NOTE TO USERS

APPLICATIONS OF PY-GC/MS TO THE STUDY OF MAILLARD REACTION: MECHANISTIC AND FOOD QUALITY ASPECTS

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PY-GC/MS ANALYSIS OF MAILLARD REACTION

ABSTRACT

The ability of Pyrolysis-GC/MS analytical system - developed earlier to study the mechanism of Maillard reaction - was further enhanced to include chemical ionization, tandem mass spectrometry and a sample pre-concentration trap essential to detect minor components. Furthermore, the sample delivery during Py-GC/MS analysis was optimized and its relevance to the study of aqueous Maillard model systems was also characterized. The results of these investigations have indicated that the use of higher values of carrier gas flow rates or high constant pressures, during Py-GC/MS analysis can increase the total number of peaks and total area counts of the pyrograms. The influence of the reaction phase on the mechanism of formation of Maillard products was studied by comparison of ^{13}C-label incorporation patterns of the common products formed in model systems consisting of labeled glycine and D-glucoses subjected to both pyrolysis and heating in aqueous solutions. Although pyrolysis reaction produced higher number of products, however, the major pathways of formation of variety of important Maillard products followed the same mechanism under both pyrolytic and aqueous systems. Furthermore, the advantages of the optimized system, were demonstrated both in the investigation of the mechanism of Maillard reaction, using L-serine and L-threonine as model systems and in the study of formation of Maillard generated carcinogens and chemical markers. Analyses of the pyrolysis products of [^{13}C-1], [^{13}C-2] and [^{13}C-3]-labeled L-serines and L-threonines have indicated the presence of three initial degradation pathways. Decarboxylation followed by deamination; a retro-aldol reaction and dehydration followed by isomerization, deamination and hydrolysis leading to the formation of pyruvic and 2-ketobutanoic acids. Interestingly, the amino carbonyl

interaction between the resulting pyruvic acid and the aminoethanol can lead to the formation of an Amadori product identical in structure to that formed from alanine and glycolaldehyde, indicating the existence of another route to this important Maillard intermediate. This route was further confirmed through Py-GC/MS analysis using variously [^{13}C]-labeled reactants and by FTIR spectroscopy. In addition, the ability of the optimized Py-GC/MS system to investigate the detailed mechanism of formation of Maillard generated carcinogens such as acrylamide and utilization of Maillard generated products such as 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-one as chemical markers for prediction of process lethality, were also demonstrated.

RÉSUMÉ

Les capacités de la pyrolyse couplée avec la CG-SM (Py-CG/SM), un système analytique intégrale, développée précédemment pour l'étude des mécanismes de la réaction de Maillard, ont été étendues et améliorées en incluant l'ionisation chimique, la spectrométrie de Masse en tandem et un piège de pré-concentration des spécimens permettant la détection des composés minoritaires. De plus, la libération des produits durant l'analyse par pyrolyse a été optimisée et son son application à l'étude de la réaction de Maillard en milieu aqueux a été également caractérisée. Il a été montré que l'utilisation d'une vitesse de flux d'hélium importante ainsi qu'une pression constante élevée lors de l'analyse Py-CG/SM, peut augmenter significativement le nombre de produits détectés et leurs concentrations. L'effet de la phase de réaction sur les mécanismes de formation des produits de Maillard a été étudié par la comparaison de l'incorporation de ^{13}C dans les plus importants produits de la réaction parvenants du système modèle des réactifs isotopiques tels que la D-glucose et la glycine, soumis à une pyrolyse ou plus classiquement chauffés dans une solution aqueuse. Bien que la réaction de pyrolyse produise un nombre de composés plus élevé, les principaux mécanismes de formation d'une variété importante de produits de la réaction de Maillard restent similaires quelle que soit la phase de réaction. De plus, le système optimisé a démontré plusieurs avantages en étudiant les mécanismes de la réaction de Maillard impliquant la L-sérine et la L-theotrine, la formation de composés cancérigènes ansi que la variation des marqueurs chimiques par la réaction de Maillard. L'analyse des produits volatils obtenus par la pyrolyse des systèmes modèles comprenant L-serines et L-theothrines isotopes lourdes a indiqué la présence de trois principales voies de leurs dégradation: il s'agit de

la décarboxilation, suivie d'une désamination; d'une réaction rétro-aldol et une perte d'eau suivie d'une isomérisation et d'une hydrolyse menant à la formation d'acide pyruvique et 2-cétobutanoique. Les interactions amino-carbonyles entre l'acide pyruvique et l'aminoethanol peuvent mener à la formation d'un composé d'Amadori identique à celui formé par la réaction d'alanine et de glycolaldehyde, indiquant l'existence d'un autre schéma réactionnel pour cet important intermédiaire de Maillard. Ce schéma a été confirmé par Py-CG/SM en utilisant plusieurs réactifs isotopes lourdes et par spectroscopie FTIR. De plus les capacités du système intégral Py-CG/SM optimisé ont été démontrées lors de l'étude des mécanismes détaillées de formation de cancérigènes générés par la réaction de Maillard tels que l'acrylamide et lors de l'utilisation de produits de la réaction de Maillard tel que 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-one comme marqueurs pour la prédiction de l'effet de chaleur accumulée (lethality) en aliments pendant le processus technologique.

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

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CONTRIBUTION OF AUTHORS

This thesis is presented in manuscript format, and consists of seven chapters. A general opening in Chapter I introduces to the concept of pyrolysis and its advantages as a novel approach to the study of Maillard reaction in conjunction with gas chromatography-mass spectrometry. It establishes the research context, rational, objectives and significance of the proposed research. Chapter II, a literature review, provides with the concept of Maillard reaction, traditional perceptions and most recent advances in the field. Chapters III, IV, V and VI constitute the main body of the thesis and are drawn based on published manuscripts and conference presentations. Connecting paragraphs provide logical bridges between the different manuscripts and chapters. This dissertation is in accordance with the guidelines for thesis preparation as published by the Faculty of Graduate Studies and Research of McGill University.

The present author was responsible for the concepts, design of experiments, experimental work, and manuscript preparation. Dr. Varoujan A. Yaylayan, thesis supervisor, had direct advisory input into the work as it progressed and as manuscript co-author critically edited the dissertation prior to its submission. The manuscript titled "Formation of Sugar-Specific Reactive Intermediates from ^{13}C-Labeled L-Serines" was co-authored with Dr. Yaylayan and Dr. Keyhani, and present author was responsible for a significant amount of experimental work, discussion of experimental concepts and interpretation of results. Some of the experimental data presented in manuscript entitled "Why Asparagine Needs Carbohydrates to Generate Acrylamide" were provided by undergraduate student, Carolina Perez-Locas, working under direct supervision of present author. Dr. Yaylayan was the first author of the following manuscripts: "Role of L-serine and L-threonine in the Generation of Sugar-specific Reactive Intermediates during the Maillard Reaction" and "The Role of beta-Hydroxyamino Acids in the Maillard Reaction - Transamination Route to Amadori Products", and the present author's contribution as a co-author in the manuscripts preparation was to peform all experimental procedures, discuss the interpretation of results and critically evaluate the articles prior to publishing.

PUBLICATIONS

Wnorowski, A., and Yaylayan, V.A. Effect of Pressure Programming on the Efficiency of Sample Transfer during Pyrolysis-GC/MS Analysis of Glucose-Glycine Mixtures. Journal of Analytical and Applied Pyrolysis, 48(2), pp.77-91, 1999.

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Maillard reaction is one of the most widely studied reactions in food, due to its important technological and medical implications. Recent efforts have been aimed at elucidating the mechanism of the reaction which predominates in model systems. Matrix complexity, multiplicity of factors affecting the reaction pathways and difficulties in extracting the reactive intermediates makes it extremely difficult to study Maillard reaction in real food systems. As a result, much of the work on Maillard reaction has been carried out on model systems, where mixtures of single amino acid and sugar(s) or sugar fragment(s) have been studied under various thermal decomposition conditions. Such "simple" systems can still provide large number of Maillard reaction volatiles to postulate their formation mechanisms. Since the first proposal on application of model systems to study mechanistic pathways of Maillard reaction by Hodge (1953), it became a favoured and widely used procedure by different researchers. To the list of advantages of using model systems, Parliment (1989) includes (1) simplicity of the system with much lower number of generated volatiles than in heated foods, (2) easier isolation and identification due to higher amounts of generated products, (3) greater possibility of elucidating the reaction pathway if substrates are known and their number is limited, and finally (4) mimicking the Maillard reaction conditions in heated foods or in physiological systems allows monitoring of the reactions which otherwise could be impossible.

First studies with model systems initially focused on variables affecting type of formed aroma and its rate. Maillard (1912) and Hodge et al. (1972) postulated that the type of generated flavour depends strictly on used amino acids and is generated in the form of its

Strecker aldehyde. However, Lane and Nursten (1983) studied more than 400 mixtures and concluded that in addition to the amino acid used, heating temperature results in different flavour profile. Changes in time resulted in differences in the amount of formed compounds, however, other variables such as moisture content, nature of sugar and inclusion of third components did not show significant influence on the formed flavours. In studying serine and phenylalanine in model systems with various sugars under different conditions, Baltes and co-workers (1989) observed that the use of fructose instead of glucose gave 50% higher yield of pyrazines. In addition, the reaction medium did not significantly influence the number and kind of formed volatiles, and that the reaction temperature was the most decisive factor for formation of aroma compounds. Influence of the reaction medium on the mechanistic pathways of glucose-glycine Maillard reaction model system was also studied by Yaylayan and Wnorowski (2000) and further to above Baltes studies they showed that not only common compounds are formed under pyrolytic and aqueous conditions but also their formation followed the same mechanistic pathways. The conditions of studying model systems can be very diverse. The temperature ranges are between 20 to 250°C, time from seconds to weeks, and further addition of third components, changes in pH, water activity and use of buffers add further complexity to the factors influencing the reaction. Since the number of compounds formed can be down to few and high to thousands (Parliment, 1989) various isolation, detection and identification techniques can be used.

1.2 Pyrolysis-GC/MS-MS a Novel Approach to Study Maillard Reaction

Capillary GC/MS has been widely used for the analysis of flavour compounds derived from Maillard reaction model systems. The most popular isolation methods used to transfer the volatiles into GC system were static and dynamic headspace, direct injection and solvent extraction techniques. However, diverse physical and chemical properties of Maillard reaction products, such as hydrophilicity and hydrophobicity, high volatility and polarity, or presence of polymeric high molecular weight compounds, discriminate among the formed compounds and those that can be isolated and analysed with GC/MS system. The use of an integrated reaction-separation-identification system, Py-GC/MS, can substantially increase the range of analysed compounds.

1.2.1 Advantages of the Integrated Py-GC/MS System

The classical approach to study Maillard reaction involves factors such as long reaction time, low temperature, aqueous reaction conditions and need for extraction/concentration, although suitable for measurements of colour or end-products formation or disappearance of reactants, do not represent accurately the conditions of a heated food system. The Maillard reaction in foods occurs at higher temperature and lower moisture level. Furthermore, this type of analysis is labour intensive and time consuming, and solvent extraction could discriminate some of the products. The employment of Pyrolysis/Gas Chromatography/Mass Spectrometry (Py-GC/MS), as an integrated reaction, separation

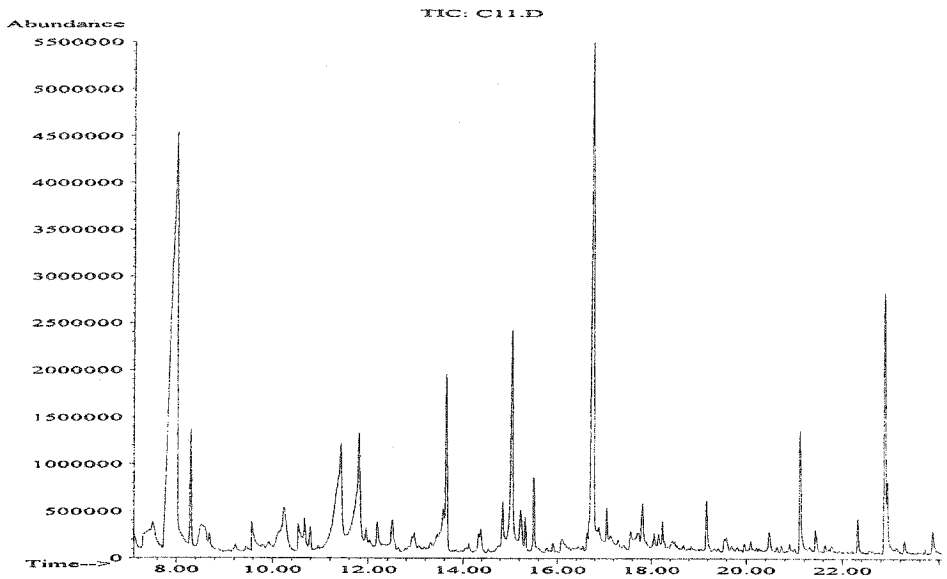


Figure 1.1 Pyrogram of Glycine/Glucose model system (pyrolyzed at 250°C for 20sec)

and identification system, not only accelerates the time of analysis to minutes, but also eliminates the need for extraction since the generated volatiles are directly transferred into the GC column. In addition, the amount of reactants are in order of 1 to 2 mg. This fact is of importance with respect to studies with expensive isotopic reactants or hard to obtain synthetic intermediates. The additional convenience and ease of pyrolysis stems from its integration with the analytical systems, such as gas chromatograph and mass spectrometer, and pyrograms obtained through Py-GC/MS can be used as fingerprints for the model systems (White et al., 2003). For example, Galletti et al., (1995) used fingerprint of pyrolyzed aspartame for fast and convenient screening of the sweetener in foods. Generally speaking, pyrolysis is the conversion of a sample into smaller fragments through application of heat (Irwin, 1982) in the absence of air. The pyrolysis may thermally extract intact molecules, but usually it results in thermal degradation leading into molecules of lower masses. However, due to intermolecular interactions it also can

produce products with increased molecular weights if the products are reactive. The optimum pyrolysis temperature for polymer analysis is determined from experiment as a temperature that produces the highest yield of monomers, and it ranges between 330-800°C (Peters, 1993). Pyrolysis begins by supplying sufficient thermal energy to break carbon-to-carbon bonds, then break down the polymer matrix into volatile, semivolatile and non-volatile components. Since bond dissociation always occurs in reproducible and predictable way under the same pyrolytic conditions, which is due to relative strengths of the bonds between the atoms, fragmentation pattern is representative of the original molecule (Yaylayan, 1999). Consequently, the same distribution of the smaller molecules will be produced each time an identical sample is heated in the same manner, and the resulting fragment will display the same information about the arrangement of the original molecule (Wampler, 1999). The resulting chromatograms are called pyrograms (Figure 1.1), which then will serve as fingerprint to identify molecules of the same model system.

A Py-GC/MS system consists of a pyrolyzer, a high resolution capillary column and a MS detector (Figure 1.2). As the sample is heated the reactants melt, degrade or react with each other to generate and initiate Maillard reaction products in the pyrolyzer oven, and consequently the volatiles formed are transported into the GC capillary column for separation. Separated compounds are analysed by mass spectrometer and identified based on the comparison of their fragmentation patterns with patterns in data stored in the Willey® and NIST® software libraries. The Py-GC/MS system gives flexibility to the study of Maillard reaction. Pyrolysis can be performed at various temperatures and carrier gas flow rates to efficiently transfer the sample into the column, and with different

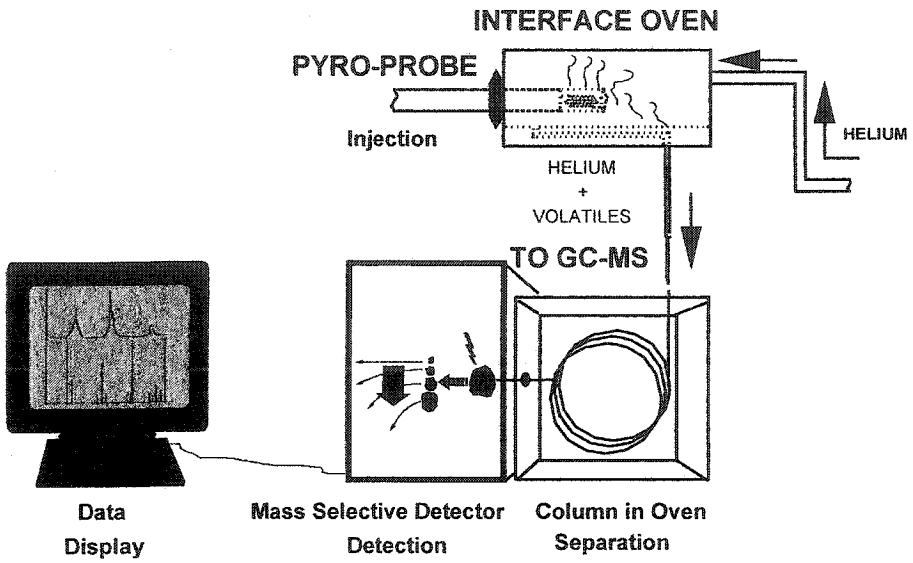


Figure 1.2 Schematic Diagram of a Pyrolysis-GC/MS System

separation programs. Different techniques of sample introduction, such as application of the sample on a platinum ribbon probe 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 advanced pyrolysis products. Less volatile pyrolysis products can be derivatized post-pyrolysis, through silylation or methylation, by replacing active hydrogens with derivatizing groups, but this technique requires an additional instrumentation (Moldoveanu, 2001). Py-GC/MS has been used so far to study precursors as well as degradation of primary and secondary products of Amadori compound (Huyghues-Despointes et al., 1994; Yaylayan and Keyhani, 1996; Keyhani and Yaylayan, 1996), generation and fate of reactive intermediates (Yaylayan et al., 1998), structural elucidation of melanoidins (Yaylayan and Kaminski, 1998), role of various forms of reactants on Maillard reaction (Yaylayan et al., 2003), or to elucidate the formation mechanisms of various groups of compounds generated from Maillard reaction (Yaylayan

and Keyhani, 2001; Yaylayan and Haffenden, 2003) through the use of labelled reactants. This technique provides specific advantages in the application of labelling techniques due to the requirement of only milligram quantities of the expensive isotopically labelled reactants (for more details on isotope labelling studies see appendix A).

1.3 Rationale and Objectives of Proposed Research

The multiplicity of factors influencing the different pathways of Maillard reaction and the complexity of the products formed, make the study of the detailed mechanism of Maillard reaction a difficult task. Pyrolysis coupled with GC/MS was proposed and consequently was demonstrated to be a convenient analytical tool to perform simultaneous generation, separation and identification of Maillard reaction products. The importance of this novel approach was further underlined due to its ability to perform isotope labelling studies with milligram quantities of expensive ^{13}C-labelled or ^{15}N-labelled starting materials. This efficient analytical system was utilized extensively to unravel the mechanistic pathways of aroma generation from model systems containing: L-proline, glycine, L-methionine and L-alanine. However, during these studies it was realized that the chemical interaction in the pyrolysis probe between the reducing sugars and amino acids occurs at the molten state and that the volatiles generated from this interaction can further interact in the gas phase during sample transfer from the pyrolysis interface to the top of the analytical column. Moreover, the ability of the Py-GC/MS integrated system to perform quantitative analysis was needed to be addressed and further tested. The above concerns have raised the issue of the influence of the variation of carrier gas pressure on the efficiency of sample delivery and of sample composition and its control through EPC

devices. More importantly, the criticism has been raised as to the relevance of the reaction mechanism occurring at the molten phase to that of aqueous systems. To investigate these concerns, the influence of carrier gas pressure, flow rates and different pulse sequences using EPC on the efficiency of sample transfer was investigated in chapter 2. In addition, design and use of a sample preconcentration trap using different adsorbents was investigated. In chapter 3, label incorporation patterns in Maillard reaction products, generated either pyrolytically or extracted from an aqueous phase were compared, in order to ascertain the existence if any, of differences between the mechanisms operating in the two different phases. In chapter 4, the advantages of the optimized conditions of Py-GC/MS was demonstrated in the study of Maillard reaction mechanism and in chapter 5 in the study of formation of Maillard generated carcinogens and chemical markers for prediction of process lethality.

