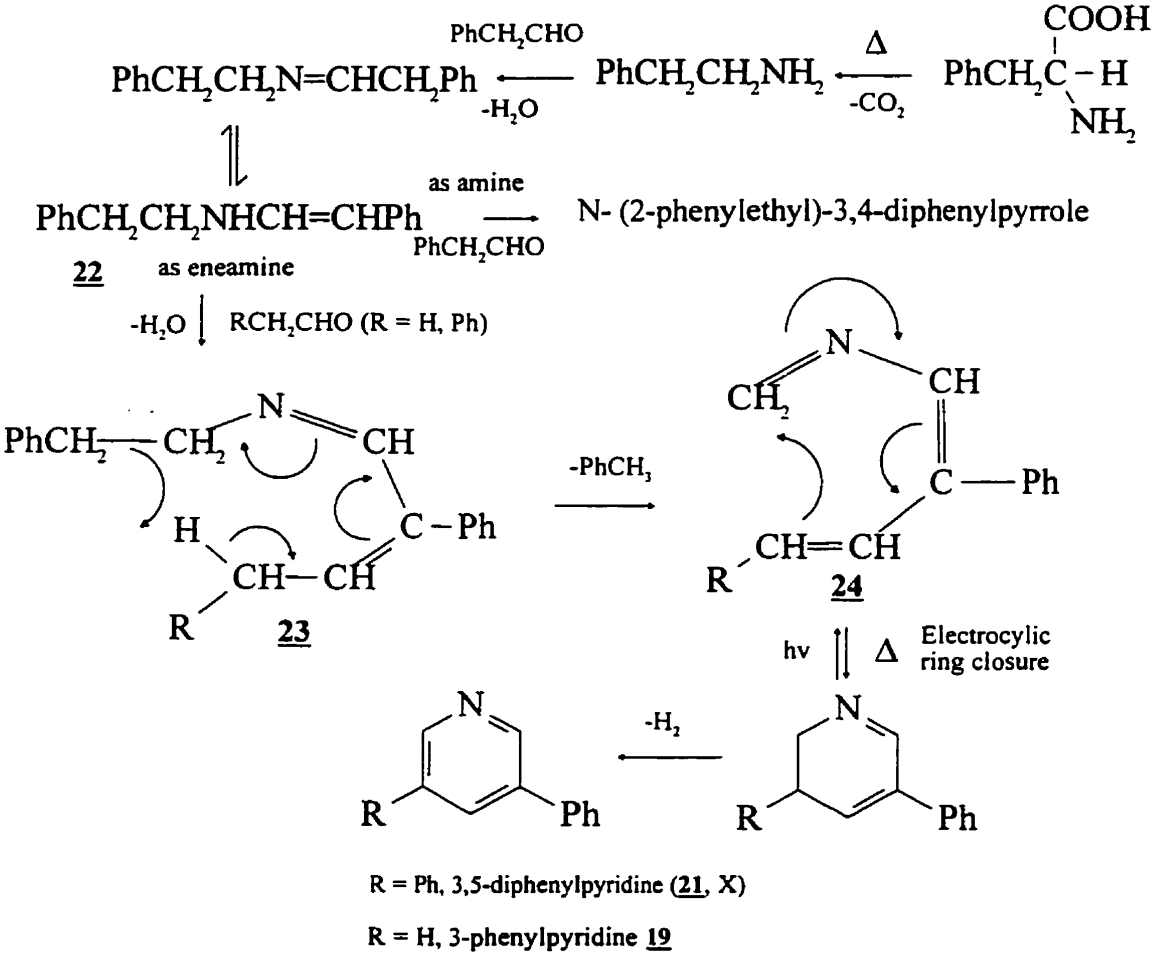
Table 4.3. Effect of temperature on the formation of pyrolysis products from Amadori phenylalanine using the ribbon probe.

Compound	MW	150 °C	200 °C	250 °C
AROMATIC				
benzaldehyde	106	-	-	+
trans-trans-1,4-diphenyl-1,3-butadiene	206	-	+	+
methylbenzene	92	-	+	+
ethylbenzene	106	-	+	+
styrene	104	-	+	+
benzenepropionic acid	150	-	+	+
benzenethanol	122	-	+	+
1,1-diphenylpropene	194	+	+	+
phenylacetaldehyde	120	+	+	+
FURANS				
2 -furanmethanol	98	-	-	+
1-(2-furanyl)-ethanone	110	-	+	+
5-methyl-2- furancarboxaldehyde	110	-	+	+
3-phenylfuran	144	-	+	+
PYRIDINE				
4-(phenylmethyl)pyridine	169	-	-	+
3-phenylpyridine	155	-	+	+
3,5 diphenylpyridine (X)	231	+	+	+
NAPHTHALENE				
2'-phenylpyrollo(4,5-A)-3,4-dihydronaphthalene	245	-	-	+
anthracenamine	193	-	+	+
N-phenylethyl-1(2)-naphthalenamine (Y)	247	-	+	+
N-phenylethyl-N-methyl-1(2)-naphthalenamine (Z)	261	-	+	+
1(2)-naphthalenamine	143	+	+	+
N-methyl-1(2)-naphthalenamine	157	+	+	+
MISCELLANEOUS COMPOUNDS				
acetic acid	60	-	+	+
2-hydroxy-propanone	74	-	+	+
pyranone*	144	+	+	+
lactone**	255	+	+	+

* 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one

** 2-(5'-hydroxymethyl-2'-1'-yl)-3-phenylpropionic acid lactone

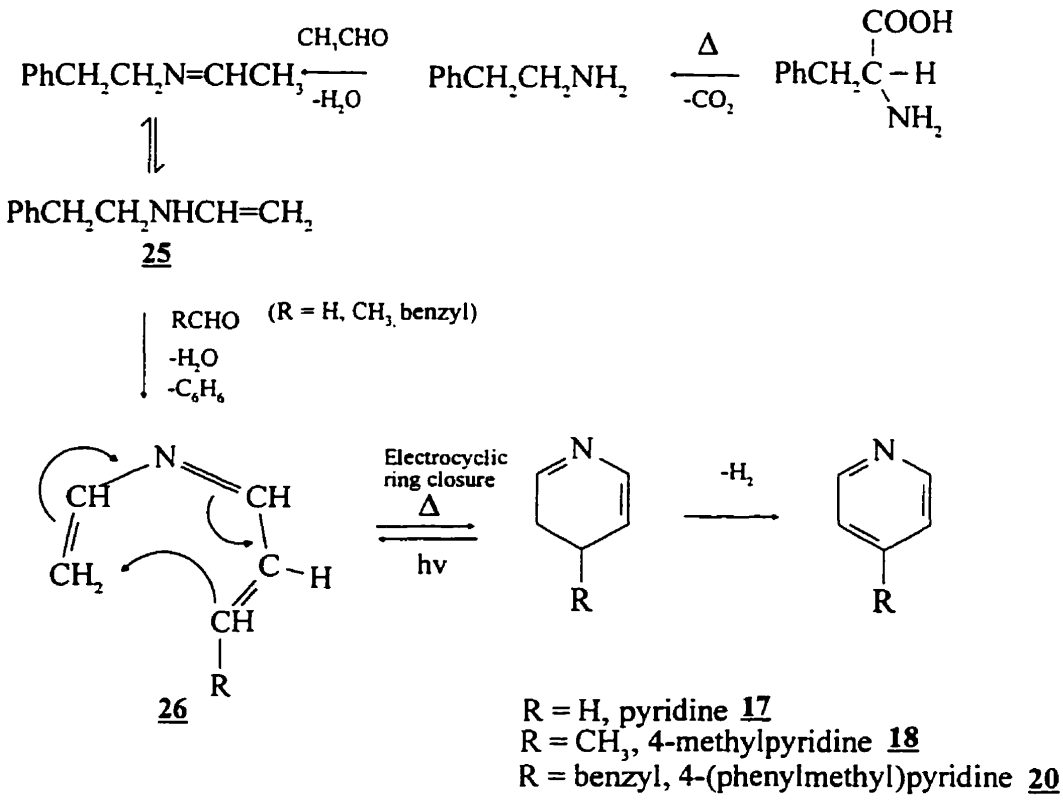
solution and mixed at room temperature for 15 hours. The solution was injected through the pyrolysis interface and also through the injection port and analyzed by GC/MS under the same conditions as mentioned under the methods section. The resulting chromatogram had a peak at the same retention time with an identical mass spectrum to that of compound X. When ARPP was dissolved in methanol and heated for 1 hour at 90 °C and analyzed by GC/MS, 3,5-diphenylpyridine (21) was observed as the predominant reaction product in the chromatogram.



Scheme 4.1. Proposed mechanism of formation of 3-substituted (19) and/or 5-substituted (21) pyridine derivatives

4.2.1.2 *Proposed mechanism of formation of 4-substituted pyridine derivatives:* The 4-substituted pyridine derivatives observed in the model systems can be envisaged to be formed by a similar mechanism depicted in Scheme 4.1. Replacing phenylacetaldehyde with acetaldehyde, which can be formed from L-phenylalanine in trace amounts (Kato et al., 1971) and more efficiently from sugars, a similar condensation product to that shown in Scheme 4.1 can be formed as shown in Scheme 4.2. The enamine (25) formed can react with either phenylacetaldehyde or any other aldehyde such as formaldehyde or acetaldehyde to form a triene (26) after losing a benzene molecule. The phenylacetaldehyde adduct for example, can form 4-(phenylmethyl)pyridine (20) after an electrocyclic ring closure and aromatization steps. When an equimolar mixture of acetaldehyde and phenethylamine was stirred at room temperature for 15 hr and subsequently heated with phenylacetaldehyde and injected through the pyrolysis interface and also through the injection port and analyzed by GC/MS under the same conditions as the pyrolysis of ARPP, the resulting chromatogram had a peak at the same retention time with an identical mass spectrum to that of 4-(phenylmethyl)pyridine (20). In addition, experiments with D-[1-^{13}C]glucose (Table 4.2) shows a large percentage of label incorporation in 4-(phenylmethyl)pyridine indicating involvement of acetaldehyde fragment coming from the sugar moiety whereas, 3,5-diphenylpyridine (21) did not show any label incorporation.

4.2.2 Naphthalene Derivatives: Naphthalene derivatives are suspected carcinogens (Orzechowski, 1992). Aminoanthracene is a recognized promutagen (Phillipson, 1983). Thermal degradation of ARPP at 250 °C, both in the quartz tube and on the ribbon probe, resulted in the formation of several naphthalene derivatives such as 1(2)-naphthaleneamine (27), N-methyl-1(2)-aminonaphthalene (28), 1-aminoanthracene and 2'-phenyl-pyrrolo[4,5- A]dihydronaphthalene (Tables 4.1 and 4.3).



Scheme 4.2. Proposed mechanism of formation of 4-substituted pyridine derivatives

At 200 °C on the ribbon probe only the pyrrolo derivative did not form and at 150 °C only naphthaleneamine and its N-methyl derivative were formed from the ARPP. On the other hand, L-phenylalanine alone on the ribbon probe did not produce any of the naphthalene derivatives mentioned above. However, in the quartz tube at 250°C all, except pyrrolo derivative, were formed. In addition to the above naphthalene derivatives, two peaks were observed in the chromatograms of various model systems containing L-phenylalanine and sugar or ARPP one at a retention time of 31.5 min. with a parent ion at

m/z 247 (compound Y) and the other at 31.7 min. with a parent ion at m/z 261 (compound Z). Both compounds exhibited characteristic fragmentation of a naphthalene derivative (see Figure 4.2 and Table 4.2) such as a fragment ion at m/z 128 (parent naphthalene) and the presence of multiply charged ions. These two compounds were present, at trace levels, among the L-phenylalanine pyrolysis products. On the ribbon probe L-phenylalanine produced only compound Y (m/z 247) at 250 °C, whereas in the quartz tube both compounds were produced at 250°C (Tables 4.4 and 4.5). Based on their molecular weight and mass spectral fragmentation patterns (Table 4.2 and Figure 4.2) naphthalene derivatives containing phenethyl groups were postulated for the structures of the two unknown compounds as shown in Figure 4.2. In general, the number of naphthalene derivatives and the efficiency of their formation (area/mole) increased for L-phenylalanine when pyrolysis was conducted in the quartz tube at 250 °C (Table 4.4). At 250°C the naphthalene derivatives produced by ARPP and L-phenylalanine in the quartz tube were similar, however the efficiency of their formation increased by eight fold based on area/mole in the case of the Amadori compound in the presence of L-phenylalanine.

4.2.2.1 *Proposed mechanism of formation of N-substituted 1-amino and 2-amino-naphthalene derivatives:* N-substituted 1-amino or 2-aminonaphthalene derivatives could be formed from corresponding aminonaphthalenes by nucleophilic substitution reaction with proper electrophiles. Protonated benzeneethanol could be one such reactant. When commercially available 2-naphthalenamine and benzeneethanol were mixed in a reaction vial in the presence of trace amounts of L-phenylalanine as the proton source and then incubated at 130 °C for 15 hours, the GC/MS analysis of the reaction mixture yielded a compound with a parent ion of m/z 247 and with the same mass spectrum and retention time as the compound Y. 1-Aminonaphthalenes and N-substituted 1-aminonaphthalenes could also have been formed in L-phenylalanine model systems by the aldol condensation of phenylacetaldehyde and acetaldehyde as illustrated in Scheme 4.3.

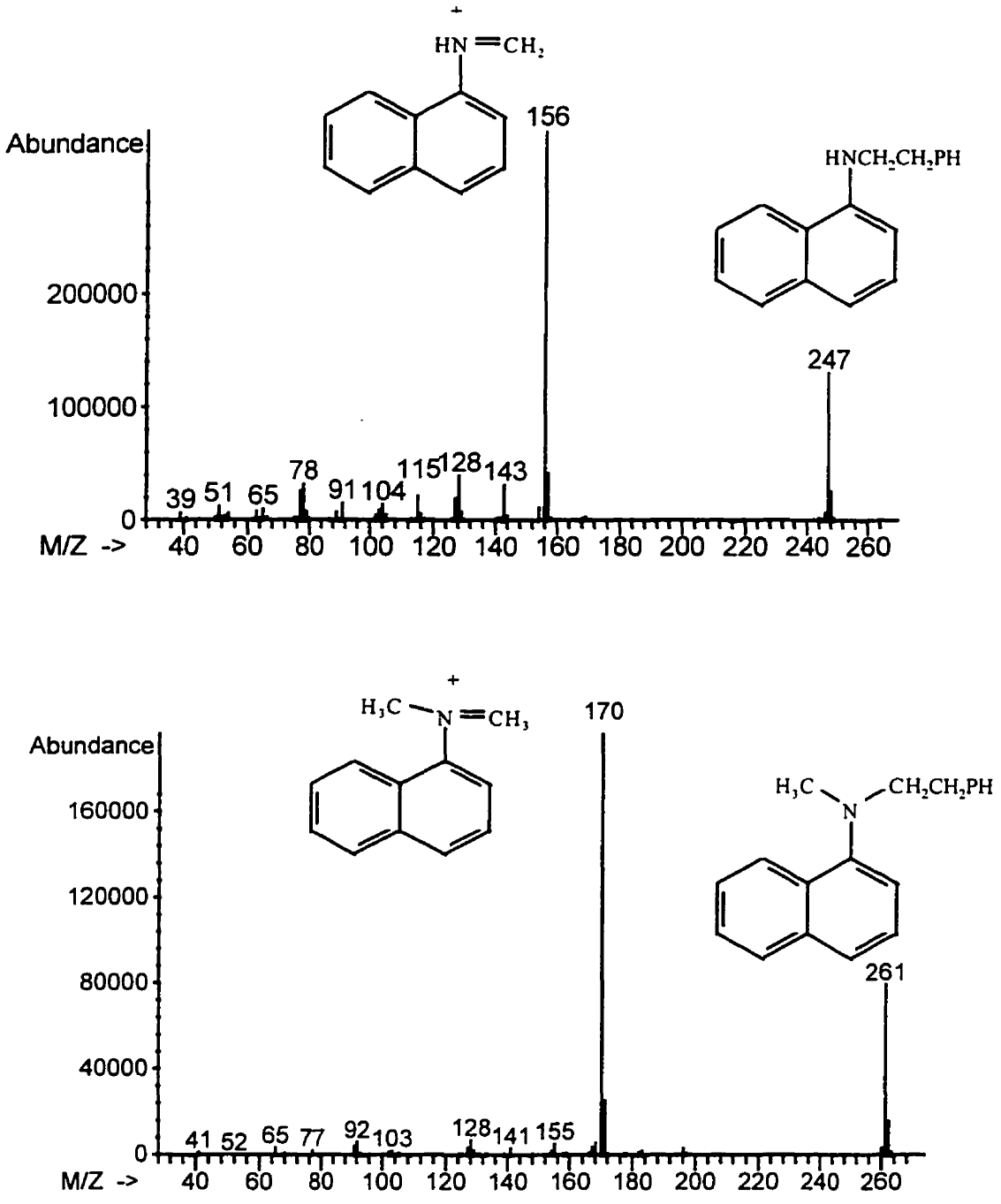


Figure 4.2. (upper) Mass spectrum of compound **Y** tentatively assigned the structure of N-phenethyl-1(2)-aminonaphthalene (27); (lower) Mass spectrum of compound **Z**, tentatively assigned the structure N-methyl-N-phenethyl-1(2)-amino-naphthalene (28).

Table 4.4. Effect of temperature on the efficiency of formation (x 10^{11}) in area/mole of molecular ions of pyridine and naphthalene derivatives and their precursors generated from ribbon pyrolysis for 20 s of L-phenylalanine and Amadori phenylalanine*

		150 °C		200 °C		250 °C	
Compound	**MW**	**Phe**	**ARPP**	**Phe**	**ARPP**	**Phe**	**ARPP**
Phenylacetaldehyde	120	0	3	0	18	0	25
Phenethylamine	121	0	0	6	0	135	0
Benzenemethanamine	107	0	0	0	0	0	0
Benzaldehyde	106	0	0	0	0	0	t
Benzenethanol	122	t	0	0	t	0	t
N-methyl-1(2)-naphthaleneamine	157	0	t	0	t	0	t
1(2)-Naphtalenamine	143	0	2	0	7	0	9
1-(N-benzyl)naphthaleneamine	233	0	0	0	0	0	0
1(2)-(N-phenethyl)naphthaleneamine (Y)	247	0	0	0	4	t	10
1(2)-(N-phenethyl-N-methyl)naphthaleneamine (Z)	261	0	0	0	2	0	5
3-Phenylpyridine	155	0	0	0	2	t	4
4-(Phenylmethyl)pyridine	169	0	0	0	0	0.5	2
3,5-Diphenylpyridine (X)	231	t	9	17	41	9	113

* *Phe L-phenylalanine, ARPP Amadori phenylalanine, t = trace*

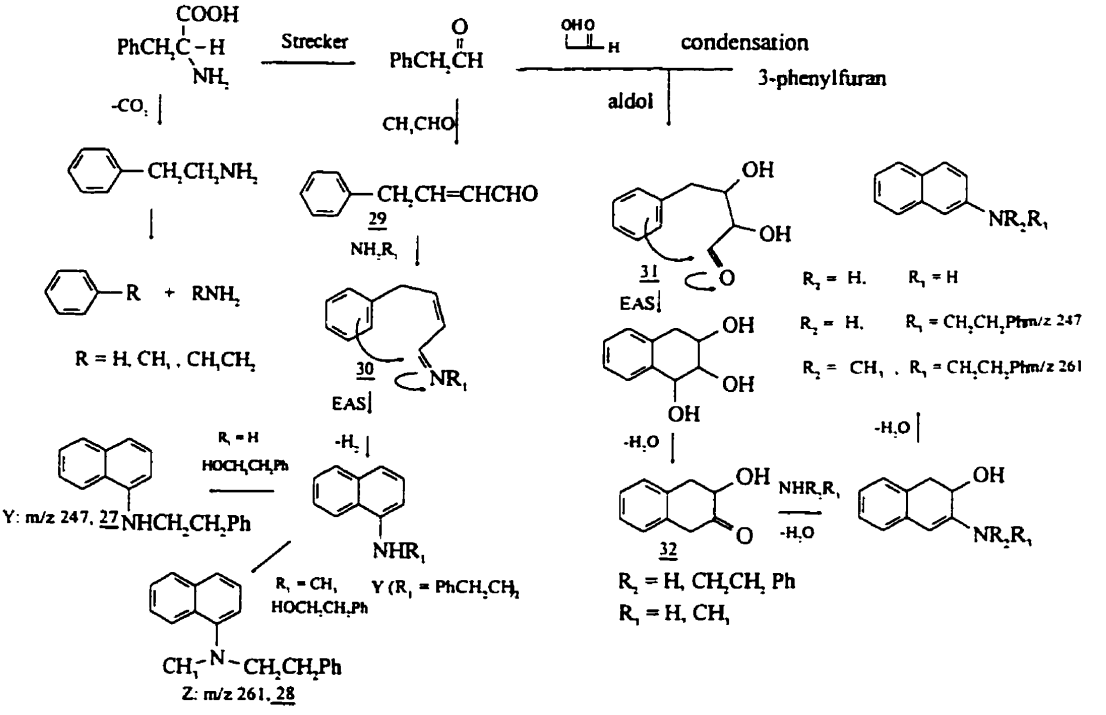
systems containing two components are equimolar

Table 4.5. Efficiency of formation (x 10^{11}) in area/mole of molecular ions of pyridine and naphthalene derivatives and their precursors generated from pyrolysis at 250 °C for 20 seconds in the quartz tube of model systems containing L-phenylalanine.*

Compound	MW	Phe	Glu/Phe	ARPP	ARPP/Phe
Phenylacetadehyde	120	0	34	27	0
Phenethylamine	121	187	0	0	340
Benzenemethanamine	107	t	0	0	t
Benzaldehyde	106	0	0	0	0
Benzenethanol	122	0	3	4	6
N-methyl-1(2)-naphthaleneamine	157	t	t	t	t
1(2)-Naphtalenamine	143	3	8	4	7
1-(N-benzyl)naphthaleneamine	233	t	t	0	0
1(2)-(N-phenethyl)naphthaleneamine (Y)	247	4	17	6	35
1(2)-(N-phenethyl-N-methyl)naphthaleneamine (Z)	261	3	9	4	20
3-Phenylpyridine	155	t	3	4	6
4-(Phenylmethyl)pyridine	169	0.5	1	3	4
3,5-Diphenylpyridine (X)	231	14	30	88	156

**Phe L-phenylalanine, Glu D-glucose, ARPP Amadori L-phenylalanine, t = trace*

systems containing two components are equimolar



Scheme 4.3. Proposed mechanisms of formation of N-substituted 1- and 2-aminonaphthalenes.

The resulting α, β-unsaturated aldehyde (29) can react with any primary amine to produce an α, β- unsaturated imine (30), this adduct can undergo an intramolecular electrophilic aromatic substitution reaction (EAS) and can aromatize to produce the target compounds. An equimolar mixture of phenylacetaldehyde and acetaldehyde was refluxed for 30 min. at the end of which phenethylamine was added and refluxing was continued for an additional 15 min. Analysis of the mixture indicated the presence of compound Y. Alternatively, phenylacetaldehyde can condense with glycoladehyde to

produce N-substituted 2-aminonaphthalene derivatives. The initial α, β-dihydroxyaldehyde intermediate (31) can undergo intramolecular electrophilic aromatic substitution reaction (EAS) and after dehydration can produce hydroxy β-tetralone (3,4-dihydro-3-hydroxy-2(1H)-naphthalenone, 32) which can react with different primary and secondary amines to form N-substituted 2-aminonaphthalene derivatives. When phenylacetaldehyde was reacted with glycoladehyde dimer for 15 hr at 120 °C, the reaction mixture contained 3-phenylfuran as the major product. Subsequently, further reaction with phenethylamine in the presence of catalytic amount of L-phenylalanine produced compound Y (m/z 247). The amino naphthalene derivatives therefore, could be a mixture of 1- and/or 2-substituted isomers.

4.3 Conclusion

Thermal degradation of phenylalanine containing model systems produced naphthalene derivatives, some of which have already been classified as mutagens (Orzechowski, 1992). Pyridines as a group are heterocyclic compounds which produce positive results with the Ames and Williams test (Sasaki et al., 1987). Py/GC/MS was demonstrated to be a useful and rapid technique in understanding the degradation pathways of Amadori compounds. The use of isotopically enriched compounds to determine reaction mechanisms is facilitated and economical since Py/GC/MS analyses can be conducted on a microscale.

CHAPTER 5

GLYCINE MODEL SYSTEMS

5.1 Introduction

Application of Py/GC/MS in generation of Maillard reaction products (MRP's) have utilized proline (Huyghues-Despointes et al., 1994) and phenylalanine (Chapter 4) and their respective Amadori products. The stability of these amino acids, upon exposure to elevated temperatures and the incorporation of their stable side chains into the volatile products detected, facilitated elucidation of the main reaction routes. However, in this chapter, advantage is taken of the low molecular weight of glycine to conduct complete labeling experiments, in an effort to trace the origin of all carbon atoms arising from both amino acid and sugar. We also demonstrate that the product profile obtained upon pyrolysis at 250°C for 20 seconds is comparable to reported products produced from glycine models subjected to temperatures between 95-180°C (Olsson, et al., 1978; Nyhammer et al., 1986; Hyase and Kato., 1985; Oh et al., 1992; Bemis-Young et al., 1992; Hwang et al., 1995).

Py/GC/MS has been demonstrated to be a fast and convenient technique for the analysis of Maillard reaction products (Huyghues-Despointes et al., 1994), especially arising from isotopically enriched compounds for mechanistic studies (Huyghues-Despointes and Yaylayan, 1996). The use of ^{13}C and ^{15}N enriched compounds for the elucidation of reaction mechanisms concerning the Maillard reaction has been well documented (Tressl et al., 1993; Hwang et al. 1994; Amrani-Hemaimi, 1995). However, this investigation of D-glucose/glycine model systems is the first comprehensive study utilizing labeled D-glucoses and glycines at all their carbon atoms and the nitrogen of glycine.

The role of glycine in the Maillard reaction as a flavor precursor has been investigated by many researchers. Rizzi (1972) showed the possible mechanism for the formation of pyrazines from glycine and alanine with diketones. Olsson et al. (1978) presented a detailed review and illustration for the possible formation pathways of various flavor compounds generated from an aqueous D-glucose/glycine model system, containing excess glucose, refluxed for 120 h. The volatiles generated included furans, pyrroles, pyridines, phenols, carboxylic acids and lactones. An aqueous equimolar D-

glucose/glycine model system refluxed at 95°C yielded diacetyl, furfuryl alcohol, pyrroles, pyranone and amides as Maillard reaction products (Hyase and Kato, 1985). Equimolar D-glucose/glycine model systems subjected to higher temperatures produce a variety of alkyl pyrazines (Amrani-Hemaimi et al., 1995; Hwang et al., 1995).

Chuyen et al. (1973) identified a series of 2-(3'-alkyl-2'-oxo-pyrazin-1'-yl) alkanoic acids in the equimolar reaction mixture of various dipeptides including glycine with glyoxal, heated at 100°C. Oh et al. (1992) detected the decarboxylated analogs of the above alkanoic acids (alkyl 2(1H)-pyrazinones) in the equimolar reaction mixture of various glycine peptides and glucose heated at 180°C and classified them as peptide specific Maillard reaction products (Scheme 1.4). Section 5.2.2 investigates the mechanism of formation of pyrazinones by pyrolysis coupled with gas chromatography/mass spectrometry (Py/GC/MS) from ^{13}C enriched glucoses and $^{15}N/^{13}C$ enriched glycines.

Some of the major products detected by GC/MS in the pyrograms of Amadori glycine and D-glucose/glycine model systems, could not be identified unambiguously and required the production of analytically pure samples for spectroscopic conformation. The ability of focused microwave irradiation under atmospheric pressure conditions to generate and extract chemical reaction products, using a two-stage microwave assisted process (MAP) (Paré et al., 1991, 1994) was successfully applied to synthesize and isolate some of the major unknown products formed when D-glucose was pyrolyzed in the presence of excess glycine (Yaylayan et al., 1996). Spectroscopic analysis of one of the major products that has been isolated using the microwave extraction, have indicated that D-glucose/glycine model systems can generate fused benzopyrazinone derivatives (quinoxalinones) in addition to alkyl substituted pyrazinones, by a related mechanism. Alkyl and tetrahydroquinoxaline derivatives have been identified in different food products such as brewed coffee (Sasaki et al., 1987), pork liver (Mussinan and Walradt 1974), and roasted filberts (Kinlin et al., 1972). Imidazoquinoxalines, specific products of creatinine/D-glucose/glycine mixture, have been identified in fried beef (Nagao et al., 1983). Section 5.2.3 reports the isolation and mechanism of formation of 5-hydroxy-1,3-dimethyl-2[1H]-quinoxalinone (33) in D-glucose/glycine model system.