1.4 Significance of the Proposed Research

The results of these studies will impact not only our understanding of the detailed mechanism of Maillard reaction but also analytical methodologies employed for its study. Controlling Maillard reaction, can facilitate aroma and flavour production or prevention of undesirable browning or generation of certain mutagenic or carcinogenic compounds. Understanding the pathways of Maillard reaction may help elucidate some of the *in vivo* processes of the reaction during ageing, cataract formation or in diabetic patients. The interest in L-serine and its analogue L-threonine results from the fact that during thermal decomposition it has the ability to form sugar-specific reactive intermediates in the absence of sugars, in addition to amino acid degradation products. Thus low-sugar foods

can be fortified with serine or threonine in order to enhance the desired characteristics of the Maillard reaction.

CHAPTER 2

LITERATURE REVIEW

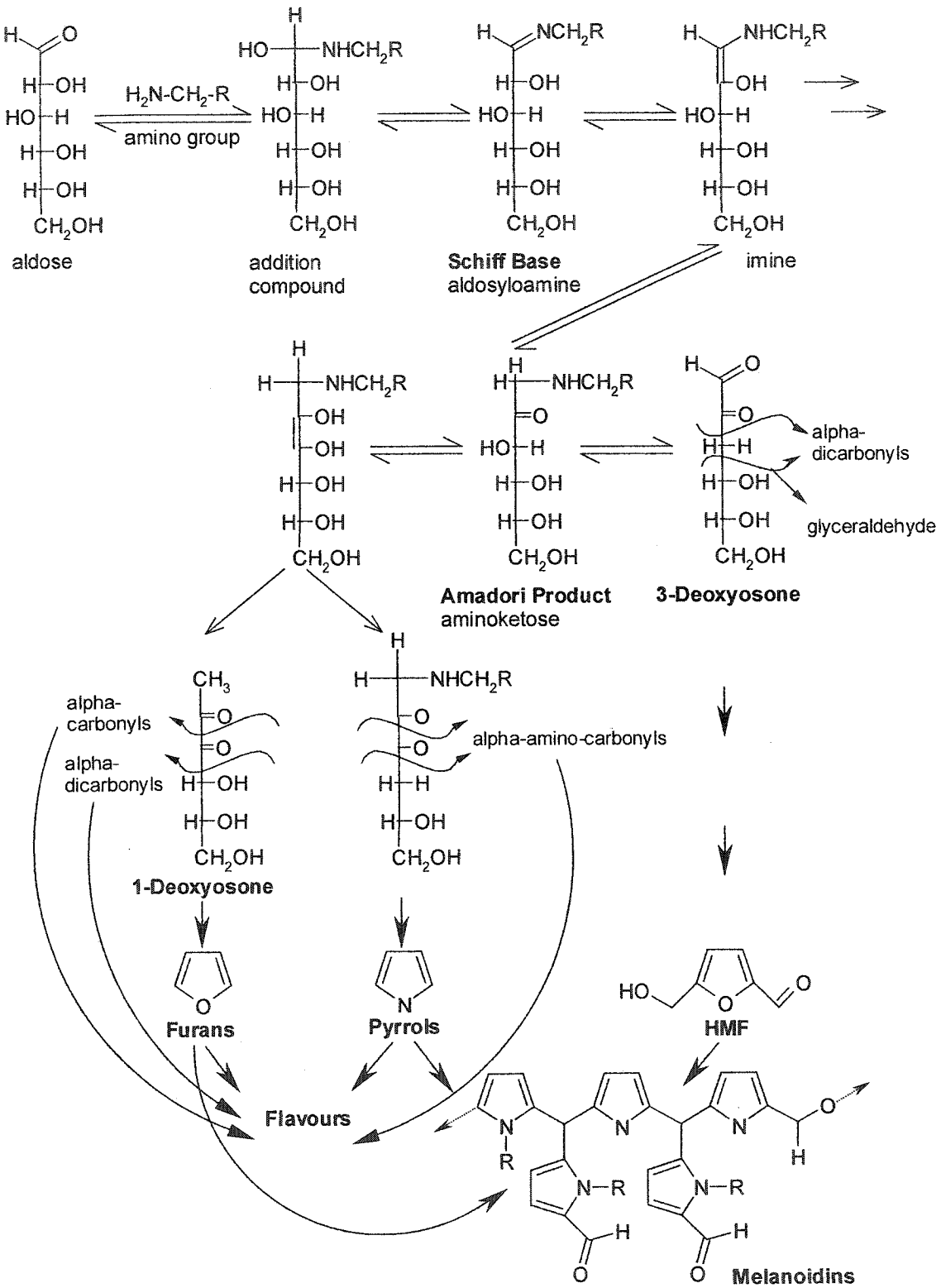
2.1 General Introduction

Maillard reaction has been intriguing researchers for almost a century. It is perceived as proceeding through complex reaction pathways, where the starting materials are carbonyl and amine moieties and final products are aroma compounds and brown melanoidins. The reaction is believed to proceed through enolization, dehydration, fragmentation, condensation, cyclization and reduction-oxidation reactions. Developments in analytical instrumentation during the last fifty years allowed more detailed study of initial Maillard reaction. Advanced and final stages of the reaction, however, are still incompletely understood. Due to the overwhelming impact of the reaction on food characteristics and quality, such as colour, flavour, texture, formation of antioxidants and antinutritional substances, studying its mechanistic pathways is of utmost interest to food chemists. Occurrence of the Maillard reaction in physiological systems and its impact on ageing, diabetes and cataract formation further underlines the importance of its study.

2.2 Historical Background

2.2.1 Traditional Perception of Maillard Reaction

The interaction of amino acids with sugars was first described in 1912 by Louis-Camille Maillard (Maillard, 1912). He observed that reducing sugars react with amino acids to form brown substances and aromas, and that the type of generated aroma depends on specific amino acid used. The first explanation of the nature and the mechanism of the sugar-amino acid condensation in initial step of the Maillard reaction was published by Hodge (1953), and practically it is still authoritative today.



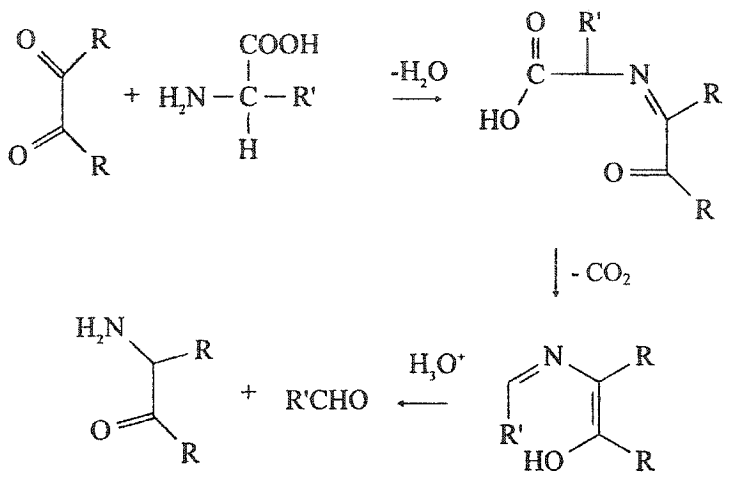
Scheme 2.1 Summary of Maillard Reaction

In relation to flavour formation, the Maillard reaction can be divided into three stages: initial, advanced and final.

2.2.2 Reaction Stages

Hodge proposed that the reaction starts by nucleophillic attack of the amino group of amino acid, peptide or protein on the carbonyl group of open chain form of reducing sugar (Scheme 2.1). Firstly, a Schiff base is formed, but due to its unstability it undergoes rearrangement into 1-amino-1-deoxy-D-fructose, which is known as Amadori Product, if glucose is used as a precursor. If fructose is used, 2-amino-2-deoxy-D-glucose will be formed, which is known as Heyn's Product. Amadori or Heyn's Products do not impart colour nor flavour, however upon further degradation they produce intermediates of over 500 flavour compounds.

Both the Amadori and the Heyn's products undergo further transformations to yield very reactive α-dicarbonyl compounds known as deoxyosones, which upon further degradation yield C_5, C_4, C_3, and C_2 α-dicarbonyl and hydroxycarbonyls (Scheme 2.1). Consequently, those reactive α-dicarbonyls can further react in Strecker degradation reactions with α-amino acids to form Schiff bases (Kobayashi and Fujimaki, 1965), which are decarboxylated and hydrolysed into α-amino carbonyls and Strecker aldehydes (Scheme 2.2). Strecker aldehyde incorporates the side chain of the original amino acid, and is considered an important flavour compound (Hodge and Mills, 1972). It can also participate in final stages of melanoidin production. α-Amino carbonyls, on the other hand, are precursors of many important flavour compounds, such as pyrazines.



Scheme 2. 2. Mechanism of Strecker Degradation of Amino Acids

In the final stage of Maillard reaction melanoidins, brown polymeric pigments are formed. Formation of melanoidins has been postulated to occur by polymerisation of various unsaturated carbonyl compounds. Condensation between 3-deoxyglucosulose and its enamine (a reductone) has been suggested as a basis for polymerization (Kato and Tsuchida, 1981; Kato et al., 1986), although units of furan and pyrrole have also been reported (Hayase, 1996). Very little is known about the formation mechanisms, structure and properties of these high molecular weight substances: some are more or less water soluble with at least 7000 Da molecular weight.

Experiments with ^{13}C- and ^{15}N-labeled sugars and/or amino acids have shown that the carboxy group of the amino acid survives in part in the melanoidin and that the signal of the α-carbon largely remains in position in the melanoidin spectrum where it appears in the amino acid spectrum (Kim et al., 1984; Kato et al., 1985). The same authors confirmed that signals from the C-1 atom of sugar can be found in all parts of the

spectrum of the melanoidin polymer. Hayase (1986), also based on analysis of glucose-glycine melanoidins enriched with ^{13}C or ^{15}N by ^{13}C- and ^{15}N-P-MAS NMR, identified six major regions in melanoidins spectrum: saturated carbons joined to carbon or nitrogen atoms, or methyl carbons, saturated carbons bound with oxygen or nitrogen, unsaturated or aromatic carbons and amide or carboxyl carbons and carbonyl carbons. The author deduced that the saturated and aliphatic carbons form the backbone of melanoidins.

Generation of C_6-, C_5-, and C_4-pyrroles and furans, as proposed crucial intermediates in melanoidins formation, from intact sugar molecules, had been reported by Tressel et al. (1998) (Scheme 2.1). By employing ^{13}C-labeled starting materials, the authors reasoned that the two generated types of melanoidins are either polycondensation products of 15-25 bridged pyrroles or furans, to form linear type I melanoidins, or up to 30 bridged pyrroles along furan nucleus, to form branched type II melanoidins. Confirming above, Caemmerer et al. (2002) reported that in the Maillard reaction under water-deficient conditions, a notable amount of intact di- and oligomeric carbohydrates are incorporated into the melanoidin back-bone with intact glycosidic bond, forming side chains at the melanoidin skeleton. However, as the moisture content increased, hydrothermolytic as well as retro-aldol reactions of the starting carbonyl components prevailed, and side chains formation decreased. Thermal degradation of various model melanoidins by Adams et al. (2003) has given characteristic profiles varying in quantity and quality depending on used carbonyl and amine sources.

The melanoidin-colour formation upon carbonyl-amino acid interaction is so important that often used synonym for Maillard reaction is the non-enzymatic browning. Thus, elucidation of the mechanism of melanoidin formation is of utmost interest to food scientists in order to control/prevent the extent of browning in baked or roasted foods.

The initial stage of the Maillard reaction is well studied and its developments can be viewed through many reviews (Danehy, 1986; Kato, 1968; Mauron, 1981; Nursten, 1981; Yaylayan, 1997 and 2003, Gerrard, 2002; Schieberle and Hofmann, 2002; Tsai et al., 2002), books (Ikan, 1996; Finot, 1990; O'Brian et al., 1998; Fayle and Gerrard, 2002) and symposium series (Waller and Feather, 1983; Fujimaki, 1986; Parliment et al., 1989; Labuza et al., 1994, Tressl, 1994; Tressl and Rewicki, 1999, Nursten, 2000). In recent years scientific literature has reported many newly isolated Maillard reaction products that can serve as chemical markers of the extend of the Maillard reaction in foods or in biological systems. Their formation mechanisms however, originating from advanced stages of Maillard reaction, often remain vaguely understood and unclear.

2.3 Recent Advances in Maillard Reaction - Maillard Reaction Products

2.3.1. Reactive Dicarbonyls from Carbohydrates and Amadori Rearrangement

The knowledge of advanced and final stages of Maillard reaction still remains incomplete. The fact that the reaction represents a series of parallel and consecutive reactions which produce a complex matrix of products, which in turn can be further used as reactants, makes it a difficult task to deal with all the aspects of the reaction. The use of model systems, a mixture of an amino acid with sugar(s), can simulate the Maillard reaction in foods or physiological systems in a controllable manner and thus has found wide applicability in studies of Maillard reaction under various conditions. Similarly, synthesis of Amadori Product would provide deeper insight into circumstances of its formation and rearrangement into intermediate phase of Maillard reaction network, thus facilitating understanding and controlling of flavour and browning generation or protein glycation and its prevention *in vivo*.

2.3.1.1 Characterisation of Amadori Product and its Rearrangement

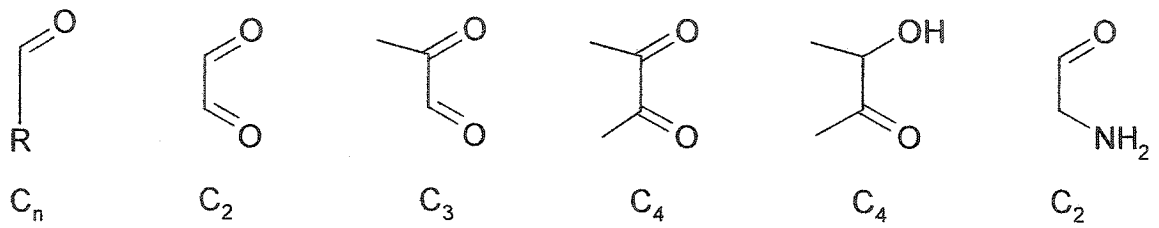
The difficulties in studying degradation of Amadori products result from commercial unavailability of Amadori products and deficiency in adequate methods for their preparation (Shigematsu et al., 1977; Birch et al., 1984). The factors interfering with the synthesis attend from chemical properties of reducing sugars, such as mutarotation, oxidation and acid- or base-catalysed transformations. Nevertheless, many successful attempts to synthesise and analyse degradation of Amadori Products have been reported (Vernin et al., 1988; Yaylayan and Sporns, 1989; Wrodnig and Eder, 2001; Manini et al.,

2001). An extensive review on synthesis methods using free and protected sugars and the analysis methods was presented by Yaylayan and Huyghues-Despointes (1994).

Many studies published recently shed some light on the advanced phase of the Maillard reaction. Based on the collective data, it has been postulated that the direction of advanced stage of Maillard reaction has been governed by Amadori rearrangement. Thus, understanding the chemistry of Amadori rearrangement provides tools to our understanding and controlling advanced stages of this reaction cascade.

Degradation of glycine-glucose Amadori product as a function of time and pH was recently investigated by Davidek et al., (2002). The authors observed that Amadori product released its parent sugar and amino acid faster as the pH increased, particularly at pH 5-7 in water with phosphate buffer. Although at higher pH values there was more amino acid regeneration, sugar liberation decreased due to its immediate degradation similarly to Amadori product that degraded to carbonyls rather than to whole sugar under these conditions. Enhanced degradation of Amadori product in the presence of phosphate buffer was attributed to the buffer ability to abstracting a proton and thus acting as a catalytic base.

Yaylayan, in his review (2003) discussed the impact of Amadori rearrangement products on Maillard reaction. Based on the published data, he compared the influence of Strecker degradation product and Amadori rearrangement product on overall aroma and colour formation during Maillard reaction. Accordingly, although the consequence of both



Scheme 2.3 Carbonyl, Dicarbonyl and α-Hydroxy Carbonyl Compounds

pathways is reductive amination of various sugar fragments by primary amine, Strecker degradation generates α-amino ketones and Strecker aldehyde, directly involved in aromagenesis, and reduces α-dicarbonyls implicated in browning. Conversely, Amadori rearrangement of amino acids with α-hydroxycarbonyls, can contribute to chromagenesis by ionic and free radical condensation pathways through generating an imino-ketose or α-dicarbonyls, and Amadori product of ammonia contributes to aromagenesis through producing α-amino carbonyls. The author concludes that C_3 and C_4 α-dicarbonyls condense much faster to form pigments than their counterpart α-hydroxycarbonyls, which are more prompt to undergo reductive amination to generate aromas.

2.3.1.2 Characterisation of Deoxyosones and their Rearrangement

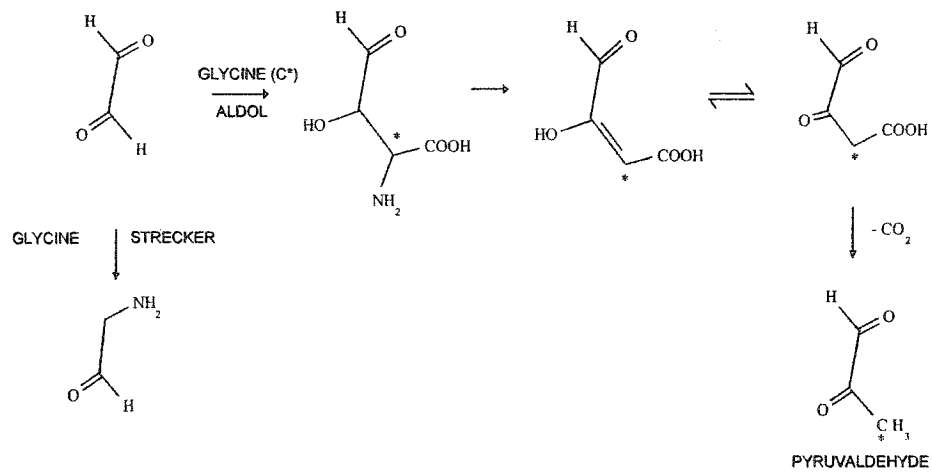
At the present state of knowledge of Amadori rearrangement, it is well accepted that Amadori product undergoes degradation reactions by several pathways including enolization, dehydration, aldol condensation and Strecker degradation to form a large number of products, and that these pathways are pH dependent (Yaylayan and Huyghues-Despointes, 1994; Davidek et al., 2002; Yaylayan, 2003). At acidic pH, the Amadori product tends to undergo 1-2 enolization to form 3-deoxyglucosone, after an elimination of the amino moiety (Scheme 2.1). While at basic pH, it follows 2,3-enolization to form

1-deoxyglucosone and 1-amino-1,4-dideoxy-2,3-dicarbonyl derivative. Boekel (2001), in his critical review on kinetic aspects of Maillard reaction, states that Amadori rearrangement represents complex kinetics due to the regeneration of amino group released from Amadori product and reacting further with available carbonyls. This regeneration is less apparent at higher temperatures and pH values, as more carbonyls from fragmentation of sugar moiety is carrying on the advanced Maillard reaction and amino acids are much faster incorporated in Strecker aldehyde and melanoidins formation (Boekel, 2001; Davidek et al., 2002).

Detailed studies on deoxyosone formation and decomposition have revealed that they can be formed upon sugar decomposition as well as during degradation of Amadori Product (Feather and Harris, 1973; Yaylayan and Huyghues-Despointes, 1994; Hofmann and Schieberle, 2000), and that they are not products of Amadori oxidation (Wells-Knecht et al., 1995), which would rather lead to corresponding Strecker aldehyde (without α-dicarbonyls presence). 3-Deoxyglucosone, a reactive α-dicarbonyl, is thought to lead to formation of 5-(hydroxymethyl)-2-furaldehyde (HMF), a block unit of brown pigment melanoidins. 1-Deoxyglucosone, on the other hand, contributes to flavour and aroma production through intermediate formation of furanones and pyrans. No products of 1-amino-1,4-dideoxy-2,3-dicarbonyl degradation have been found, but the product can further react and form different heterocyclic compounds.

2.3.1.3 Characterisation of Reactive Intermediates

The Amadori products, deoxyosones, sugar derivatives as well as products of autoxidation of sugars and lipids and Strecker degradation reactions can undergo retro-aldol reactions, especially under basic conditions, to produce C_2, C_3 and C_4 sugar fragments containing α-dicarbonyls that can react more efficiently with amino acids than sugars (Yaylayan et al., 1998; Glomb and Tschirnich, 2001; Gerrard, 2002). Because of their high reactivity towards amines, α-hydroxy carbonyl, carbonyl and α-dicarbonyl compounds generated in advanced stages of Maillard reaction play a crucial role in the overall reaction leading to the generation of many significant flavour compounds (Feather, 1994; Weenen and Van der Ven, 2001), and *in vivo* protein glycation (Miller et al., 2003). Some of the most common sugar fragments containing α-dicarbonyls are presented in Scheme 2.3, and examples include glyoxal (C_2), pyruvaldehyde (C_3) and 2,3-butanedione (C_4).



Scheme 2.4 Carbon Chain Elongation (through aldol condensation)

Another interesting and important characteristic of dicarbonyls is their ability to undergo chain elongation reactions. It was only recently reported that amino acids not only act as

N-nucleophiles, but can be also involved in carbon chain elongation of sugar fragments as electrophiles (Keyhani and Yaylayan, 1996), thus contributing to the "sugar fragmentation pool". The results of this study clearly showed that amino acids, by interaction with an α-ketoaldehydes, can transform them into α-diketones, resulting in the alkylation and subsequent chain elongation (Scheme 2.4). This discovery was possible only as a result of application of PYR-GC/MS as a chemical reactor combined with isotopic studies.

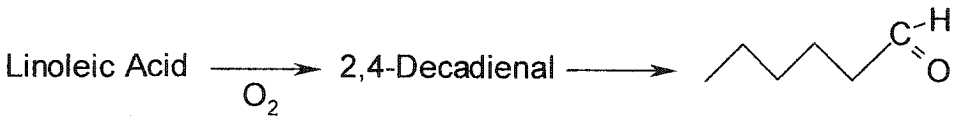
The significance of α-dicarbonyls and α-hydroxy carbonyls was likewise underlined through further study (Yaylayan and Haffenden, 2003) where the authors showed their crucial role in pyrazole formation. The authors proposed that α-hydroxy carbonyls upon dehydration and elimination generate β-diketones, serving as backbone for pyrazoles and that their formation mechanism through oxidation is assisted by α-dicarbonyls.

2.4 Autoxidation of Food Constituents and Resulting Participation in Maillard Flavour Formation

A common characteristic of the most reactive carbohydrates and lipids is their susceptibility to oxidation, consequently, food chemists have recognised that control of the Maillard reaction requires control of both, the oxidation of lipids and carbohydrates. Initiation of the oxidation of either carbohydrates or lipids stimulates the oxidation of the other by generating reactive oxygen species.

2.4.1 Lipids Autoxidation

Numerous studies have shown that flavour of processed foods originates not only from interaction of sugars with amino acids but also from the presence of the degradation products of lipids (aldehydes, ketones, alcohols and acids) and through their participation in Maillard reaction (Mottram and Edwards, 1983; Whitefield, 1992; Gandemer, 1999; Mottram and Elmore, 2002). These degradation products can be formed through hydrolysis, oxidation and thermal decomposition of lipids. Among those, carbonyl compounds originating from lipid oxidation contribute most to the generation of Maillard reaction flavours.

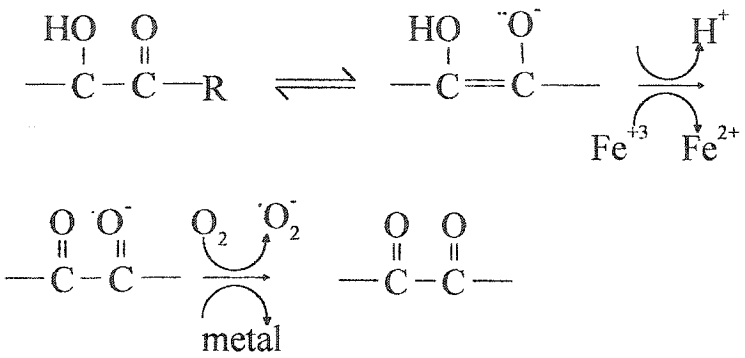


Scheme 2.5 Lipids Oxidation and Formation of Carbonyl Intermediates (Ho, 1996)

Scheme 2.5 shows a general pathway of oxidation of fatty acids. As the fatty acids are released from lipids, they are degraded to hydroperoxides, and then to corresponding aldehydes. Ho (1996) showed that 2,4-decdienal and its retro-aldolization product, hexanal, react directly with Maillard reaction intermediates to form heterocyclic compounds.

2.4.2 Carbohydrates Autoxidation

Similarly, sugars (Scheme 2.6) under specific conditions and in presence of oxygen and transition metals, can be oxidized to dicarbonyls (Yaylayan and Huyghues-Despointes, 1994). Both reactive species, aldehydes from lipids and dicarbonyls from sugars, are



Scheme 2.6 Carbohydrate Oxidation and Formation of Dicarbonyl Intermediates (Yaylayan and Huyghues-Despointes, 1994)

active intermediates in reactions with amines through Strecker reaction to form flavour compounds or to cyclize, dehydrate and polymerize to form brown melanoidins. Most common flavour compounds originating from lipid-Maillard reaction interaction are pyridines, thiazoles, oxazoles, triothiolanes and pyrazines (Tang et al., 1983; Carlin et al., 1986).

2.4.3 Autoxidation *in vivo*

Sugar and lipid oxidative fragmentation does not occur in foods only, it is also believed to play a big role in metal-catalysed protein autoxidative glycation *in vivo* (Brownlee and Cerami, 1981; Curtiss and Witztum, 1985; Mullarkey et al., 1990; Wells-Knecht et al., 1995; Linton et al., 2001). Studies of glycated proteins by oxidation products of carbohydrates have shown exactly same composition of the amino-carbonyl adduct as in the case of Amadori adduct to a protein, with at least 45% contribution to the total protein glycation (Wold and Dean, 1987). In addition, carbonyl-amine adduct formation through carbonyls generated from lipid peroxidation, was accelerated by 25 fold compared to carbohydrate glycoxidation pathway, implying that polyunsaturated fatty acids (PUFA)

could be the major source of protein glycoxidation *in vivo* even during hyperglycemia (Fu et al., 1996). Moreover, it had also been observed, that Amadori product of a protein, is able to enolize and autoxidize in a similar fashion as its parent sugar molecule (Gascoyne, 1980; Fu et al., 1996), consequently carrying on further oxidative Maillard reaction and protein damage.

2.5 Factors Affecting the Maillard Reaction

The complexity of Maillard reaction results not only from the fact that literally thousands of reactions and compounds are involved but also due to the susceptibility of formation pathways to various factors such as temperature and time of heating, concentration and nature of reactants, water activity, pH, oxygen, exercised pressure and finally presence of catalysts and inhibitors. Again, due to the overwhelming complexity of the Maillard reaction most, if not all, of the information available on effects of various factors on the reaction pathways relates to observations of heated model systems.

2.5.1 Temperature, Time and Activation Energy Effect

The temperature and duration of the heating are considered by many researchers the critical key factors in the formation of flavour compounds and colour (Baltes et al., 1989). An increase of both would increase the yield of Maillard reaction products for up to six fold (Ledl and Schleicher, 1990). In his review on chemical analysis of deterioration of foods, Labuza (1984) showed that for non-enzymatic browning Q_{10}, an increase in rate for every 10°C increase in temperature, ranges from 2 to 8. Analysis of kinetic data of formation of some Maillard reaction products also shows a wide range of activation

energy, 10-160 kJ/mole (Lingnert, 1990). Reineccius (1990) concluded that lower temperature cannot produce the same flavour profile and that time influences only the amount of each flavour constituent. Application of higher temperature will then consequently result in flavour profile changes, favouring the formation of high activation energy compounds, such as alkyl-pyrazines and pyridines (Leahy and Reineccius, 1989). An increase of the reaction temperature can also selectively change mechanistic pathway of formation of the same compound (Huyghues-Despointes et al., 1994; Cerny and Davidek, 2003). Moreover, generated labile compounds will decompose faster and become substrates for heat resistant flavour constituents.

To underline the complexity of the interdependence of many factors, Eichner and co-workers (1985) showed that formation energy can be increased by lower water activities. Lee and co-workers (1984) observed changes in activation energy as a function of pH between different stages of the Maillard reaction such as melanoidins and Amadori product formations. They concluded that as the reaction temperature rises, the pH influence diminishes. The temperature of the Maillard reaction is also influenced by the participating reactants. Holmes (1970) reported differences in temperature sensitivity between different amino acids.

2.5.2 Moisture Content Effect

Moisture content has a significant effect on the quantity and quality of Maillard reaction products formed in heated model systems (Reineccius, 1990). It plays a multiple role and critically impacts the browning rate by acting as a solvent for reactants, a plasticizer for

matrix mobility and a chemical reactant (Sherwin and Labuza, 2003). It is generally recognized that the reaction climax is at water activity of 0.3-0.8 depending on the reaction system. For example, Shu and Ho (1989) showed that water content has a pronounced effect on the yield of the reaction of cystine and hydroxy-furanone (maximum at 75% water content), and influenced the formation selectivity of the flavouring compounds, i.e. formation of thiazoles decreased but hexanedione and hydroxy-pentanone increased as water content increased. To go further, using labeled studies and Py-GC/MS technique, Wnorowski and Yaylayan (2000) showed that there is no significant difference in the mechanism of formation of the common Maillard products between aqueous phase reactions compared with molten or dry phase.

The effect of water is complex and depends not only on the water content and its activity but also on the state of bound water, mobility of the reactants, presence of water-binding agents, etc. Based on the research conducted on dry foods, Kaanane and Labuza (1989) concluded that optimum generation of Maillard reaction products could be achieved by adjusting the ratio of product moisture to the monolayer moisture content to unity. Below this value, solute mobility is greatly limited and above it, due to solute dilution and inhibitory effect of water generated through dehydration, the reaction rate drops.

2.5.3 Type of Substrates Effect

The influence of the type of sugar and amino acid in the system on the flavour and colour formation was studied by many workers. The generalisation of the results allows to conclude that sugar has less influence on sensory properties of the final mixture than

amino acid. The sugar choice, on the other hand, dictates the total amount of the flavour formed. Higher reactivity of aldopentoses, compared to aldohexoses or even reducing disacharides, results from the higher concentration of open-chain form, increasing availability of carbonyl groups for nucleophillic attack of amines. While monitoring reducing properties of Maillard reaction, Rizzi (2003) confirmed higher reactivity of pentoses by producing more electrochemical activity than hexoses. Yeboah et al., (1999) however, suggest that although ketoses are characterized by higher concentration of open-chain forms, but still aldoses are more reactive because initial kinetics of glycation depends rather on electrophilicity of carbonyl to nitrogen source, then on proportion of open form.

It is well known that during Maillard reaction amino acids containing sulfur will yield meat-like flavours, methionine leads to vegetable notes, and proline produces cracker and bread aromas. Type of amino acid also influences the browning extent and quantity of Maillard reaction products. Piloty and Baltes (1979) observed that basic and hydroxy amino acids have higher affinity to react with α-dicarbonyls, compared to non-polar and acidic amino acids. Ashoor and Zent (1984), based on the magnitude of browning with common reducing sugars, concluded that amino acids can be divided into three categories. Lysine, glycine, tryptophan and tyrosine produce highest extent of browning; intermediate browning producers are proline, leucine, isoleucine, alanine, hydroxyproline, phenylalanine, methionine, valine, glutamine and asparagine; and least browning give cysteine, histidine, threonine, aspartic acid, arginine and glutamic acid. The ratio of sugar to amino acid is also important. Wolfrom et al. (1974) reported that as there was more

amino acid compared to sugar (5:1) more colour was developed upon heating of D-glucose-glycine mixtures. Many published studies relating to isotope distribution of Maillard reaction products have proven that some compounds are formed only in excess of amino acid compared to sugar (Keyhani and Yaylayan, 1996 and 1997).

2.5.4 pH Effect

Many stages of the Maillard reaction are catalysed by acid or base (e.g. Amadori rearrangement). The base catalysis increases the amount of open-chain tautomer (Canter and Peniston, 1940), rearrangement and fragmentation of sugars and reactivity of the amino groups toward the carbonyls, while acid catalysis increases the polarity of the oxo group of the open chain. The evaluation of pH influence on the Maillard reaction is not an easy task since the reaction itself influences the pH. While heating of unbuffered various amino acids and ribose systems, Whitfield et al. (1988) observed a pH drop from 7.0 to 4.5. Various studies have confirmed that Maillard reaction is favoured by alkaline degradation of sugars, producing more browning and volatiles (Bates, 1996; Ames, 2001). In most cases, the Maillard reaction is favoured with increasing pH, but there are some exceptions depending on the character of the reactants and formed products. According to Lee (1984) optimum pH for lysine/glucose system heated at various temperatures ranges from 4 to 8. Leahy and Reineccius (1989) found that pyrazine formation from lysine/glucose system was 500 times higher at pH 9 than at pH 5. The meaty flavour compounds generation from cystine and hydroxy-furanone gave highest yield at pH 4.7 and best aroma at pH 2.4 (Shu and Ho, 1989). While studying volatiles formation from cysteine and 5`-IMP Madruga et al., (1998) showed that sulfur-substituted

furans, mercaptoketones and alkylfurans were formed mainly at acidic pH, while pyrazines were preferred at higher pH (Ames et al., 1996; Tai et al., 1998).

2.5.5 High Pressure Effect

Effect of pressure on non-enzymatic browning has been studied in detail by many researchers, especially after recent introduction of High Pressure Processing of food, with browning as a function of pH and volatiles generation studied most extensively. The general observation, while applying high pressure to Maillard reaction model systems, is that browning is slower at acidic pH and faster at alkaline pH but accompanied by reduced volatiles formation. In more detailed study (Isaacs and Coulson, 1996), a comparison of reaction rates of individual reaction products formed from tryptophan and glucose or xylose showed that the early Maillard reaction is accelerated with pressure, while advanced reaction steps are slowed down. It seems that application of high pressure slows down degradation of Amadori product rearrangement product and thus reduces generation of volatiles (Hill et al., 1999; Brostow and Isaacs, 1999). Most likely explanation is that high pressure promotes reactions characterized by negative volumes of activations (Ames, 1998), such as aldol condensation, and suppresses retro-aldol reduction or elimination types. Moreover, in another study (Hill et al., 1996), browning at lower than 6.5 pH values was reduced due to the fact that under high pressure the ionic form of carboxylic groups was prevalent, thus by dropping pH the Maillard reaction slowed down. On the other hand, at higher pH values, where there is a balance of amino ionic groups, pressure has no effect on pH change, thus it favours Maillard reaction (Heremans, 1995).

2.5.6 Inhibitors Effect

Food industry has long been looking for ways to inhibit the Maillard reaction with regard to colour and off-flavour formation. In the past, best results were obtained using hurdle effect of combining pH, temperature and oxygen availability control. Addition of bisulfite, which reacts with carbonyl groups and thus prevents sugars from participating in Maillard reaction, has also been shown to be effective, where allowed for use (Feather, 1994). Recently, use of aminoguanidine (Brownlee et al., 1986) showed to be very effective mean for inhibiting Maillard protein crosslinking at physiological conditions, brown colour formation and fluorescent materials associated with ageing. Feather et al. (1996) showed that aminoguanidine reacts with dicarbonyl intermediates of later stage of Maillard reaction and converts them to unreactive triazine derivatives.

2.6 Importance of Maillard Reaction in Foods

Maillard reaction plays an important role in food acceptance through affecting the quality factors such as flavour, odour, texture and nutritional value. Volatile and soluble products contribute to aroma and taste, while less soluble materials are associated more with colour and texture. The colour, flavour and taste of food stuffs is often so strongly associated with Maillard reaction, that it determines the product's distinctive characteristics and its acceptability, as for example in bakery products, chocolate and coffee, grilled meats, etc. Undesirable consequence of Maillard reaction is discoloration, formation of off-flavours and toxic or mutagenic compounds, reduction of essential amino acids availability and decrease of protein digestibility by inhibiting enzymes or blocking access to peptide bonds.

2.6.1 Desired and Undesired Impact of Maillard Reaction Products on Food Quality

Food industry has been long zealous in applying Maillard reaction to generate desired flavours of roasting and baking and to enhance particular aromas of processed foods, or to prevent browning, especially in stored foods. Moreover, development of umami and savoury flavours (Schlichtherle-Cerny et al., 2002; Beksan et al., 2003), or efforts in enhancing the flavour characteristics of low-carbohydrate and low-calories or microwavable (Yaylayan, 1996; Yu et al., 1998) products by the controlled Maillard reaction are also most recent research area applications. However this is a very difficult task due to the fact that variety of compounds arise under different reaction conditions, especially if hundreds of compounds are involved in producing a characteristic aroma. That is why understanding the reactivity of Amadori products and the mechanism of their further reactions are of crucial importance in controlling the outcome of Maillard reaction both in food and physiological systems.

Besides aroma, taste and colour, which were already partially discussed in previous sections and which can be further explored through available literature (Herz and Shallenberger, 1960; Tressl et al., 1981; Reineccius et al., 1972; Fors, 1983; Ledl and Schleicher, 1990; Ames, 1998; Tressl and Rewicki, 1999; Schieberle et al., 2002), Maillard reaction imparts other very important characteristics to foods.

Under certain conditions the Maillard reaction does not impart favourable characteristics to foods. For instance discoloration and off-flavours can be induced by Maillard reaction during food storage. Eichner et al. (1996) observed deterioration of quality due to the

Maillard browning proportional to the water content in stored tomato powder and flakes. Lee and Nagy (1996) have shown that Maillard reaction occurs commonly during storage of fruits and their juices. Ascorbic acid degradation, a source of carbonyls, and acid-catalysed hydration-dehydration result in a loss of freshness, discoloration and formation of off-flavours in citrus products. Kamimura and Kaneda (1992) presented comprehensive summary on formation of stale flavour while storing beer. They associate formation of furfuryl ethyl ether as product of ethanol and furfuryl alcohol reaction. Furfuryl alcohol is common Maillard reaction product formed by hydrolysis of furfuryl acetate.