5.2 Results and Discussion

5.2.1 Overview of Py/GC/MS analysis of glycine model systems

The products identified in the pyrograms obtained from quartz tube pyrolysis (250°C, 20s) of glycine model systems are listed in Table 5.1. The majority of the products identified can be categorized as furans, pyrazines, pyrroles, pyrazinones, quinoxalinones and ketones. The product profiles for Amadori glycine and D-glucose/glycine were similar, however, the formation efficiencies (integrated area/per mole of reactant) of several compounds were different (Table 5.2). This was especially noticable in systems containing excess glycine.

The D-glucose/glycine model system contains an appreciable number of free glucose pyrolysis products such as furfural and hydroxymethylfurfural. (Table 5.1). Pyrroles are closely related in structure to the furans, and they are speculated to be formed in a related manner from the reaction of a 3-deoxyketose with ammonia or an amino compound followed by dehydration and ring closure. At higher temperatures (180°C vs. 120°C) pyrrole formation has been reported to increase substantially (Reese and Baltes, 1992). As the aldose carbon chain decreased, the variety of furans and pyrroles detected in the glycine model systems also decreased (Table 5.1).

Pyrazine formation, on the other hand, increases with increasing glycine availability in the reaction system. This is in accord with published reports which link pyrazine formation to the concentration and nature of the nitrogen source available in the system (Wong and Bernhard, 1988). Haung et al. (1989) found that different amounts of the same pyrazine were produced by different amino acids that had been thermally reacted with D-glucose. They reported that the predominant pyrazine in D-glucose/glycine models was 2,3,5-trimethylpyrazine which correlates with results obtained by Py/GC/MS.

Alkyl 2(H)-pyrazinones have been reported as peptide specific MRP's. The three pyrazinones identified in this study have been detected in aqueous reaction mixtures

containing equimolar concentrations of glycylglycine and D-glucose (Ho et al., 1992). The pyrazinones were identified in models containing excess glycine. In the DL-glyceraldehyde/glycine system only 1,5,6-trimethylpyrazinone (34) was detected. An in depth investigation to determine the mechanism of formation of these pyrazinones in models containing free glycine is presented in section 5.2.2.

In addition, two new compounds, subsequently confirmed to be quinoxalinones were also identified in model systems containing excess glycine. The mechanism of their formation, isolation and structural characterization are reported in section 5.2.3.

To study the effect of temperature on the decomposition products, a solution of glycine Amadori compound dissolved in methanol/water was deposited on the ribbon probe and heated sequentially, first to remove the solvent and then to pyrolyze the product. A high flow rate of carrier gas was used to sweep the initial pyrolysis products onto the GC column without extensive secondary product formation. The Amadori product was pyrolyzed at 100, 150, 200 and 250°C. Table 5.3 lists the products identified. Secondary pyrolysis products were observed at 250°C. Between 100-200°C the main degradation event, based on the observed product profile, was carbon-nitrogen bond cleavage reactions.

Table 5.1. Products Identified During Pyrolysis/GC/MS of Aldose/Glycine Model Systems, Using Quartz Tube At 250°C for 20s.

Glycine Models[a]		ARP	ARP/GC	GL/GC	GL/GC	RI/GC	RI/GC	GD/GC	GD/GC
molar ratio			1/1	1/1	1/3	1/1	1/3	1/1	1/3
Furans	**MW**								
1-(2-furanyl)-ethanone	110	+	-	+	+	+	+	-	-
2(3H)-furanone	84	+	-	-	-	-	-	-	-
2-furancarboxaldehyde	96	-	-	+	-	+	+	+	-
2-furancarboxylic acid	112	+	-	-	-	-	-	-	-
2-furanmethanol	98	-	-	+	-	+	-	-	-
2-methylfuran	82	-	-	-	-	+	+	+	-
5-hydroxy-2-furancarboxaldehyde	126	-	-	+	-	-	-	-	-
5-methyl-(2H)-furanone	98	+	-	-	-	-	-	-	-
5-methyl-2(H)-furanone	98	-	-	+	-	-	-	-	-
5-methyl-2-furancarboxaldehyde	110	+	-	+	-	-	-	+	-
dihydro-2-methyl-3(2H)-furanone	100	-	-	-	-	+	-	-	-
Protoanemonin	96	+	-	+	+	-	-	-	-
Pyrazines									
2,3-dimethylpyrazine	108	-	-	-	+	-	+	-	-
2,6-dimethylpyrazine	108	-	-	+	+	-	-	+	+
tetramethylpyrazine	136	-	+	-	+	-	-	-	-
trimethylpyrazine	122	+	+	+	+	-	+	+	+
Pyrroles									
1-(1H-pyrrol-2-yl)-ethanone	109	+	+	+	+	-	-	-	-
1-(2-furanylmethyl)-1H-pyrrole	147	-	-	-	-	+	-	-	-
1-methyl-1H-pyrrole	81	+	+	+	+	+	+	-	-
1H-pyrrole	67	+	-	-	-	+	+	-	-
2,5-dimethyl-1H-pyrrole	95	-	+	+	-	+	-	+	-
2-formyl-1-methylpyrrole	109	-	-	-	-	+	+	-	-
5-methyl-1H-pyrrole-2-carboxaldehyde	109	+	-	-	-	-	-	-	-
Pyrazinones									
1,5,6-dimethyl-2(1H)-pyrazinone[b]	138	-	-	-	+	-	+	+	+
1,5-dimethyl-2(1H)-pyrazinone[b]	124	-	+	-	+	-	+	-	+
1,6-dimethyl-2(1H)-pyrazinone[b]	124	-	+	-	+	-	+	-	+
1,7-dimethyl-cyclopenta-2[1H]-pyrazinone[c]	165	-	-	-	+	-	-	-	-
Quinoxalinones									
5-hydroxy-1,3,7-trimethyl-2-[1H]-quinoxalinone[d]	204	+	+	-	+	-	-	-	-
5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone[d]	190	+	+	-	+	-	-	-	-
Ketones									
1-hydroxy2-propanone	74	+	-	+	+	+	-	+	+
2,3-butanedione	86	+	-	+	+	-	-	-	+
2,3-dihydro-3,5-dihydroxy-6-methyl-(4)H-pyran-4-one	144	+	+	+	+	-	-	-	-
2,3-pentanedione	100	-	-	-	+	-	+	-	-
2-propanone	58	-	-	-	-	-	-	+	+
3-hydroxy-2-butanone	88	-	-	-	+	-	-	-	-
Other									
1,3-dimethyl-1H-pyrazole	96	-	-	+	-	-	-	-	+
1-[2-pyridinyl]-ethanone	121	+	-	-	-	-	-	-	
acetic acid	60	+	+	+	+	+	+	+	+
levilunic acid	116	-	-	+	-	+	-	+	-
N-methylmethanamine	82	+	-	-	-	-	-	-	-

[a] +, present; -, absent; Glycine Models: ARP, Amadori Glycine; GC, Glycine; GL, D-Glucose; RI, D-Ribose; GD, DL-Glyceraldehyde, [b]reported in Oh et al., 1992; [c]reported in Section 4.2.2, [d]reported in Section 4.2.3.

Table 5.2. Efficiency of Formation (x 10^{11}) in area/mole of selected pyrolysis products of glycine model systems.

1,5 and 1,6-Dimethylpyrazinone	(area/mole) x 10^{11}
D-Glucose/Glycine (1:1)	6.2
D-Glucose/Glycine (1:3)	80
Amadori Glycine	10
Amadori Glycine/Glycine (1:1)	20
1,5,6-Trimethylpyrazinone	
D-Glucose/Glycine (1:1)	17
D-Glucose/Glycine (1:3)	225
Amadori Glycine	t
Amadori Glycine/Glycine (1:1)	50
5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone	
D-Glucose/Glycine (1:1)	t
D-Glucose/Glycine (1:3)	350
Amadori Glycine	52
Amadori Glycine/Glycine (1:1)	80

Table 5.3. Effect of Temperature on the Formation of Pyrolysis Products from Glycine Amadori Compound Using the Ribbon Probe.

Compounds[a]	**MW**	**Temperature °C**			
		100	150	200	250
Pyrroles					
1-methyl-1H-pyrrole	81	-	-	-	+
1-(1H-pyrrol-2-yl)-ethanone	109	+	+	+	+
Pyrazine					
trimethylpyrazine	122	-	-	-	+
Furans					
5-methyl-2-furancarboxaldehyde	110	+	+	+	+
1-(2-furanyl)-ethanone	110	+	+	+	+
2-furanmethanol	98	-	+	+	+
1-(3-pyridinyl)-ethanone	121	-	-	+	+
Carbonyl					
1-hydroxy-2-propanone	74	-	-	+	+
2,3-butanedione	86	-	-	+	+
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	144	+	+	+	+
Quinoxalinones					
5-hydroxy-1,3,7-trimethyl-2-[1H]-quinoxalinone[b]	204	-	-	-	+
5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone[b]	190	-	-	-	+
Carboxylic Acids					
Acetic acid	60	+	+	+	+
propionic acid	70	-	-	-	+
Other					
protoanemonin	96	+	+	+	+

[a] *+, present;-, absent,* [b] *compounds identified in section 5.2.3.*

Table 5.4. Products Identified During Pyrolysis/GC/MS of carbonyl/glycine 1:3 Model Systems, Using Quartz Tube At 250°C for 20s.

carbonyl/glycine models[a]		AC/GC	BU/GC	GY/GC	GX/GC	PY/GC
Molar Ratio (1:3)	**MW**					
Ketones						
1-hydroxy-2-butanone	88	+	-	-	-	-
2,3-pentanedione	100	-	+	-	-	-
2-propanone	74	+	-	-	-	-
Pyrroles						
2,3,5-trimethylpyrrole	109	+	-	-	-	-
2,3-dimethyl-1H-pyrrole	95	+	-	-	-	-
2,5-dimethyl-1H-pyrrole	95	-	+	-	-	-
3-methyl-pyrrole	81	-	-	-	-	-
Pyrazines						
2,3-diethyl-5-methyl-pyrazine	150	+	-	-	-	-
2,3-dimethylpyrazine	108	-	-	+	-	-
2,5-dimethylpyrazine	108	-	-	-	-	+
2,6-dimethyl-3-propylpyrazine	150	+	-	-	-	-
2,6-dimethylpyrazine	108	+	-	-	-	-
2-ethyl-3,5-dimethylpyrazine	136	+	-	-	-	-
3-ethyl-2,5-dimethylpyrazine	136	+	-	-	-	-
ethenylpyrazine	106	-	-	-	+	-
methylpyrazine	94	-	-	-	+	-
pyrazine	80	-	-	-	+	-
tetramethylpyrazine	136	+	+	-	-	-
trimethylpyrazine	122	+	+	+	+	+
Pyrazinone						
1,5,6-dimethyl-2(1H)-pyrazinone[b]	138	+	+	+	+	+
1,5-dimethyl-2(1H)-pyrazinone[b]	124	+	-	+	+	-
1,6-dimethyl-2(1H)-pyrazinone[b]	124	+	-	+	+	-
Other						
1,3,5-trimethylpyrazole	110	-	+	-	+	-
1H-imidazole	68	-	-	-	+	-
acetamide	59	-	-	-	+	-
acetic acid	60	+	+	+	+	+
levalunic acid	116	+	-	-	-	-
trimethyloxazole	111	-	+	-	-	-

[a] +, present; -, absent; Carbonyl/glycine models: GC, glycine; AC, acetol; BU, 2,3-butadione; GY, glycoaldehyde dimer; GX, glyoxal trimer; PY, pyruvaldehyde
[b] reported in Oh et al., 1992

5.2.2 Pyrazinone formation in glycine model systems.

The reaction of various dipeptides, including glycylglycine, with glyoxal at 100 °C have yielded a series of 2-(3'-alkyl-2'-oxo-pyrazin-1'-yl)alkanoic acids (Chuyen et al., 1973) that have been characterized by different spectroscopic techniques. Oh et al. (1992) obtained alkyl 2(1H)pyrazinones, the decarboxylated analogs of the alkanoic acids, when glycine dipeptide was reacted with pyruvaldehyde or D-glucose at a higher temperature (180°C). The authors referred to alkyl 2(1H)pyrazinones as peptide-specific Maillard reaction products, since in an equimolar mixture of D-glucose and glycine, pyrazinones were not detected. In the course of our study of different Amadori products and mixtures of D-glucose and amino acids by Py/GC/MS, it was found that trace amounts of dimethyl-2-(1H)-pyrazinones (35 and 36) and 1,5,6-trimethyl-2(1H)-pyrazinone (34) were formed from the glycine Amadori product and from an equimolar D-glucose/glycine mixture when pyrolyzed at 250 °C for 20 seconds. The efficiency of formation of these compounds increased when an equimolar amount of glycine was added to the glycine Amadori compound or when a two or three-fold excess of glycine was reacted with D-glucose (Table 5.1). Table 5.5 shows the mass spectrometric data of pyrazinones.

To confirm the origin of the carbon atoms incorporated into the pyrazinone structures and to elucidate the role of excess glycine in the formation of pyrazinones, D-[^{13}C]glucoses labeled at [1-^{13}C], [2-^{13}C], [3-^{13}C], [4-^{13}C], [5-^{13}C] and [6-^{13}C] positions were systematically reacted with glycine. In addition, [1-^{13}C], [2-^{13}C], [1,2-^{13}C] and [^{15}N]glycines were also reacted with D-glucose and the glycine Amadori compound to confirm the incorporation of carbon and nitrogen atoms originating from glycine, in the pyrazinone structure.

Table 5.5. Mass spectrometric data of selected pyrazinones.

Pyrazinone	m/z (relative intensity)
1,5-dimethyl-2(1H)pyrazinone[a]	124(80), 95(100), 81(22), 68(34), 54(19), 42(47), 41(46), 39(42)
1,5-dimethyl-2(1H)pyrazinone	124(80), 95(100), 81(22), 68(39), 54(22), 42(45), 41(12), 39(18)
1,6-dimethyl-2(1H)pyrazinone[a]	124(95), 95(100), 81(14), 68(27), 56(48), 42(31), 39(31)
1,6-dimethyl-2(1H)pyrazinone	124(98), 95(100), 81(15), 68(30), 56(56), 42(29), 39(25)
1,5,6-trimethyl-2(1H)pyrazinone[a]	138 (75), 109(100), 95(33), 82(8), 68(20), 56(42), 42(30)
1,5,6-trimethyl-2(1H)pyrazinone	138 (80), 109(100), 95(42), 82(8), 68(22), 56(43), 42(28)

[a] *Oh et al., 1992*

Table 5.6. Percent label distribution in 1,6- and 1,5-dimethylpyrazinone mixture (4:1) formed from labeled D-glucoses and glycines.

Model	M	M+1	M+2	M+3	M+4
Glycine/Glucose	100	0	0	0	0
D-[1-^{13}C]Glucose/Glycine	68	30	2	0	0
D-[2-^{13}C]Glucose/Glycine	68	31	1	0	0
D-[3-^{13}C]Glucose/Glycine	91	9	0	0	0
D-[4-^{13}C]Glucose/Glycine	88	12	0	0	0
D-[5-^{13}C]Glucose/Glycine	42	58	0	0	0
D-[6-^{13}C]Glucose/Glycine	38	57	4	0	0
D-Glucose/[^{15}N]Glycine (90%)	0	0	100	0	0
D-Glucose/[1-^{13}C]Glycine	0	98	2	0	0
D-Glucose/[2-^{13}C]Glycine (92%)$_a$	0	0	30	70	0
D-Glucose/[2-^{13}C]Glycine (92%)	0	0	40	60	0
D-Glucose/[2-^{13}C]Glycine(92%)$_b$	15	14	35	36	0
D-Glucose-[1,2-^{13}C]Glycine	0	0	0	30	70

Ratio of amino acid /sugar (3:1) unless otherwise specified.

[a] *ratio (2:1)*

[b] *includes 10% w/w of the unlabeled dipeptide glycylglycine*

The distribution of the isotope incorporation in reaction products was calculated from the intensities of their parent ions. The results were corrected to account for the natural ^{13}C content of the corresponding unlabeled reference compounds (see Appendix B). Tables 5.6 and 5.7 summarize the results obtained. Table 5.6 indicates that dimethylpyrazinones (1:4 mixture of 1,5- to 1,6-dimethylpyrazinones) mostly incorporate two carbon units originating from glucose; C1-C2 (~30 %), C3-C4 (~10 %) and C5-C6 (~60 %). The reactive two carbon sugar fragment could be either glyoxal (Chuyen et al., 1973) or glycoaldehyde. Inspection of Table 5.7 on the other hand, reveals that trimethylpyrazinone is predominantly formed by the incorporation of three carbon units from glucose; C1-C2-C3 (~ 25 %) and C4-C5-C6 (~ 75 %). It has already been demonstrated that a three carbon unit, pyruvaldehyde, reacts with glycylglycine to produce both di- and trimethylpyrazinones (Oh et al., 1992). The authors speculated that pyruvaldehyde is converted into 2,3-butanedione through a free radical mechanism, however, as we demonstrate below, this conversion is effected by glycine through aldol type condensation.

Table 5.7. Percent label distribution in 1,5,6-trimethylpyrazinone formed from labeled D-glucoses and glycines.

Model	M	M+1	M+2	M+3	M+4
D-Glucose/Glycine	100	0	0	0	0
D-[1-^{13}C]Glucose/Glycine	77	23	0	0	0
D-[2-^{13}C]Glucose/Glycine	77	23	0	0	0
D-[3-^{13}C]Glucose/Glycine	70	30	0	0	0
D-[4-^{13}C]Glucose/Glycine	27	73	0	0	0
D-[5-^{13}C]Glucose/Glycine	27	73	0	0	0
D-[6-^{13}C]Glucose/Glycine	26	74	0	0	0
D-Glucose/[^{15}N]Glycine (98%)	0	0	100	0	0
D-Glucose/[1-^{13}C]Glycine	0	98	2	0	0
D-Glucose/[2-^{13}C]Glycine (92%)[a]	0	0	20	80	0
D-Glucose/[2-^{13}C]Glycine (92%)	0	0	30	70	0
D-Glucose/[2-^{13}C]Glycine (92%)[b]	2	10	38	50	0
D-Glucose-[1,2-^{13}C]Glycine	0	0	0	20	80

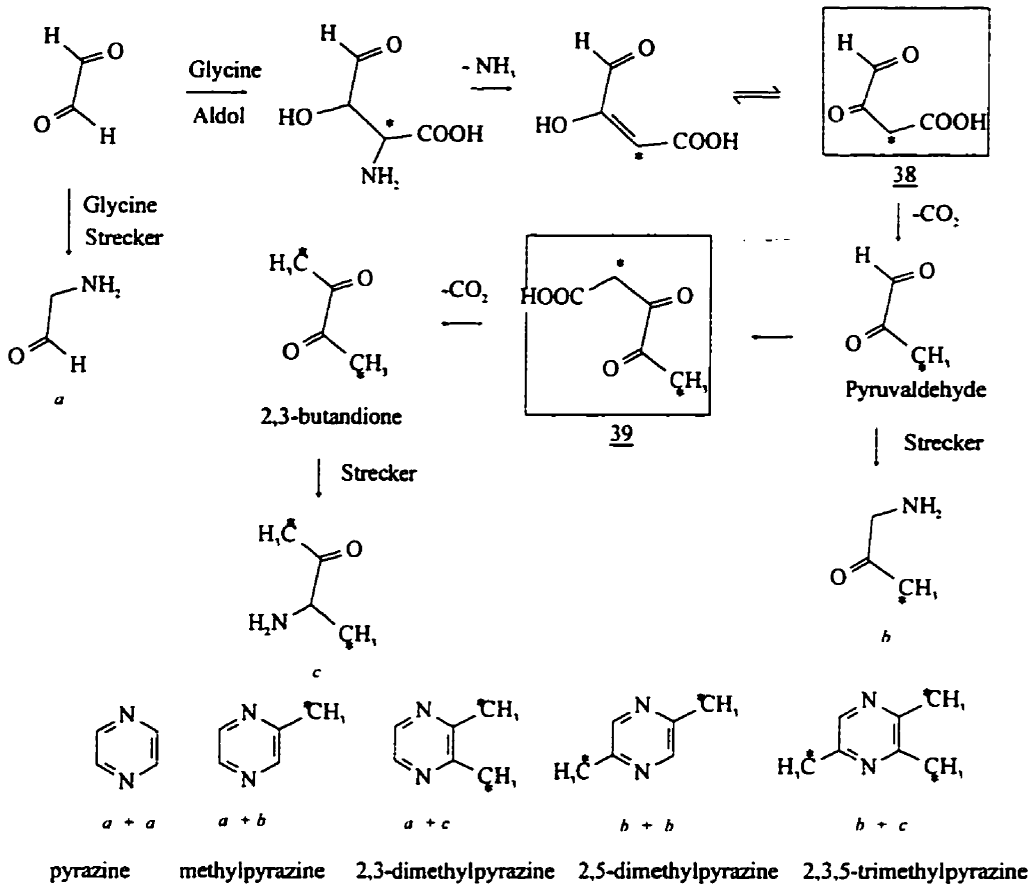
Ratio of amino acid to sugar (3:1) unless otherwise specified.
[a] ratio 2:1, [b] includes 10% w/w of the unlabeled dipeptide glycylglycine

Previous studies (Huyghues-Despointes and Yaylayan, 1996) have indicated that pyruvaldehyde can be produced by the interaction of glyceraldehyde with amino acids and that glyceraldehyde itself can be generated by retro-aldol reaction of Amadori compounds. To verify whether glyoxal, glycoaldehyde and glyceraldehyde produce pyrazinones when they interact with glycine, model systems containing unlabeled and [2-^{13}C]glycines were analyzed by Py/GC/MS and the results are shown in Table 5.8. The data in this table indicate that indeed pyrazinones can be produced by the two and three carbon sugar fragments in the presence of free glycine. In addition, the model studies have indicated that both glyoxal and glycoladehyde can be converted into a three carbon reactive unit (pyruvaldehyde) by the incorporation of C-2 of glycine through an aldol condensation reaction as described below and outlined in Scheme 5.1.

Table 5.8. Percent distribution of unlabeled and labeled pyrazinones formed from glycine and glycoladehyde, glyoxal or glyceraldehyde mixtures*

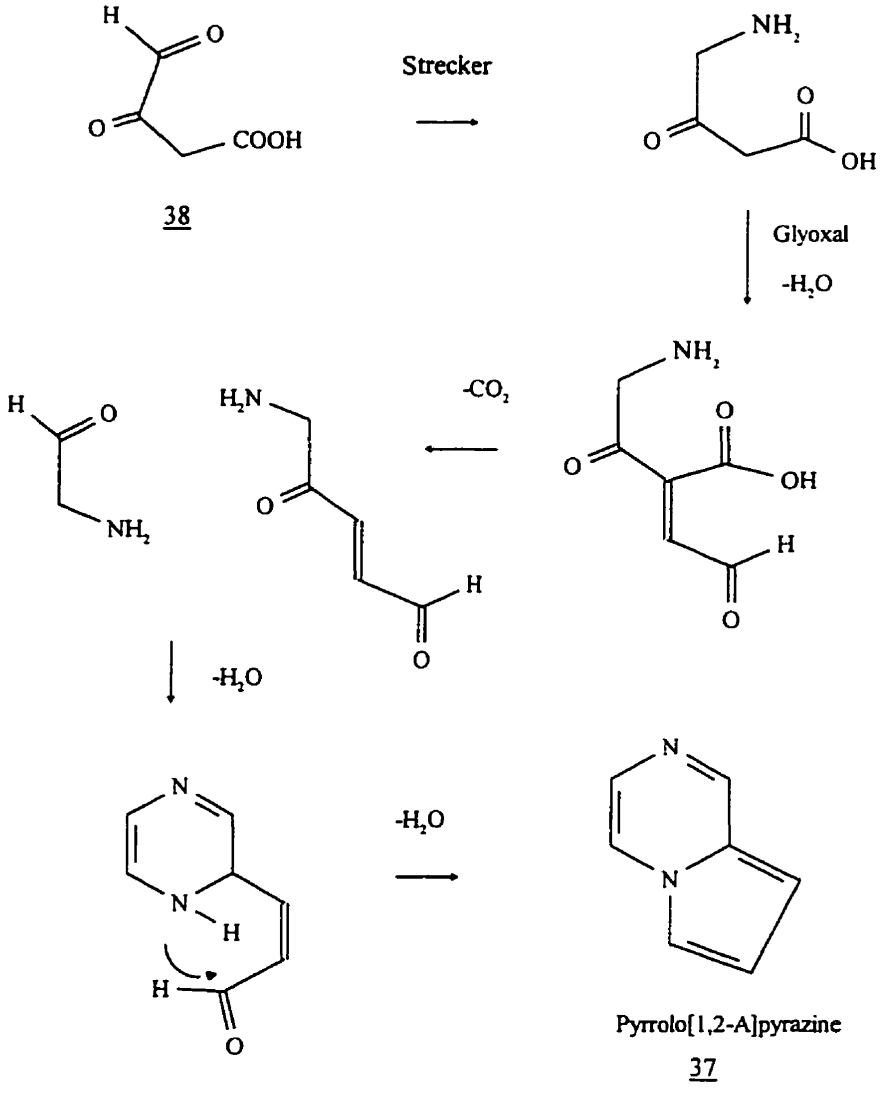
Model	M	M+1	M+2	M+3	M+4	M+5
[^{13}C-2]glycine + Glycoaldehyde						
dimethyl pyrazinone	0	2	3	95	0	0
trimethyl pyrazinone	0	2	29	48	17	4
[^{13}C-2]glycine + Glyoxal						
dimethyl pyrazinone	0	1	10	89	0	0
trimethyl pyrazinone	0	0	3	21	73	2
[^{13}C-2]glycine + Glyceraldehyde						
dimethyl pyrazinone	0	0	0	0	0	0
trimethyl pyrazinone	0	0	14	82	4	0

**Ratio of amino acid to dicarbonyl compounds (3:1) [2-13C]glycine 92% enriched.*



Scheme 5.1. Proposed mechanism of formation of methyl pyrazines in glyoxal/glycine model systems. Asterisk indicates 2-^{13}C of glycine.

5.2.2.1 Pyrazine and pyrazinone formation in glyoxal model systems: To investigate the mechanistic relationship between the structurally related pyrazines and pyrazinones, the model system containing glyoxal and excess glycine, was also analyzed for the formation of pyrazines. The system produced the parent pyrazine and pyrrolo[1,2-A]pyrazine (37) in addition to five methylated pyrazines; methyl-, 2,3-dimethyl, 2,5-dimethyl, 2,3,5-trimethylpyrazines and trace amounts of tetramethylpyrazine. Analysis of the isotopic distribution of the resulting methylated pyrazines from glyoxal/[2-^{13}C]glycine model system indicated that all the methyl groups incorporated on to the pyrazine rings arose from the C-2 of glycine (100 % incorporation in all methylpyrazine derivatives). Scheme 5.1 illustrates a plausible mechanism of formation of pyrazines from glyoxal/glycine model system. According to Scheme 5.1, glyoxal can undergo two types of interaction with glycine one is the commonly accepted Strecker degradation that generates α-amino acetaldehyde (a in Scheme 5.1) and the other is the aldol condensation followed by deamination and formation of intermediate 38 which decarboxylates to form pyruvaldehyde. Pyruvaldehyde, in turn, can undergo a similar reaction with glycine to form 2,3-butandione, through the intermediate 39 (Scheme 5.1). Both pyruvaldehyde and 2,3-butandione were detected in the glyoxal/[2-^{13}C]glycine model systems with the expected label incorporation. The net result of this newly observed transformation is the chain elongation of reactive C_2 and C_3 α-dicarbonyl fragments by one carbon unit originating from C-2 atom of glycine. If the universality of this transformation can be verified with other amino acids, they could be viewed also as alkyl donors during the Maillard reaction. Glyoxal, pyruvalaldehyde and 2,3-butanedione can react subsequently with glycine through Strecker degradation and produce α-amino carbonyls (*a*, *b* and *c* in Scheme 5.1) which eventually can interact and generate all the observed alkylpyrazines by condensation as illustrated in Scheme 5.1. Pyrrolo[1,2-A]pyrazine (37) on the other hand, incorporated only one ^{13}C-2 atom of glycine and a plausible mechanism for its formation, starting with intermediate 38, is presented in Scheme 5.2.