Numerous studies have shown that Maillard reaction can also impart beneficial aspects to the food, in the sense of microbial control (Einarsson et al., 1983 and 1988). In his extensive review, Eklund (1980) discussed inhibitory effect of Maillard reaction products on microorganisms and proposed three possible targets of antimicrobial compounds: cellular membranes, genetic material and enzymes. While studying the mode of action of antibacterial Maillard reaction products, Einarsson (1990) observed that Maillard products did not impair mutagenic effect on the microorganisms (in agreement with observations of Aeschbacher et al., 1981; Omura et al., 1983) but rather they interfere with cation intake (Fe^{+3}) by reducing its solubility at elevated pH values and lowered temperatures. To draw a conclusion, Maillard reaction products indirectly inhibit the uptake of oxygen needed for cell metabolism, they also reduce availability of carbohydrates necessary for enzymes of respiratory chain (Banerjee et al., 1981).

Takahashi et al., (2000) showed that some of the Maillard reaction products characterized by antimicrobial properties also display ability to improve the functional properties of proteins in the course of Maillard reaction. The Maillard-type protein-polysaccharide conjugates comprise mainly of lysozyme derivatives and display superior emulsyfying, heat stability and antimicrobial properties in various of added foods (Kato, 2002).

Antioxidant properties of some of the Maillard reaction products (Griffith et al., 1957; El-Massary et al., 2003) also help in preserving stored foods. This desirable characteristic is influenced by many factors, such as type of amino acid and sugar involved, their ratio, temperature, pH and water activity (Lingnert et al., 1986; Obretenov et al., 1986). The mechanism of antioxidant inhibition proposed by Aeschbacher (1990) is based on a few possible mechanisms: inhibition of nitrosamine formation (competition with electrophilic reaction of nitrite with secondary amines), scavenging of reactive molecules (trap positively charged electrophilic metabolites or scavenge oxygen radicals), modulation of monooxygenase - "radical defence" enzyme system (through alteration of electrons of NADPH), and antipromotion (free radicals are involved in tumour promotion). Some of the heated food products, for instance meat, milk or cereals have been shown to possess improved oxidative stability. Flavour of stored foods was greatly preserved by antioxidative components produced via Maillard reaction (Kayati et al., 1998; Lee and Shibamoto, 2002).

2.6.2 Impact of Maillard Reaction Products on Food Nutritional Attributes

Numerous studies have shown that the formation of Maillard reaction products in foods decreases their nutritional values (Hurrell, 1990; Velisek, 1995; Arnoldi, 2002; Rerat, 2002). The interaction of carbonyl groups of reducing sugars with amino groups of amino acids, peptides or proteins results in loss of available essential amino acids, protein crosslinking and lowered digestibility, and formation of toxic or mutagenic substances. In addition, some premelanoidins can react with and destroy certain vitamins (Ford et al., 1983) and influence trace element metabolism (Finot and Furniss, 1986; Finot, 1990).

Most widely documented nutritional consequence of Maillard reaction is reduction of available lysine. This labile essential amino acid is considered a limiting amino acid in many foods, and thus prevention of its modification during processing and storage of foods is important (Hurrell et al., 1983; Finot 1990; Matsuda and Kato, 1996). Formation of carcinogenic or mutagenic compounds has been studied by many workers. Although the authors findings are often in part contradictory and not comparable, they agree that Maillard reaction in processed and stored foods results in formation of food toxicants (Wong and Shibamoto, 1996; Lee and Shibamoto, 2002) including kidney-damaging compounds (Friedman et al., 1984), growth inhibitors (Lipka and Ganowiak, 1994), mutagenic (DNA-damaging) (Shibamoto, 1983; Hiramoto et al., 1997), clastogenic (chromosome-damaging) and carcinogenic compounds (Tanaka, et al., 1977; Lee et al., 1982; Finot, 1990; Friedman, 1996, Skog et al., 1998).

2.7 Impact of Maillard Reaction on Biological Systems

The importance of the study of Maillard reaction results from its impact on food quality and its impact on human pathology. Only in late 1970's, the significance of non-enzymatic reactions *in vivo* and its chemical alterations of proteins and other biomolecules during ageing and diseases such as cataract, diabetes, Alzheimer's and Parkinson diseases or atherosclerosis (Kato and Oimomi, 1991; Brownlee, 1994; Vlassara et al., 1994; Prakash et al., 2002), have begun to raise questions and concerns among scientists. At first, the evidence indicated that oxidation processes moderate the Maillard reaction *in vivo* (Baynes, 1991), however some studies pointed that Maillard reaction of simpler carbohydrates could also proceed efficiently in the absence of oxygen (Litchfield et al., 1999). However, most recent studies on Maillard-type reaction, have certainly confirmed that the reaction *in vivo* is controlled by oxidative processes of body carbohydrates and lipids, and is catalyzed by Reactive Oxygen Species (Linton et al., 2001). Thus many researchers uniformly agree that pathways of the Maillard reaction *in vivo* are initiated by metal-catalyzed autoxidative glycosylation (Wolff et Dean, 1987) and oxidative fragmentation of Schiff base or Amadori adducts to proteins (Thornalley et al., 1999, Sell et al., 2001) or peroxidation of lipids, followed by continuing reaction of sugar- and lipid-derived oxidation products with proteins (Fu et al., 1996). The best studied examples of Advanced Glycation End Products (AGE) are pentosidine and carboxymethyllysine, derived from arabinose (crosslinking lysine and arginine) and glyoxal respectively (Wells-Knecht et al., 1995; Miller et al., 2003).

In vivo, one of the pioneering studies (Koening, et al., 1977) had revealed that glucose-Amadori Product was attached to the terminal amino acid valine of the β-chain of haemoglobin (minor haemoglobin Hb_{IC}). Further studies confirmed that the presence of Amadori compound was not restricted to Hb only, but was also observed to occur on the terminal amino acids of different proteins; such as immunoglobulins, LDL and serum albumin (Brownlee et al., 1985). As in the case of Hb, the amount of Amadori product was increased by three fold in the diabetic state (Ledl and Schleicher, 1990).

The ability of Amadori product to condense with other amino acids of proteins results in proteins crosslinking, affecting their structure, recognition and function. This is particularly true for long-lived proteins, such as collagen and lens crystallines, which accumulate the modification over significant periods of time. This leads to alteration of collagen elasticity and changes in permeability of renal and vascular membranes (Kohn et al., 1984). These changes again, are accelerated in diabetes and are further implicated in cardio-vascular diseases (Cerami et al., 1979). Also, yellow-brown fluorescent pigments were noted to accumulate in the lens with age and induced cataract formation (Frye et al., 1998). Despite very intensive research, today there is still no medically satisfying antidote inhibiting the reaction *in vivo* that would be efficient and do not interfere with other vital body functions. Application of aspirin, sulfite, chelators, aminoguanidine or other dicarbonyls trapping chemicals indicated that, although prevented the reaction *in vitro*, did not protect protein functionality (Tsai et al., 2002; Miller et al., 2003). Sell et al., (2001) conducted long-term rat feeding experiments with aminoguanidine. The results of their research suggest that α,β-dicarbonyls do not play a major role in collagen

crosslinking. They suggest that rather post-Amadori reactions involving oxidative or nonoxidative fragmentation of the Amadori product are more likely responsible for collagen ageing. There have been some promising discoveries concerning development of a new class of enzymes termed as Amadoriases, which catalyze the oxidative deglycation of glycated Amadori products to yield corresponding amino acids, glucosone, and H_2O_2, unfortunately with no detected activity on glycated proteins (Monnier et al., 1995; Gerhardinger, 1995; Wu et al., 2000).

Concerning the positive impact of Maillard reaction products on physiological systems, it has been shown, in studies on rats (Miura and Gomyo, 1990), that dietary melanoidins suppressed the elevation of cholesterol level of plasma and liver in rats, and that possibly melanoidins affected intestinal metabolism of cholesterol. The antioxidant potential of Maillard reaction products might also result in protection from cancer, since other substances with antioxidant property showed cancer inhibition (Aeschbacher, 1990; Elizalde et al., 1991). The desmutagenicity of Maillard reaction products has been widely presented (Kim et al., 1987; Lee et al., 1994; Chuyen, 1998), although mutagenic compounds generation was reported as well.

2.8 Traditional Analytical Approaches to Study Maillard Reaction

In the advent of studies of Maillard reaction, heated foods or model system mixtures were extracted with various solvents to recuperate volatile compounds. Often present in ppm amounts, the extracted compounds had to be concentrated before separation and identification. This was certainly very laborious and time consuming. Accurate identification was also not possible until 1960's when first prototype gas chromatograph interfaced to mass spectrometer was applied to separate volatiles from fructose degradation (Shaw et al., 1968). The development of high resolution capillary gas chromatograph/mass spectrometer (GC/MS) had significantly accelerated studies of the Maillard reactions by 1970's, and basically until today GC coupled with various detectors is the primary instrument of choice.

2.8.1 Classical Techniques and Methodologies to Study Maillard Reaction

The most commonly followed procedure in model system studies of Maillard reaction is illustrated in Figure 2.1. In this procedure, amino acid is mixed with sugar(s) or sugar fragment(s) under specific ratio and dissolved in water, mixture is buffered and heated at specific temperature and time. Once the reaction is terminated, its products are extracted with an organic solvent, concentrated, if needed derivatized and finally injected to GC for separation and MS (IR) characterisation. The amount of extracted volatiles depends on model system and applied conditions, and can vary from 0.5% to 55%. The remaining mixture, thus, presents major part of the reaction. It can be further fractionated in several separation steps using traditional chromatographic methods (ion exchange, adsorption, partition, exclusion) to give pure substances. High Performance Liquid Chromatography

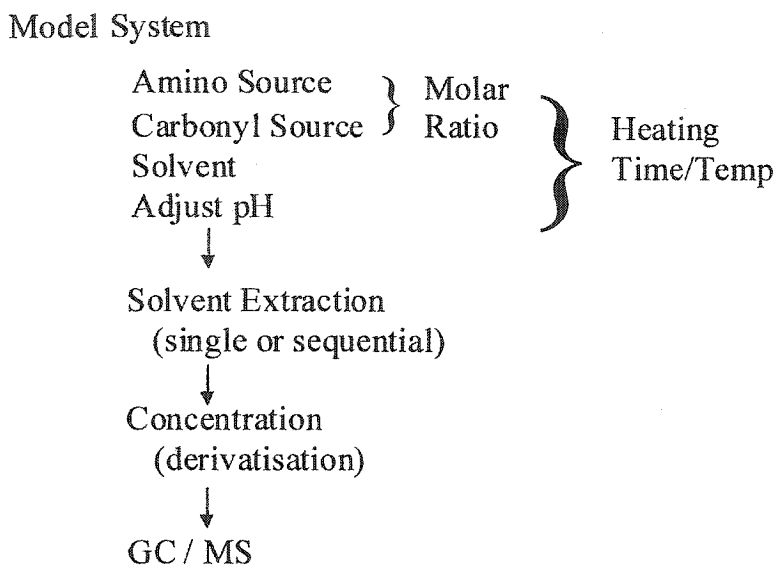


Figure 2.1 Classical Approach for Analysis of Volatiles produced during the Maillard Reaction

(HPLC) has been successfully applied to fractionate the water-soluble mixture, and application of various detectors (UV, fluorescence, light scattering) simplified detection of numerous classes of substances (Moll and Gross, 1983; Eichner et al., 1994) or even single compounds in interfaced LC/MS (Milon et al., 1987; Hsuehli et al., 1990). Non-volatile fractions can also be analyzed, after derivatization step, with an application of various mass spectrometric methods. In a recent review Yeboah and Yaylayan (2001) discuss successful use of mass spectrometric methods, namely ESI and MALDI, and the type of information that can be obtained as well as their advantages and disadvantages in qualitative and quantitative analysis of glycated proteins.

2.8.2 Elucidation of Mechanistic Pathways Using Isotopes

In order to understand and be able to control the Maillard reaction in food and biological systems, identification of its mechanistic pathways is of utmost importance. The

complexity of the reaction, often involving several pathways, to generate the same product, necessitates the use of isotopically labeled starting materials, such as amino acids and sugars or their fragmentation products, to establish the origin and fate of the intermediates and products, thus to gain information on how substrates decompose and how products are formed. Since Koehler and colleagues (1969) applied labeled material to elucidate the formation of pyrazines, this methodology became essential to other researchers in studying Maillard reaction pathways (Nyhammar et al., 1983; Tressl et al., 1998; Huyghues-Despointes and Yaylayan, 1996; Schieberle and Hofmann, 1998; Cerny and Davidek, 2003). While most researchers use singly labeled carbohydrates or amino acids, Schieberle et al. (2002) and Frank and Hofmann (2002) propose the use of all-carbon labeled carbohydrate moiety, in a technique called CAMOLA, an acronym for "carbohydrate module labeled". This labeled variation helps in determining the extent at which the precursor skeleton remains intact during flavourings formation, and the role of its breakdown products. The use of isotopically labeled material not only allows identification of which substrate atoms are incorporated into product molecule, or where they are located in the product, but also allows classification of reactive intermediates which are formed from sugar and amino acid moieties and serve as universal building blocks for stable end-products.

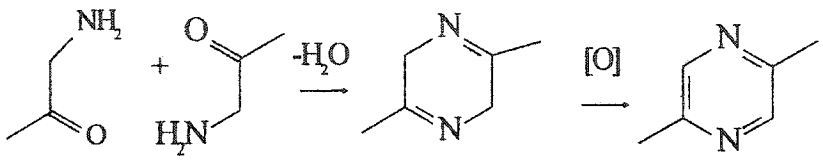
Labeled studies particularly permitted the identification of C_2, C_3, C_4, C_5 and C_6 carbonyls and dicarbonyls as universal intermediates (i.e. same compounds generated through various model systems) of sugar fragments or amino acids methylated sugar fragments or degradation products of Amadori, Heyn's or lipids oxidation, formed in

advanced stages of Maillard reaction undergoing enolization, elimination, retro aldol and aldol condensation reactions to cyclize, dehydrate and yield flavourings and pigments (Tressl et al., 1993; Amrani-Hemaimi, et al., 1995; Weenen, 1998; Yaylayan et al., 1998; Yaylayan and Haffenden, 2003). Classification of the common reactive intermediates was possible by trapping them into stable end-products, then by tracing back the origin of each labeled atom from the stable end-product to its substrate a corresponding reactive intermediate could be elucidated (Yaylayan et al., 2000). Similarly, identification of mechanism of protein glycation and crosslinking *in vivo*, became possible with *in vitro* studies using labeled materials (Tressl et al., 1994; Biemel et al., 2002; Schoevaart and Kieboom, 2002). The conclusions were the same for both food-scientists and bio-chemists, the Maillard-type reaction is carried on by reactive dicarbonyls, such as glyoxal, methylglyoxal, pyruve aldehyde, glycoaldehyde, 1,3-butanedione, glyceraldehyde and deoxyglucosones, in contrary to their early research focus on glucose as the major participant in oxidative deamination reactions.

2.8.3. Mechanistic Studies of Selected Maillard Reaction Products by Labeling Techniques

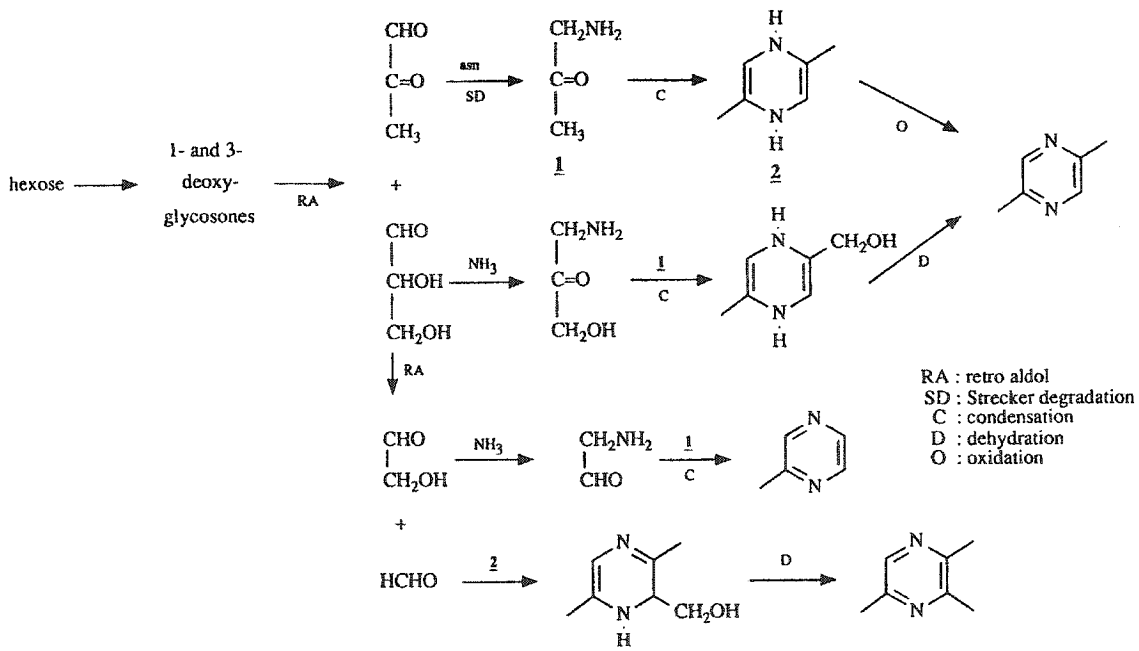
2.8.3.1 Investigation of Mechanism of Pyrazine Formation

Pyrazines, the major volatile flavour chemicals produced in Maillard reaction (Maga, 1973 and 1992), are heterocyclic, nitrogen-containing compounds present in almost all processed foods imparting roasted, toasted and nutty flavour notes. The abundance of pyrazines is explained by their straightforward formation through condensation of two



Scheme 2. 7. Reaction Scheme for the Formation of Pyrazines from α-aminoacetones (Baltes, 1990)

molecules of α-amino ketones, generated either by the interaction of α-dicarbonyls with α-amino acids through Strecker degradation (Scheme 2.7) (Baltes, 1990) or by the reaction of free ammonia with α-hydroxyketones (Ho, 1996). The dicarbonyls and hydroxyketones originate through retro-aldolization from 1- and 3-deoxyosones (Scheme 2.1), which are in turn generated as a consequence of sugar and Amadori product enolization or oxidation.