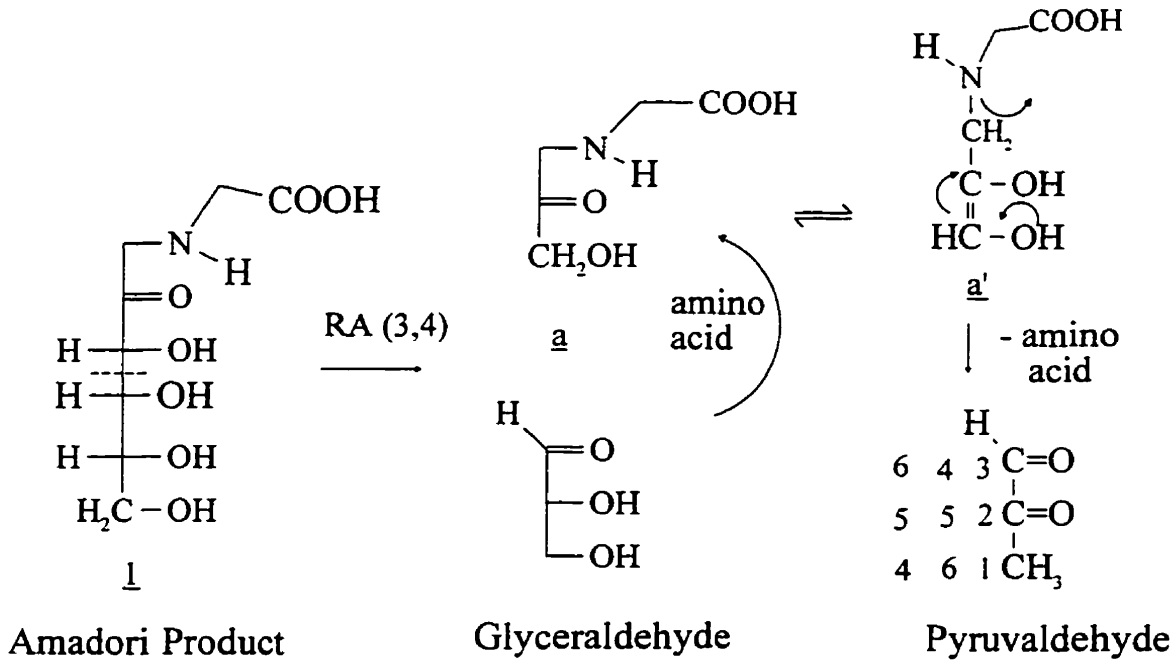
38

37

Scheme 5.2. Proposed mechanism of formation of pyrrolo[1,2-A]pyrazine (37) in glyoxal/glycine model system. Asterisk indicates C-2 of glycine.

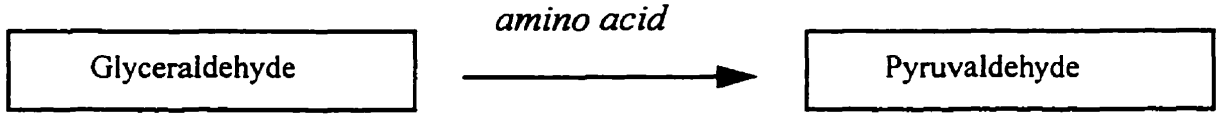
5.2.2.2 Mechanism of alkyl(1H)pyrazinone formation: From the model and labeling studies it was evident that the main sugar fragments incorporated into the dimethyl- and trimethylpyrazinones were glyoxal and pyruvaldehyde, respectively. Scheme 5.3 illustrates the mechanism of formation of pyruvaldehyde from the glycine Amadori compound (1). According to this Scheme, the Amadori compound undergoes a retro aldol cleavage at C3-C4 to generate compound a , Scheme 5.3 (from the first three carbon atoms of glucose) and glyceraldehyde (from the last three carbon atoms of glucose) which can react with free glycine by Amadori rearrangement and produce more of compound a that subsequently undergoes a β-elimination from its tautomeric form (a') to form pyruvaldehyde and releases free glycine. In effect, this process can convert glyceraldehyde into pyruvaldehyde (see Scheme 5.4) through the catalytic action of amino acid, which was also observed with proline Amadori compound (Huyghues-Despointes and Yaylayan, 1996). Glyoxal, on the other hand, can be formed by similar retro aldol cleavages of glucosone and 1-deoxyglucosone as illustrated in Scheme 5.5.

Tables 5.6 and 5.7 also indicate that pyrazinones incorporate two nitrogen atoms, one C-1 and three or two C-2 atoms from glycine. These data clearly show that there is more than one pathway of formation of alkylpyrazinones, utilizing either two or three glycine molecules per pyrazinone. The major pathway of formation of pyrazinones incorporates three C-2 atoms of glycine (70 % in the case of dimethylpyrazinone mixture and 80 % in the case of trimethylpyrazinone) and the minor pathway incorporates two C-2 atoms of glycine (94 % in the case of 1,5-dimethylpyrazinone (35), 40 % in the case of 1,6-dimethylpyrazinone (36), and 20 % in the case of 1,5,6-trimethylpyrazinone 34). Scheme 5.6 illustrates the formation of pyrazinones through both pathways A and B. Pathway B requires pyruvaldehyde to form dimethylpyrazinones and 2,3-butandione to form trimethylpyrazinone. On the other hand, pathway A requires glyoxal and pyruvaldehyde to generate dimethyl and trimethyl pyrazinones respectively and consequently it incorporates three glycine molecules in the pyrazinone structures, whereas in pathway B the number of glycine molecules incorporated depends on whether pyruvaldehyde and 2,3-butandione originate from Amadori compound or are formed through transformation of glyoxal and pyruvaldehyde by glycine as shown in Scheme 5.1.

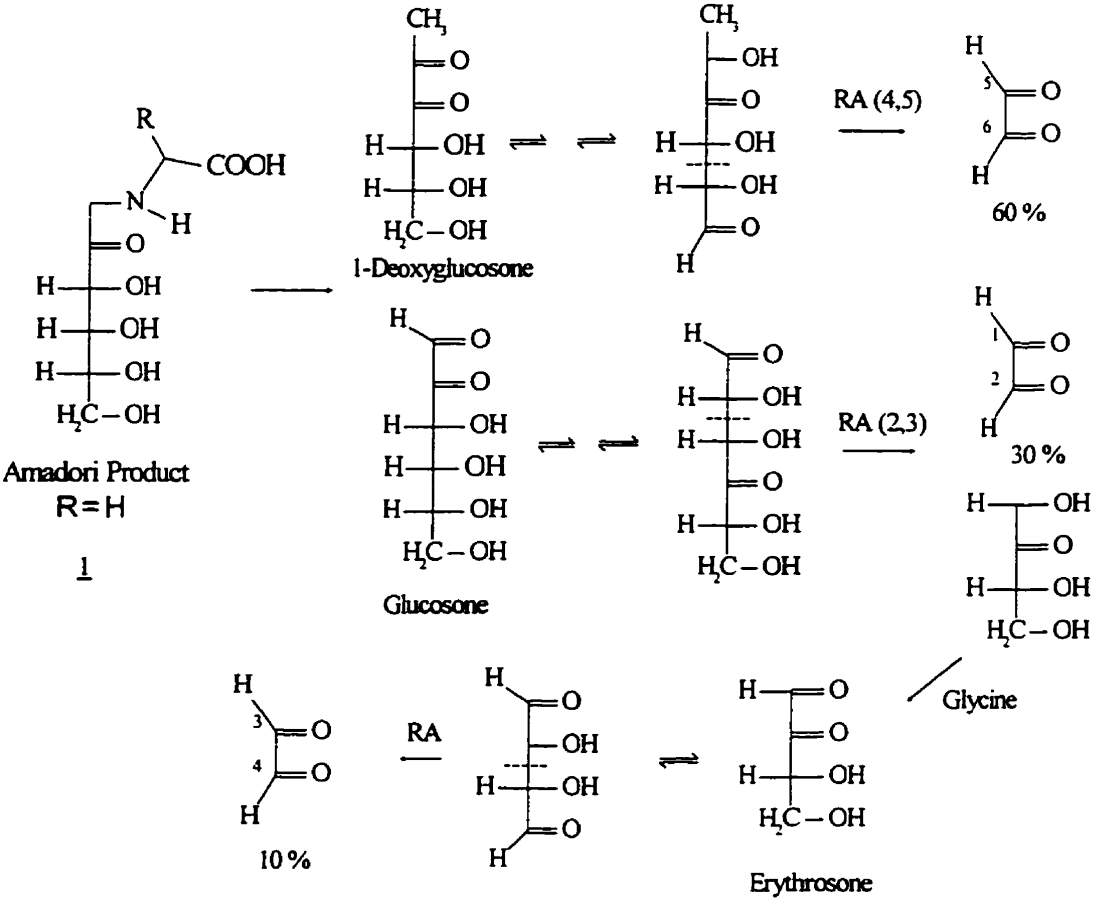


Amadori Product — Glyceraldehyde — Pyruvaldehyde

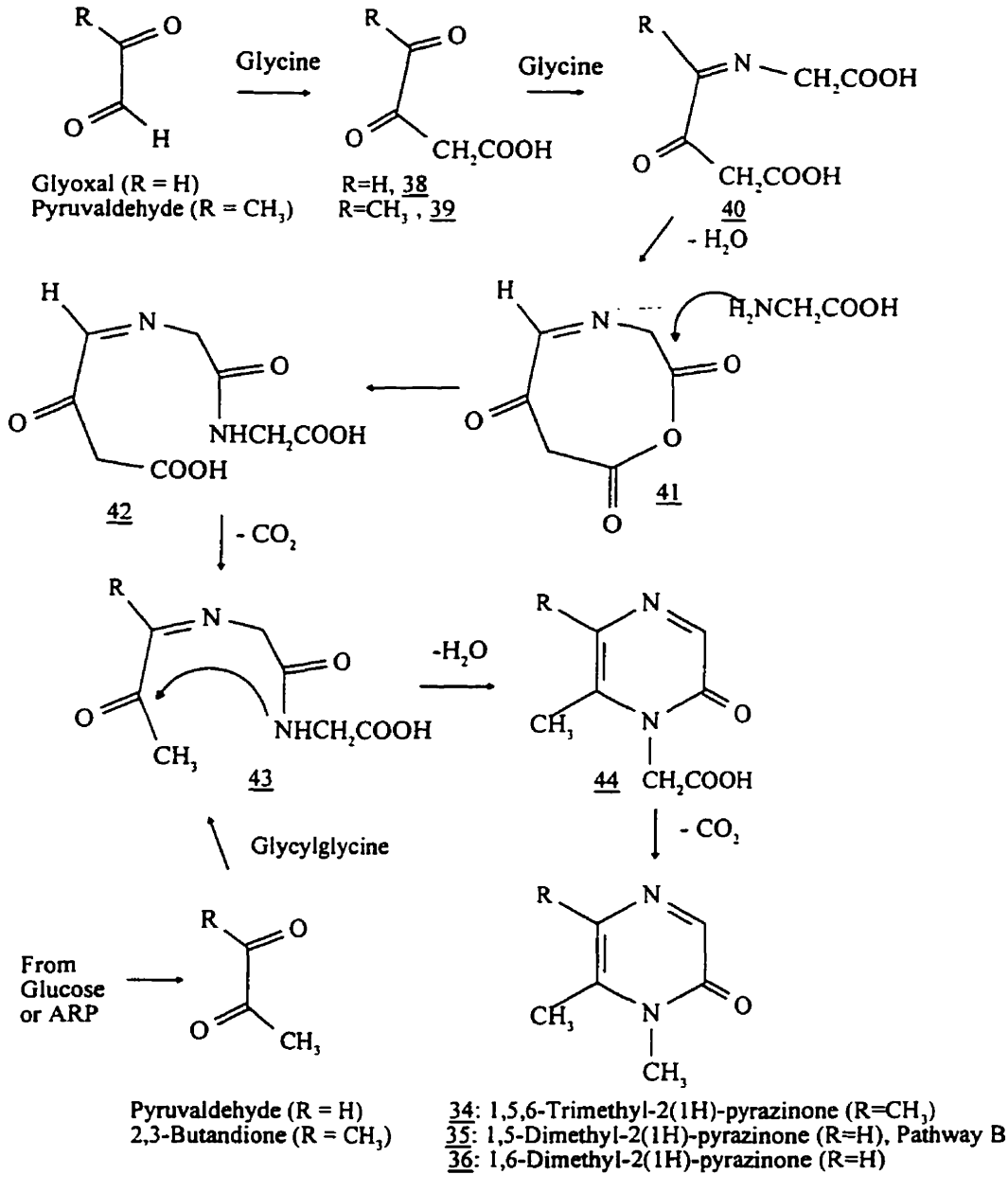
Scheme 5.3. Proposed mechanism of formation of D-glyceraldehyde from Amadori product and its subsequent transformation into pyruvaldehyde. Carbon numbers indicate original D-glucose carbon locations. RA[x,y]=retro-aldol cleavage at Cx-Cy.



Scheme 5.4. Conversion of D-glyceraldehyde into pyruvaldehyde by the action of amino acids.



Scheme 5.5. Proposed mechanism of formation of glyoxal from Amadori product. RA[x,y]=retro-aldol cleavage at Cx-Cy.



Scheme 5.6. Proposed Mechanisms of Formation of Pyrazinones[a]

[a] *ARP, Amadori rearrangement product.*

5.2.2.2.1 *Pyrazinone formation through incorporation of three glycine molecules - pathway A:* Intermediates 38 and 39 shown in Scheme 5.1 can be considered as the common mechanistic link between pyrazines and pyrazinones. These intermediates that are formed by aldol condensation of glycine with glyoxal and pyruvaldehyde respectively, can lead to 1,6-dimethyl- and 1,5,6-trimethylpyrazinones but not to 1,5-dimethyl isomer, as described in Scheme 5.6. Intermediates 38 and 39 can react with a second mole of glycine to produce the diacid 40 which upon dehydration can be converted into the acid anhydride 41 containing an activated carbonyl group which reacts with a third mole of glycine to produce 42. After decarboxylation and cyclization steps, intermediate 43 produces 2-(2'-oxo-pyrazin-1'-yl)alkanoic acids (44) which have been isolated and identified previously from model systems containing glyoxal and equimolar amounts of glycylglycine (Chuyen et al., 1973). Upon decarboxylation intermediate 44 produces 1,6-dimethyl- (36, R = H, glyoxal) and 1,5,6-trimethyl-2(1H)-pyrazinone (34, R = CH_3; pyruvaldehyde). This pathway can not produce 1,5-dimethyl pyrazinone (35) isomer.

5.2.2.2.2 *Pyrazinone formation through incorporation of glycylglycine - pathway B:* Evidence from labeling experiments indicates that in the mixture of excess glycine with D-glucose, glycine can form the dipeptide glycylglycine and that added glycylglycine (unlabeled) in the reaction mixture of [^{13}C-2]glycine/glucose can hydrolyze to form free glycine as evidenced by observing M and M+1 pyrazinone species (Tables 5.6 and 5.7). The formed dipeptide can eventually react with pyruvaldehyde and 2,3-butandione to produce pyrazinones according to a previously published mechanism (Chuyen et al., 1973) through the common intermediates 43 and 44 as shown in Scheme 5.6. A model system containing 2,3-butanedione and glycine produced 1,5,6-trimethyl-2(1H)-pyrazinone (35). In the case of pyruvaldehyde, the amino terminal of the dipeptide can react either with the *aldehydo* carbonyl or the *keto* carbonyl of the pyruvaldehyde to produce 1,6- and 1,5-dimethylpyrazinones, respectively. This pathway requires two glycine molecules as a dipeptide to produce pyrazinones, if the reacting dicarbonyls (pyruvaldehyde and 2,3-butandione) are formed directly from the Amadori product and

not through the intermediates 38 and 39. However, if they are produced through the intermediates 38 and 39 (as outlined in Scheme 5.1) that already incorporates one C-2 of glycine, than the total number of C-2 incorporated into the pyrazinone structures will be three, similar to pathway A. In order to assess the extent of pyruvaldehyde formation through intermediate 38, label incorporation from [2-^{13}C]glycine into the methylpyrazine and 2,5-dimethylpyrazine identified in [2-^{13}C]glycine/glucose model system was calculated (Table 5.9) and was found to be 10 and 5 % respectively, which indicates that for every mole of pyruvaldehyde formed through intermediate 38, approximately 9 moles are produced through retro aldol reaction of the Amadori intermediate (Scheme 5.3).

5.2.2.2.3 *Evidence for the release of intact amino acid from the Amadori products:* 2,3-Enolization of Amadori products followed by β-elimination, are known to produce 1-deoxyglucosones and free amino acid. Analysis of model systems containing unlabeled Amadori product and labeled free glycine (see Table 5.10) can confirm the release of free amino acid from Amadori products, if they can be trapped in a volatile product that can be detected. As was demonstrated above, pyrazinones are formed by incorporation of at least two glycine molecules of which one remains intact as part of the pyrazinone structure, and as such could be used as indicator compound for the presence of free glycine (released from Amadori product) in a model system containing unlabeled Amadori product and labeled free glycine. Inspection of Table 5.10 shows that mixtures of labeled free glycine and unlabeled Amadori products produce appreciable amounts of unlabeled pyrazinones indicating the presence of released amino acid from the Amadori product. In addition, the percent of unlabeled dimethylpyrazinones were relatively higher than unlabeled trimethylpyrazinone which indicates that dimethylpyrazinones are formed at a later stage, on the decomposition time scale of Amadori products, than the trimethylpyrazinones, since higher concentrations of unlabeled glycine released from Amadori product can compete more effectively with the already present labeled free glycine to produce unlabeled pyrazinones.

Table 5.9. Percent label distribution in dimethylpyrazinones* and selected** pyrazines formed from D-glucose and [2-^{13}C]glycine (92% enriched).

Compound	M	M+1	M+2	M+3
1,5-dimethylpyrazinone	0	0	94	6
1,6-dimethylpyrazinone	0	0	40	60
methylpyrazine	90	10		
2,3-dimethylpyrazine	25	70	5	

**The isomers were separated using the following temperature programming of the GC column: 40 - 260 °C at 2 °C/min. and held at 260 °C for 40 min.*
*** methyl and 2,3-dimethylpyrazines are formed by condensation of 1-amino-2-propanone, fragment b in Scheme 5.1 (Strecker degradation product of pyruvaldehyde)*

Table 5.10. Percent distribution of unlabeled and labeled pyrazinones formed from unlabeled glycine Amadori compound and variously labeled free glycines.

Model	M	M+1	M+2	M+3
[^{15}N]glycine (98%) + ARP (2:1)				
dimethyl pyrazinone	18	40	42	
trimethyl pyrazinone	10	30	60	
[^{13}C-1]glycine + ARP (1:1)				
dimethyl pyrazinone	46	54		
trimethyl pyrazinone	23	77		
[^{13}C-2]glycine (92%)+ ARP (1:1)				
dimethyl pyrazinone	30	24	26	20
trimethyl pyrazinone	9	10	31	50

5.2.3 Formation of 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone and related compounds in glycine model systems.

Multiple addition reactions of amino acids to sugar dicarbonyl fragments have not been studied extensively, despite their importance in elucidating cross-linking of proteins. Model studies (Section 5.2.2.) using D-[^{13}C]glucoses and a series of C_2, C_3, C_4 dicarbonyl compounds with labeled [^{15}N] or [^{13}C]glycines, have indicated that methyl substituted pyrazinones in these model systems, are formed through such addition reactions. Quinoxalinone could also be formed through similar additions as described in this section.

5.2.3.1 *Prediction, confirmation and identification of products arising from multiple additions of glycine during Maillard reaction:* Products arising from the incorporation of more than one amino acid moiety into a sugar fragment could be identified by a method based on Py/GC/MS utilized as an integrated reaction, separation and identification system and by the use of labeled sugars and amino acids as reactants. In this approach, sugars are reacted in the pyrolysis probe with increasing concentrations of the amino acid relative to the sugar, consequently, chromatographic peaks arising from multiple additions of the amino acid will increase and thus could be identified and further confirmed by reacting the sugar with ^{15}N- and ^{13}C-labeled amino acids and observing the incorporation of multiple labels into the product by Py/GC/MS analysis as discussed earlier. Performing such experiments with D-glucose/glycine, revealed the participation of three glycine molecules in the formation of alkyl substituted pyrazinones. In addition, other chromatographic peaks (Table 5.11) such as m/z 176, m/z 190 and m/z 204 also showed increased intensity by the addition of excess amino acid. Experiments performed with ^{13}C-labeled D-glucoses (independently labeled at each carbon atom) and ^{15}N- and ^{13}C-labeled glycines, indicated the incorporation of all the six carbon atoms of the sugar and two nitrogens, one C-1 and three C-2 atoms of glycine into m/z 190 (see Scheme 5.7 and Table 5.12). However, m/z 176 contained only two C-2 atoms of glycine and m/z 204 contained up to four C-2 atoms of glycine. Comparison of their mass spectra (Figure

5.1) indicated that they are structurally related compounds, differing only in the number of methyl group substituents, arising mainly from C-2 atom of glycine. The chromatographic peak related to m/z 190 was the most abundant followed by m/z 204 and m/z 176 respectively. When excess glycine was reacted with synthetic Amadori glycine, the peak due to m/z 190 was the most intense in the pyrogram. In addition, due to the similarity of the their substitution pattern to that of alkyl pyrazinones (Section 5.3.3.; Oh et al., 1992), and intense molecular ions in their mass spectra, it was predicted that they should possess aromatic pyrazinone structures, and as such could be extracted into non-polar solvents such as hexane. All attempts to extract these compounds from heated D-glucose/glycine systems failed to produce sufficient quantities for spectroscopic analysis, however, when the synthesis and extraction with hexane was performed by focused microwave irradiation under atmospheric pressure conditions (Yaylayan et al., 1996), a simple mixture was obtained containing the compound having the ion m/z 190 as the major component. Further purification by preparative chromatography yielded the pure compound which was assigned the structure of 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone (33, Scheme 5.7), based on spectroscopic analysis.

Table 5.11. Mass spectrometric data of quinoxalinones and pyrazinones

5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone (from glycine / D-glucose)
191(11), 190(100), 162(32), 161(23), 134(18), 133(42), 119(23), 106(6), 93(12), 92(19), 56(27)
5-hydroxy-1,3,7-trimethyl-2-[1H]-quinoxalinone (from glycine / D-glucose)
205(12), 204(100), 176(21), 175(36), 161(11), 148(17), 147(48), 133(11), 92(14), 56(28)
5-hydroxy-1-methyl-2-[1H]-quinoxalinone (from glycine / D-glucose)
177(100), 176(100), 148(30), 120(21), 119(23), 105(24), 92(11), 79(15), 51(15)

Table 5.12. Percent distribution of molecular ion m/z 190 generated from labeled D-glucoses or excess labeled glycines.

D-Glucose / excess glycine	m/z 190	m/z 191	m/z 192	m/z 193
D-Glucose/Glycine	99	1	0	0
D-[1-^{13}C]Glucose/Glycine	0	100	0	0
D-[2-^{13}C]Glucose/Glycine	0	100	0	0
D-[3-^{13}C]Glucose/Glycine	0	100	0	0
D-[4-^{13}C]Glucose/Glycine	0	100	0	0
D-[5-^{13}C]Glucose/Glycine	0	100	0	0
D-[6-^{13}C]Glucose/Glycine	0	95	5	0
D-Glucose - [1-^{13}C]Glycine	0	100	0	0
D-Glucose - [2-^{13}C]Glycine[a]	0	0	16	84
D-Glucose - [^{15}N]Glycine[b]	0	2	98	0
ARP glycine / excess glycine	**m/z 190**	**m/z 191**	**m/z 192**	**m/z 193**
ARP Glycine - [1-^{13}C]Glycine	63	37	0	0
ARP Glycine - [2-^{13}C]Glycine[a]	64	28	8	0
ARP Glycine - [^{15}N]Glycine[b]	35	52	15	0

ARP Amadori rearrangement product
a [2-^{13}C]glycine 90% enriched
b [^{15}N]glycine 98% enriched

Table 5.13. Incorporation of labels in selected mass spectral fragments of 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone.

D-Glucose/excess glycine	m/z 190	m/z 162	m/z 161	m/z 133	m/z 56
D-Glucose/Glycine	190	162	161	133	56
D-[1-^{13}C]Glucose/Glycine	191	163	162	134	57
D-[2-^{13}C]Glucose/Glycine	191	163	162	134	56
D-[3-^{13}C]Glucose/Glycine	191	163	162	134	56
D-[4-^{13}C]Glucose/Glycine	191	163	162	134	56
D-[5-^{13}C]Glucose/Glycine	191	163	162	134	56
D-[6-^{13}C]Glucose/Glycine	191	163	162	133	56
D-Glucose/[1-^{13}C]Glycine	191	162	161	133	56
D-Glucose/[2-^{13}C]Glycine[a]	193	165	164	136	58
D-Glucose/[^{15}N]Glycine[b]	192	164	163	135	57

a [2-^{13}C]glycine 90% enriched
b [^{15}N]glycine 98% enriched

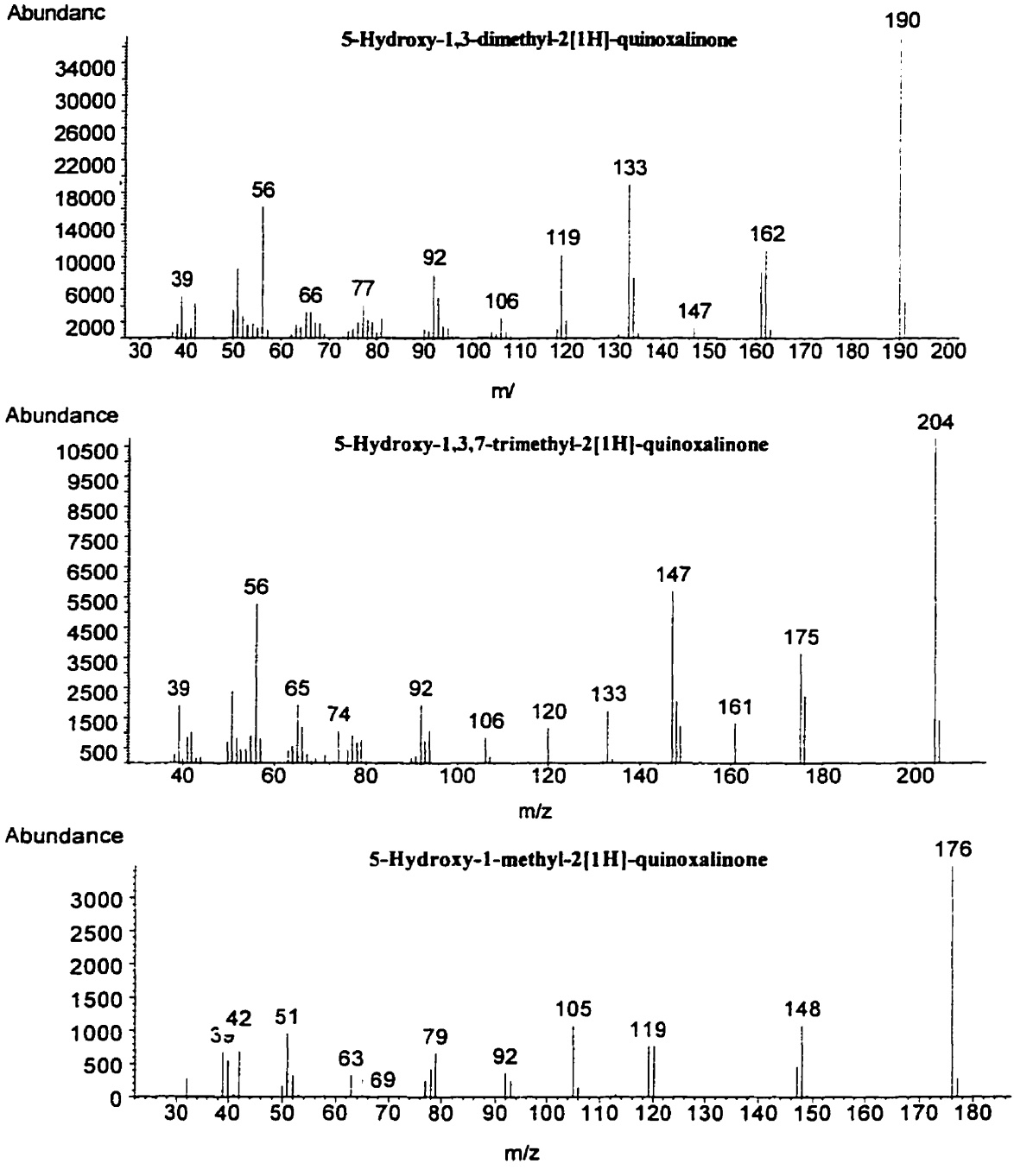
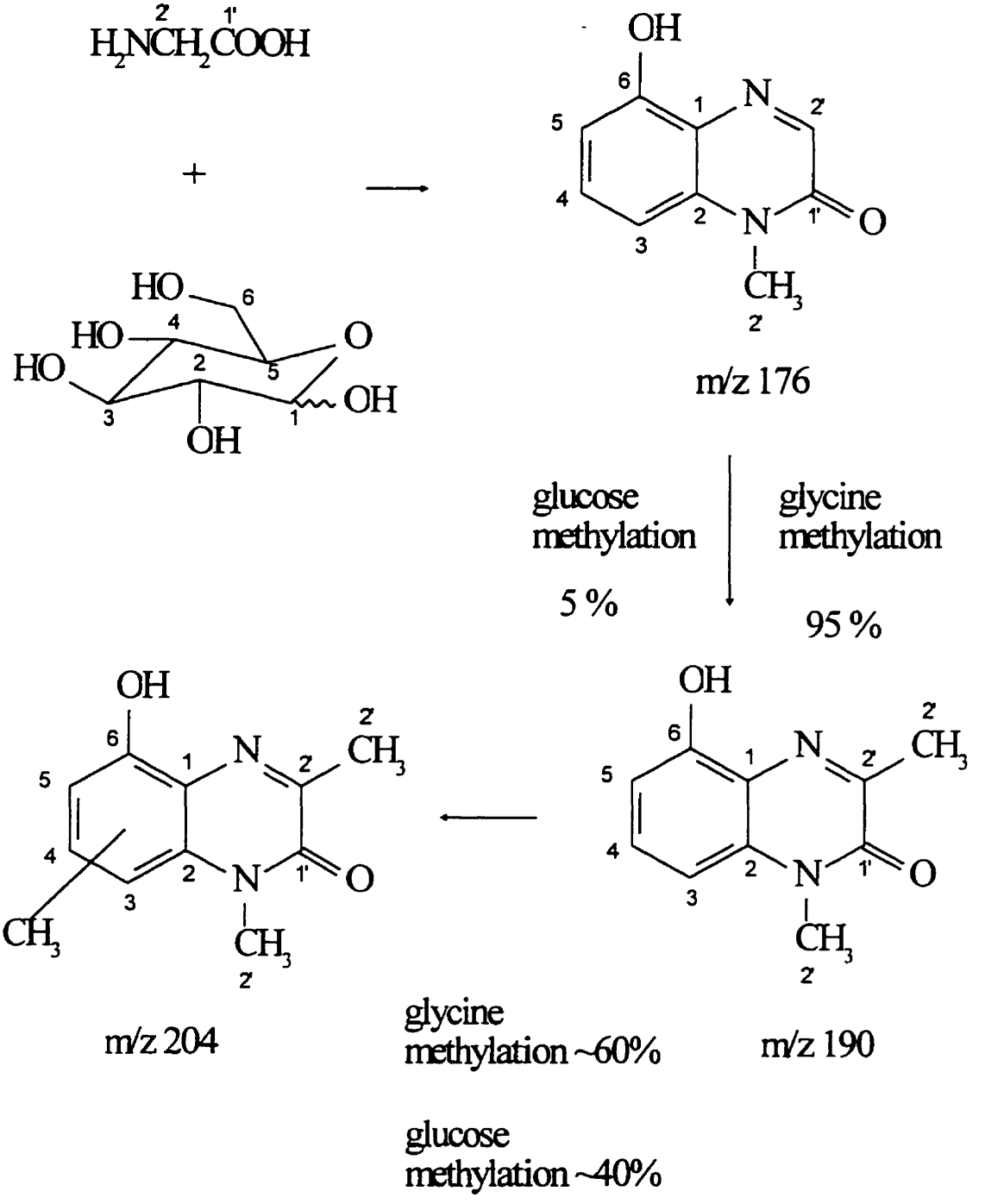