Scheme 2.8 Formation of Pyrazines from Hexose and Asparagine (Weenen et al., 1994)

Most of the pyrazines are highly substituted with methyl-, ethyl-, propyl-, vinyl-, allyl- and propenyl groups. Application of labeled reactants allowed more detailed studies on

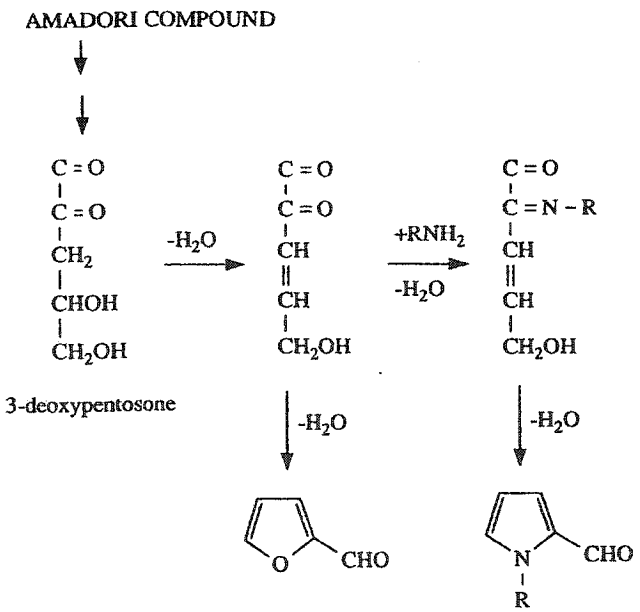
the influence of sugar and amino acid on alkylpyrazine formation. Koehler with co-workers (1969) used ^{13}C-labeled amino acids with sugars to analyse formation of methyl- and dimethylpyrazines. They concluded that sugar was the sole carbon donor and amino acids could provide only nitrogen atoms, and that different reaction rates of various amino acids with carbonyls result in different alkylation patterns of pyrazines. Shibamoto et al. (1979) and Coleman et al. (1999) proposed however, that the addition of Strecker aldehydes to the dihydropyrazines followed by dehydration and aromatisation result in multiple alkylations of pyrazine rings. Similarly, other studies with isotopically labeled reactants (Amrani-Hemaimi, 1995; Yaylayan et al., 1998) showed that amino acids also contribute to the alkyl side chain of some alkylpyrazines. Amrani-Hemaimi using [3-^{13}C] alanine and [2-^{13}C] glycine proposed that alanine donated C_2 unit (from C-2 and C-3) in the ethyl groups of ethylmethyl-, ethyldimethyl- and diethylmethylpyrazines, and glycine one of the methyl groups in trimethylpyrazine.

Contribution of α- and ε-amino and amide nitrogen atoms to pyrazine formation has been investigated by Hwang et al. (1993 and 1994). Based on the distribution of ^{15}N in the pyrazines, they concluded that deamination of amide initiated during Maillard reaction can significantly contribute to formation of pyrazines (more than 50%), and that both amino groups of lysine can reductively aminate sugars. Thermal degradation of β-hydroxy amino acids alone can also generate methyl- and ethyl-substituted pyrazines (Yaylayan and Wnorowski, 2001) through Strecker degradation or Amadori rearrangement of generated carbonyls. While pyrolyzing various amino acids alone with hexamethyldisilazane at 600°C, Chiavari et al. (2001) detected 2,5-trimethyl silylated

pyrazine from glycine and 3,6-dimethyl-2,5-trimethyl silylated pyrazine from alanine and serine as a result of their self-condensation followed by dehydration and oxidation.

2.8.3.2 Investigation of Mechanism of Pyrrole Formation

Pyrroles, representing at least fifty members in the group, are the building blocks for porphyrins and biologically active compounds (Jones, 1990), have also been detected in various food products and have been associated with undesirable odour, although some are characterised as pleasant corn-like or sweet (pyrrole-2-carboxyaldehyde), or caramel-like (2-acetylpyrrole) flavours. Traditionally, pyrroles are synthesised through Paal-Knorr synthesis by refluxing γ-dicarbonyls with primary amines (Amarnath et al., 1991) or



Scheme 2.9 Formation of Pyrroles from Intermediates of the Maillard Reaction (Weenee, et al, 1994)

through Hantzsch (Roomi and MacDonald, 1970) and Knorr syntheses (Hamby and Hodges, 1993) by reacting α-amino carbonyls with β-diketones in the presence of a base or α-halo carbonyls in the presence of primary amine.

A simple model system consisting of rhamnose and ammonia produced eight pyrrole derivatives (Shibamoto and Bernhard, 1978). Studies with D-glucose/amine (Olsson et al., 1977; Beck et al, 1989) and with D-glucose/amino acid mixtures (Olsson et al, 1978; Nyhammar et al., 1983) allowed to propose that pyrrols are formed from the reaction of a 3-deoxyketose with ammonia or an amino compound followed by dehydration and ring closure (Scheme 2.9). Extensive studies with application of ^{13}C in D-glucoses, D-arabinoses and D-fructoses with ^{15}N in 4-aminobutyric acid gave much more insight into the formation of pyrroles (Tressl et al., 1994; Tressl et al., 1995). Their results, representative for primary α-amino acids, support 3-deoxyaldoketoses as intermediates of 2-formylpyrroles and disqualify 4-deoxy- and 1-deoxydiketose routes to 2-acetylpyrroles as lacking labeled on the acetyl moiety, proposed by Njorge (1988), which are rather formed from 1,3-dideoxy-1-amino-2,4-diketose through cyclization, dehydration and enol tautomerization. In addition, presence of double labeled, [5-^{13}C] and [^{13}CHO], indicated two possible pathways of 5-formylpyrrole formation. All the above results were confirmed by Wnorowski and Yaylayan (2000) who observed in 2-formyl-5-methylpyrrole 100% incorporation of all the six carbon atoms of D-glucose (authors used labeled of all glucose carbon atoms reacting systematically with glycine) including glucose ^{13}C-1 in the same pyrrole position [^{13}CHO]. Yaylayan and Wnorowski (2001) reported that dicarbonyls formed upon thermal degradation of serine or threonine alone, after chain elongation by amino acid-assisted alkylation or aldol condensation, can generate 2-substituted pyrroles following Paal-Knorr synthesis. Comparison of isotope incorporation in C-2 and C-3 substituted pyrroles generated from variously labeled pyrolyzed serines allowed Yaylayan and Keyhani (2001) to deduce common source of α-

amino carbonyl to be glycoaldehyde, a Strecker aldehyde of serine. The authors also confirmed that ethyl group attaches to pyrrole ring only through C-2 atoms of amino acid, after aldol condensation or amino acid assisted chain elongation of glycoaldehyde.

2.8.3.3 Investigation of the Role of Amino Acids in Chain Elongation Reactions

Until recently carbon chain elongation of C_2, C_3 and C_4 fragments was attributed to aldol type condensation of smaller sugar fragments. Application of ^{13}C-labeled precursors to mechanistic studies initiated by Simon and Heubach (1965) and Koehler et al. (1969), developed by Tressl et al. (1993), finally expended and modified by Yaylayan and Keyhani (1996), gives enough evidence to trace back the origin of all Maillard reaction products, including the most reactive intermediates.

Labeled studies allowed Amrani-Hemaimi et al. (1995) to discover that glycine was able to incorporate its second carbon and alanine its two-carbons into dihydropyrazines to form alkylpyrazines. Yaylayan et al. (1998), went even further and utilized labeled glucose and glycine at all their carbon atoms and confirmed the ability of amino acids to donate their carbon atoms. According to Yaylayan et al. (1998), amino acids can interact with the aldehydo moiety of α-ketoaldehyde, or α-hydroxy aldehyde, and transform them into an α-diketones (Scheme 2.4), thus resulting in carbonyl chain elongation. This alkylation process is in direct competition with Strecker-type interaction that generates Strecker aldehydes and α-aminocarbonyl compounds, and, in contrary to Amrani-Hemaimi findings, occurs before pyrazine formation.

Recent studies (Keyhani and Yaylayan, 1996) on the formation of alkyl-2(1H)-pyrazinones have also confirmed the occurrence of an addition of glycine carbon atoms to glycine-Amadori product. This remarkable discovery sheds new light on the origin of most reactive dicarbonyl intermediates in Maillard systems. For instance, acetic acid a C_2 precursor of N-acetylpyrolidine, was confirmed to originate from a C1-C2 or C5-C6 fragments of glucose; pyruvaldehyde (Scheme 2.3), a C_3 precursor of di- and trimethylpyrazines, was confirmed to be formed by a minor pathway from glyoxal chain elongation, and could be further elongated to generate 2,3-butandione, precursor of trimethylpyrazine (Yaylayan et al., 1998). Through the use of model systems in labeled studies of formation of substituted pyrrols, Yaylayan and Keyhani (2001) proposed that alanine, through aldol condensation donates its C-2 and C-3 atoms to glycoaldehyde to extend its backbone to 2-aminobutanaldehyde and 1-amino-2-butanone, which upon consecutive condensation with acetaldehyde, cyclizes and dehydrates to generate methyl- and ethyl-substituted pyrroles.

Utilisation of model systems in studying Maillard reaction not only simplifies the identification of the reaction products, compared to complex matrix of real foods, but also allows to precisely control and manipulate the reaction conditions, such as pH, redox potential, water activity, transition metals, or inhibitors – factors which would be difficult to control in real food systems. The results of these chemically defined experiments can then be related to the less defined processes occurring in real foods or biological systems. Some of the laboratory simulations are the only way to gain any knowledge on the Maillard reaction mechanism, as for example while taking place in biological systems. It

was only with the assistance of model systems that discovery of lipid peroxidation products impact on non-enzymatic browning was possible (Toyomizu and Chung, 1968).

CHAPTER 3

OPTIMIZATION OF PYROLYSIS-GC/MS PARAMETERS: ELECTRONIC PRESSURE CONTROL AND THE USE OF SAMPLE PRECONCENTRATION TRAP

3.1 Introduction

Auxiliary sample introduction devices such as pyrolyzers, are commonly interfaced to the GC to extend the range of sample matrices and the analyte types beyond of those that can be handled by the conventional inlets. Pyrolysis involves the rapid and controlled thermal decomposition of a few milligrams of the sample in the pyrolyzer. The volatile products are then swept onto the column for separation and eventual identification. While using a pyrolyzer, the overall conditions and objectives are the same for these analysis as in more conventional work with GC inlets, however, due to longer sample introduction time, column head pressure may be programmed differently through electronic pressure control (EPC) devices, to improve the efficiency of sample transfer onto the analytical column. In addition, during Py-GC/MS analysis, analytes are produced continuously (during total heating time), that have different polarities and volatilities and are introduced into the liner as a vapour, the composition of which changes over time. Under those conditions of sample introduction, analyte discrimination inside the liner is greatly pronounced and the efficiency of sample transfer into the column becomes a primary concern. Using EPC devices and temperature programming, analyses could be performed either under constant pressure (decreasing flow rate as temperature is increased) or at a constant flow rate (increasing pressure as temperature is increased). Alternatively, a programmed pressure pulse can be introduced at the beginning of the run, which allows high carrier gas flow through the liner during sample introduction, thus allowing rapid and efficient transfer of volatiles from the liner into the GC column. This technique is known as electronic pressure programming (EPP). In general, the use of EPC devices increases the sensitivity and reduces the decomposition due to longer residence time in the heated liner, of the

semi-volatiles and the labile analytes. Numerous studies have been published on the effect of pressure programming with split-splitless GC inlets using EPC devices (Stafford, 1995; Vincenti et al., 1995), however, no studies have been reported with auxiliary devices such as pyrolyzers.

In our laboratories, a resistively heated Py-GC/MS system has been extensively employed as an integrated reaction, separation and identification unit (Yaylayan and Keyhani, 1996; Yaylayan and Keyhani, 1998; Keyhani and Yaylayan, 1996a; Keyhani and Yaylayan, 1996b). The reactants are heated at the desired temperature for a precisely controlled period of time in the pyrolysis probe and the generated products are swept by the carrier gas into the GC column for separation and eventual identification by the MS. This system was mainly used to study the mechanism of volatile formation (Keyhani and Yaylayan, 1997) from mixtures of ^{13}C- and ^{15}N-labeled amino acids and reducing sugars through Maillard reaction (Yaylayan, 1997). Conventionally, this type of analyses are performed through classical reflux, followed by solvent extraction, concentration and GC/MS analysis. The Py-GC/MS system allows the use of expensive labeled starting materials in milligram quantities, reduces the time of analysis and eliminates the need for solvent extraction. During such analysis, where expensive labeled reactants are used to perform chemical reactions in the pyrolysis probe, it is important to run the chromatographic system as efficiently as possible to be able to calculate label incorporation free from interferences from overlapping peaks. The objective of the present study was to examine the effect of carrier gas pressure, flow rate and different pulse sequences on the efficiency of sample transfer and on the chromatographic

resolution during Py-GC/MS analyses. A model Maillard system consisting of D-glucose and glycine, that has been studied extensively in our laboratories (Keyhani and Yaylayan, 1997; Keyhani and Yaylayan, 1996), was used in this investigation.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Glycine and D-glucose were purchased from Aldrich Chemical Company (Milwaukee, WI).

3.2.2 GC/MS Analysis

A Hewlett-Packard GC/mass selective detector (5890 series II GC/5971B MSD, Palo Alto, CA) interfaced to a CDS Pyroprobe 2000 unit (CDS Analytical Inc., Oxford, PA), through a quartz-lined and valved interface (CDS 1500), was used for the Py-GC/MS analysis. In all experiments, solid samples of a mixture of D-glucose-glycine (molar ratio 1:3; 3.3 mg) were introduced inside the quartz tube (0.3 mm thickness) and plugged with quartz wool and inserted inside the coil pyrolysis probe. The pyroprobe was set at 250°C at a heating rate of 50°C/s with a total heating time of 20 sec. The pyroprobe interface temperature was set at 250°C. The samples were analysed under constant pressure (12, 22, 26, 30, 40, 50 and 60 psi) or constant flow rate (0.7, 1.0, 1.3, 2.0, 3.0, 4.0 mL/min) or under pulsed pressure (Table 3.1). The samples were introduced under splitless mode (valve was on after 1 min). The pressure was regulated by an Electronic Pressure Controller (Hewlett-Packard, Palo Alto, CA). Capillary direct MS interface temperature was 280°C; ion source temperature was 180°C. The ionisation voltage was 70 eV and

the electron multiplier was 2047 volts. The mass range analysed was 30-350 AMU. The column was a fused silica DB-5MS (60 m x 0.25 mm x 0.33 μm; Supelco, Inc.). The column initial temperature was set at –5°C for 2 min and then increased to 50°C at a heating 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 five minutes. The structures of the products were identified through library (Willey6n ™) search facility of the HP software ChemStation ® ver. C.03.00.

Table 3.1 Pulse Sequences

Delayed Pulse	**Pulse**
(S1) constant pressure of 17.2 psi for 8sec, increase at a rate of 99 psi/min to 60 psi for 60sec, drop to 17.2 psi at a rate of 99 psi/min, to establish a constant flow of 1.5 mL/min	(S5) constant pressure of 60 psi for 30sec, drop to 17.2 psi at a rate of 99 psi/min, to establish a constant flow of 1.5 mL/min
(S2) constant pressure of 17.2 psi for 20sec, increase at a rate of 99 psi/min to 60 psi for 60sec, drop to 17.2 psi at a rate of 99 psi/min, to establish a constant flow of 1.5 mL/min	(S6) constant pressure of 60 psi for 120sec, drop to 17.2 psi at a rate of 99 psi/min, to establish a constant flow of 1.5 mL/min
(S3) constant pressure of 21 psi for 20sec, increase at a rate of 99 psi/min to 60 psi for 20sec, drop to 17.2 psi at a rate of 99 psi/min, to establish a constant flow of 1.5 mL/min	(S7) constant pressure of 60 psi for 300sec, drop to 23.4 psi at a rate of 99 psi/min, to establish a constant flow of 1.5 mL/min

Table 3.2 Selected indicator reaction products

Analyte	Code	Origin	Retention Time [1] (min)
Acetic acid	a	1° pyrolysis	5.8 - 7.5
2,3-Dimethylpyrazine	b	2° pyrolysis	9.3 - 12.9
Trimethylpyrazine	c	2° pyrolysis	11.0 - 14.8
Pyranone derivative	d	1° pyrolysis	14.2 - 18.2
Dimethyl pyrazinone	e	2 ° pyrolysis	18.7 - 23.2
Trimetyl pyrazinone	f	2 ° pyrolysis	20.6 - 25.1
Quinoxalinone derivative	g	2 ° pyrolysis	26.8 - 31.7

[1] Depending on the operational parameters

3.3 Results and Discussion

The principle of chromatographic separation is based on the variation in the rate at which different analytes migrate through a stationary phase under the influence of a mobile phase. Rates of migration may vary, because of differences in distribution ratios between the two phases. Besides the affinity of the analytes to the stationary phase, temperature and pressure (and resulting carrier flow rate) have a great influence on the distribution ratios. Effect of temperature has been described extensively in the literature (Jennings and Adam, 1975; Berger, 1995; Grob, 1995). It is commonly known that as the column temperature increases, the carrier gas viscosity increases as well, resulting in a decrease in flow rate over time, if the pressure is not regulated by EPC devices. It is undesirable to maintain a drop in carrier flow as the column temperature increases, because it results in loss of resolution, peak broadening and in undesirable increase in separation time and temperature (Zlatkis et al., 1965). In addition, decreasing flow rate over time greatly affects the MS vacuum pressure which should be maintained at a specific value range. Spectral reproducibility and sensitivity can be maximised by maintaining a proper

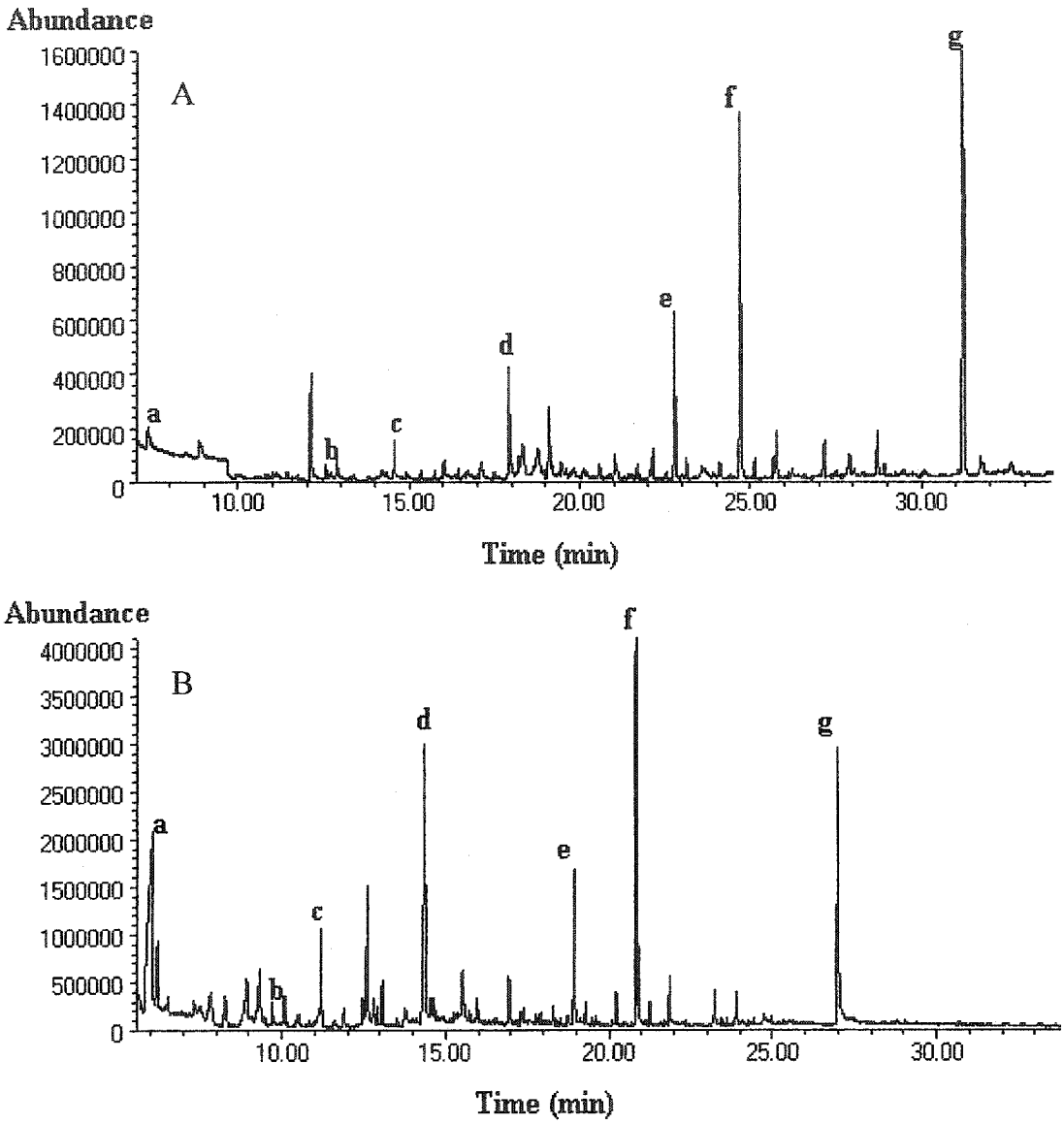


Figure 3.1 Py-GC/MS Chromatograms of D-Glucose-glycine mixture; (A) Without EPC Control-column Inlet Pressure of 22 psi and (B) Constant Pressure of 60 psi. See Table 3.2 for peak designations

vacuum. Precise and accurate control of gas flow is therefore essential for both chromatographic separation and MS detection (Stafford, 1995).

To investigate the effect of pressure on the efficacy of sample transfer from the pyrolyzer to the column, experiments were carried out in which either the pressure or the flow rate was kept constant during the chromatographic runs. In addition, the effect of different pulse sequences was also studied using the pyrolysis of D-glucose in the presence of glycine as a model. Total area counts and total number of peaks were used as indicators of efficiency of sample transfer. Selected pyrolysis products (see Table 3.2) from the beginning, the middle and the end of the chromatogram were also used to monitor changes in retention times, resolution (R_S) and the separation number (TZ). TZ value (Bakeas and Siskos, 1996) expresses the efficiency as a function of the difference in retention times (t_R) of two homologues α and β differing by one methylene group and the sum of the corresponding peak widths (w) at half-height as shown by equation (Eq.3.1).

$$TZ = [(t^{\beta}_R - t^{\alpha}_R)/(w^{\alpha} + w^{\beta})] - 1 \qquad \text{(Eq.3.1)}$$

The TZ value is also used (Ettre, 1975) to calculate the resolution (R_S) of the homologues (see Eq.3.2)

$$TZ = (R_S/1.77) - 1 \qquad \text{(Eq.3.2)}$$

Two sets of homologous compounds were selected to monitor changes in resolution as a function of operating parameters. Pyrazine analogues (compounds b and c in Table 3.2) were used to monitor resolution in early eluting peaks and pyrazinone analogues (compounds e and f in Table 3.2) for the later eluting peaks.

3.3.1 Effect of Constant Pressure

Presence of auxiliary devices such as a pyrolyzer, generally causes an additional pressure drop in the GC/MS system. This necessitates the use of higher pressures during analysis to compensate for the drop in total pressure. The use of EPC devices can facilitate such a control throughout the chromatographic run. Figure 3.1 compares the best chromatogram obtained without EPC (henceforth referred to as the control) with a chromatogram obtained under constant pressure maintained by EPC. Comparison of pyrograms A and B in Figure 3.1 indicates the advantage of constant high pressure run in the recovery of early eluting, low molecular weight volatile components. The differences in sensitivity and resolution are apparent in the first 15 min of the chromatographic run.

The effect of different constant pressure runs (from 12 psi to 60 psi) on the total number of peaks, total peak area counts and on the retention times of selected peaks is summarised in Table 3.3. Inspection of Table 3.3 indicates that at higher pressures, the total number of peaks and the total peak area counts are increased. As expected, the retention times of the separated peaks decreased with increasing pressure. However, the decrease in retention times was more pronounced for the later eluting peaks.

Table 3.3 Effect of Constant Column Head Pressure on the Total Area Counts, Total Number of Peaks and Retention Times of Selected Analytes

Pressure (psi)	**Total number of peaks**	**Relative Total area**	**Total area (x 10^8)**	**t^a_R (min)**	**t^d_R (min)**	**t^g_R (min)**
12	20	1	0.8	7.5	18.2	31.7
22	50	2.1	1.7	7.2	17.5	30.8
26	62	2.4	1.9	6.8	16.9	30.1
30	91	4.5	3.6	6.6	16.5	29.5
40	96	4.7	3.8	6.2	15.5	28.4
50	117	8.6	6.9	6.0	14.8	27.5
60	135	11.4	9.1	5.8	14.2	26.8
Control [1]	87	3.6	2.9	7.4	17.9	31.2

[1] without EPC (column inlet pressure 22 psi)

As a result of this decrease in retention times, the separation occurs at lower temperatures (provided the temperature programming does not change), consequently, the resolution increases. Elution of compounds at lower column temperatures can often improve resolution, extend the operating range of high-temperature applications or provide milder conditions for sensitive analytes (David and Stafford, 1995). This increase in resolution at higher pressures (Table 3.4) was reflected in the detection of higher number of separated compounds at higher pressures (see Table 3.3). According to Berger (1995), above a certain optimum pressure, resolution should decrease since it is proportional to column efficiency (N) and column efficiency increases with increase in retention time (t_R) according to equation (Eq.3.3).

$$N = 5.54\left[\frac{t_R}{w}\right]^2 \qquad \text{(Eq.3.3)}$$

According to Table 3.4, the optimum pressure for the highest resolution for the early eluting peaks was around constant pressure of 30 psi, however, increasing the pressure further will increase the resolution of the later eluting peaks up to a value of 50 psi. According to Grob (1987), a 1 % decrease in retention time will effect in a 1% drop of peak width and an increase of a 1 % in peak height.

Table 3.4. Effect of Various Constant Pressures on the Separation Parameters [1]

Pressure (psi)	Pyrazine analogues			Pyrazinone analogues		
	TZ	d (min)	R	TZ	d	R
12	24.34	1.87	29.8	5.46	1.9	7.6
26	27.70	1.84	33.8	11.02	1.89	14.1
30	29.82	1.82	36.3	19.88	1.89	24.6
40	28.57	1.77	34.8	26.41	1.89	32.3
50	22.36	1.73	27.5	28.94	1.88	35.2
60	18.45	1.69	22.9	27.01	1.87	33.0
Control [2]	21.85	1.87	26.9	22.45	1.92	27.6

[1] TZ = separation number, R = resolution (Eq.3.2), d = $t^{\beta}_{R} - t^{\alpha}_{R}$

[2] without EPC (column inlet pressure 22 psi)

3.3.2 Effect of Constant Flow Rate

The advantage of using constant flow with MS detection is the improved quality of mass spectrometric data. In addition, flow could be adjusted to attain the best Van Deemter value for the eluent linear velocity. Keeping the flow rate constant with increasing column temperature, implies increasing pressure over the chromatographic run (Stafford et al., 1991). Figure 3.2 compares the chromatograms obtained at 4 and 2 mL/min constant flow rates. The comparative data on the effect of different constant flow rates on the chromatographic separation are listed in Tables 3.6 and 3.7. Similar to constant

pressure experiments, increasing the flow rate increases the total number of peaks and total area counts, at the same time, decreasing the retention times. The drop in retention times of the later eluting peaks was again higher than the earlier eluting peaks. The optimum resolution for the earlier peaks was achieved at 2 mL/min and was at 4 mL/min for the later eluting peaks. At this flow rate the total area (compare Tables 3.3 and 3.6) was similar to that of constant pressure run at 30 psi (3.6×10^8) but with lower number of peaks. Table 3.5 compares total peak area counts of selected pyrolysis products (Table 3.2) under constant flow and constant pressure conditions.

Table 3.5 Integrated Peak Areas ($x10^6$) of Selected Indicator Compounds [1] as a Function of Pressure and Flow Rate

Flow rate (mL/min)	**a**	**b**	**c**	**d**	**e**	**f**	**g**
1	13	0.25	1.2	12	1.8	5.2	8.5
2	25	0.50	3.2	14	3.9	4.2	15
3	54	1.60	9.5	24	4.9	7.8	24
4	40	2.0	13	20	7.2	17	27
Pressure (psi)							
12	20	0.61	3.3	12	3.2	8.8	17
26	28	1.07	6.7	18	4.6	16	22
40	43	1.48	10	22	8.8	26	46
50	81	2.8	21	33	17	43	46
60	85	3.00	19	67	20	51	48
Control [2]	3.5	0.07	2.2	9.7	14.7	33.4	38.4

[1] see Table 3.2

[2] without EPC (column inlet pressure 22 psi)

Table 3.6. Effect of Constant Flow Rates on the Total Area Counts, Total Number of Peaks and Retention Times of Selected Analytes

Flow rate (mL/min)	Total number of peaks	Relative total area	Total area (x 10^8)	t^a_R (min)	t^d_R (min)	t^g_R (min)
1	27	1	0.5	7.6	17.5	29.7
2	41	2.6	1.3	6.4	15.8	27.8
3	68	5.8	2.9	5.9	14.9	26.8
4	73	7.2	3.6	5.7	14.3	26.2
Control [1]	87	5.8	2.9	7.4	17.9	31.2

[1] without EPC (column inlet pressure 22 psi)

Table 3.7. Effect of Various Constant Flow Rates on the Separation Parameters [1]

Flow rate (mL/min)	**Pyrazine**			**Pyrazinone**		
	TZ	d	R	TZ	d	R
1	22.07	1.73	27.1	5.21	1.713	7.3
2	32.08	1.687	38.9	15.60	1.743	19.5
3	30.88	1.658	37.5	17.70	1.739	22.0
4	29.28	1.635	35.6	23.54	1.742	28.9
Control [2]	21.85	1.87	26.9	22.45	1.92	27.6

[1] TZ = separation number, R = resolution, d = $t^\beta_R - t^\alpha_R$

[2] without EPC (column inlet pressure 22 psi)

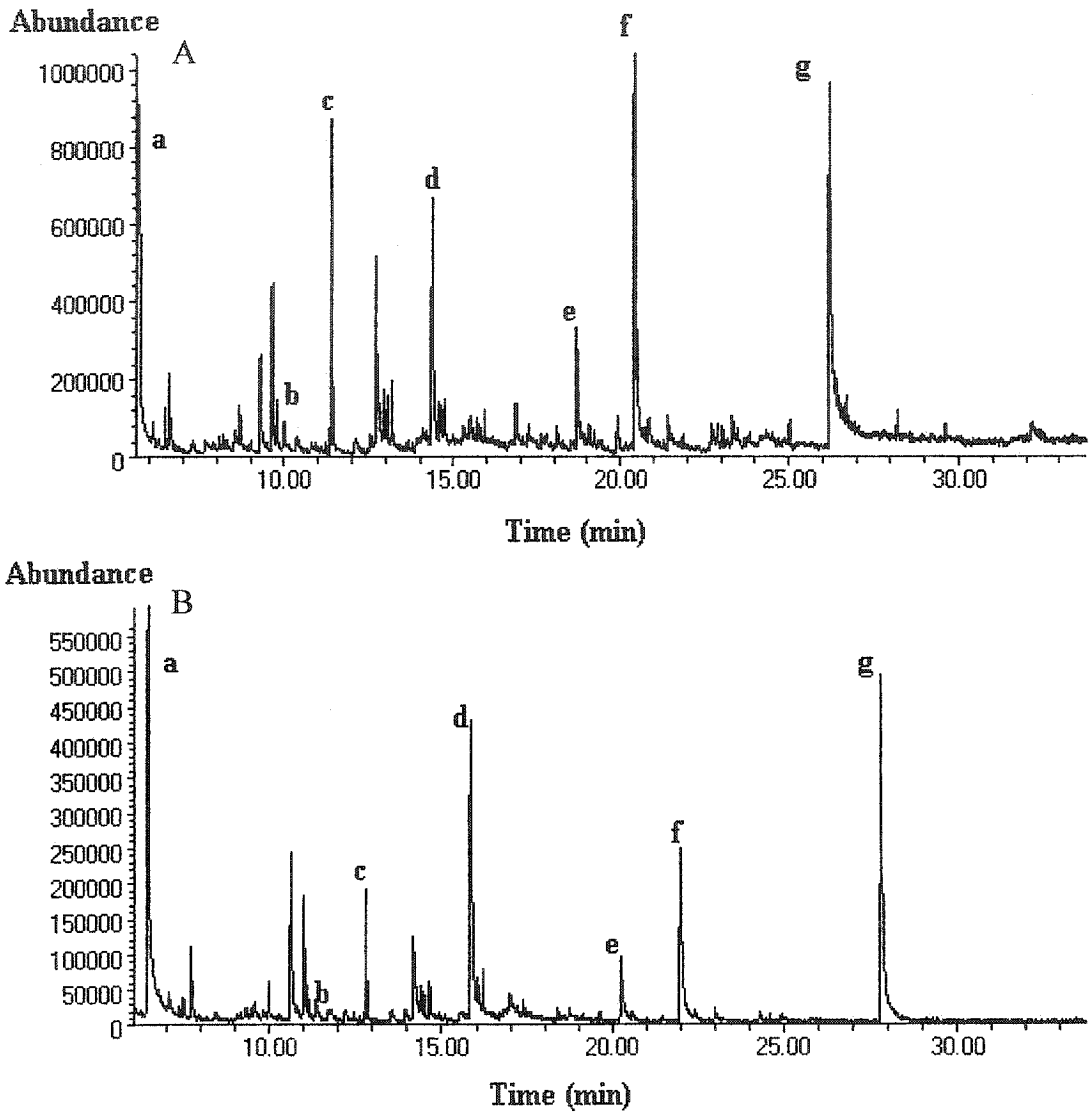


Figure 3. 2 Py-GC/MS Chromatograms of D-glucose-glycine Mixture; (A) Constant Flow Rate of 4mL/min and (B) Constant Flow Rate of 2 mL/min. See Table 3.2 for Peak Designations

3.3.3 Effect of Electronic Pressure Programming (EPP)

The disadvantage of using constant flow throughout the separation is associated with the beginning of the chromatographic run when flow may be too low to achieve efficient sample transfer. This is especially true for a splitless type of injection, where a higher pressure is required for a fast transfer of volatile compounds into the column. Too long residence times in the inlet, causes loss of labile compounds by thermal degradation and/or interactions among the compounds (Wylje et al., 1992). To overcome this problem, pressure pulse techniques could be used. During such analyses, at the time of injection the inlet pressure can be rapidly increased, then returned to its optimum flow rate for the rest of the analysis. The effect of pulse duration was investigated using a 60 psi pressure pulse for 30, 120 and 300 sec. In addition, two experiments were performed where a pulse of 60 psi (20 sec duration) was delayed for 8 and 20 sec. Tables 3.8 to 3.10 summarise the results.

The effect of pulse duration on the efficiency of sample transfer is clearly seen in the increase of the total area counts and total number of peaks as the pulse duration increases. This increase in intensity and the higher recovery may reflect the faster transfer rate of volatiles from the interface to the column and shorter residence time of heat sensitive components in the liner, consistent with literature data (Stafford, 1995). Inspection of Table 3.9 also indicates that the later eluting peaks are more enhanced compared to the early peaks. However, there was no significant change in the resolution of later eluting peaks as a function of pulse duration, compared to early eluting peaks where the resolution decreased with increasing pulse duration. The particular advantage of the

delayed pulse technique over the pulse technique was the increased resolution of the early eluting peaks and in the retaining of two volatile gases; CO_2 and methyl amine, generated from the decarboxylation of glycine (see Figure 3.3).

Table 3.8 Effect of Various Pulse Sequences on the Total Area Counts and Total Number of Peaks [1]

Pulse Sequence	Total number of peaks	Total area count (x 10^8)
Delayed pulse		
S1	147	13.6
S2	138	8.4
S3	116	5.6
Pulse		
S5	68	12.9
S6	120	16.3
S7	142	35.3
Control [2]	87	2.9

[1] see Table 3.1
[2] without EPC (column inlet pressure 22 psi)

Table 3.9. Peak Area Counts (x10^7) of Selected Indicator Compounds as a Function of Pulse Sequence [1]

Pulse Sequence	a	b	c	d	e	f	g
S1	8.47	0.819	3.04	1.83	5.10	8.66	2.31
S2	4.23	0.440	2.03	2.46	5.14	9.58	3.52
S3	2.70	0.237	1.02	1.46	3.71	7.44	1.97
S5	26.3	0.211	1.97	16.3	0.210	0.131	1.01
S6	43.3	0.717	4.29	20.9	3.05	4.35	1.78
S7	84.4	1.23	6.63	39.7	6.20	9.21	6.34
Control [2]	0.35	0.007	0.22	0.97	1.47	3.34	3.84

[1] see Tables 3.1 and 3.2
[2] without EPC (column inlet pressure 22 psi)

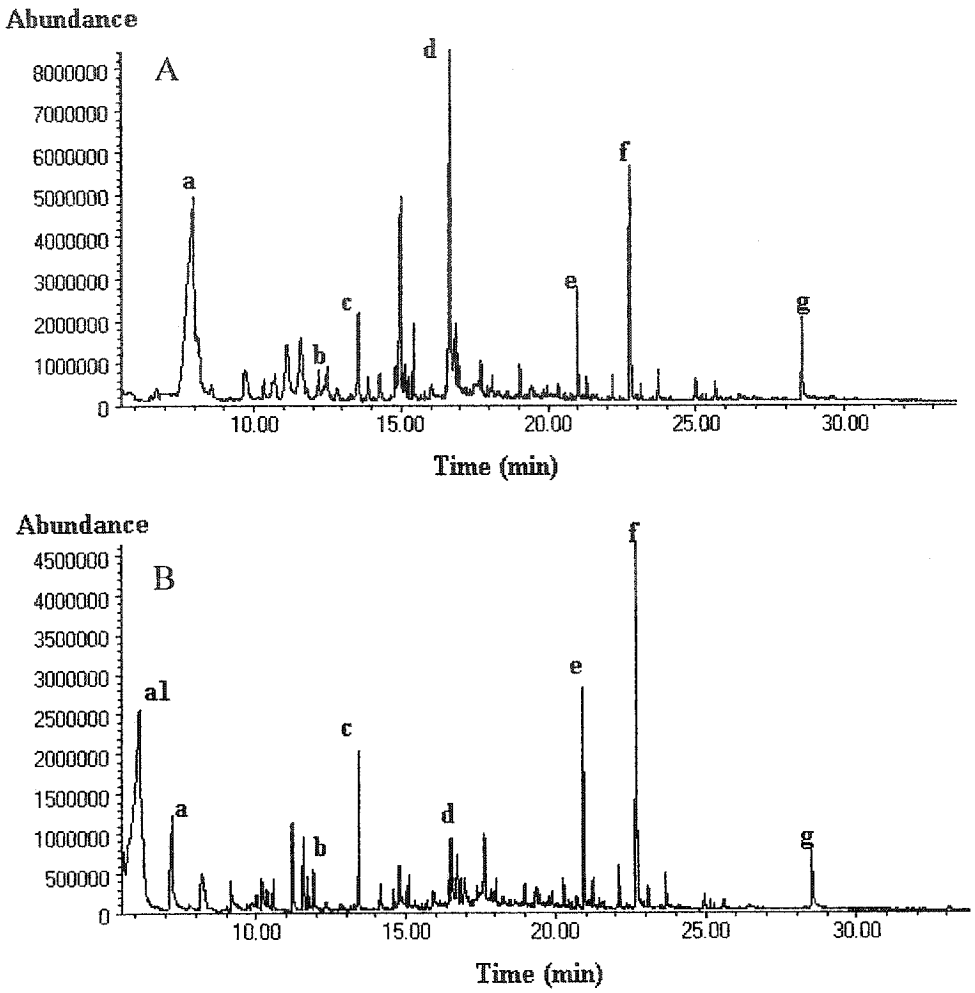


Figure 3. 3 Py-GC/MS Chromatograms of D-glucose-glycine Mixture; (A) 60 psi Pulse for 300 sec Followed by Constant Flow of 1.5 mL/min – Pulse S7; (B) 60 psi Delayed (8 sec) Pulse for 60 sec Followed by Constant Flow of 1.5 mL/min – Pulse S1. See Table 3.2 for Peak Designations, a1 = CO_2 and Methyl Amine

Table 3.10 Effect of Various Pulse Sequences on the Separation Parameters [1]

Pulse Sequence	Pyrazines			Pyrazinones		
	TZ	d	R	TZ	d	R
S1	28.50	1.71	34.72	25.10	1.78	30.72
S2	28.34	1.70	34.54	19.43	1.78	24.04
S3	29.50	1.71	35.90	26.28	1.77	32.10
S5	23.94	1.70	29.36	27.31	1.76	33.32
S6	18.91	1.69	23.43	26.50	1.76	32.37
S7	11.93	1.73	15.22	25.65	1.76	31.37
Control [2]	21.85	1.87	26.9	22.45	1.92	27.6

[1] TZ = separation number, R = resolution, d = $t^{\beta}_{R} - t^{\alpha}_{R}$,

[2] without EPC (column inlet pressure 22 psi)

3.3.4 Comparison of the Various Operational Parameters

The most efficient sample transfer protocols investigated under different operational parameters, are listed in Table 3.11. Application of a pressure pulse of 60 psi for 300 sec (S7) increased the efficiency of sample transfer by more than an order of magnitude compared to the control. Investigation of Table 3.12 indicates a dramatic increase in the total area counts of products a to d and specifically for product a where the increase was more then 200 fold. Although the resolution of early eluting peaks drops (see Table 3.13) due to the peak width broadening (see Figure 3.3A), however, the resolution of later eluting peaks increases.