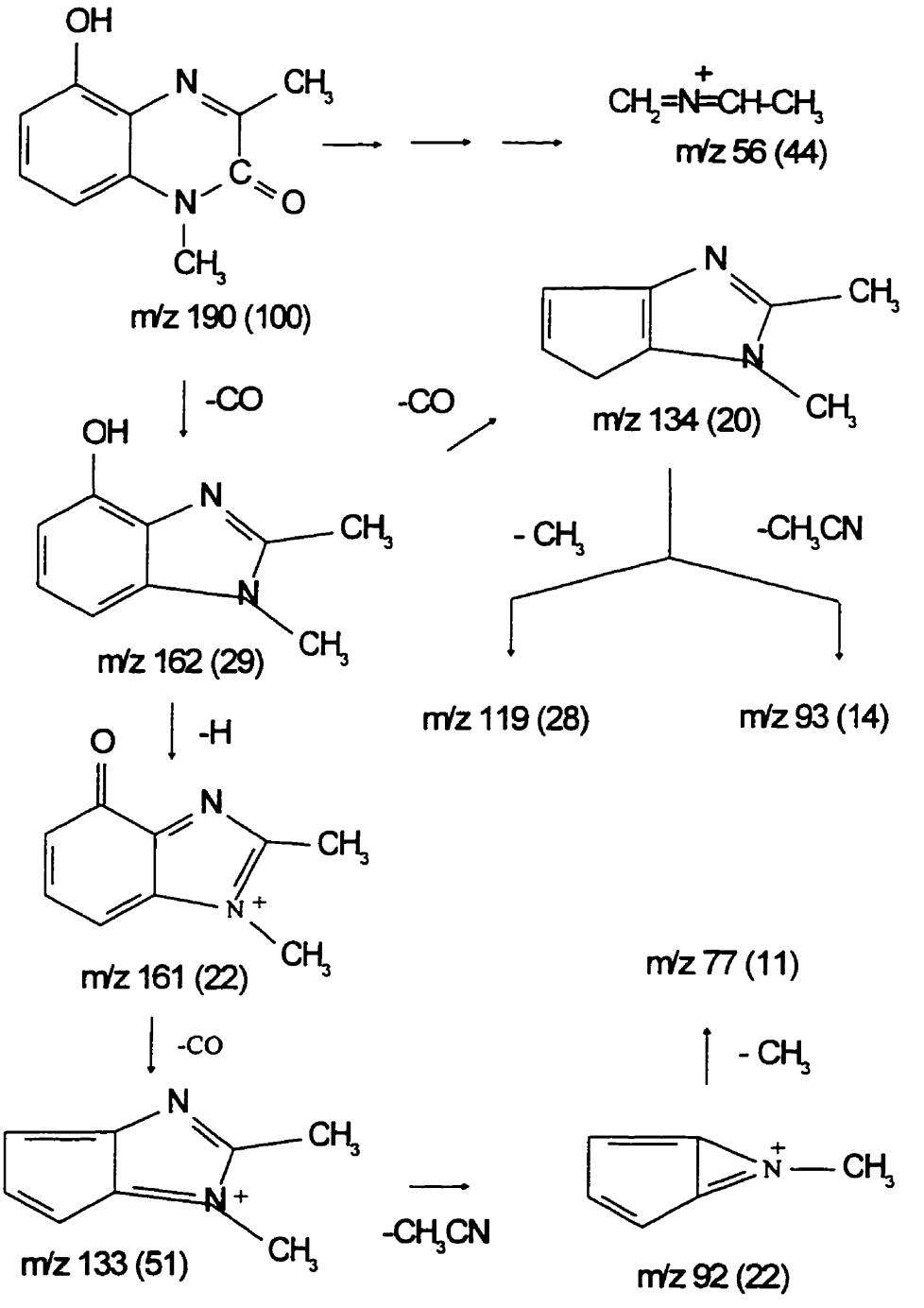
Figure 5.1. Mass Spectra of selected quinoxalinone derivatives formed in D-glucose/glycine models.



Scheme 5.7. Origin of carbon atoms in quinoxalinones

5.2.3.2 *Spectroscopic characterization of 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone (33):* The mass spectra of compound m/z 190 obtained by reacting separately labeled D-[^{13}C]glucoses at each carbon atom with unlabeled glycines and reacting separately labeled [^{15}N] and [^{13}C]glycines with unlabeled D-glucose, revealed the incorporation of ten carbon atoms (six from sugar, one C-1 atom of glycine, and three C-2 atoms of glycine) and two nitrogens into the structure of m/z 190. This combination of carbons and nitrogens can produce only the following elemental formula $C_{10}H_{10}N_2O_2$. Analysis by 2-D NMR (COSY) and ^{1}H NMR confirmed the presence of three mutually coupled aromatic protons, one phenolic proton and two methyl groups. FTIR analysis indicated the presence of phenolic, alkyl, carbonyl, and aromatic functional groups. The parent 2-[1H]-quinoxalinone shows carbonyl stretching absorption in the region of 1660-1690 cm^{-1} and uv maxima (H_2O) at 343, 287, 254 and 228 nm (Cheeseman and Cookson, 1979). These values are consistent with those reported under experimental section. In addition, the parent 2-[1H]-quinoxalinone fragments under electron impact conditions by successive losses of carbon monoxide and hydrogen cyanide from the molecular ion (Kovacik et al., 1973). According to Scheme 5.8 and Table 5.13, the compound m/z 190 shows similar fragmentations. Since position C-3 is substituted with a methyl group, a loss of methyl cyanide (41 amu) is observed rather than that of a hydrogen cyanide.

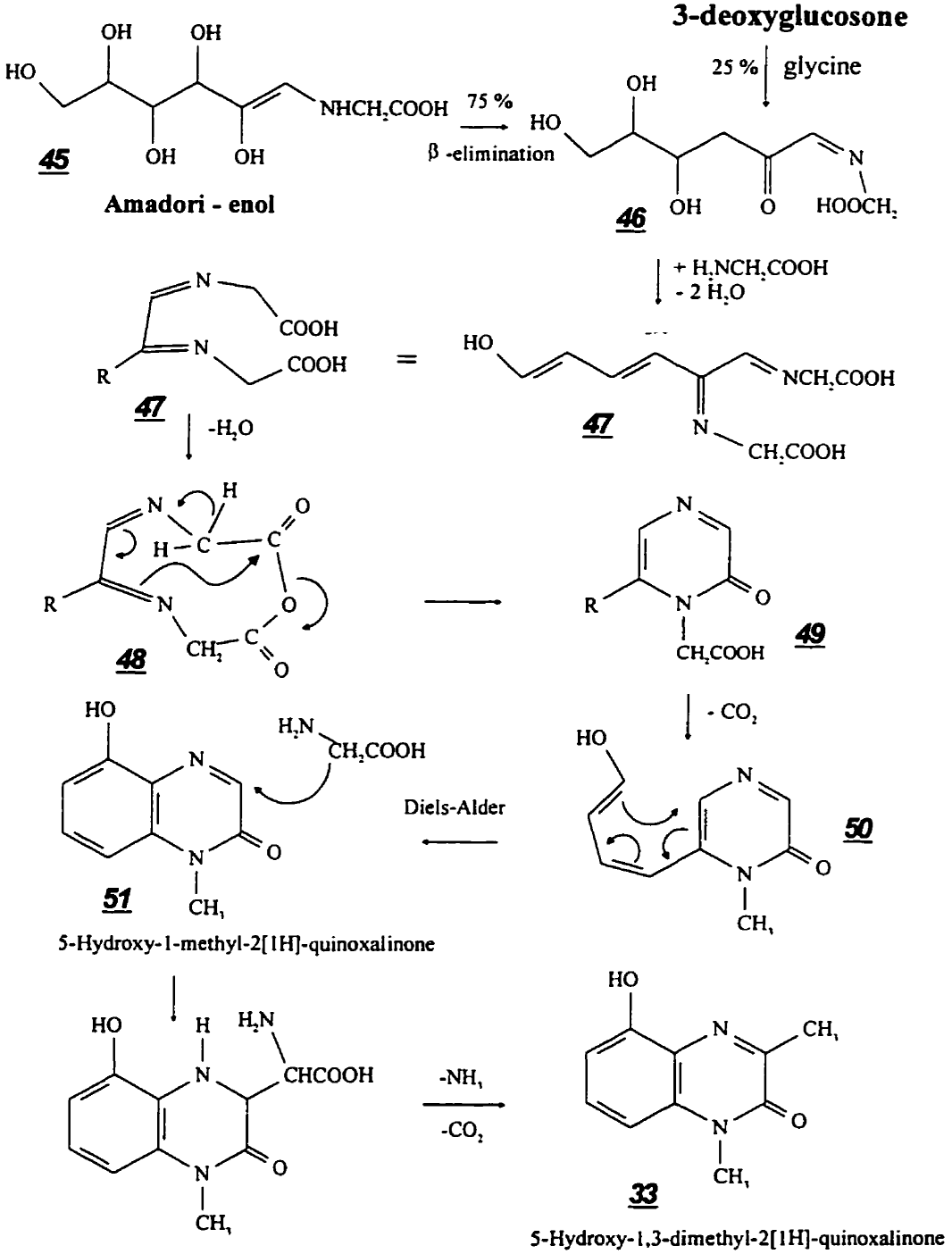
One of the advantages of using labeled reactants is that all the atoms of a product can be traced back to their origin in the starting material. This fact, not only facilitates elucidation of their mechanism of formation, but also the assignment of their mass spectral fragments. The molecular ion m/z 190 (100 %) loses carbon monoxide (originating from C-1 of glycine) to produce m/z 162 (29 %) which in turn loses another carbon monoxide (originating from C-6 of glucose) to produce m/z 134 (20 %) which either loses a methyl or methyl cyanide group to produce m/z 119 (28 %) and m/z 93 (14 %) respectively.



Scheme 5.8. Electron impact fragmentation pathways of 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone (46), based on labeling studies.

Ion at m/z 162 (29 %) can also lose a hydrogen atom to produce m/z 161 (22 %) which in turn can lose carbon monoxide to produce m/z 133 (51 %) that undergoes successive losses of methyl cyanide and a methyl group to produce m/z 92 (22%) and m/z 77 (11 %) successively, as illustrated in Scheme 5.8. Labeling experiments indicate that fragment m/z 56 (44 %) incorporates one C-1 atom of D-glucose, one nitrogen atom and two C-2 atoms of glycine. The importance of this fragment lies in the fact that it establishes the connectivity of nitrogen atom to C-1 atom of D-glucose, indicating the possibility of 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone (33) formation directly from the Amadori product.

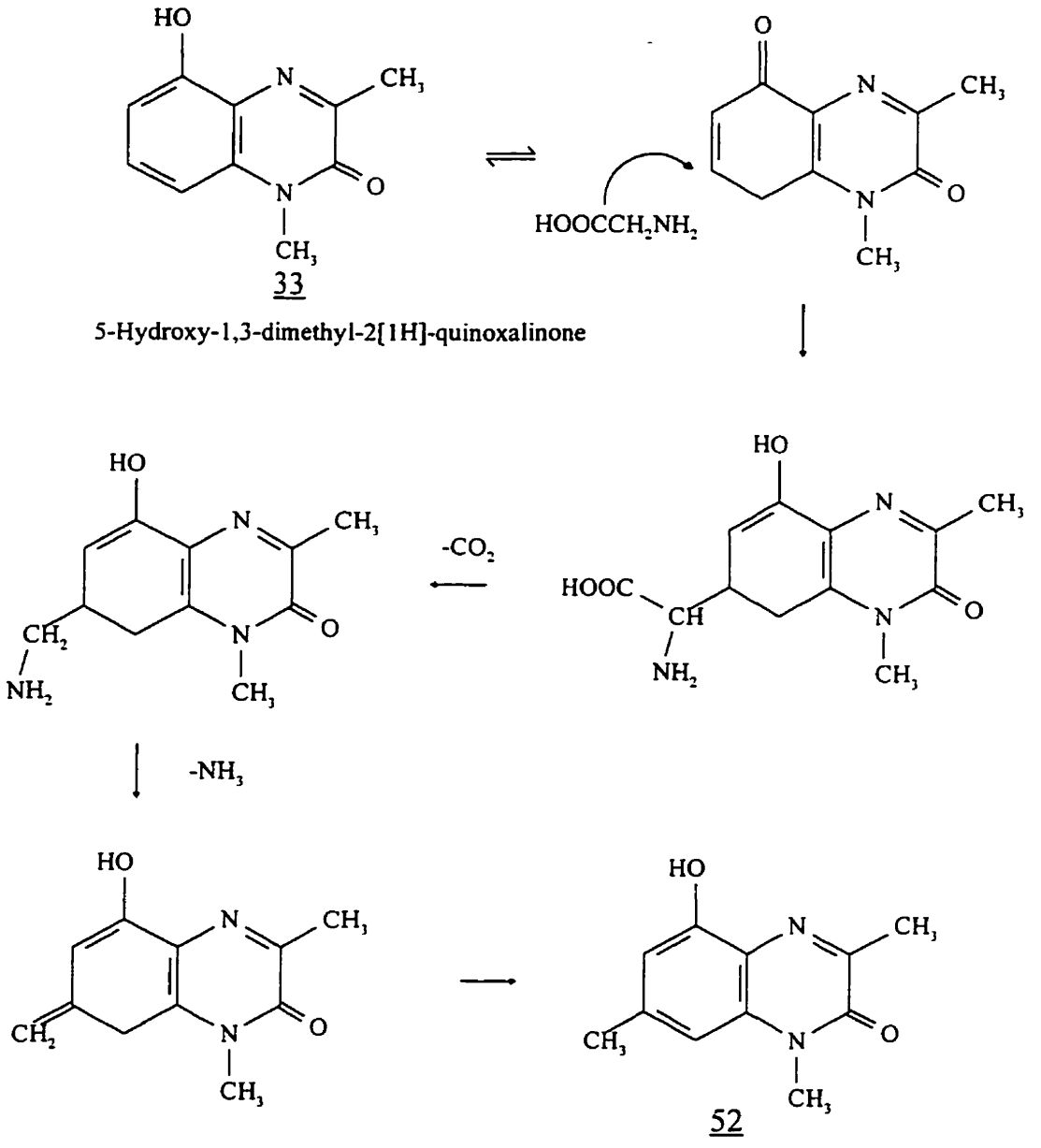
5.2.3.3 *Mechanism of formation of 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone:* To confirm the above assertion that 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone (33) could be formed directly from glycine Amadori compound, two fold molar excess [^{15}N]glycine was reacted with unlabeled glycine Amadori product (ARP) and label distribution was analyzed for the parent ion m/z 190 and fragment m/z 56. If the compound is formed only through 3-deoxyglucosone (2, 3DG) pathway, by reacting with three moles of free glycine, it is expected to observe a high percentage of ^{15}N incorporation into the product due to the presence of excess [^{15}N]glycine, with the formation of ions at m/z 192 and m/z 57. For example, in the same reaction mixture, trimethylpyrazinone (m/z 138), which is formed by the reaction of three moles of glycine with 2,3-butandione through a similar pathway, generates 60 % of doubly labeled parent ion (m/z 140), 30 % singly labeled and 10 % unlabeled. However, only 15 % of the parent ion of 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone was doubly labeled (m/z 192, 3DG + 2 x [^{15}N]glycine), 52 % was singly labeled (m/z 191, ARP + [^{15}N]glycine or 3DG + [^{15}N]glycine + glycine) and 35 % was unlabeled (m/z 190, ARP + glycine). In addition, only 25 % of m/z 56 was labeled (m/z 57), indicating 75 % of the 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone was formed by direct addition of glycine (either released from ARP or added) to the Amadori product when it was pyrolyzed in the presence of excess [^{15}N]glycine (Table 4.12).



Scheme 5.9. Proposed mechanism of formation of 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone (33) from D-glucose and glycine, based on labeling studies.

The proposed mechanism of formation of 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone (33) is illustrated in Scheme 5.9. The enol form of Amadori glycine (45) undergoes a β-elimination reaction to produce an α-keto imine derivative (46) which could be generated also from 3-DG through reaction with glycine. Reaction with a second mole of glycine and subsequent dehydrations, produce a conjugated diimine (47) which undergoes a dehydration reaction between the two carboxylic acid groups of the glycine to generate a cyclic anhydride (48). The cyclic anhydride (48), then undergoes an intramolecular cyclization (49), followed by decarboxylation to form a 6-substituted N-methyl pyrazinone (50). The latter, can undergo an aldol-type intramolecular condensation and aromatization to produce 5-hydroxy-1-methyl-2-[1H]-quinoxalinone (51), which has been detected in the same reaction mixture (Table 5.11, Figure 5.10). The imine functionality in 2-[1H]-quinoxalinones is known to react with carbon nucleophiles (Cheesman and Cookson, 1979), the C-2 carbon of glycine or acetic acid generated from either glycine or glucose, can methylate this position to form 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone (33) .

The initially formed 5-hydroxy-1-methyl-2-[1H]-quinoxalinone (51) can undergoe methylation at the imine site to produce 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone (33) which in turn can produce a 5-hydroxy-1,3,7-trimethyl-2-[1H]-quinoxalinone (52) by methylation, through, for example, Michael type addition, as proposed in Scheme 5.10. However, the position of the methylation site is not confirmed. Table 5.14 lists the isotopic distribution of a compound m/z 204 (Figure 5.2) which has been tentatively identified as 5-hydroxy-1,3,7-trimethyl-2-[1H]-quinoxalinone (52).



Scheme 5.10. Proposed mechanism of formation of 5-hydroxy-1,3,7-trimethyl-2-[1H]-quinoxalinine from D-glucose and glycine based on labeling studies.

Table 5.14. Percent distribution of molecular ion m/z 204 generated from labeled D-glucoses or excess labeled glycines.

D-Glucose / excess glycine	204	205	206	207	208
D-Glucose/Glycine	98	2	0	0	0
D-[1-^{13}C]Glucose/Glycine	0	85	15	0	0
D-[2-^{13}C]Glucose/Glycine	0	97	3	0	0
D-[3-^{13}C]Glucose/Glycine	0	92	8	0	0
D-[4-^{13}C]Glucose/Glycine	0	100	0	0	0
D-[5-^{13}C]Glucose/Glycine	0	97	3	0	0
D-[6-^{13}C]Glucose/Glycine	0	92	8	0	0
D-Glucose/[1-^{13}C]Glycine	11	91	0	0	0
D-Glucose/[2-^{13}C]Glycine[a]	0	3	11	34	52
D-Glucose/[^{15}N]Glycine[b]	0	2	98	0	0
ARP glycine / excess glycine	**204**	**205**	**206**	**207**	**208**
ARP Glycine/[1-^{13}C]Glycine	67	33	0	0	0
ARP Glycine/[2-^{13}C]Glycine[a]	0	42	41	17	0
ARP Glycine/ [^{15}N]Glycine[b]	0	0	97	3	0

ARP = Amadori rearrangement product

[a] [2-^{13}C]glycine 90% enriched, [b] [^{15}N]glycine 98% enriched

5.3 Conclusion

Carbon chain elongation in Maillard reactions so far has been thought to occur by aldol type condensations of smaller sugar fragments, this study provides evidence that amino acids could be also involved in such processes by interaction with the *aldehydo* end of an α-ketoaldehyde and subsequent transformation into an α-diketone. This process is in direct competition with Strecker type interaction that generates Strecker aldehydes and α-amino carbonyl compounds.

Quinoxalinones and pyrazinones are formed in Maillard systems by multiple additions of amino acids to different dicarbonyls, as such, they could be viewed as *in situ* indicators of formation of dicarbonyl compounds in Maillard model systems. Elucidation of their mechanism of formation was possible through the use of labeled sugars and amino acids utilizing Py/GC/MS.

CHAPTER 6

ALANINE MODEL SYSTEMS

6.1 Introduction

Alanine is an aliphatic amino acid differing in structure from glycine by an extra methyl group in the side chain. Volatile compounds detected in a D-glucose/DL-alanine and Amadori alanine subjected to roasting conditions include alkyl and acyl substituted pyrroles, alkyl pyrrole-2-aldehydes, alkyl substituted pyrazines, furfuryl pyrroles, pyranones and furanoid compounds (Shigematsu et al., 1972, 1977). The objective in studying L-alanine models was to identify products arising in alanine model systems and to confirm the common mechanistic pathways followed between glycine and alanine. To confirm the origin of atoms incorporated in the pyrolysis products, D-glucoses labeled at [1-^{13}C], [2-^{13}C], [3-^{13}C], [4-^{13}C], [5-^{13}C], [6-^{13}C] were systematically reacted with alanine; L-[1-^{13}C], DL-[2-^{13}C], DL-[3-^{13}C] and L-[^{15}N]-alanines were reacted with glucose, pyruvaldehyde and glyoxal .

The origin of the volatile and reactive short chain carbon fragments such as glyoxal (or glycoaldehye), pyruvaldehyde, 2,3-butanedione, and 2,3-pentanedione formed in Maillard reaction mixtures is relatively difficult to determine due to the multiple origin of these components. Some could arise directly from the sugar or Amadori product, others could be formed by chain elongation through aldol condensation with fragments arising from either sugar or the amino acid. In addition, detection of these components could also pose some difficulties due to their volatility and reactivity. To overcome the problem of direct detection of the short chain reactive species in the model systems, tracing back the origin of each carbon atom in a product could be successfully achieved by GC/MS analysis of model reaction mixtures performed with separately ^{13}C-labeled sugars and amino acids at each of their carbon atoms. By utilizing Py/GC/MS to generate and identify the stable end-products, the incorporation of these fragments into their molecular structure becomes possible. This approach entails, for example, the analysis of ten model reaction mixtures in the case of L-alanine with D-glucose.

The employment of Py/GC/MS technique developed in our laboratory, as an integrated reaction, separation and identification system, to perform such studies, not only diminishes the time required to accomplish the labeling studies but also reduces the

high cost associated with expensive labeled starting materials since only few milligrams are required to perform each Py/GC/MS analysis.

6.2 Results and Discussion

Quartz tube pyrolysis of L-alanine (250°C, 20s) yields 1,4-dimethyl-2,5-diketopiperazine (alanine related dimer) as the main product. Table 6.1 lists the quartz tube pyrolysis products identified in different D-glucose/L-alanine model systems at two pyrolysis temperatures (250°C and 210°C) and with ratios 1:1; 1:2 and 1:3 of D-glucose to L-alanine. The compounds identified were classified mainly as furans, pyridines, pyrroles, pyrazines, carbonyls and carboxylic acids.

6.2.1 Carbon Chain Elongation: Carbon chain elongation in Maillard reactions until recently has been attributed to aldol type condensations of smaller sugar fragments. Chapter 5, provided evidence that amino acids could be also involved in such processes by interaction with the *aldehyo* end of an α-ketoaldehyde and their subsequent transformation into an α-diketone. Blank and Fay (1996), demonstrated chain elongation in pentoses by the incorporation of glycine and alanine carbons as Strecker aldehydes, leading to substituted furanone formation. They speculated that the C_5 moiety of the sugar is prolonged by a C_1 (formaldehyde) or C_2 (acetaldehyde) fragment formed by Strecker degradation of the corresponding amino acids. In model systems containing D-glucose/L-alanine; 2,3-pentanedione was observed as a pyrolysis product. Table 6.2 indicates the isotopic distribution of 2,3-pentanedione in systems utilizing labeled alanine. Analysis of the isotopic distribution of 2,3-pentanedione in the various model systems indicated that ~90 % is formed through the addition of C-2 and C-3 atoms of alanine to C_3 units from Amadori compound and ~10 % results from condensation of a C_2 and C_3 units from Amadori degradation (Scheme 6.1). Glyoxal (or glycoaldehyde) and pyruvaldehyde resulting from Amadori product degradation (Scheme 5.3 and 5.4) can undergo reduction (Huyghues-Despointes and Yaylayan, 1996) and aldol condensation to produce 2,3-pentanedione (Scheme 6.2). Kim and Baltes (1996), report 2,3-pentanedione

formation from the degradation of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (12), however, a formation mechanism is not proposed.

In the major pathway for 2,3-pentanedione formation in D-glucose/L-alanine model system a C_3 from the sugar (pyruvaldehyde) adds C-2 and C-3 atoms of L-alanine to produce 2,3-pentanedione. Scheme 6.3 illustrates a plausible mechanism of formation of 2,3-pentanedione and 2-ketobutanal by the addition of L-alanine to glyoxal (or glycoaldehyde) and pyruvaldehyde respectively. This scheme is similar to Scheme 5.1 except in this case the amino acid is L-alanine rather than glycine. 2-Ketobutanal, a reactive intermediate, could not be detected in the D-glucose/L-alanine models, however evidence from stable end products that incorporate 2-ketobutanal (see section 6.2.2) indicate its probable availability in the reaction pool. Carbon chain elongation arising solely from amino acid carbons is also observed in the formation of 2-butenal in the D-glucose/L-alanine models. Table 6.2 indicates the isotopic distribution of 2-butenal and Scheme 6.4 proposes a reaction route for its formation based on aldol condensation of two acetaldehydes. Py/GC/MS analysis of a pyruvaldehyde/acetaldehyde model system indicated the formation of 2-butenal, while 2,3-pentanedione was not detected. These results highlight the importance of amino acids carbons in the formation of C_4 and C_5 aldehydes and ketones.

6.2.2 Pyrazine Formation: Glyoxal (or glycoaldehyde), pyruvaldehyde, 2-ketobutanal and 2,3-pentanedione generated from the D-glucose/L-alanine model systems can undergo Strecker degradation with alanine producing acetaldehyde, and the respective α-aminoketones and α-aminoaldehydes of the above mentioned dicarbonyls (Figure 6.1). These amino derivatives can be converted via dimerization or condensation to yield pyrazines (Figure 6.2, Scheme 1.7).

Table 6.1. Products identified from Py/GC/MS of D-glucose/L-alanine models.

Compounds	MW	Temperature °C					
		250	250	250	210	210	210
ratio		1:1	1:2	1:3	1:1	1:2	1:3
Furans							
2-furancarboxaldehyde	96	+	+	+	-	-	+
2-furanmethanol	93	+	-	-	+	+	-
5-methyl-2(3H)-furanone	98	t	-	-	+	-	-
1-(2-furanyl)-ethanone	110	+	+	+	+	+	+
5-methyl-2-furancarboxaldehyde	110	+	+	+	+	+	+
Pyridines							
2-methylpyridine	93	t	t	t	t	-	+
4-methylpyridine	93	+	+	+	+	+	+
Pyrroles							
1H-pyrrole	67	t	+	+	t	t	-
1-ethyl-1H-pyrrole	95	+	+	+	+	+	+
2-methyl-1H-pyrrole	81	t	+	+	-	-	t
1-(1H-pyrrol-2-yl)-ethanone	109	+	+	+	+	+	+
Pyrazines							
methylpyrazine	94	+	+	+	t	-	+
2,5-dimethylpyrazine	108	+	+	+	+	+	+
2-ethyl-5-methyl-pyrazine	122	+	+	+	+	+	+
trimethylpyrazine	122	t	t	t	t	t	t
2-ethyl-6-methyl-pyrazine	122	t	t	t	t	t	t
5-ethyl-2-methyl-pyridine	121	-	+	+	-	+	+
2-ethyl-3,5-dimethylpyrazine	136	+	+	+	+	+	+
2-ethyl-3,6-dimethylpyrazine	136	-	t	t	t	t	t
Carbonyls							
2,3-butanedione	86	-	t	-	-	+	+
2-butenal	70	+	+	+	+	+	+
1-hydroxy-2-propanone	74	+	+	+	+	+	+
2,3-pentanedione	100	+	+	+	+	+	+
3-hydroxy-2-butanone	88	+	t	-	+	+	+
2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one	144	+	+	+	+	+	+
Carboxylic Acids							
acetic acid	60	+	+	+	+	+	+
propanoic acid	74	+	+	+	+	+	+
Other							
N-methylmethanamine	45	+	-	-	-	-	-
2-ethylaziridine	71	-	+	-	-	-	-
N-ethylacetamide	87	-	-	+	-	-	-
protoanamoin	96	+	+	+	+	+	+
1-ethyl-3,5(6)-dimethylpyrazinone$_a$	152	+	+	+	+	+	+
1,4-dimethyl-2,5-diketopiperazine	142	+	+	+	-	-	-
5-hydroxy-1-ethyl-3-methyl-2-[1H]-quinoxalinone$_a$	204	+	+	+	t	t	t
179$_a$	179	-	-	-	+	+	+
192$_a$	192	-	-	-	+	+	+

a: tentatively identified based on mass spectra (Table 6.9) and isotopic distribution Tables 6.10, 6.11 and 6.12. t=trace

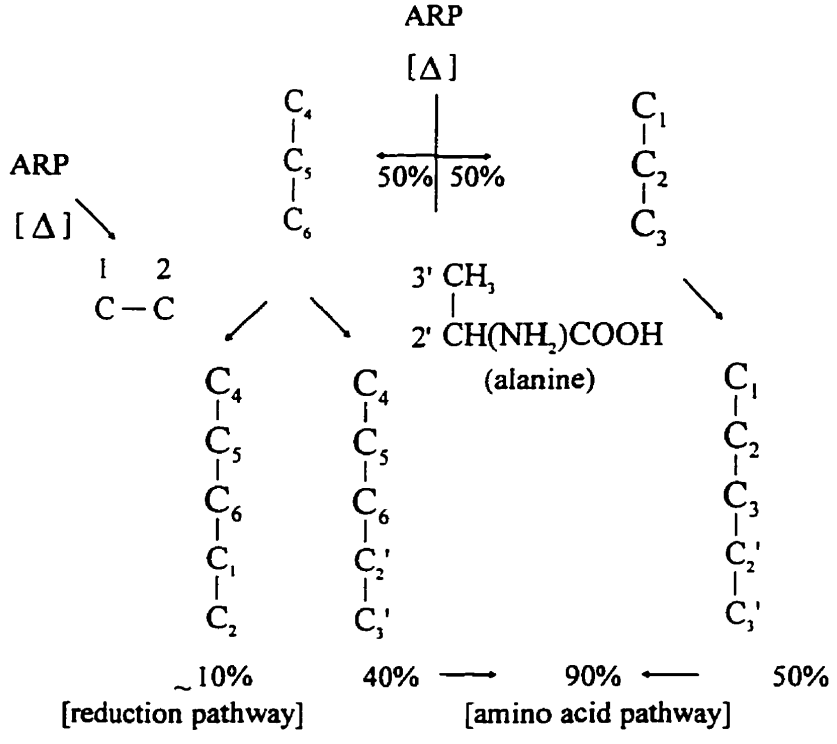
Table 6.2. Percent label distribution in 2,3-pentanedione and 2-butenal formed from labeled D-glucoses and alanines.

Model	2,3-pentanedione			2-butenal		
	100	101		70	71	72
	M	M+1		M	M+1	M+2
D-Glucose/L-Alanine	100	0		100	0	0
D- [1-^{13}C]Glucose/L-Alanine	38	62		100	0	0
D- [2-^{13}C]Glucose/L-Alanine	38	62		100	0	0
D- [3-^{13}C]Glucose/L-Alanine	44	56		100	0	0
D- [4-^{13}C]Glucose/L-Alanine	51	42		100	0	0
D- [5-^{13}C]Glucose/L-Alanine	56	54		100	0	0
D- [6-^{13}C]Glucose/L-Alanine	51	49		100	0	0
D-Glucose/L-[^{15}N]Alanine	100	0		100	0	0
D-Glucose/L-[1-^{13}C]Alanine	100	0		100	0	0
D-Glucose/DL-[2-^{13}C]Alanine *	15	85		1	9	90
D-Glucose/L-[3-^{13}C]Alanine	9	91		0	0	100
Pyruvaldeyde/DL-[2-^{13}C]Alanine*	0	100		0	0	100

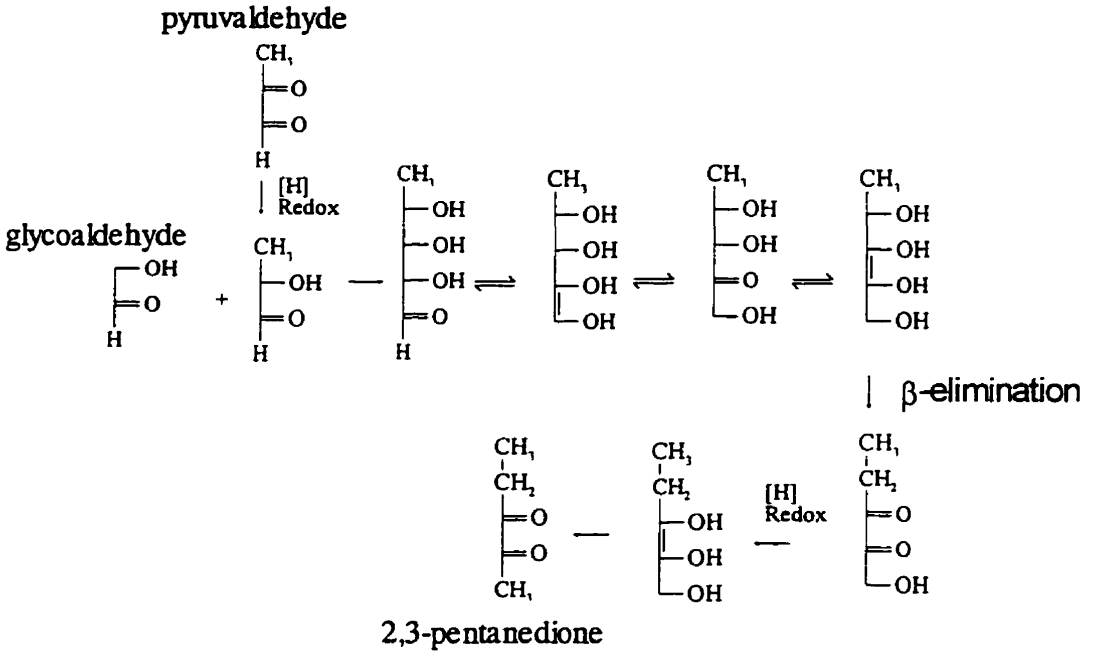
DL-[2-^{13}C]alanine 92% enriched*

The reactions that lead to pyrazine formation have been extensively studied in a wide range of model systems consisting of different sugars and amino acids (Maga, 1992). Arnoldi et al.(1988), heated fructose with different amino acids at 120°C for 3 hours, considered α-amino compounds such as α-aminoacetone important intermediates for the formation of dihydropyrazines. These intermediates are oxidized to the corresponding pyrazines (Shibimoto and Bernhard, 1977). Cerny and Grosch (1994) determined L-alanine to be an important precursor for the aroma-active trialkylated pyrazines, 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine. Amrani-Hemaimi et al. (1995) investigated alkyl substituted pyrazine formation using D-glucose and D-fructose with [2-^{13}C]glycine and [3-^{13}C]alanine. Based on the isotopic distribution of the mass spectrum, they provided evidence that the carbon atoms from the amino acids are incorporated during the formation of different pyrazines. They propose that methyl or ethyl substituents add to the dihydropyrazine ring leading to alkyl substituted pyrazines (Scheme 1.8). However, evidence from the label distribution in 2,3-pentanedione in D-glucose/alanine and pyruvaldehyde/alanine models is indicative of a reaction route in

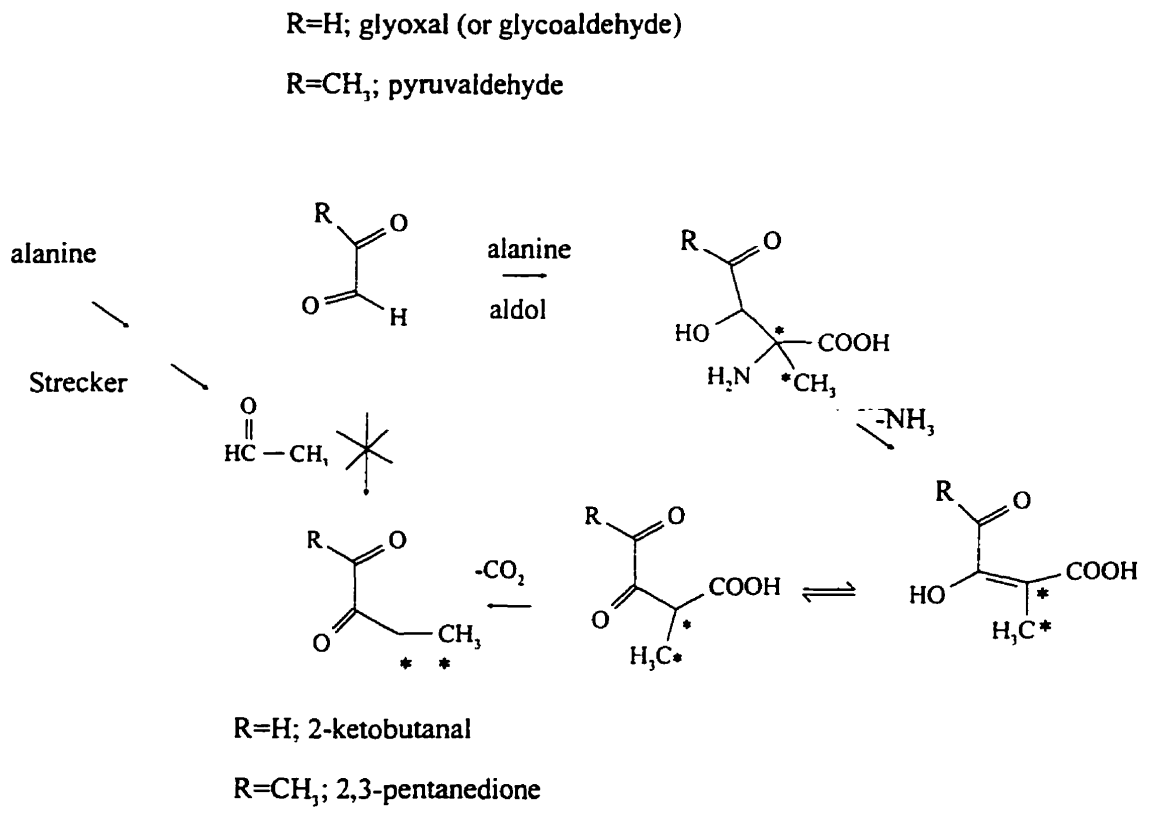
which carbon chain elongation occurs prior to pyrazine formation. In addition, since the 2,3-pentanedione formation was not detected in pyruvaldehyde/acetaldehyde models indicates that the amino acid itself rather than its Strecker aldehyde, apparently initiates the elongation reactions of dicarbonyls. The main pyrazines observed in D-glucose/L-alanine models are methyl, 2,5-dimethyl (or 2,6-dimethylpyrazine), 2-ethyl-5-methyl and 2-ethyl-3,5-dimethylpyrazine (or 3-ethyl-2,5-dimethylpyrazine). Tables 6.3-6.6 list the percent label distribution of these compounds in D-glucose/ alanine model systems.



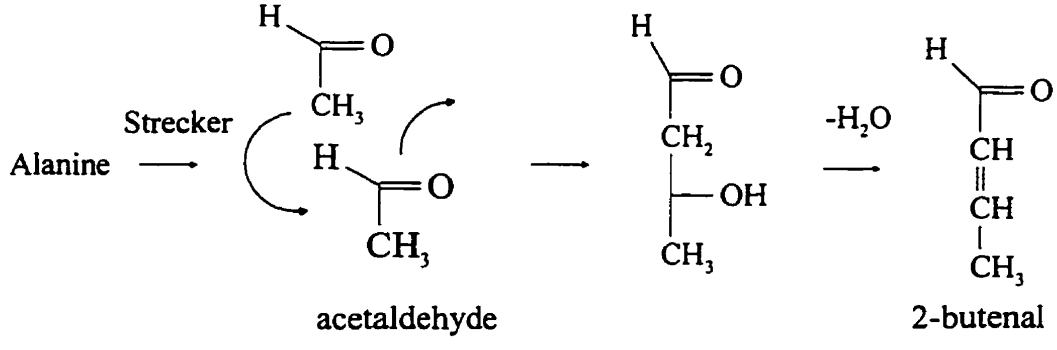
Scheme 6.1. Proposed origin of 2,3-pentanedione carbons produced in D-glucose/L-alanine model systems based on isotopic distributions (Table 6.2). Numbers indicate D-glucose carbon locations and primed numbers that of alanine..



Scheme 6.2. Reduction pathway for formation of 2,3-pentanedione formation from dicarbonyls (pyruvaldehyde and glycoaldehyde).



Scheme 6.3. Proposed mechanism of formation of 2-ketobutanal and 2,3-pentanedione from glyoxal and pyruvaldehyde respectively, similar to reaction proposed in Scheme 5.1. Asterisks indicates C-2 and C-3 atoms of L-alanine



Scheme 6.4. Mechanism of 2-butenal formation in D-glucose/L-alanine model systems.

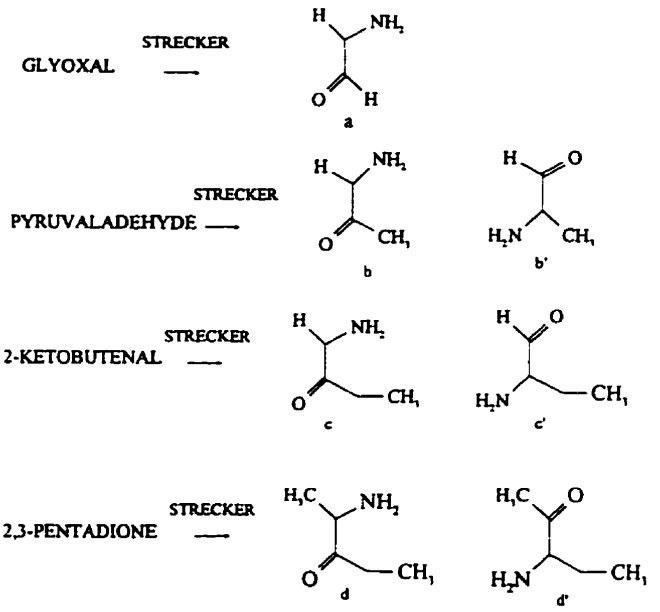


Figure 6.1. α-Aminoketones and α-aminoaldehydes produced upon Strecker degradation of selected dicarbonyls.

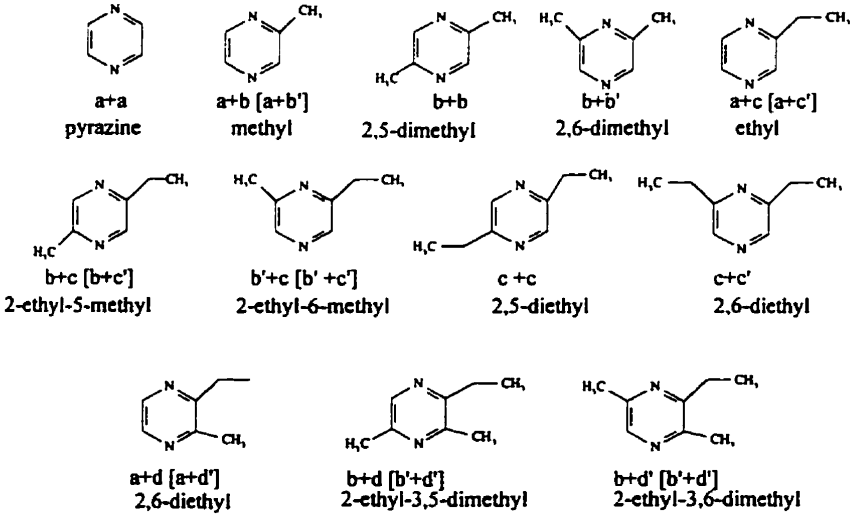


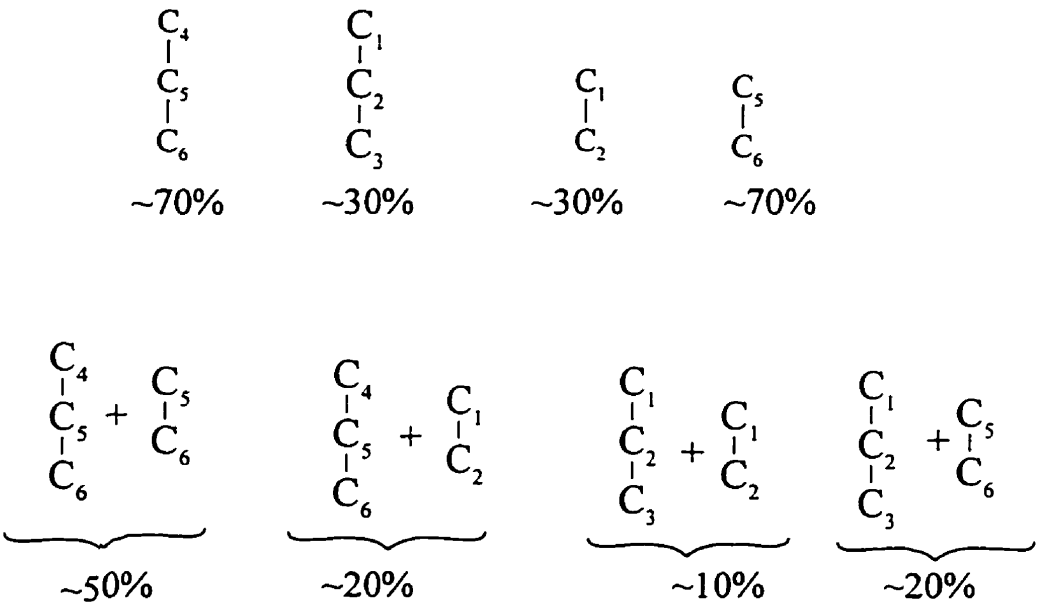
Figure 6.2. Pyrazines which can theoretically form by dimerization or condensation and oxidation of the α-aminoketones and α-aminoaldehydes listed in Figure 6.1.

6.2.2.1 *Methylpyrazine:* The label distribution in methylpyrazine indicates incorporation of carbons solely from D-glucose (Table 6.3, Scheme 6.5). Methylpyrazine requires the condensation of α-aminoacetaldehyde and α-aminopropionaldehyde (Figure 6.1 and 6.2). Retro-aldol cleavage of Amadori proline and Amadori glycine to form glyoxal was demonstrated to be favored between C-4 and C-5 atoms of the D-glucose chain through the 1-deoxyglucosone (3) intermediate (Huyghues-Despointes and Yaylayan, 1996; Chapter 5, Scheme 5.5). The majority (~ 70 %) of the methylpyrazine formed in D-glucose/L-alanine model systems includes C-5 and C-6 atoms of D-glucose indicating the significance of the above mentioned pathway towards glyoxal (glycoaldehyde) formation in D-glucose/L-alanine systems.

Table 6.3. Percent label distribution in methylpyrazine formed from labeled D-glucoses and alanines.

Model	94	95	96
	M	M+1	M+2
D-Glucose/L-Alanine	100	0	0
D-[1-^{13}C]Glucose/L-Alanine	57	34	9
D-[2-^{13}C]Glucose/L-Alanine	57	34	9
D-[3-^{13}C]Glucose/L -Alanine	69	30	1
D-[4-^{13}C]Glucose/L-Alanine	30	69	1
D-[5-^{13}C]Glucose/L-Alanine	11	37	52
D-[6-^{13}C]Glucose/L-Alanine	11	37	52
D-Glucose/L-[^{15}N]Alanine	0	0	100
D-Glucose/L-[1-^{13}C]Alanine	100	0	0
D-Glucose/DL-[2-^{13}C]Alanine *	100	0	0
D-Glucose/DL-[3-^{13}C]Alanine	100	0	0

* DL-*[2-^{13}C]-alanine 92% enriched*



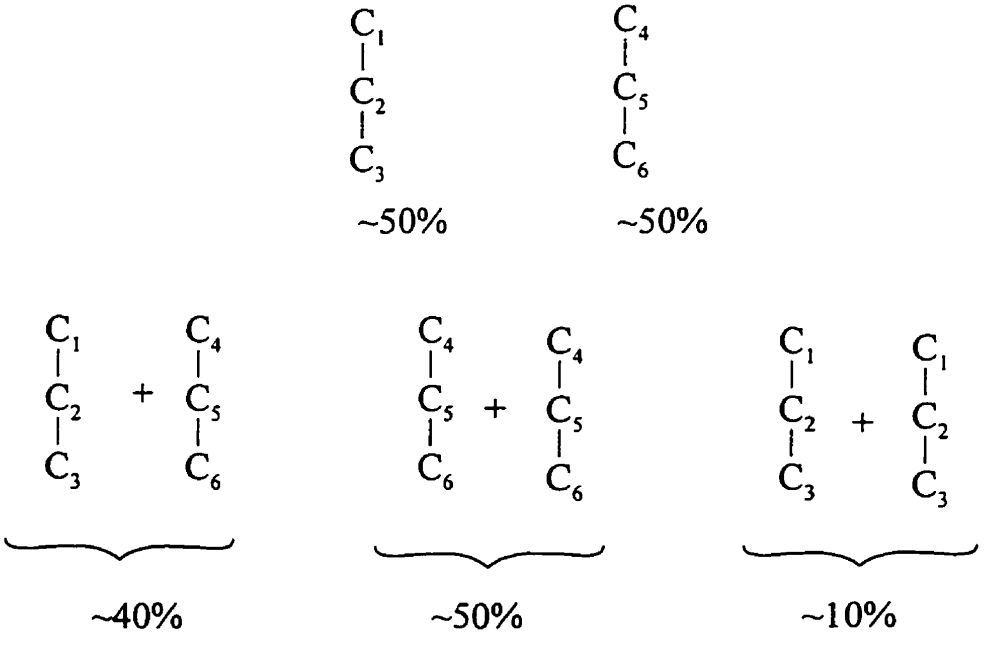
Scheme 6.5. D-glucose carbons incorporated in methylpyrazine generated from D-glucose/ alanine model systems. (Based on isotopic distribution and intermediates listed in Figure 6.1 and 6.2.)

6.2.2.2 *2,5(6)-dimethylpyrazine*: The similarity of the mass spectra of 2,5-dimethyl- and 2,6-dimethyl pyrazines prevented the confirmation of which isomer had been produced in the D-glucose/L-alanine model systems. Scheme 5.3 illustrates the formation of glyceraldehyde from Amadori glycine through a retro-aldol cleavage between position C-3 and C-4 of the glucose carbons and its subsequent transformation into pyruvaldehyde by the catalytic action of glycine. The isotopic distribution of the title compound (Table 6.4), indicated its formation from condensation of two α-aminopropionaldehydes, with the majority (~ 90 %) incorporating carbon atoms C-4, C-5 and C-6 of the hexose chain (Scheme 6.6).

Table 6.4. Percent label distribution in 2,5(6)-dimethylpyrazine formed from labeled D-glucoses and alanines.

Model	108	109	110
	M	M+1	M+2
D-Glucose/L-Alanine	100	0	0
D-[1-^{13}C]Glucose/L-Alanine	53	39	8
D-[2-^{13}C]Glucose/L-Alanine	53	39	8
D-[3-^{13}C]Glucose/L -Alanine	53	39	8
D-[4-^{13}C]Glucose/L-Alanine	10	40	50
D-[5-^{13}C]Glucose/L-Alanine	10	40	50
D-[6-^{13}C]Glucose/L-Alanine	10	40	50
D-Glucose/L-[^{15}N]Alanine	0	0	100
D-Glucose/L-[1-^{13}C]Alanine	100	0	0
D-Glucose/DL-[2-^{13}C]Alanine *	100	0	0
D-Glucose/DL-[3-^{13}C]Alanine	100	0	0

* *DL-[2-^{13}C]-alanine 92% enriched*



Scheme 6.6. D-glucose carbons in 2,5(6)-dimethylpyrazine generated in D-glucose/ alanine model systems, through dimerization of α-aminopropionaldehyde.

6.2.2.3 *2-ethyl-5-methylpyrazine:* The percent label distribution in 2-ethyl-5-methyl pyrazine detected in D-glucose/L-alanine models, indicate that there is more than one pathway for its formation (Table 6.5). Amrani-Hemaimi et al. (1995), propose a mechanism based on the incorporation of the C-3 atom of alanine as acetaldehyde into the precursor dihydropyrazine structure (Scheme 1.8). They propose the condensation of α-aminoacetaldehyde with α-aminopropionaldehyde to produce methyldihydropyrazine followed by the addition of acetaldehyde, the Strecker aldehyde, of alanine to the pyrazine ring concluding with dehydration steps to produce 2-ethyl-5-methylpyrazine. The formation of glyoxal (or glycoaldehyde) in D-glucose/L-alanine model systems was demonstrated based on the labeling distribution of methylpyrazine (Table 6.3, Scheme 6.5). Carbon chain elongation of pyruvaldehyde with atoms C-2 and C-3 of alanine to produce 2,3-pentanedione was demonstrated earlier in this chapter (Table 6.2, Scheme 6.1 and 6.3). The major isotopomer of 2-ethyl-5-methylpyrazine (~70 %) incorporates C-2 and C-3 atoms of alanine into its structure. Therefore, it is proposed that glyoxal can be converted to 2-ketobutanal (Scheme 6.3) similar to the transformation of pyruvaldehyde to 2,3-pentanedione under Maillard reaction conditions in the presence of L-alanine, and upon Strecker degradation produce the corresponding α-aminoketones (or α-aminoaldehydes, Figure 6.1) which subsequently condense to produce the ethyl-methyl substituted pyrazines (Figure 6.2). Therefore, by determining the origin of the carbons in the 2-ethyl-5-methyl pyrazine; dicarbonyl chain elongation through L-alanine is evident (Scheme 6.3). Approximately 25 % of the 2-ethyl-5-methylpyrazine did not incorporate C-2 and C-3 atoms of alanine which indicates the formation of 2-ketobutanal directly from the hexose chain by an alternate pathway.