Table 3.11 Effect of Selected [1] Operational Parameters on the Total Area Counts and Total Number of Peaks

Operational Parameter	Total number of peaks	Relative Total area	Total area count ($x10^8$)
Pulse (S7)	142	12.2	35.4
Delayed pulse (S1)	147	4.7	13.6
Constant pressure (60 psi)	135	3.1	9.1
Constant flow (4 mL/min)	73	1.2	3.6
Control [2]	87	1	2.9

[1] selected based on highest total area in the category
[2] without EPC (column inlet pressure 22 psi)

Table 3.12. Effect of Selected [1] Operational Parameters on the Peak Area Counts ($x10^6$) of Indicator Compounds Listed in Table 3.2

Operational Parameter	a	b	c	d	e	f	g
Pulse (S7)	844	12.3	66.3	397	62	92	63
Delayed pulse (S1)	84	8.2	30.4	18.3	51	86.6	23.1
Constant pressure (60 psi)	85	3.0	19	67	20	51	48
Constant flow (4 mL/min)	40	2	13	20	7.2	17	27
Control [2]	3.5	0.07	2.2	9.7	14.7	33.4	38.4

[1] selected based on highest total area count in the category
[2] without EPC (column inlet pressure 22 psi)

Table 3.13. Effect of Selected [1] Operational Parameters on the Separation Efficiency [2]

Operational Parameter	Pyrazines			Pyrazinones		
	TZ	d	R	TZ	d	R
Pulse (S7)	11.93	1.73	15.22	25.65	1.76	31.37
Delayed pulse (S1)	28.50	1.71	34.72	25.10	1.78	30.72
Constant pressure (60 psi)	18.45	1.69	22.9	27.01	1.87	33
Constant flow (4 mL/min)	29.28	1.63	35.6	23.54	1.742	28.9
Control [3]	21.85	1.87	26.9	22.45	1.92	27.6

[1] selected based on highest total area count in the category
[2] TZ = separation number, R = resolution, d = $t^{\beta}_{R} - t^{\alpha}_{R}$
[3] without EPC (column inlet pressure 22 psi)

3.4 Effect of Sample Pre-concentration during Py-GC/MS Analyses

3.4.1 Introduction

Application of Sample Pre-concentration Trap (SPT) in gas chromatographic analysis has been a well established and a suitable technique for trace analysis (Baltussen et al., 1998; Kirshen, 2003). It allows the application of a large volume of diluted gaseous sample and its subsequent concentration on a convenient adsorbent material prior to injection onto GC. Trapping of volatiles usually occurs on adsorbents such as charcoal, glass beads and/or porous polymers at ambient temperature. The sample is then desorbed onto the head of the GC column by reverse flushing with the carrier gas at elevated temperatures in order to release the analytes from adsorbent. High heating rate of 40°C/sec allows an instantaneous desorbtion and formation of a compact sample band on the cold analytical column head. The pre-concentration of a sample on SPT not only allows to drastically decrease the sample volume necessary for high resolution GC analysis, but also affects its separation and sensitivity. Since, the critical factor during sample introduction into GC is

to inject the sample as a short band or as a compact plug, so it reaches the column without broadening (Grob, 1994). Consequently, SPT allows to focus sample bands and to improve peak height and separation. This is a very important factor in mechanistic studies where isotopically labeled products have to be studied in great detail and good resolution is a key factor in investigating the ion mass fragmentation and localization of labels without interference form neighbouring bands. Moreover, since pyrolysis is a continuous process, volatiles are generated and are carried onto GC column over a period of time (seconds to minutes), resulting in broad peaks and poor resolution without the use of SPT. Thus, volatile trapping prior to GC analysis is crucial for high resolution Py-GC/MS analysis.

Furthermore, during pyrolysis of amino acids and sugars, relatively large quantities of water are generated as a consequence of dehydration reactions. Selection of appropriate SPT material allows to selectively retain compounds of interest and eliminate the undesired ones, such as water, which over time may damage the analytical column and undesirably increase ion protonation in the ion trap type detectors. The experimental variables such as time, temperature and flow rates are crucial to be optimized for every specific sample system (Jennings et al., 1997).

Although application of SPT for sample pre-concentration in trace and environmental analyses on GC has been well established, the application of SPT as an integrated Py-GC/MS pre-concentration device, has not been reported. In collaboration with Varian company, we have adapted their SPT system, initially designed for concentration of ambient air samples, to the Py-GC/MS through a valving system that can be controlled by

the resident GC/MS software that can direct the generated pyrolytic volatiles to SPT for trapping and then to desorb onto GC column head. Figure 3.4 shows the schematic of the SPT valving system in its two operational modes, (A) when volatiles from pyrolysis are swept for trapping and pre-concentration on SPT, and (B) carrier gas reverse flushing of volatiles and desorbtion onto the GC column. Unretained volatiles are purged out through the Front Split Vent. Various model systems have been studied to test the effect of sample pre-concentration. Glucose-glycine model system will be discussed as a representative sample.

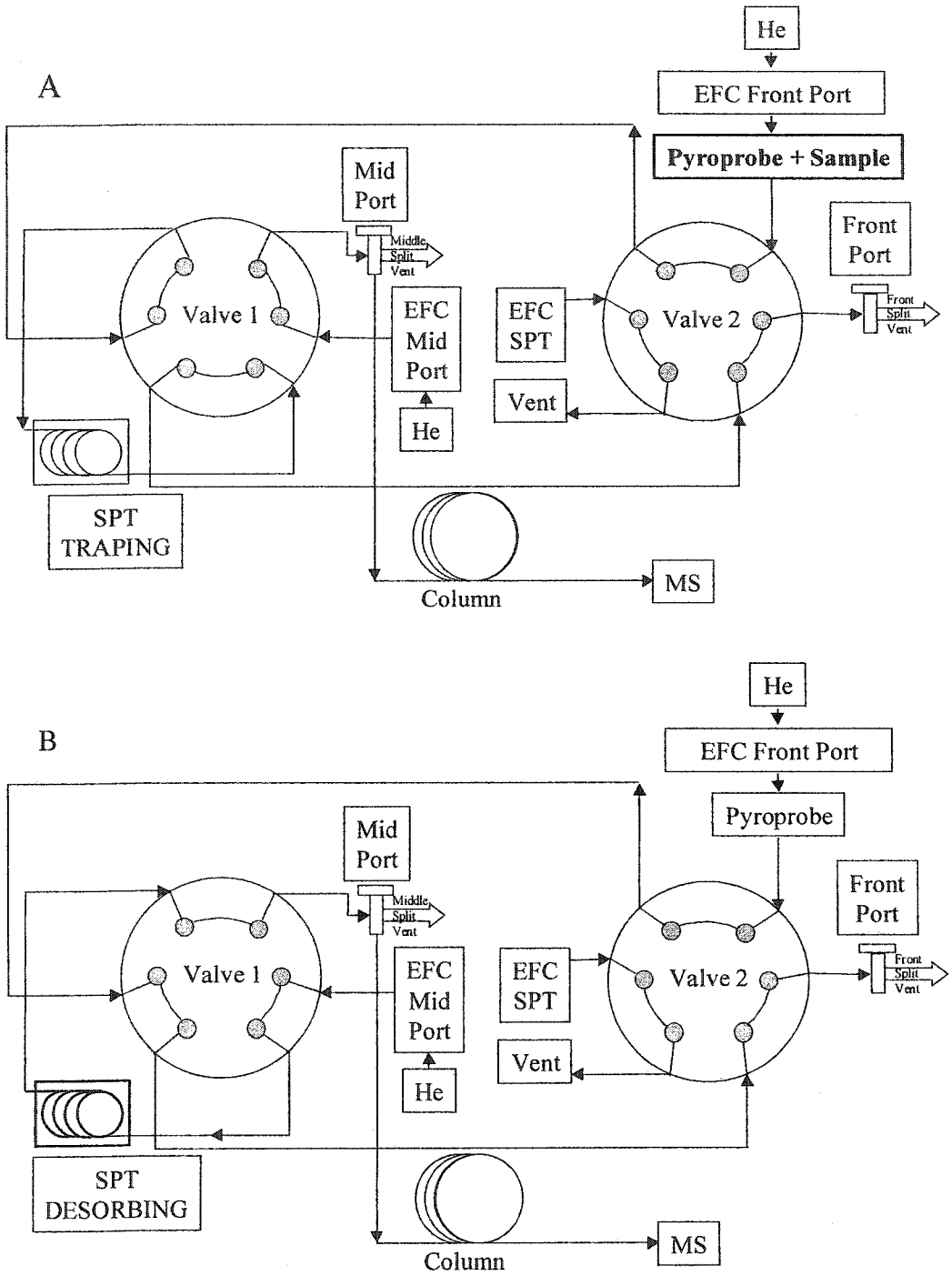


Figure 3.4 Schematic of SPT Valving System; **A** Position to Trap Volatiles from Pyrolysis, **B** Position to Desorb Volatiles into GC

3.4.2 Materials and Methods

3.4.2.1 Reagents and Chemicals

Glycine (99%) and D-glucose (96%) were purchased from Aldrich Chemical Company (Milwaukee, WI).

3.4.2.2 Py-GC/MS Conditions

A Varian CP-3800 gas chromatograph (Varian Inc., Walnut Creek, CA, USA) equipped with a sample pre-concentration trap (SPT) (Varian Tenax GR, 60-80 mesh, 29.2 cm active bed length) and coupled to a Varian Saturn 2000 mass selective detector and interfaced to a CDS Pyroprobe 2000 unit (CDS Analytical Inc., Oxford, PA), through a valved interface (CDS 1500) was used for Py-GC/MS analysis. The column used was a fused silica DB-5MS (50m length x 0.2 mm i.d. x 33 um film thickness; J&W Scientific, ON). Solid samples of mixtures of glycine and glucose at a ratio 3:1 and the size of 0.2mg were mixed with silica gel (Merck, grade 60) and inserted inside the quartz tubes (0.3mm thickness) for pyrolysis at 250°C for 20 sec. Generated volatiles were either concentrated on SPT at 50°C during 2 minutes and subsequently directed to GC column for separation and MSD analysis, or were directed directly to the column colled at –5°C. After 5 minutes, the SPT was purged at 250°C for the rest of the run. The GC column flow rate was regulated by Electronic Pressure Controller and set at pressure pulse of 60 psi for 2 min during SPT trapping, and 70 psi for 2 min during SPT desorbing, then maintained at constant flow of 1.5mL/min. Capillary direct MS interface temperature was set at 250°C, manifold at 50°C and ion trap temperature at 175°C. The ionization voltage of 70eV was used and EMV was set at 1750 V. The GC oven initial temperature was set at -5°C for 5

minutes and then increased to 50°C at a rate of 50°C/min.; after that the temperature was increased to 250°C at a rate of 8°C/min and kept at 250°C for 5 min. The MS fragmentation and peaks identification was estimated with the use of Varian software SatView (ver. 5.52) and NIST Library (ver.1.7).

3.4.3 Results and Discussion

Continuously generated volatiles during pyrolysis require a pre-concentration and re-focusing step prior to chromatographic separation, to prevent peak broadening. To study the contribution of SPT to the enhancement of sensitivity of Py-GC/MS analysis, a sample of glucose-glycine was studied with and without activation of SPT. In general, the application of sample pre-concentration prior to GC/MS analysis has shown to increase the number of detected volatiles and their abundances with improved separation and shape of chromatographic peaks (Figure 3.5).

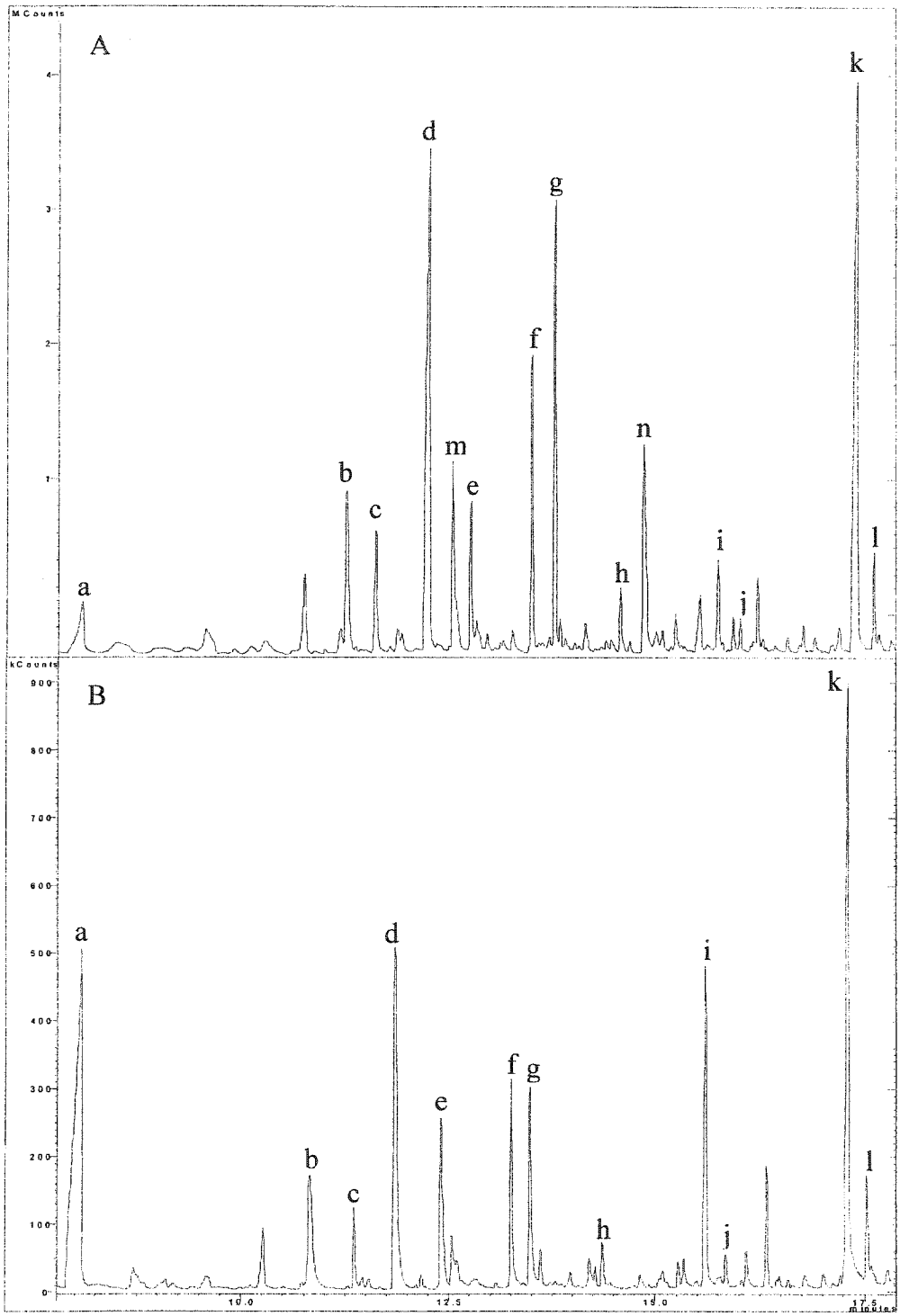


Figure 3.5 Pyrograms of Glucose-Glycine Mixtures with the (**A**) Application of SPT and without SPT (**B**)

Table 3.14 Effect of Sample Pre-concentration Prior GC Analysis on the Area Count of Selected Products from Glucose-Glycine Model

Analyte	Code	No SPT		with SPT	
		Total Peak Area Count	Relative Concentration [%]	Total Area Count	Relative Concentration [%]
Acetic Acid	a	1,217,010	27	437,259	1
Furfural	b	285,157	6	3,739,092	7
2-Furan Methanol	c	84,501	2	2,246,901	4
2-Cyclopentene-1,4-dione	d	637,437	14	12,400,849	25
1-(2-furanyl)-Ethanone	e	230,886	5	2,184,882	4
5-methyl-1H-Imidazole-4-methanol	f	246,348	5	4,158,363	8
5-methyl-2-Furancarboxaldehyde	g	284,010	6	8,446,571	17
trimethyl-Pyrazine	h	61,958	1	874,654	2
1-(1H-pyrrol-2-yl)-Ethanone	i	416,243	9	1,290,985	3
1-(1-methyl-1H-pyrrol-2-yl)-Ethanone	j	35,807	1	462,075	1
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one	k	941,352	21	13,015,371	26
4-amino-2-Hydroxytoluene	l	137,794	3	1,194,767	2
Total Area		***4,578,504***	***100***	***50,451,769***	***100***
Total number of peaks		***30***		***83***	

Inspection of Table 3.14 indicates that application of sample pre-concentration not only resulted in an increased number of detected peaks (from 30 to 83), but also provided increased concentration of generated volatiles. The concentration of some of the selected compounds (major compounds in the chromatogram) increased by more than eleven fold. In addition, there were numerous compounds, not present in the experiment without SPT (Table 3.15 lists the two most abundant).