6.2.2.4 *2-ethyl-3,5-dimethylpyrazine (or 3-ethyl-2,5-dimethylpyrazine):* The similarity of the mass spectra of 2-ethyl-3,5-dimethyl- and 3-ethyl-2,5-dimethylpyrazines prevented the distinction between the isomers. Approximately 85 % of the isotopomers incorporated C-2 and C-3 atoms of alanine. Analysis of the isotopic distribution of labeled carbon atoms of glucose in 2-ethyl-3,5-dimethylpyrazine (or 3-ethyl-2,5-dimethylpyrazine) indicated retro-aldol cleavage between C-3 and C-4 atoms of glucose

to produce pyruvaldehyde (Scheme 5.6). With the detection of 2,3-pentanedione in D-glucose/L-alanine models and with expected isotopic distribution of labeled atoms (Table 6.6), there is direct evidence for dicarbonyl elongation prior to Strecker degradation (Scheme 6.3) and formation of α-aminoketones (or α-aminoaldehydes), the precursors of pyrazines (Figure 6.1 and 6.2). The detection of 100 % singly labeled 2,3-pentanedione (Table 6.2) and ~98 % singly labeled 2-ethyl-3,5-dimethylpyrazine in pyruvaldehyde/[2-^{13}C]alanine models (Table 6.6), indicates that the major pathway for the formation of this pyrazine results from dicarbonyl elongation by the action of L-alanine rather than acetaldehyde addition to the dimethyldihydropyrazine ring (Shibimoto et al., 1977; Amrani-Hemaimi et al., 1995).

Table 6.5. Percent label distribution in 2-ethyl-5-methylpyrazine formed from labeled D-glucoses and alanines.

Model	122	123	124
	M	M+1	M+2
D-Glucose/L-Alanine	100	0	0
D-[1-^{13}C]Glucose/L-Alanine	45	41	14
D-[2-^{13}C]Glucose/L-Alanine	45	41	14
D-[3-^{13}C]Glucose/L -Alanine	40	50	10
D-[4-^{13}C]Glucose/L-Alanine	24	55	21
D-[5-^{13}C]Glucose/L-Alanine	13	41	46
D-[6-^{13}C]Glucose/L-Alanine	13	41	46
D-Glucose/L-[^{15}N]Alanine	0	0	100
D-Glucose/L-[1-^{13}C]Alanine	100	0	0
D-Glucose/DL-[2-^{13}C]Alanine *	34	64	2
D-Glucose/DL-[3-^{13}C]Alanine	26	72	1

* DL-*[2-^{13}C]-alanine 92% enriched*

Table 6.6. Percent label distribution in 2 (3)-ethyl-3 (2),5-dimethylpyrazine formed from labeled D-glucoses and alanines.

Model	136	137	138
	M	M+1	M+2
D-Glucose/L-Alanine	100	0	0
D-[1-^{13}C]Glucose/L-Alanine	46	40	14
D-[2-^{13}C]Glucose/L-Alanine	46	40	14
D-[3-^{13}C]Glucose/L -Alanine	46	40	14
D-[4-^{13}C]Glucose/L-Alanine	13	38	49
D-[5-^{13}C]Glucose/L-Alanine	13	38	49
D-[6-^{13}C]Glucose/L-Alanine	13	38	49
D-Glucose/L-[^{15}N]Alanine	0	0	100
D-Glucose/L-[1-^{13}C]Alanine	100	0	0
D-Glucose/DL-[2-^{13}C]Alanine *	20	80	0
D-Glucose/DL-[3-^{13}C]Alanine	10	85	5
DL-[2-^{13}C]Alanine/Pyruvladehyde	12	86	1

* *[2-^{13}C]-alanine 92% enriched*

6.2.2.5 *Pyrazine formation in glyoxal/^{13}C alanine model system*: Model studies were also conducted with glyoxal/alanine and DL-[2-^{13}C], [3-^{13}C]alanine/glyoxal to determine the major pyrazines formed and to confirm the mechanisms proposed above. Pyrazine, 2,3-dimethylpyrazine, ethylpyrazine and 2,6-diethylpyrazine were the major pyrazines observed in L-alanine/glyoxal model systems. Table 6.7 indicates the labeling distribution for the substituted pyrazines. These pyrazines, except 2,3-dimethylpyrazine, could all be theoretically produced in these systems based on Figures 6.1 and 6.2. The major pathway for the formation of doubly labeled 2,6-diethylpyrazine (~85 %) can be explained through the dimerization of α-aminoketones arising from labeled 2-ketobutanal (Figures 6.1 and 6.2, Scheme 6.3). While in the case of ethylpyrazine the major pathway (~95 %) is the condensation of α-aminoacetaldehyde with the α-aminoketone originating from 2-ketobutanal (Scheme 6.3).

The isotopic distribution of 2,3-dimethylpyrazine indicates the incorporation of C-2 and C-3 atoms of alanine in the structure. While 2,3-dimethylpyrazine was detected in much lower concentration compared to the other two pyrazines, its formation requires the condensation of α-aminoacetaldehyde with the aminoketone of 2,3-butanedione. The mechanism of this transformation has yet to be determined.

Table 6.7. Labeling distribution of pyrazines identified in alanine/glyoxal models.

Model	M	M+1	M+2
2,3-Dimethylpyrazine	**108**	**109**	**110**
L-alanine/Glyoxal	100	0	0
DL-[2-^{13}C]Alanine/Glyoxal *	18	82	0
[3-^{13}C]Alanine/Glyoxal	0	100	0
Ethylpyrazine	**108**	**109**	**110**
L-alanine/Glyoxal	100	0	0
DL-[2-^{13}C]Alanine/Glyoxal *	15	85	0
[3-^{13}C]Alanine/Glyoxal	6	94	0
2,6-diethylpyrazine	**136**	**137**	**138**
L-alanine/Glyoxal	100	0	0
DL-[2-^{13}C]Alanine/Glyoxal *	4	28	68
[3-^{13}C]Alanine/Glyoxal	2	14	84

* *[2-^{13}C]-alanine 92% enriched*

6.2.3 Pyrazinone Formation: Pyrazinone formation from L-alanine model systems has not been reported in the literature. Based on the information gained from the analysis of the glycine model systems, the pyrograms of D-glucose/L-alanine models were analyzed to detect pyrolysis products which could be tentatively identified as pyrazinones. A compound with the molecular ion 152 (Table 6.8) has been tentatively identified as 1-ethyl-3,5(or 6)-dimethylpyrazinone (53) based on the similarities of its mass spectral fragmentation pattern to other pyrazinones and its isotopic distribution in models utilizing labeled alanines and D-glucoses (Table 6.9). Based on this analysis a mechanism is proposed in which pyruvaldehyde reacts with two molecules of L-alanine to form a pyrazinone moiety (Scheme 6.7). To further test this assumption models of alanine/pyruvadehyde and L-[1-^{13}C], L-[^{15}N], DL-[2-^{13}C]-alanine/pyruvaldehyde were subjected to Py/GC/MS analysis. A product with the same mass spectrum was generated and the observed isotopic distribution (Table 6.9) added further evidence to the mechanism proposed in Scheme 6.7.

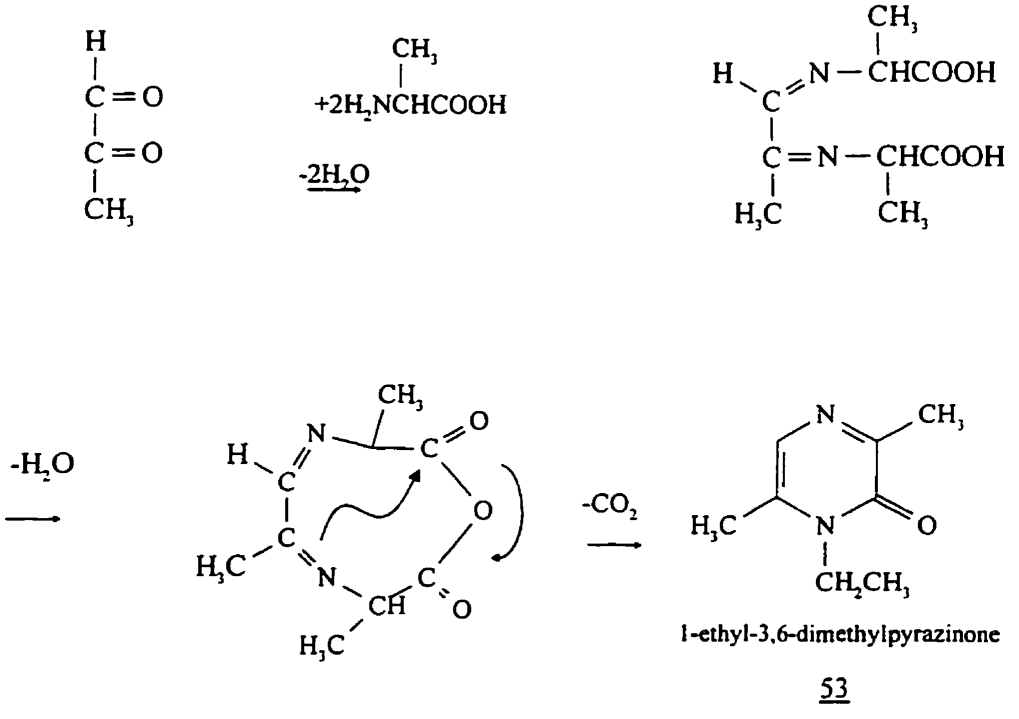
Table 6.8. Mass spectra of tentatively assigned and selected unknown compounds from D-glucose/alanine model systems.

Compounds	Mass Spectra
1-ethyl-3,5(6)-dimethylpyrazinone	153(11), 152(100), 124(64), 123(51), 109(51), 96(33), 95(48), 68(53)
5-hydroxy-1-ethyl-3-methyl-2-[1H]quinoxalinone	205(13), 204(100), 176(34), 148(24), 147(30), 120(22), 119(14), 106(15), 92(17), 79(14), 65(13)
compound m/z 179	180(11), 179(100), 135(17), 134(45), 122(21), 108 (10),106(44), 79(21), 66(13), 65(19)
compound m/z 192	193(13), 192(100), 91(91), 178(12), 177(69), 163(11), 149(16), 122(19), 121(19), 120(47), 108(29), 94(27)

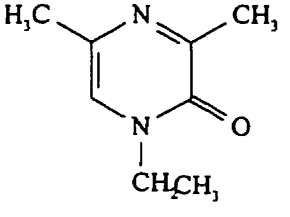
Table 6.9. Percent label distribution in 1-ethyl-3,5(6)-dimethylpyrazinone formed from labeled D-glucoses, alanines and pyruvaldehyde.

Model	152	153	154
	M	M+1	M+2
D-Glucose/L-Alanine	100	0	0
D-[1-^{13}C]Glucose/L-Alanine	60	40	0
D-[2-^{13}C]Glucose/L-Alanine	60	40	0
D-[3-^{13}C]Glucose/L -Alanine	60	40	0
D-[4-^{13}C]Glucose/L-Alanine	40	60	0
D-[5-^{13}C]Glucose/L-Alanine	40	60	0
D-[6-^{13}C]Glucose/L-Alanine	40	60	0
D-Glucose/L-[^{15}N]Alanine	0	0	100
D-Glucose/L-[1-^{13}C]Alanine	2	98	0
D-Glucose/DL-[2-^{13}C]Alanine *	0	15	85
D-Glucose/DL-[3-^{13}C]Alanine	0	5	95
L[^{15}N]Alanine/Pyruvaldehyde	0	0	100
L-[1-^{13}C]Alanine/Pyruvaldehyde	2	98	0
DL-[2^{13}C]Alanine/Pyruvaldehyde *	1	12	87

* *DL-[2-^{13}C] alanine 92% enriched*



Or depending on the position of pyruvaldehyde during the reaction 1-ethyl-3,5-dimethylpyrazinone can also be formed.



Scheme 6.7. Proposed mechanism of formation of 1-ethyl-3,5(or 6)-dimethylpyrazinone.

6.2.4 Quinoxalinone Formation: Pyrazinones and quinoxalinones can be formed during Maillard reaction, by multiple additions of glycine to an α-dicarbonyl compound. This interaction can lead to the formation of different derivatives, depending on the structure of the dicarbonyl fragment. It has been demonstrated that pyruvaldehyde and 2,3-butanedione can generate alkyl substituted pyrazinones, and Amadori products or 3-DG can generate alkyl substituted quinoxalinones. In the case of glycine model systems the initially formed 5-hydroxy-1-methyl-2-[1H]-quinoxalinone (51) can undergo methylation at the imine site to produce 5-hydroxy-1,3-dimethyl-2-[1H]-quinoxalinone (33) which in turn can produce a 5-hydroxy-1,3,7-trimethyl-2-[1H]-quinoxalinone (52) by methylation, through, for example, Michael type addition, as proposed in Scheme 5.10. However, the position of the methylation site is not confirmed. To demonstrate the generality of this reaction with other amino acids containing alkyl side chains, L-alanine reaction was investigated, similar to that of glycine, with variously labeled starting materials. The main quinoxalinone structure, tentatively identified by labeling studies was 5-hydroxy-1-ethyl-3-methyl-2-[1H]-quinoxalinone (Table 6.8 and 6.10), the equivalent product to that of 5-hydroxy-1-methyl-2-[1H]-quinoxalinone from glycine (Chapter 5, Section 5.2.3). It is worth mentioning that when D-glucose was replaced with a *keto* sugar, such as D-fructose or D-tagatose, the peaks associated with quinoxalinone structures increased significantly, both in glycine and L-alanine systems. This observation could be related to the fact that Heyn's product is more reactive towards the second amino acid attack, considering the carbonyl group being reacted is an aldehyde.

6.2.5 Unknown major products containing an intact glucose moiety: Two compounds, with molecular ions m/z 179 and m/z 192, were detected in the D-glucose/alanine models at 210°C but could only be detected in trace amounts when pyrolyzed at 250°C. There mass spectra (Table 6.8) and isotopic distribution of these

compounds have been included, however identification of their structure requires further investigation (Table 6.11 and 6.12).

Table 6.10. Percent label distribution of molecular ion m/z 204 tentatively identified as 5-hydroxy-1-ethyl-3-methyl-2[1H]quinoxalinone generated from labeled D-glucoses or excess labeled alanine.

D-GLUCOSE / L- ALANINE	204	205	206
D-Glucose/L-Alanine	98	2	0
D-[1-^{13}C]Glucose/ L-Alanine	0	98	2
D-[2-^{13}C]Glucose/ L-Alanine	0	98	2
D-[3-^{13}C]Glucose/ L-Alanine	0	98	2
D-[4-^{13}C]Glucose/ L-Alanine	0	100	0
D-[5-^{13}C]Glucose/L-Alanine	0	100	0
D-[6-^{13}C]Glucose/L-Alanine	0	100	0
D-Glucose/ L-[1-^{13}C]Alanine	12	88	0
D-Glucose/ DL-[2-^{13}C]Alanine (92 % enriched)	1	18	81
D-Glucose/DL-[3-^{13}C]Alanine	0	5	95
D-Glucose/ L-[^{15}N]Alanine	0	1	99

Table 6.11. Percent distribution of molecular ion m/z 179 generated from labeled D-glucoses and alanine models.

D-GLUCOSE / L- ALANINE	179	180
D-Glucose/L-Alanine	100	0
D-[1-^{13}C]Glucose/ L-Alanine	0	100
D-[2-^{13}C]Glucose/ L-Alanine	0	100
D-[3-^{13}C]Glucose/ L-Alanine	0	100
D-[4-^{13}C]Glucose/ L-Alanine	0	100
D-[5-^{13}C]Glucose/L-Alanine	0	100
D-[6-^{13}C]Glucose/L-Alanine	0	100
D-Glucose/ L-[1-^{13}C]Alanine	11	89
D-Glucose/ DL-[2-^{13}C]Alanine (92 % enriched)	11	89
D-Glucose/DL-[3-^{13}C]Alanine	0	99
D-Glucose/ L-[^{15}N]Alanine	0	100

Table 6.12. Percent distribution of molecular ion m/z 192 generated from labeled D-glucoses or excess labeled alanine.

D-Glucose/L-Alanine	192	193	194
D-Glucose/L-Alanine	100	0	0
D-[1-^{13}C]Glucose/ L-Alanine	3	97	0
D-[2-^{13}C]Glucose/ L-Alanine	7	93	0
D-[3-^{13}C]Glucose/ L-Alanine	0	100	0
D-[4-^{13}C]Glucose/ L-Alanine	0	100	0
D-[5-^{13}C]Glucose/L-Alanine	0	100	0
D-[6-^{13}C]Glucose/L-Alanine	0	100	0
D-Glucose/ L-[1-^{13}C]Alanine	7	93	0
D-Glucose/ DL-[2-^{13}C]Alanine (92 % enriched)	17	83	0
D-Glucose/DL-[3-^{13}C]Alanine	4	96	0
D-Glucose/ L-[^{15}N]Alanine	0	0	100

6.3 Conclusion

Py/GC/MS of alanine model systems produces a wide range of products most of which have been reported by previous investigators. Utilization of enriched precursors (alanines and glucoses) provided evidence that the carbon chain elongation of α-dicarbonyls by amino acid carbons observed in glycine models is also a predominant reaction pathway in alanine model systems. Formation of ethyl-substituted pyrazines and tentative identification of a pyrazinone and a quinoxalinone in alanine model systems, indicates the occurrence of common mechanistic pathways among aliphatic amino acids.

CHAPTER 7

L-SERINE

7. 1 Introduction

L-Serine and L-threonine are β-hydroxy amino acids that have been shown to produce a variety of heterocyclic compounds upon thermal degradation in the absence of sugars. Several volatile compounds, including pyrazines, were produced from the thermal degradation of L-serine and L-threonine at high temperatures (Kato et al., 1970; Wong and Odell, 1973). Approximately 350 volatiles have been identified from roasting mixtures of sucrose, serine and threonine (Baltes and Bochmann, 1987; Reese and Baltes, 1992). The compounds are categorized as alkyl-, alkenyl-, and acyl- substituted furans, pyrroles, pyrazines, pyridines, oxazoles and pyrrolylalkanols. With the exception of furans and oxazoles, most of these compounds were formed from heating L-serine and L-threonine in the absence of sucrose.

Thermal degradation of L-serine (Figure 7.1) has been reported to produce, alanine, glycine, formaldehyde, 2-amino-1-ethanol and pyruvic acid as its initial products (Baltes, 1990). The formation mechanism of heterocyclic compounds by L-serine has not yet been reported. In this chapter we study degradation of L-serine by Py/GC/MS and utilize L-[3-^{13}C]serine to investigate the main mechanisms of degradation.

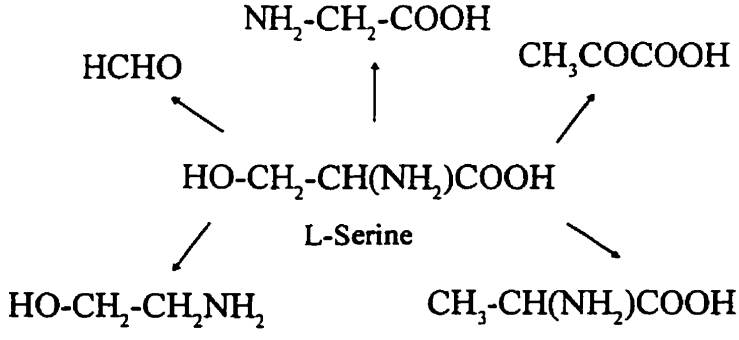


Figure 7.1. Primary thermal decomposition products of L-Serine, according to Baltes, (1990).

7.2 Results and Discussion

Thermal degradation of L-serine (120°C-360°C) produces a wide range of heterocyclic compounds such as pyrazines, pyrroles and pyrrolylalkanols (Kato et al., 1970; Mussinan and Walradt, 1973; Wang and Odell, 1973 and Baltes and Bochmann, 1987; Reese and Baltes, 1992). However, the mechanistic pathways which lead to the variety of compounds observed, have not been investigated. Pyrolysis of L-Serine at 250°C for 20s (Figure 7.2) produces similar compounds to those reported by other investigators (Table 7.1). Utilization of L-[3-^{13}C]serine and calculation of the percent label incorporation (Table 7.1) in the pyrolysis products enabled the proposal of mechanistic pathways which lead to the formation of several of these heterocyclic compounds from L-serine.

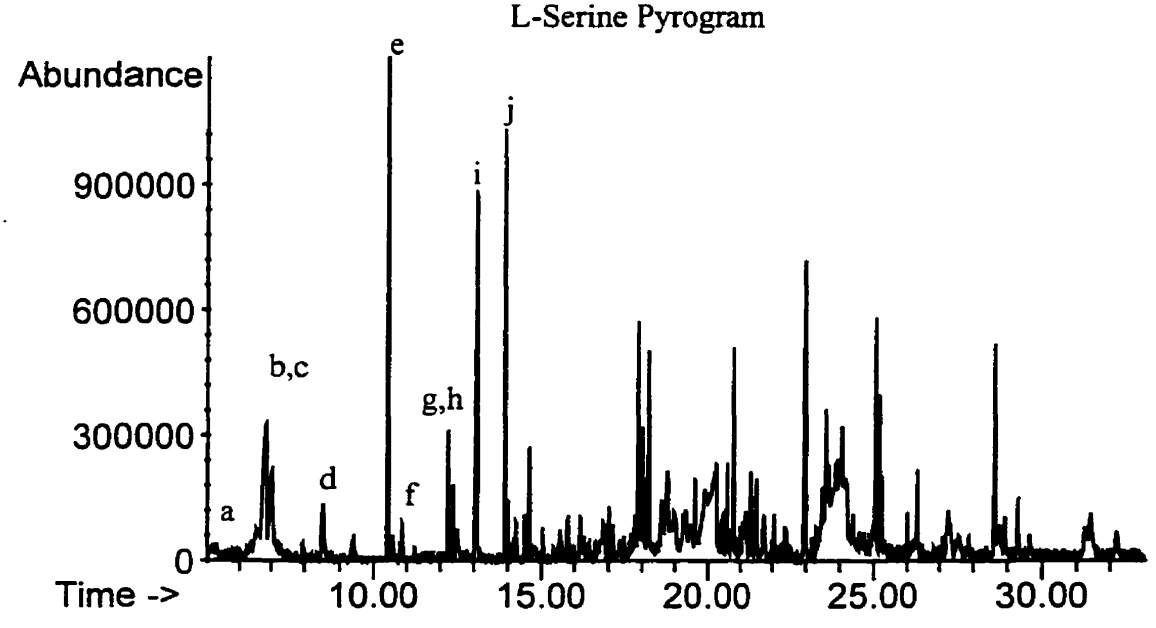


Figure 7.2. Pyrogram of L-serine from quartz tube pyrolysis 250°C 20s
a:2-aminoethanol; b: pyrazine; c: 1H-pyrrole; d: methylpyrazine; e: ethylpyrazine; f: ethenylpyrazine; g: 2-ethyl-6-methylpyrazine; h: 2-ethyl-5-methylpyrazine; i: 2-(1-pyrrolyl)ethan-1-ol (Baltes and Bochmann, 1987); j: 2,6-diethylpyrazine

Table 7.1. Selected compounds identified in upon Py/GC/MS (250°C, 20s) of L-serine and percent label incorporation upon utilization L-[3-^{13}C]serine.

L-Serine					
Compounds	MW	Percent [3-^{13}C] incorporation			
		M+1	M+2	M+3	M+4
Pyrazines					
pyrazine	80	0	100	0	0
methylpyrazine	94	0	33	67	0
ethylpyrazine	108	0	0	100	0
dimethylpyrazine	108	0	10	66	24
ethenylpyrazine	106	0	0	100	0
2-ethyl-6-methylpyrazine	122	0	0	27	73
2-ethyl-5-methylpyrazine	122	0	0	27	73
2,6-diethylpyrazine	136	0	0	0	100
2,5-diethylpyrazine	136	0	0	0	100
dimethyl-2-vinylpyrazine*	134	0	0	0	100
Pyrroles					
1H-pyrrole	67	0	100	0	0
2-methyl-1H-pyrrole	81	0	55	45	0
2-ethyl-1H-pyrrole	95	0	0	100	0
2-(1-pyrrolyl)ethan-1-ol*	111	0	0	100	0
Other					
2-aminoethanol	61	100	0	0	0
acetamide	59	100	0	0	0
propanamide	73	100	0	0	0
1,4-dimethyl-2,5-diketopiperazine	142	0	100	0	0

**Baltes and Bochmann, 1987*

7.2.1 Initial thermal degradation products of L-serine

Several initial thermal degradation products of L-serine have been reported previously by Baltes (1990), however the method of detection of these products and mechanistic pathways were not reported.

7.2.1.1 *L-Alanine and glycine formation from L-serine:* 1,4-Dimethyl-2,5-diketopiperazine, an indicator compound for L-alanine formation was identified from the Py/GC/MS analysis of L-alanine (Table 6.1) and also in L-serine (Table 7.1). Utilization

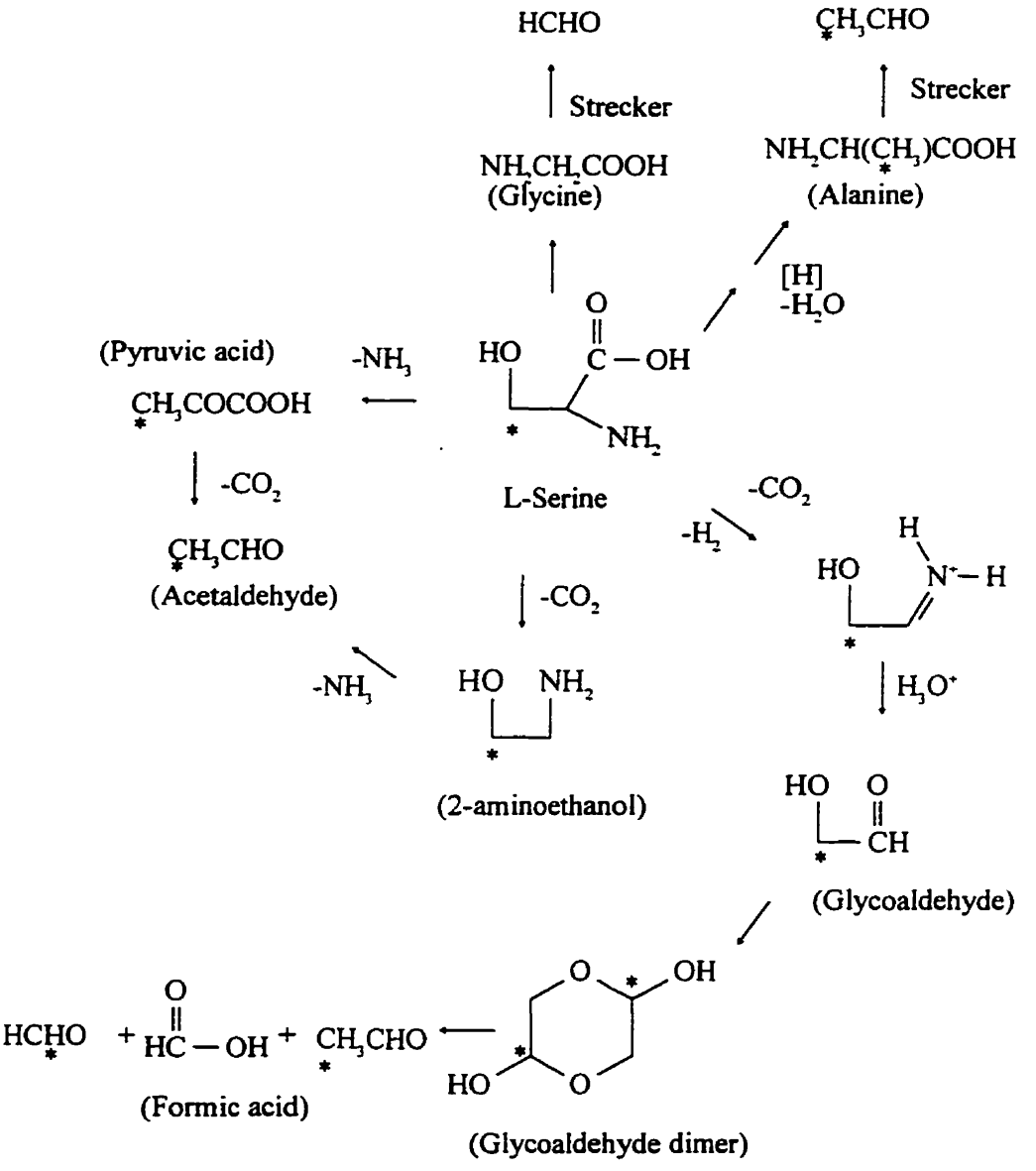
of L-[3-^{13}C]serine produced 100% doubly labeled 1,4-dimethyl-2,5-diketopiperazine (Table 7.1). This provided indirect evidence for the production of L-alanine from the thermal degradation of L-serine (Scheme 7.1). To determine whether L-alanine and glycine indeed form from L-serine, a D-glucose/L-serine model system was analyzed by Py/GC/MS. If alanine and glycine are formed from serine, then the D-glucose/L-serine model system should generate glycine and alanine specific compounds, such as pyrazines and pyrazinones, that were identified in D-glucose/L-alanine and D-glucose/L-serine model systems (see Chapters 5 and 6). Table 7.2 lists common glycine and L-alanine specific products found in D-glucose/L-serine model, providing further evidence for the formation of L-alanine and glycine from thermal degradation of L-serine.

Table 7.2. Indicator compounds for the formation of glycine and L-alanine from the thermal degradation of L-serine.

Compounds	D-glucose/L-serine	D-glucose/glycine	D-glucose/L-alanine
1,5,6-dimethyl-2(1H)pyrazinone	+	+	-
1,6-dimethyl-2(1H)pyrazinone	+	+	-
1,5-dimethyl-2(1H)pyrazinone	+	+	-
2(3)-ethyl-3(2),5-dimethylpyrazine	+	-	+
1-ethyl-3,5(6)-dimethylpyrazine	+	-	+
1,4-dimethyl-2,5-diketopiperazine	+	-	+

7.2.1.2 *Glycoaldehyde formation from L-serine:* The Strecker aldehyde of L-serine is glycoaldehyde. However, to initiate Strecker degradation, the presence of a dicarbonyl is required. In the absence of a sugar source, it could be concluded that the glycoaldehyde detected should arise directly from the thermal degradation of L-serine (Scheme 7.1). The ability of carbonyl compounds to form stable end products with phenylhydrazine ($H_2NNHC_6H_5$) was utilized in a model study, to provide evidence that glycoaldehyde is formed from thermal degradation of L-serine. The major product of the pyrolysis of glycoaldehyde in the presence of phenylhydrazine was N-ethylidene-N'-phenylhydrazine.

Py/GC/MS analysis of L-serine in the presence of phenylhydrazine also produced the same compound. In addition, by trapping the glycoaldehyde produced from L-serine, the wide variety of heterocyclic compounds requiring glycoaldehyde as an intermediate were prevented from forming.



Scheme 7.1. The proposed initial thermal degradation products of L-serine. Asterisk indicates [3-^{13}C] atom of L-serine.

7.2.1.3 *Acetaldehyde formation from L-serine*: Baltes (1990), reported pyruvic acid formation from the deamination of L-serine. Subsequent decarboxylation of pyruvic acid can result in the formation of acetaldehyde (Scheme 7.1). Strecker degradation of L-alanine and deamination of 2-aminoethan-1-ol (Table 7.1) are other possible sources for acetaldehyde formation (Scheme 7.1) from L-serine. Evidence for the formation of acetaldehyde is provided in this chapter by observing its incorporation in pyrazines (see section 7.2.2) and pyrroles (see section 7.2.3).

7.2.1.4 *Formaldehyde formation from L-serine*: Formaldehyde and glycine have also been reported to form upon thermal degradation of L-serine (Baltes, 1990). In addition, formaldehyde is the Strecker aldehyde of glycine. According to Scheme 7.1, formation of formaldehyde from Strecker degradation of glycine would not generate a labeled formaldehyde upon Py/GC/MS of L-[3-^{13}C]serine, while its formation directly from L-serine would generate both labeled and unlabeled formaldehyde. Huyghues-Despointes and Yaylayan (1996), reported on cyclic dimer formation of α-hydroxy carbonyl compounds followed by thermally allowed electrocyclic ring opening during Maillard reaction. This process can explain the formation of formaldehyde and formic acid from glycoaldehyde (Scheme 7.1). While formaldehyde could not be detected by Py/GC/MS analysis, its incorporation into the products of L-serine, as demonstrated below, indicates its availability in the reaction pool.

7.1.3 Pyrazine formation from L-serine

Baltes and Bochmann (1987), propose that upon thermal degradation, L-serine can decarboxylate and react directly by dimerization to yield pyrazines. While this proposed mechanism explains parent pyrazine formation from L-serine, however, it can not not explain the variety of methyl and ethyl substituted pyrazines observed from L-serine degradation. The formation of a variety of alkylsubstituted pyrazines from the degradation of L-serine requires the production of C_2, C_3 and C_4 α-aminoaldehydes.

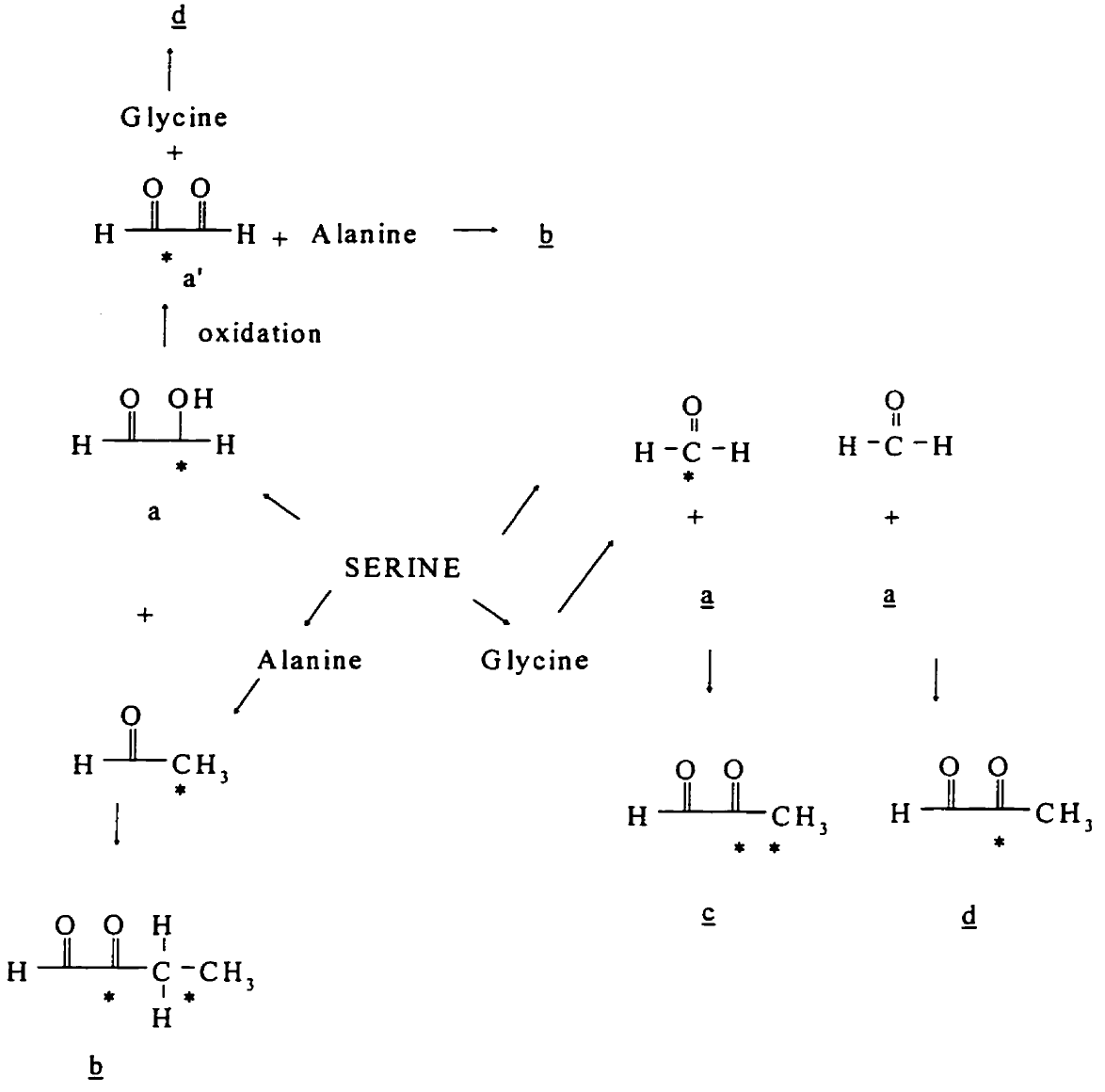
Their dicarbonyl precursors can be formed from L-serine according to the mechanism shown in Scheme 7.2.

Glycoaldehyde (glyoxal) can react with glycine according to previously described mechanism (Scheme 5.1) to produce pyruvaldehyde (C_3), therefore, incorporating the C-2 atom of L-serine. An aldol condensation between glycoaldehyde and formaldehyde (Schemes 7.2 and 7.3) is also a possible route to pyruvaldehyde (C_3) formation from L-serine. The formaldehyde can result from either the C-3 or C-2 atom of L-serine as shown in Schemes 7.1 and 7.2. 2-Ketobutanal (C_4) can result from the addition of C-2 and C-3 atoms of alanine to glycoaldehyde according to a previously described mechanism (Scheme 6.3) therefore incorporating both C-2 and C-3 atoms of L-serine. It can also form from aldol condensation of acetaldehyde and glycoaldehyde (Scheme 7.3).

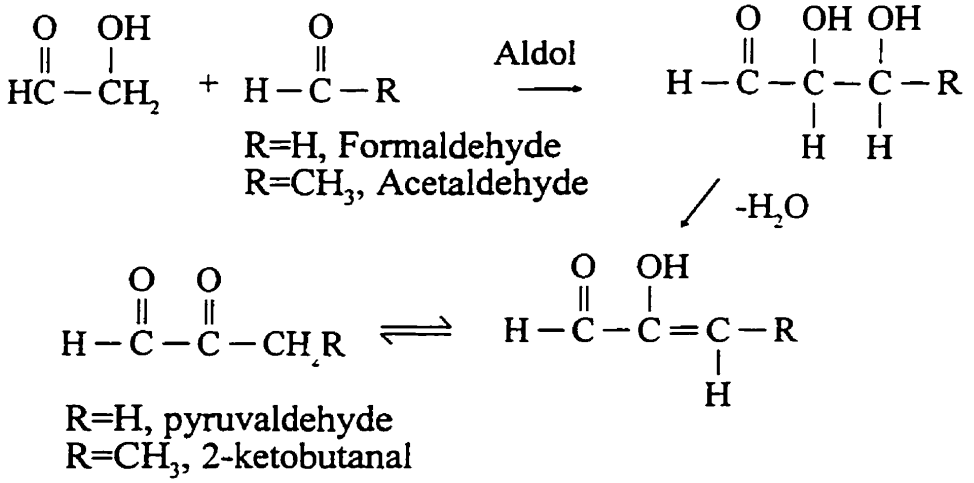
Addition of methyl and ethyl groups to the parent dihydropyrazine to produce alkylsubstituted pyrazines have been reported previously (Scheme 1.7 and 1.8; Shibomoto et al., 1977; Amrani-Hemaimi, 1995). Although, the information gained from use of L-[3-^{13}C]serine can not be used to dispute the existence of this pathwa, however, addition of formaldehyde and acetaldehyde to dihydropyrazines requires carbon chain elongation prior to α-aminoaldehyde formation to produce the variety of alkylpyrazines observed from Py/GC/MS analysis of L-serine.

The label distribution of the alkylsubstituted pyrazines discussed below are based on the designations a, b, c, d in Scheme 7.2. Structures of the required α-aminocarbonyl intermediates and the resulting pyrazines are shown in Figures 6.1 and 6.2.

Methylpyazine: The production of methylpyrazine from the degradation of L-serine alone requires the condensation of aminoacetaldehyde and α-aminoacetone. Doubly labeled methylpyrazine (~ 33 %) results from the corresponding α-aminoaldehydes of a and d, and triply labeled methylpyrazine (~ 67 %) results from the corresponding α-aminoaldehydes of a and c (see Scheme 7.2).



Scheme 7.2. Mechanism of dicarbonyl formation from L-[3-^{13}C]serine. a=glycoaldehyde (singly labeled), b=2-ketobutanal(doubly labeled), c=pyruvaldehyde (doubly labeled), d= pyruvaldehyde (singly labeled). Asterisk indicates [3-^{13}C] atoms of serine.



Scheme 7.3. Mechanism of aldol condensation between aldehydes formed from L-serine, to produce 2-ketoaldehydes.

Dimethylpyrazine: This pyrazine arises from the dimerization of α-aminoacetone. Doubly labeled dimethylpyrazine (~ 10 %) results from the corresponding α-aminoacetones of d, and the triply labeled (~ 66 %) dimethylpyrazine results from the corresponding α-aminoacetones of c and d . Dimethylpyrazine with four label incorporation results from the condensation of the corresponding α-aminoacetones of c.

Ethylpyrazine: This pyrazine which forms from the condensation of α-aminoacetaldehyde and α-aminobutanal (or α-aminobutanone) incorporates only three labels and results from the condensation of the corresponding α-aminoaldehydes of b and a.

Ethylmethylpyrazines: These pyrazines can form from the condensation of α-aminoacetone and α-aminobutanal (or α-aminobutanone). Triply labeled ethylmethylpyrazine (~27 %) results from the corresponding α-aminoaldehydes of b and

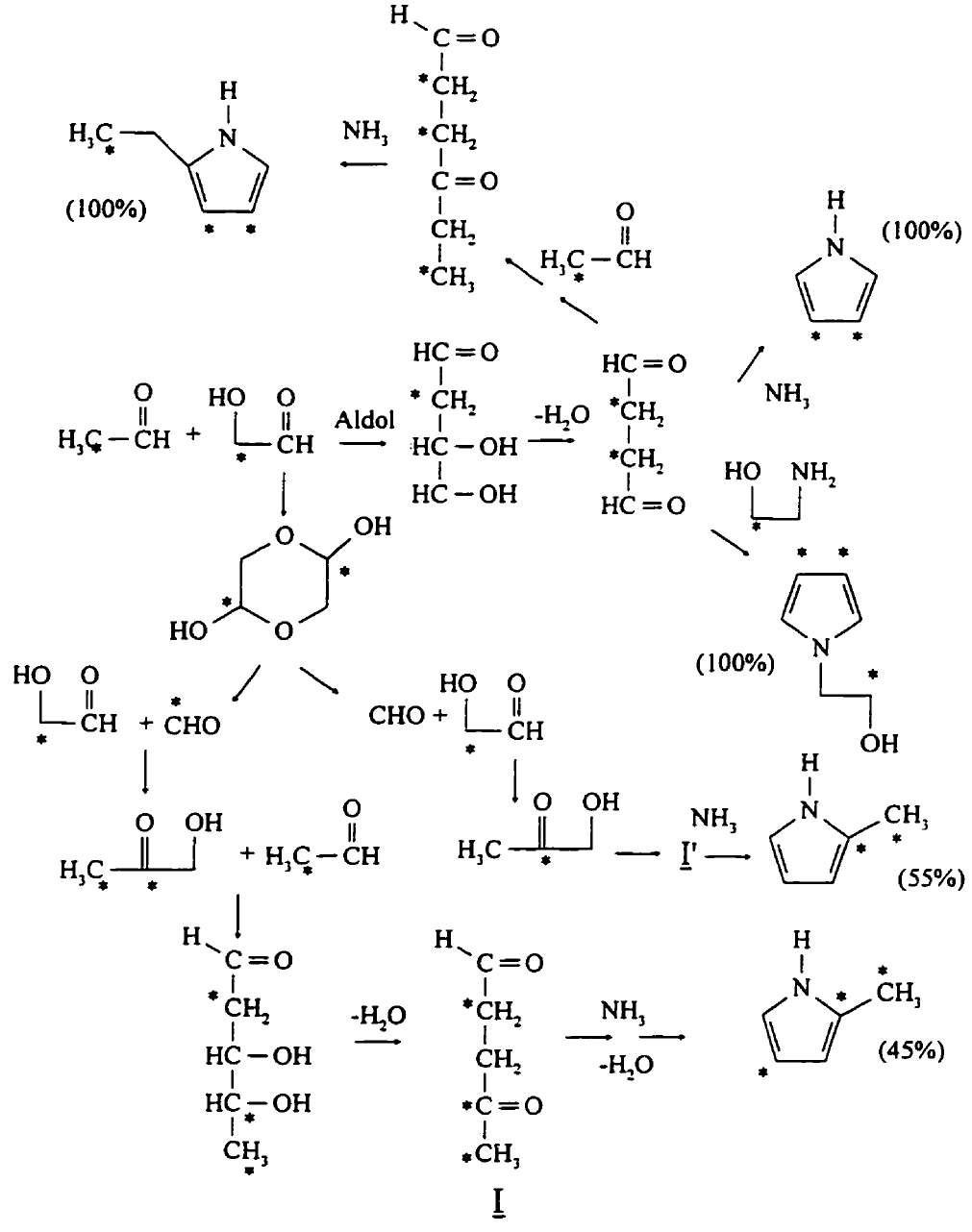
d, and those incorporating four labels (~73 %) results from the condensation of the corresponding α-aminoaldehydes b and c.

Diethylpyrazines: The condensation of α-aminobutanal produces diethylpyrazines which were detected only in one isotopomeric form incorporating four labels. This observation can be explained by dimerization of the corresponding α-aminoaldehyde of b.

The observed isotopic distribution of the alkylpyrazines detected in L-serine pyrolysis products, was accurately predicted by the mechanism outlined in Scheme 7.2. Therefore, providing indirect evidence for the formation of glycoaldehyde, formaldehyde and acetaldehyde from the thermal degradation of L-serine and occurrence of carbon chain elongation.

7.2.3 Mechanism of pyrrole formation from L-serine

1H-Pyrrole, 2-ethyl-1H-pyrrole and 2-(1-pyrrolyl)ethan-1-ol were identified in the pyrogram of L-serine. The label distribution of these compounds are listed in Table 7.1. The major pyrrole observed was 2-(1-pyrrolyl)ethan-1-ol. The proposed reaction mechanism of these compounds, based on the observed isotopic distribution, and the intermediates available in the reaction pool is presented in Scheme 7.4. In L-serine, pyrroles are formed by the interaction of 1,4-dicarbonyl intermediates with amino compounds, similar to classical Paul-Knorr pyrrole synthesis. Differently substituted 1,4-dicarbonyls are formed from smaller C_2 carbon units as shown in Scheme 7.4. The detection of these intermediates, indicate the ability of C_2 units to form larger units such as 4-ketohexanal and 4-ketopentanal (I). 1H-pyrrole and 2-(1-pyrrol)ethan-1-ol were detected as one isotopomeric form, while 2-methyl-1-H-pyrrole which requires formaldehyde for its formation, was detected as two isotopomers, one doubly labeled (~ 55 %) and the other triply labeled (~ 45 %).



Scheme 7.4. Proposed mechanism of pyrrole formation from of L-serine. Asterisk indicates C-3 atoms of serine. I' is the doubly labeled isotopomer of I.

This provides further evidence that formaldehyde can be produced from C-2 and C-3 atoms of L-serine according to mechanism shown in Scheme 7.1.

7.3 Conclusion

Unlike other amino acids Py/GC/MS analysis of L-serine in the absence of sugar indicates the formation of a multitude of heterocyclic compounds. L-Serine is a unique amino acid that generates in the absence of sugar, glycoaldehyde which is, at the same time, its own Strecker aldehyde. In addition, it can produce two other amino acids, glycine and alanine. As was demonstrated in earlier chapters glycine and alanine are able to effect chain elongation of glycoaldehyde to form C_3 and C_4 dicarbonyl compounds. In effect L-serine alone can be viewed as a potential mixture of glycine, alanine, serine, formaldehyde and C_2, C_3 and C_4, C_5 and C_6 dicarbonyls.

CHAPTER 8

L-METHIONINE MODEL SYSTEMS

8.1 Introduction

Methionine has been widely used in the flavor and food industries to produce reaction flavors such as baked potato, fried potato, coffee and meat flavors (Balance ,1961; Chen, 1968; Fan and Yeuh, 1980, Tressl et al., 1989). The contribution of methionine to flavor formation arises mainly through thermal degradation or through thermal interactions with other food ingredients, especially reducing sugars. Investigations on the contribution of methionine to flavor and aroma have been conducted at temperatures between 100-190°C (El-Ode et al., 1966; Shigematsu et al., 1977). Sulfur containing amino acids are assumed to be the most important contributors to roast aroma. Dimethyldisulfide, a product of methionine, is a well known aroma compound. In this chapter a preliminary investigation was conducted to compare the main degradation products of methionine model systems using Py/GC/MS quartz tube and ribbon.

8.2 Results and Discussion

Products identified from Py/GC/MS analysis of models containing L-methionine are listed in Tables 8.1 and 8.2. Figure 8.1 demonstrates the changes in the pyrogram of Amadori methionine with increasing pyrolysis temperatures. Figure 8.2 compares the pyrograms obtained from quartz tube pyrolysis of Amadori methionine and an equimolar mixture of D-glucose/L-methionine. Amadori methionine produces a more complex profile than the corresponding equimolar glucose/methionine system. This can be attributed to the thermal instability of methionine itself which can decompose prior to Amadori rearrangement. Amadori methionine was pyrolyzed at temperatures ranging between 100-250°C to determine the effect of temperature on the pyrograms. The pyrograms in Figure 8.1 and products listed in Table 8.1 demonstrate the temperature dependence of the degradation of Amadori methionine. The initial pyrolytic event appears to be carbon-nitrogen bond cleavage to release methionine. Methional (56, Scheme 8.1), the Strecker aldehyde of methionine, is formed at all temperatures under ribbon probe conditions. The decarboxylated amino acid, 3-(methylthio)propanamine

(55) is observed at 100°C and 250°C but could not be detected in the pyrograms generated at 150°C and 200°C indicating its reaction with methional. The intensity of 3-(methylthio)propanamine (55) increases significantly at 250°C. It appears that at this temperature the main pyrolytic event following carbon-nitrogen bond cleavage is decarboxylation of methionine rather than Strecker degradation. Under Py/GC/MS conditions Strecker degradation appears to be favored below 250°C. This is also consistent with the increased detection of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one at 250°C. With the increased formation of this compound fewer forms of reactive carbonyls are available in the system to initiate Strecker degradation of methionine. Based on this information; it is likely that primary pyrolysis products of the thermal degradation of ARP methionine include reactive dicarbonyls; whose formation is favored between 150-200°C. Methionine also undergoes deamination to produce 3-(methylthio)propanoic acid methyl ester since it was identified in all the pyrograms.

8.2.1 Formation of Sulphur containing compounds in methionine model systems.

Thermal degradation of non-sulfur-containing amino acids leads to the formation of the corresponding amines via decarboxylation. However, thermal degradation of sulfur containing amino acids such as methionine produces additional reactive breakdown products. Upon quartz tube pyrolysis at 250°C for 20s; in addition to 3-(methylthio)-1-propanamine (54), thermal degradation of methionine produces methanethiol, dimethyldisulfide and 2-propen-1-amine (55, Scheme 8.1). Yu and Ho (1995) reported methional (56, Scheme 8.1) as a thermal degradation product of methionine produced in an aqueous system heated at 180°C for one hour. Methional (56) was not detected during Quartz tube Py/GC/MS of free methionine possibly due to its reactivity with amino compounds, such as 3-(methylthio)-1-propanamine (54) and 2-propen-1-amine (55).

Table 8.1. Effect of temperature on the formation of Pyrolysis Products from 1 mg Amadori Methionine using the Ribbon Probe.

Ribbon Probe-Amadori Methionine[a]		Temperature (°C)			
	MW	100	150	200	250
Sulphur Containing products					
1,3-bis(methylthio)-propane	136	-	-	-	+
1-(methylthio)-propane	90	-	-	+	+
3-(methylthio)propanol	106	-	-	-	+
3-(methylthio)-propanal	104	+	+	+	+
3-(methylthio)propanoic acid methyl ester	134	+	+	+	+
methanethiol	48	-	+	+	+
dimethyldisulfide	94	+	+	+	+
thiobismethane	62	-	+	+	-
Nitrogen and Sulphur Containing Products					
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	144	-	-	+	+
2,3-dimethy-5-[(methylthio)propyl]pyrazine[b]	122	-	-	-	+
2,5-dimethy-3-[(methylthio)propyl]pyrazine[b]	122	-	-	-	+
3-(methylthio)-1-propanamine	105	+	-	-	+
3-[(methylthio)methyl]pyridine	139	-	-	-	+
Nitrogen Containing Products					
1-(1H-pyrrol-2-yl)-ethanone	109	-	-	-	+
3-methylpyridine	93	-	-	-	+
trimethylpyrazine	122	-	-	-	+
Other					
acetic acid	60	-	+	+	+
nonanal	142	-	+	-	-

[a] *+,present;-,absent*

[b] *Tentatively identified based on mass spectra, Yu and Ho (1995)*

Table 8.2. Products Identified during Pyrolysis of L-Methionine Model Systems, Using Quartz at 250°C for 20s.

Methionine Models[a]	MW	ME	GLU/ME	GLU/ME	MEP	ME/MEP	GYC/ME
Molar Ratio			1/1	1/3		1/1	1/3
Sulphur Containing Products							
(methylthio)cyclopentane	116	-	+	-	-	-	-
1,3-bis(methylthio)-propane	136	-	-	-	+	-	-
1-(methylthio)-propane	90	-	+	+	+	+	+
2,4-dithiapentane	108	-	+	-	-	-	-
2-[(methylthio)methyl]-5-(methylthio)-2-pentenal*	190	-	+	-	+	-	-
2-fufurylmethylsulfide	128	-	+	+	+	+	+
2-furancarbodithioic methyl ester	158	-	+	-	-	-	-
3-(methylthio)-1-propene	88	-	-	-	+	-	+
3-(methylthio)-propanal*	104	-	+	t	+	t	t
3-(methylthio)propanoic acid methyl ester	134	-		-	+		+
3-(methylthio)propanol	106	-	-	+	+	+	+
5-methylester-2-furancarbothioic acid	142	-	+	-	-	-	-
dimethyldisulfide*	94	+	+	+	+	+	+
dimethyltrisulfide	126	-	+	-	-	-	-
methanethiol*	48	+	+	+	+	+	-
thiobismethane	62	-	-	-	+	-	-
Nitrogen & Sulphur Containing Products							
2,3-dimethy-5-[(methylthio)propyl]pyrazine[b]	196	-	-	+	+	+	-
2,5-dimethy-3-[(methylthio)propyl]pyrazine[b]	196	-	-	+	+	+	-
3-(methylthio)-1-propanamine*	105	+	-	-	-	+	+
3-[(methylthio)methyl]pyridine*[a]	139	-	+	+	+	+	-
2-[(methylthio)ethyl]-1,2-thiazine*[c]	177	-	+	+	+	t	-
Nitrogen Containing Products							
1-(1H-pyrrol-2-yl)-ethanone	109	-	-	-	+	-	-
2,3-dimethylpyrazine	108	-	+	t	t	t	-
2,5-dimethyl-pyrazine	108	-	+	+	+	-	-
2-methyl-1H-pyrrole	81	-	-	-	+	-	-
2-propen-1-amine	57	+	-	-	-	-	+
3-methylpyridine	93	-	+	+	+	+	+
ethylpyrazine	108	-	+	-	-	-	+
methylpyrazine	94	-	+	-	+	-	-
trimethylpyrazine	122	-	-	+	+	-	-
Other							
1-hydroxy-2-propanone	74	-	+	t	+	-	-
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	144	-	+	+	+	-	-
2-furancarboxaldehyde	96	-	+	-	-	-	-
2-furanmethanol	98	-	+	-	-	-	-
acetic acid	60	-	+	t	+	+	-
protoanemoin	96	-	+	-	+	-	-

[a]+, present;-,absent; t,trace. Models: ME, methionine; GLU/ME, glucose/methionine; ARPM, Amadori Methionine; ME/ARPM, methionine/Amadori Methionine; GYC/ME, glycoaldehyde/methionine
*[b]Tentatively identified based on mass spectra, isotopic enrichment, Yu and Ho (1995) [C] Tentatively identified, Scheme 8.2, 57, *Amadori Methionines six major pyrolysis products.*

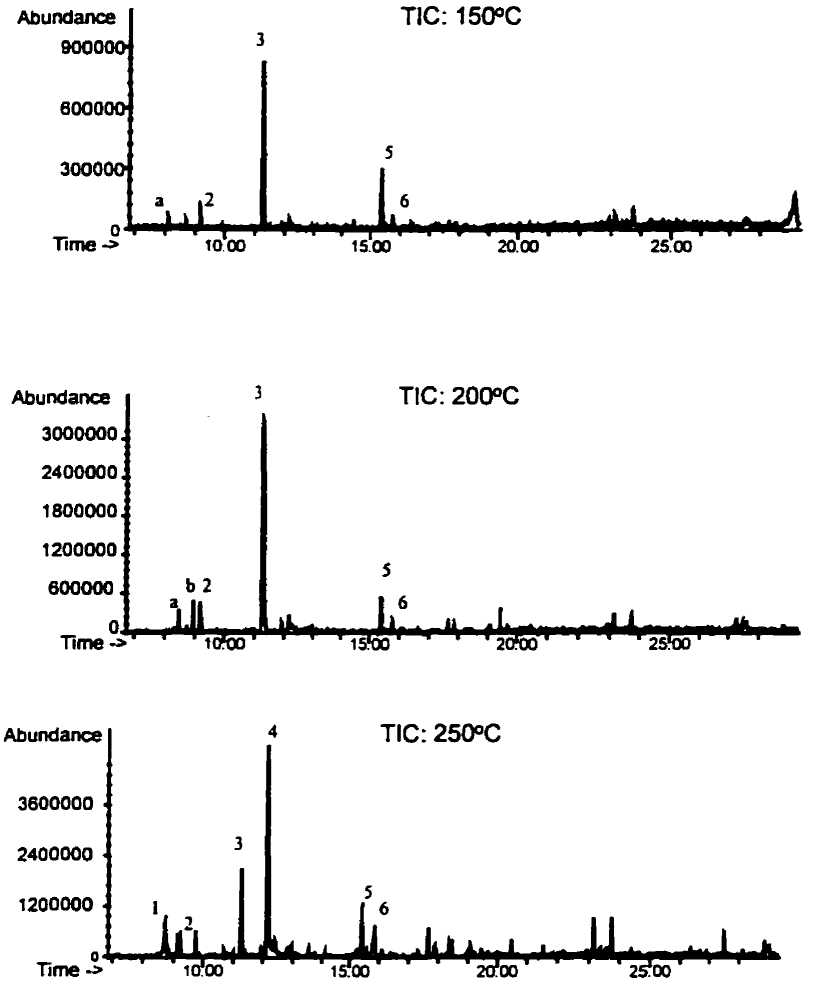


Figure 8.1. Pyrograms of Amadori methionine using the ribbon probe at different temperatures for 20s. *Peaks: (a)acetic acid; (b) 1-hydroxy-2-propanone;(1)1-(methylthio)propane; (2)dimethyldisulfide; (3)3-(methylthio)propanal;(4)3-(methylthio)propanal; (5)2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-(4)-one; (6)3-(methylthio)propanoic acid methyl ester*

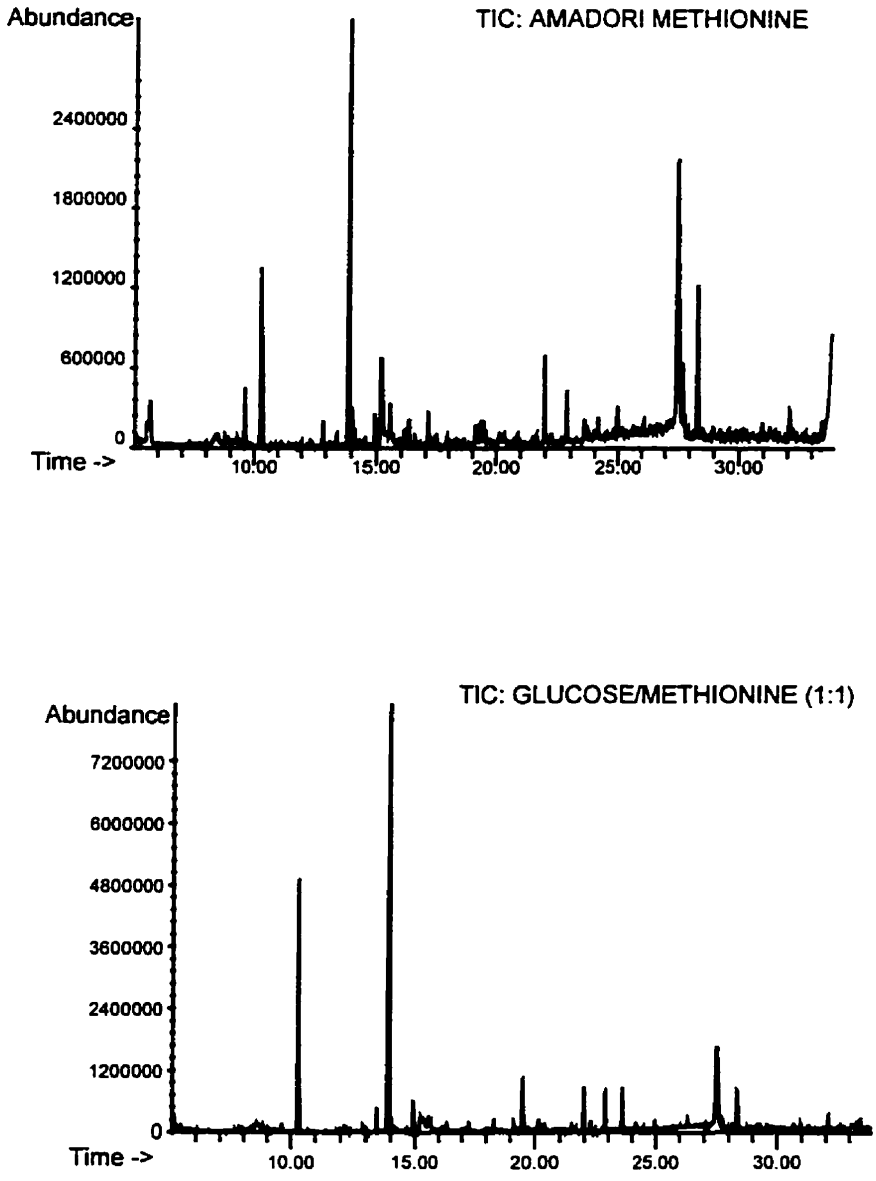
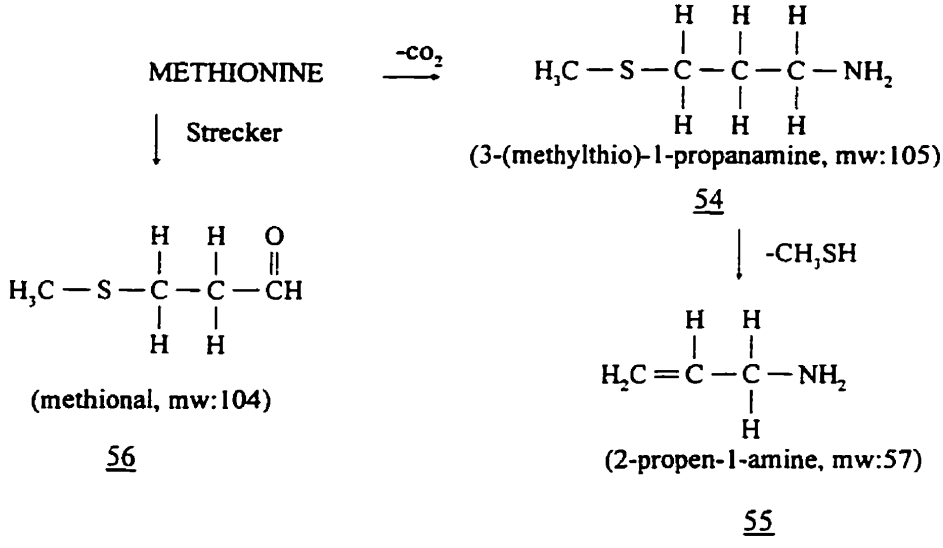
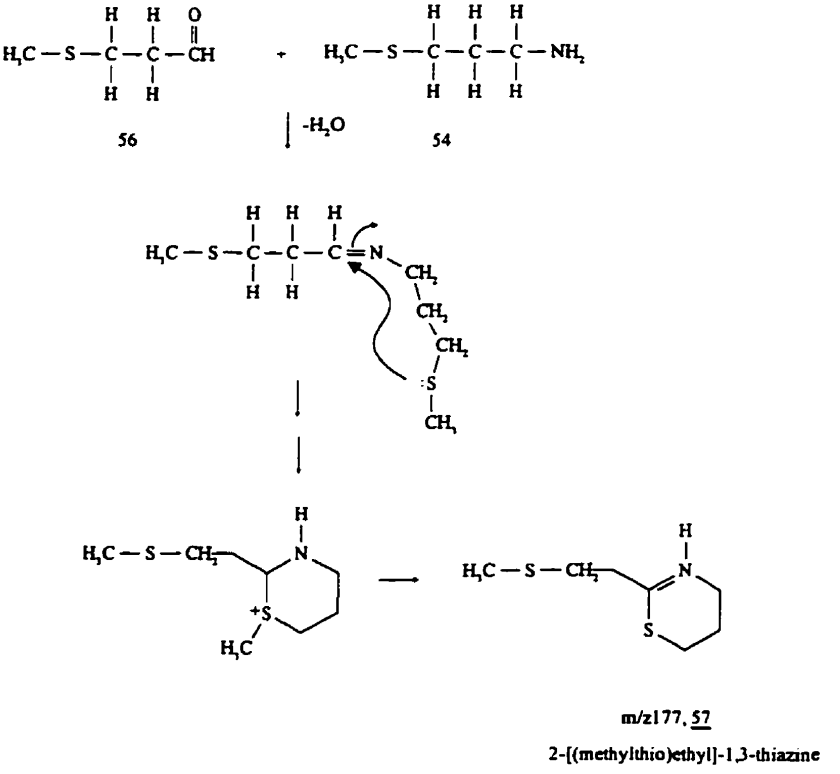


Figure 8.2. Pyrograms of Amadori methionine and an equimolar glucose/methionine mixture using the quartz tube at 250°C for 20s.



Scheme 8.1. Initial thermal degradation products of L-methionine

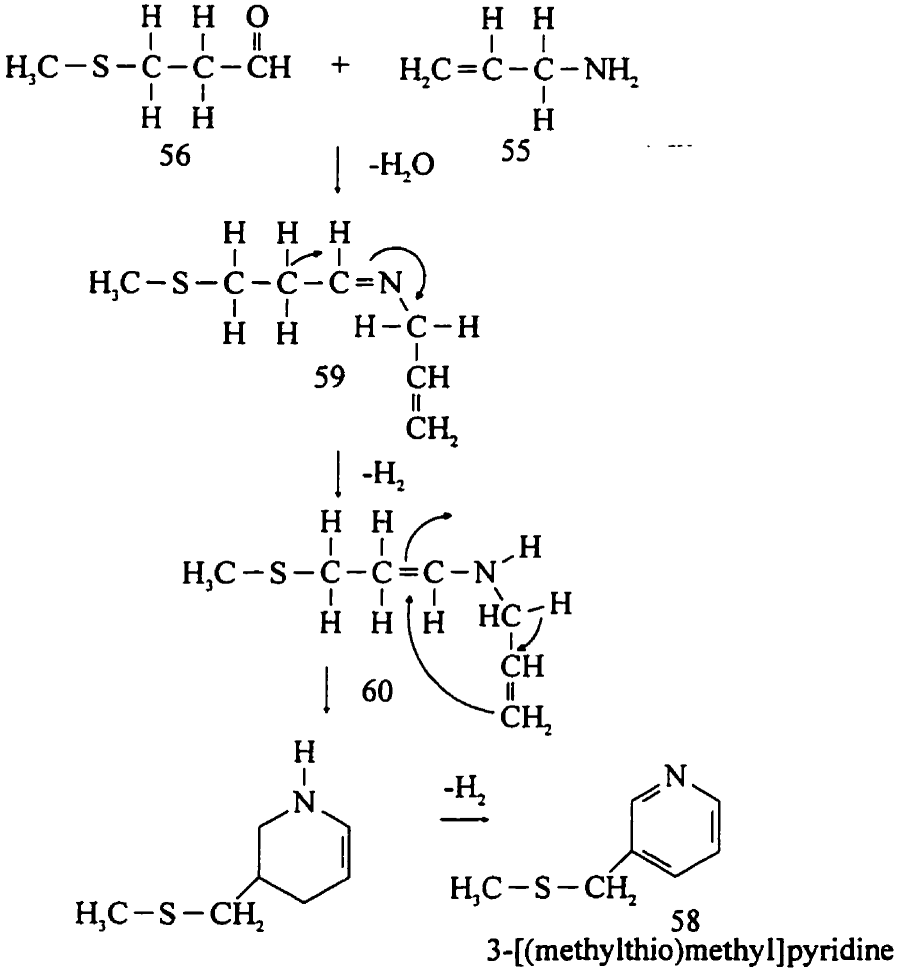
One of the main pyrolysis products of Amadori methionine and D-glucose/L-methionine systems was a compound with *m/z* 176 (Table 8.2). In an attempt to identify the structure of the unknown compound; L-methionine was pyrolyzed in the presence of labeled D-glucoses (1-^{13}C, 2-^{13}C and 5-^{13}C), none of the pyrograms indicated the incorporation of labeled carbons in the structure of compound 57. Based on these preliminary investigations and it mass spectral fragmentation pattern, compound 57 has been tentatively classified as, 2-[(methylthio)ethyl]-1,3-thiazine, a methional (56) and 3-(methythio)-1-propanamine (54) adduct. Scheme 8.2 demonstrates the proposed reaction mechanism between methional (56) and 3-(methythio)-1-propanamine (55) to produce 2-[(methylthio)ethyl]-1,3-thiazine (57).



Scheme 8.2. Proposed reaction mechanism for the formation of 2-[(methylthio)ethyl]-1,3-thiazine (57).

3-[(Methylthio)methyl]pyridine (58) was detected as a product of the thermal degradation of Amadori methionine at 250°C, 20s. The proposed reaction mechanism shown in Scheme 8.3, indicates a condensation between methional (56) and 2-propen-1-amine (55) to produce intermediate 59 which dehydrogenates to produce a triene 60 followed by thermally allowed electrocyclic ring closure to produce 3-[(methylthio)methyl]pyridine (58). 3-Phenylpyridine (19) and 3,5-diphenyl pyridine (21) detected in phenylalanine model systems (see chapter 4) are also proposed to require the formation of a similar triene intermediate (24) for their formation (Scheme 4.1).

Therefore, it is possible that substituted pyridine derivatives produced under Maillard reaction conditions require a common triene intermediate for their formation.



Scheme 8.3. Proposed reaction mechanism for the formation of 3-[(methylthio)methyl]pyridine (58).

8.3 Conclusion

Py/GC/MS analysis of L-methionine and Amadori methionine yielded product profiles similar to those reported by other investigators. Conducting a complete labeling study utilizing [^{13}C] D-glucoses and L-methionines and L-[^{15}N]methionine, to determine the main reaction routes under Maillard reaction conditions will be the subject of future investigations.

CHAPTER 9

CONCLUSIONS

The principal advantages gained by the utilization of the pyrolysis probe as a chemical reactor are the reduction in the analysis time (from hours to minutes) and elimination of the need for solvent extraction since the volatiles generated from the non-volatile precursors are directly transferred into the GC column. In addition, cooling the GC column to trap the pyrolysate at the head of the column enabled the separation and detection of small molecular weight compounds. Consequently, the resulting pyrograms can provide the Total Ion Chromatograms of all the intermediates and products produced by the thermal degradation of the model under study.

The use of enriched precursors and complete labeling studies are essential for the elucidation of reaction mechanisms. The role of amino acid carbons in pyrazine formation eluded investigators until the use of labeled precursors. The amount of reactants required for Py/GC/MS analysis is in the order of few milligrams, therefore facilitating mechanistic studies with expensive isotopically labeled reactants.

The mass spectra of unknown compounds in conjunction with labeling studies enabled the proposition of structures for the target compounds structure and the intermediates involved in their formation. This approach enabled the differentiation between two pathways of pyrazinone formation, one from the free amino acid and the other from its dipeptide.

The present investigation concentrated on specific compounds and classes of compounds to determine their reaction mechanisms and precursors. However, the pyrograms generated can be viewed as a rich database which needs only be analyzed, to extract further information regarding other classes of compounds. A priority should be given to complete the database with all $^{13}C/^{15}N$ amino acids and D-[^{13}C]glucoses. Once the data are analyzed for various classes of compounds, as demonstrated in our study with pyrazines, pyrroles and pyridines, the Maillard reaction could be classified in terms of the intermediates necessary for certain reaction pathways to predominate. This could lead the way for a "Rational Reaction Flavor Design" (similar to rational drug design being used in the pharmaceutical industry). The information could be used to predict the thermal flavor profile of a given set of precursors. While the use of pure amino acids in flavor

development may not be cost effective, the use of a hydrolyzed protein with a high alanine content for example, could be a low cost alternative to enhance pyrazine formation.

The effect of pH on the Maillard reaction products by addition of basic or acidic silica to the reactants could be investigated, using the same Py/GC/MS system.

Introduction of liquid samples into the system could be achieved through the use of the ribbon probe, however, to induce chemical reactions between two liquid samples, for example pyruvaldehyde and acetaldehyde, they could be absorbed on inert silica and inserted into the Quartz tube, however, homogenous binding onto the silica was not possible.

The development of the parameters for the use of the pyrolysis probe as a chemical reactor for the Maillard reaction, opens the door to study other chemical reactions that require the use of expensive reactants and/or labeled precursors.

APPENDICES

APPENDIX A

Table A.1. Information regarding amino acids utilized in this investigation

Amino Acid	Formula	MP °C	Amadori MP °C	Strecker Aldehyde
Glycine	$CH_2(NH_2)COOH$	240	157	Formaldehyde
Alanine	$CH_3CH(NH_2)COOH$	291	178	Acetaldehyde
Serine	$HOCH_2CH(NH_2)COOH$	222	110	Glycoaldehyde
Methionine	$CH_3SCH_2CH_2CH(NH_2)COOH$	280	100	Methional
Phenylalanine	$C_6H_5CH_2CH(NH_2)COOH$	270	143	Phenylacetaldehyde

APPENDIX B

Experiments with isotopically labeled precursors are essential in clarifying formation pathways of Maillard reaction products. However the cost of these compounds is prohibitive in conducting comprehensive studies. Table B.1 lists the cost of 500 mg of several labeled sugars and amino acids based on prices in the Cambridge Isotope Labs catalogue. Table B.2 lists recent published investigations utilizing labeled precursors. It is evident that cost is prohibitive in conducting comprehensive studies on a given model. The cost in the case of [^{13}C]-D-glucoses per model increases dramatically when [5-^{13}C]-D-glucose is used as compared to [1-^{13}C]-D-glucose. Therefore studies have utilized the labeled precursors which are relatively inexpensive. The results of the analysis are incomplete since the target compounds are extracted from the reaction mixture prior to GC/MS analysis. Therefore depending on the solvent and extraction procedure only a given class of compounds can be analyzed for in a given GC/MS run. This prohibits the tracing of intermediates incorporated in the target Maillard reaction product.

Py/GC/MS analysis of Maillard models utilizes at maximum 1-3 mg of labeled precursors. The analysis provides the complete profile of volatiles produced during a given time/temperature combination. The total ion chromatogram can be used to trace the genealogy of the atoms that make up a given structure. The pyrolyzer itself is a large fixed expenditure, however, its utilization with labeled sugars and amino acids and analysis of the resulting pyrograms can provide a comprehensive picture in determining the mechanistic events during the Maillard reaction.

Table B.1. Prices of 500 mg of enriched compounds as listed in Cambridge Isotope Labs catalogue (1996)

Compound	~$ Cost 500 mg
D-[1-^{13}C]glucose	260
D-[2-^{13}C]glucose	260
D-[3-^{13}C]glucose	870
D-[4-^{13}C]glucose	1000
D-[5-^{13}C]glucose	1100
D-[6-^{13}C]glucose	680
D-[1-^{13}C]fructose	60
D-[2-^{13}C]fructose	510
[1-^{13}C]-glycine	55
[2-^{13}C]-glycine	135
[^{15}N]-glycine	50
L-[1-^{13}C]alanine	165
L-[2-^{13}C]alanine	925
L-[3-^{13}C]alanine	180
DL-[1-^{13}C]serine	315
DL-[2-^{13}C]serine	895
DL-[2-^{13}C]serine	1740
L-[1-^{13}C]phenylalanine	135
L-[2-^{13}C]phenylalanine	1660

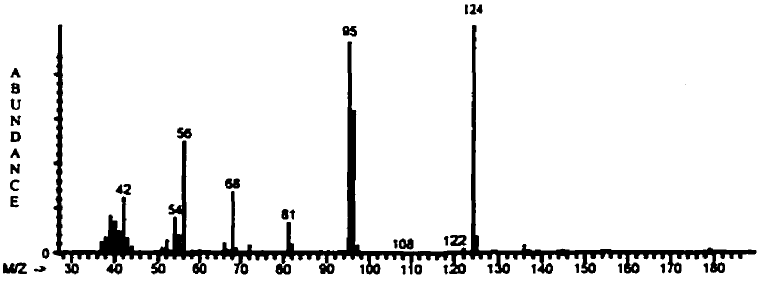
Table B.2. Approximate cost of enriched compounds utilized in one model in recent investigations.

Investigation	Model	~ $ Cost of Enriched Compound Utilized
Tressl et al., 1995	D-[1-^{13}C]glucose/L-isoleucine	131
Tressl et al., 1995	D-[1-^{13}C]fructose/L-isoleucine	140
Tressl et al., 1993c	D-[1-^{13}C]glucose/4-aminobutyric acid	131
Tressl et al., 1993	D-[1-^{13}C]glucose/proline	131
Tressl et al., 1993	D-[1-^{13}C]glucose/hydroxyproline	131
Hwang et al., 1994	D-glucose/[^{15}N]glycine	100
Amrani-Hemaimi et al., 1995	D-glucose/[2-^{13}C]-glycine	48
Amrani-Hemaimi et al., 1995	D-glucose/[3-^{13}C]alanine	60

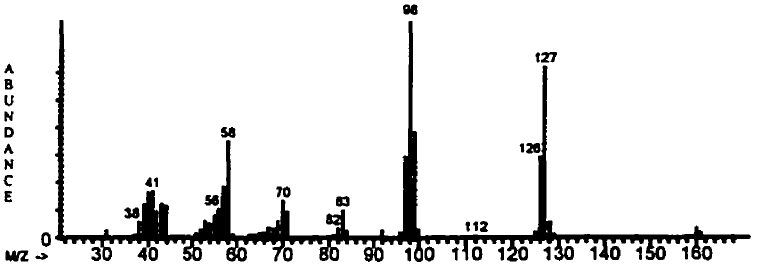
APPENDIX C

1. General procedure to calculate % label incorporation in multiply labeled ions

When a fragment ion or a molecular ion incorporates more than one ^{13}C labeled atoms, due to their formation through multiple mechanistic pathways (Figure C.1 and C.2), the following mathematical procedure could be used to calculate corrected abundances of the resulting isotopomers.



A



B

Figure C.1. A: mass spectrum of 1,6-dimethylpyrazinone produced from D-glucose/glycine model system. B: mass spectrum of 1,6-dimethylpyrazinone produced from D-glucose/[2-^{13}C]glycine.

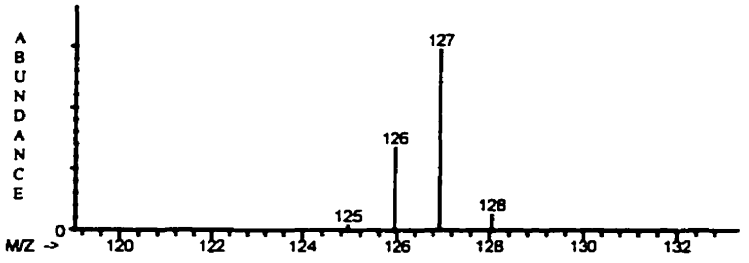


Figure C.2. Mass spectrum of 1,6-dimethylpyrazinone (From D-glucose/L-[2-^{13}C]glycine) showing m/z 120-132 region only.

For a given molecular ion exhibiting (n + 1) different isotopomeric ions, assume A^o_{M+n} (where n = integer) are the observed abundances and A^c_{M+n} are the corresponding corrected abundances.

For the lowest molecular ion, M (where n = 0), corrected abundance = observed abundance;

$$A^c_M = A^o_M$$

Consequently, for an ion at m/z (M + n), the corrected abundance is given by the following equation:

$$A^c_{M+n} = A^o_{M+n} - [(A_{M+1}/A_M) \times A^c_{M+(n-1)}]$$

Using the above equation sequentially, starting from A^c_M, all corrected abundances of the observed isotopomeric molecular ions can be calculated. The total abundance of corrected ions are given by:

$$A^c_T = \Sigma\ A^c_{M+n}.$$

The percentage of corrected abundances for each ion are given by the equation below:

$$\%\ A^c_{M+n} = [(A^c_{M+n}) / A^c_T] \times 100$$

2. General procedure to calculate % label incorporation in ions whose M-1 peak intensities are higher than M.

When M-1 peak is higher in intensity than the M peak (such as in aldehydes and some alkylsubstituted heterocyclic compounds such as pyrazines), the naturally abundant ^{13}C atoms of M-1 influences significantly the observed intensity of M ions in the normal spectrum (Figure B.3). Therefore in the labeled spectra each ion should be corrected for both natural abundance and M-1 interference. Ions, first can be corrected for M-1 interference due to ions one mass unit higher, and then for natural abundance due to ions smaller by one mass unit.

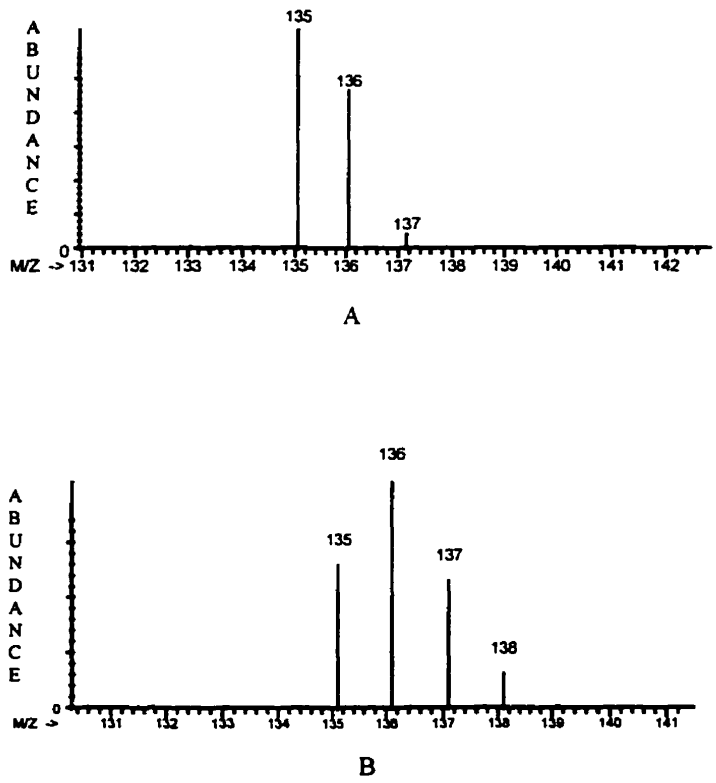


Figure C.3. (A) Mass spectrum of 2(3)-ethyl-3(2),5-dimethylpyrazine showing m/z 131-142 region (B) Mass spectrum of 2(3)-ethyl-3(2),5-dimethylpyrazine (From D-[2-^{13}C] glucose/L-alanine) showing m/z 131-142 region only.

In order to take into account for this interference during the calculation of corrected abundances in the labeled spectra, a correction factor F is calculated from normal spectrum, as follows:

F = abundance of M-1/ abundance M* (after correction for M-1 contribution of natural abundance, by using the number of carbon atoms multiplied by 0.011)

M* = [(M - 1) x (Carbon atoms x 0.011)]

Using the observed abundances from labeled spectra, the abundance of M can be corrected for interference from M-1 ion by the following formula:

$$R\,A^{\circ}_{M} = A^{\circ}_{M-1} / F = \text{corrected for interference of M-1}$$

For the highest ion (N) in the series M+n

$$R\,A^{\circ}_{N} = A^{\circ}_{N}$$

For the ions in between, the following general formula can be applied:

$$R\,A^{\circ}_{M+n} = \{(A^{\circ}_{M+(n-1)}) - (RA^{\circ}_{M+(n-1)})\} / F$$

Each interference corrected ion than can be corrected for natural abundance as shown below:

$$A^{c}_{M+n} = R\,A^{o}_{M+n} - (A_{M+1}/A_{M}) \times R\,A^{c}_{M+(n-1)})$$

$$\text{Total real corrected abundances} = R\,A^{c}_{T} = \Sigma\ A^{c}_{M+n}.$$

$$\%\ \text{label incorporation} = \%\ R\,A^{c}_{M+n} = [(A^{c}_{M+n}) / R\,A^{c}_{T}] \times 100$$

3. General procedure to calculate % label incorporation in singly labeled compounds using molecular ion

O = observed in labeled experiments, C = corrected values in labeled experiments, A = abundance, A with no superscript = absorbance from normal spectrum

Ratio of $(A_{M+1})/(A_M)$ from normal spectrum = total number of carbon atoms x 0.011

$$A^c_{M+1} = (A^o_{M+1}) - (A^o_M)(A_{M+1}/A_M)$$

$$\% \text{ Label incorporation} = \{(A^c_{M+1})/[(A^c_{M+1}) + (A^o_M)]\} \times 100$$

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IMAGE EVALUATION TEST TARGET (QA-3)

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