Table 3.15 New Major Volatiles Generated from Glucose-Glycine Model with Sample Pre-Concentration

Analyte	Code	Total Area Count
1-methyl-1H-Pyrrole-2-carboxaldehyde, rt=13.801 min	m	4,007,472
Unknown, Mass 124, rt=16.227 min	n	4,657,240

3.5 Conclusion

Pressure programming and sample pre-concentration have a considerable influence on the quality and efficiency of sample transfer during Py-GC/MS analyses and resolution of resulting pyrograms. Optimum constant flow rate provides uniform conditions for separation throughout the analysis, shortens analysis time and increases the resolution, especially for the early eluting peaks. However, because of low pressure at the beginning of the run, sample loads could be low. Constant pressure, on the other hand, improves the resolution for the later eluting peaks and increases the sample load. Application of a pulse programming, high pressure at the beginning of the separation, followed by drop in pressure and constant flow rate throughout the rest of analysis, gives best sample transfer, highest resolution and uniform separation conditions. The choice of particular operational parameters will invariably depend on the specific objectives of the study. Certainly, pressure programming simultaneously with temperature programming allows more flexibility to achieve optimum conditions for the desired separation. Application of sample pre-concentration trap, with optimized trapping and desorbtion temperatures and flow rates, for pyrolyzates concentration prior to GC analysis results in higher abundances and increased number of detected compounds, improved peaks shape and resolution, and

consequently allows to use lower sample sizes or enable the detection of trace compounds.

CONNECTING PARAGRAPH

The results presented in Chapter 3 have shown that optimization of Py-GC/MS system, can be achieved through electronic pressure control and by the use of sample pre-concentration trap. Such enhancements in sensitivity and sample delivery are crucial to carry out before any development of specific applications of Py-GC/MS to the study of Maillard reaction, so that full advantage of the system can be taken. In the following chapter, the relevance of the mechanism of Maillard reaction under pyrolytic conditions to that occurring under aqueous systems will be studied to provide justification in relating information generated from pyrolysis to food systems.

CHAPTER 4

THE RELEVANCE OF THE MECHANISM OF MAILLARD REACION UNDER PYROLYTIC CONDITIONS TO THAT OF AQUEOUS SYSTEMS

4.1 Introduction

Maillard reaction can occur in liquid and solid phases during various thermal treatments of foods and/or manufacture of "reaction flavors". During roasting or baking, Maillard reaction products are mainly formed through solid phase interactions in the presence of moisture. This process can be mimicked through pyrolysis experiments (Baltes and Mevissen, 1988). Aqueous phase Maillard reactions can be encountered during domestic cooking and manufacture of "reaction flavors" that in turn can be mimicked through aqueous model system studies (Tressl et al., 1993a). Thermal processing under pressure or closed containers can also cause some interactions of the volatile intermediates to occur in the gas phase. The influence of the reaction phase on the course of Maillard reaction is manifested in the differences of the perceived flavors of roasted versus boiled food products.

In general, solid phase reactions are faster than their liquid phase counterparts (Laidler et al., 2003) due mainly to the more efficient energy transfer and the concentration effect and partially due to substantial loss of energy to the solvent in the liquid phase reactions. Furthermore, in solid phase, the energy absorbed is directly expended through chemical transformations and degradations. However, in aqueous phase reactions solvent assisted isomerizations and attainment of equilibrium conditions are more pronounced than in solid phase reactions. In addition, chemical transformations with polar transition states are more favored in liquid phase than in solid phase due to solvent stabilization effect. It is expected therefore solid phase reactions to produce a higher number of products in a shorter period of time compared with aqueous reactions under comparable reaction

conditions. In our laboratories, Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS) system has been extensively employed as a micro-scale reactor to study the mechanism of aroma formation from mixtures of ^{13}C or ^{15}N-labeled amino acids and ^{13}C-labeled reducing sugars through Maillard reaction (Yaylayan and Keyhani, 1996). Although the mechanistic pathways identified through pyrolysis experiments are relevant to roasting conditions, questions could be raised regarding its relevance to aqueous reaction conditions. Preliminary analysis of literature data (Yaylayan and Keyhani, 1999) has already indicated that mechanistic pathways operating under pyrolytic conditions are similar to that of aqueous reactions. Confirmation of the relevance of pyrolytic mechanisms to that of aqueous conditions is of practical importance. Since carrying out the reaction in the pyrolysis probe, can substantially reduce the analysis time (from hours to minutes) and eliminate the need for solvent extraction. In addition, the amount of reactants required to perform the experiments, is in the order of few milligrams. This property can facilitate studies with expensive isotopically labeled reactants. One of the advantages of using labeled reactants is that all the atoms of a reaction product can be traced back to their origin in the starting material (if properly labeled reactants are available). This fact facilitates elucidation of their mechanism of formation and also the assignment of their mass spectral fragments (Yaylayan and Huyghues-Despointes, 1996). Huyghues-Despointes et al. (1994) demonstrated that the pyrolysis of the proline Amadori compound in the quartz tube for 20 sec at 250°C, is comparable to autoclaving of proline/glucose mixture at 150°C for 1.5 h in water. Huyghues-Despointes and Yaylayan (1996) using ^{13}C-labeled glucoses elucidated several mechanistic pathways of formation of proline-specific products through retro-aldol reactions using Py-GC/MS.

Due to the convenience of carrying out such experiments to obtain mechanistic information regarding the Maillard reaction, it is informative to explore similarities and differences, if any, between the aqueous phase and pyrolytic reactions. In this paper we compare label incorporation patterns of selected Maillard reaction products generated under pyrolytic and aqueous reaction conditions.

4.2 Materials and Methods

4.2.1 Chemicals and Reagents

D-[1-^{13}C]glucose, D-[3-^{13}C]glucose, D-[4-^{13}C]glucose, D-[5-^{13}C]glucose, D-[6-^{13}C]glucose and [2-^{13}C]glycine were purchased from Cambridge Isotope Laboratories (Andover, MA), all other chemicals and D-[2-^{13}C]glucose were purchased from Aldrich Chemical Company (Milwaukee, WI).

4.2.2 Aqueous Reactions

Labeled [^{13}C-2]glycine or unlabeled glycine (6.0×10^{-4}M) and labeled (at [^{13}C-1]-, ([^{13}C-2]-, ([^{13}C-3]-, ([^{13}C-4]-, ([^{13}C-5]-, ([^{13}C-6]-) or unlabeled D-glucose (2.0×10^{-4}M) in 100 μL of water was placed in a 0.1 mL crimp-cap vial (Labcor, Anjou, QC). The samples were then heated in an oil bath (Fisher Scientific, Canada) at a temperature of 120°C for three hours and immediately quenched by placing the vials into an ice bath. The samples were subsequently extracted with ethyl ether (3 x 100μL) and concentrated before analysis.

4.2.3 GC/MS Analysis of Aqueous Extracts

The analysis was performed using a Hewlett-Packard GC/mass selective detector (5890 series II GC/5971B MSD). Samples (1μL) were injected using cool on-column injector and a fused silica capillary needle. An HP-PLOT-Q capillary column (30m x 0.32 mm x 0.20 μm, Hewlett-Packard, Mississagua, Canada) was used. The 3mL/min constant flow of helium was maintained by Electronic Pressure Controller (Hewlett-Packard). The column initial temperature (30°C) was held for 2 min then increased to 100°C at a rate of 30°C/min, then further increased to 250°C at a rate of 7°C/min, and held for 35 min. Capillary direct MS interface temperature was 280°C, ion source temperature was 180°C. The ionization voltage was 70eV, and the electron multiplier was 2047V. The mass range analyzed was 28-200 amu, with 3.7 scans/sec.

4.2.4 Pyrolysis GC/MS Analysis

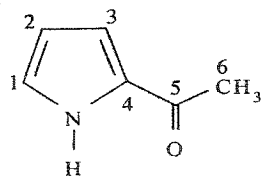
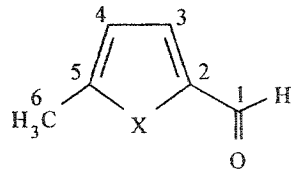
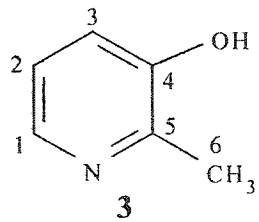
A Hewlett-Packard GC/mass selective detector (5890 series II GC/5971B MSD) interfaced to a CDS Pyroprobe 2000 unit, through a valved interface (CDS 1500), was used for Py-GC/MS analysis. In all experiments, solid samples of a mixture of labeled or unlabeled D-glucose-glycine (1:3 molar ratio, 3.5 mg) were introduced inside the quartz tube (0.3 mm thickness) and plugged with quartz wool and inserted inside the coil probe. The pyroprobe was set at 250°C at a heating rate of 50°C/s with a total heating time of 20 sec. The pyroprobe interface temperature was set at 250°C. The samples were introduced under splitless mode. The 3 mL/min constant flow was maintained by an Electronic Pressure Controller (Hewlett-Packard). 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 2047 volts. The mass range analyzed was 17 - 200 amu. The column was an HP-PLOT-Q (30 m x 0.32 mm x 0.20 μm (Hewlett-Packard, Mississagua, ON). The column initial temperature (30°C) was held for 2 min then increased to 100°C at a rate of 30°C/min, then further increased to 250°C at a rate of 7°C/min, and held for 35 min. The reported percent label incorporation values are the average of triplicate analyses.

4.3 Results and Discussion

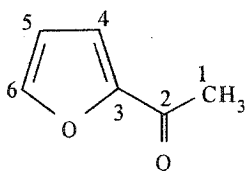
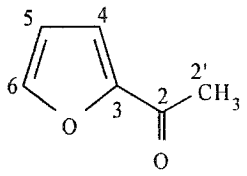
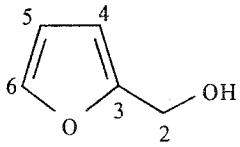
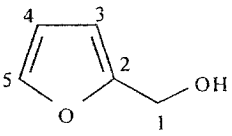
The impact of the reaction phase on the mechanism of formation of Maillard reaction products was studied by comparison of ^{13}C-label incorporation pattern of the selected common products formed in model systems consisting of labeled glycine and D-glucose subjected to both pyrolysis and heating in aqueous solutions. Analysis of the two samples have indicated that around 40 compounds from pyrolysis and 11 compounds from the extract of the aqueous sample were isolated by the column. Even though the pyrolysis reactions were shorter in duration relative to aqueous phase reactions (20 sec vs 3 h), yet they produced more reaction products. Such a difference is not surprising and consistent with the above discussion on the differences between the two phases. In addition, pyrolysis produced most of the compounds observed in the aqueous extract. Utilization of PLOT-Q column during pyrolysis allowed the detection of carbon dioxide, water, methyl amine and acetic acid, indicating facile decarboxylation and deamination of amino acid and dehydration of sugar under pyrolytic conditions.

Table 4.1. Position[1] and Percent Label Distribution of D-glucose Carbon Atoms in Pyrrole and Pyridine Derivatives

		
1	**2**	**3**
pyrolysis (100%)	X = NH **a**, O **b**	pyrolysis (100%)
aqueous (100%)	pyrolysis (100%)	aqueous (100%)
	aqueous (100%)	

[1]Numbers indicate original D-glucose carbon atoms

Table 4.2. Position[1] and Percent Label Distribution of D-glucose Carbon Atoms in Furan Derivatives

			
4	**4'**	**5**	**5'**
major	*minor*	*major*	*minor*
pyrolysis (100%)	pyrolysis (0%)	pyrolysis (90%)	pyrolysis (10%)
aqueous (70%)	aqueous (30%)	aqueous (100%)	aqueous (0%)

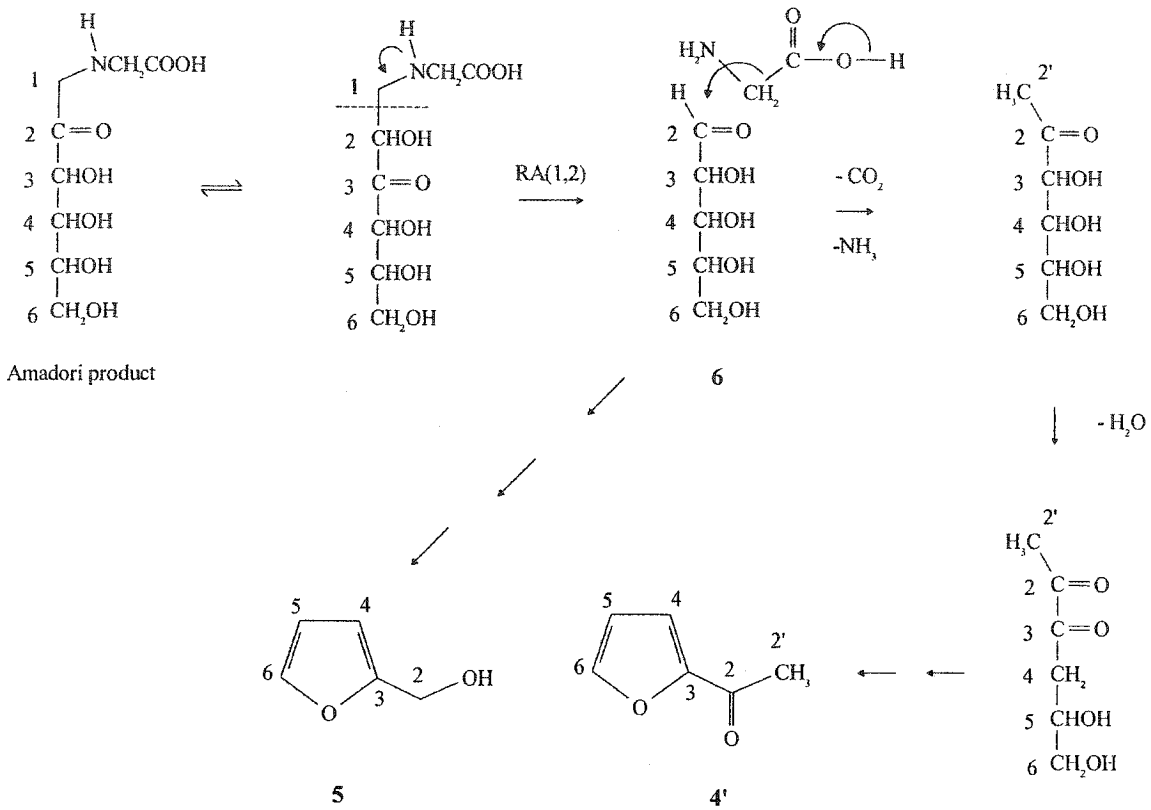
[1]Numbers indicate original D-glucose and glycine (primed) carbon atoms

4.3.1 Formation of Pyrrole and Pyridine Derivatives

Careful comparison of chromatograms resulting from pyrolysis and aqueous systems showed that one pyridine and two pyrrole derivatives were commonly identified for both systems (Table 4.1). All the three products; 1-(1H-pyrrol-2-yl)ethanone (**1**), 2-formyl-5-methylpyrrole (**2a**) and 2-methyl-3-pyridinol (**3**) showed 100 % incorporation of all the six carbon atoms of D-glucose in both pyrolysis and aqueous systems. Analysis of their mass spectral fragmentation patterns indicated that the sequence of D-glucose carbon atoms incorporated in these structures did not change and that intact hexose skeleton was involved in their formation. The position of D-glucose carbon atoms in these structures are indicated in Table 4.1. The mechanism proposed by Tressl et al., (1994) for the formation of compounds **1**, **2a** and **3** from 3-deoxyglucosone is consistent with this observation. In addition, the position of labeled [^{13}C-1]glucose atom in these products also corresponds to that observed by Tressl et al. (1994).

4.3.2 Formation of Furan Derivatives

Generation of 2-formyl-5-methylfuran (**2b**), 2-acetylfuran (**4**) and furanmethanol (**5**) was confirmative under both aqueous and pyrolytic conditions (Table 4.2). Similar to its nitrogen counterpart, 2-formyl-5-methylfuran (**2b**) showed 100 % incorporation of all the six carbon atoms of D-glucose in both pyrolysis and aqueous systems. Analysis of the mass spectral fragmentation pattern indicated that the sequence of D-glucose carbon atoms incorporated did not change and that intact glucose skeleton was involved in their formation. The positions of incorporated glucose carbon atoms are indicated in Table 4.1. The position of labeled [^{13}C-1]glucose atom also corresponded to that observed by



Scheme 4.1 Proposed Mechanism of Formation of Furanmethanol (5) and 2-acetylfuran (4'). [RA (x,y) = retro aldol cleavage between C-x and C-y atoms of D-glucose. Numbers indicate original D-glucose carbon locations]

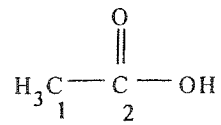
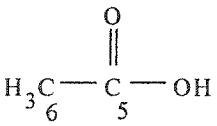
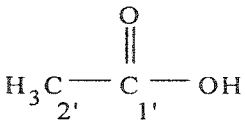
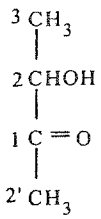
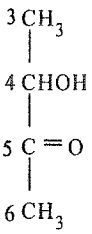
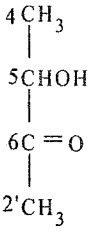
Tressl et al. (1993a). The obvious precursor of furan **2b** is 3-deoxyglucosone. Analysis of label incorporation in 2-acetylfuran (**4**) indicated existence of two pathways of formation in the aqueous system (Table 4.2). A major pathway (70%) involved incorporation of all the six carbon atoms of D-glucose with the retention of the original sequence of the carbon atoms and a minor pathway (30%) involving C-2' atom of glycine and formation of isotopomer **4'** (see Table 4.2). Pyrolysis mixture on the other hand, generated only **4**. Tressl et al. (1993a) identified furanmethanol (**5**) from [^{13}C-1]glucose model systems with no label incorporation which is consistent with the data shown in Table 4.2. However, the pyrolysis mixture also generated **5'** through a minor pathway

(10%) incorporating glucose carbon atoms one through five. Scheme 4.1 proposes the formation of **4'** and **5** through a common pentose intermediate (**6**) originating from the Amadori product through a retro-aldol reaction. The aldehyde **6** can either cyclize and eventually form furanmethanol (**5**) or react with glycine to form **4'** via 1-deoxy-2-ketohexose. Although the important reaction of amino acids as C-nucleophiles with aldehydes has been demonstrated earlier during pyrolytic reactions (Keyhani and Yaylayan, 1996; Yaylayan and Keyhani, 1999) this is the first observation of this transformation in an aqueous medium.

4.3.3 Formation of Acetic acid, 2,3-Butanedione and 3-Hydroxy-2-butanone

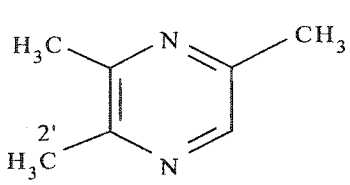
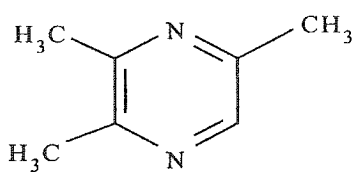
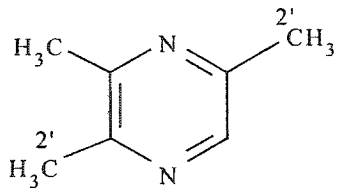
Acetic acid formation under both conditions followed the same two pathways. The major counted for 80%, where C-1 and C-2 atoms of glucose were used to generate acetic acid (Table 4.3). A minor, comprising 20% in the case of aqueous reaction and 5% in the case of pyrolytic reaction, involved C-5 and C-6 atoms of glucose. In the special case of glycine model system, deamination of the amino acid under pyrolysis can also generate acetic acid (15 %). In a previous study on the origin of α-dicarbonyl compounds in glucose model systems (Yaylayan and Keyhani, 1999), three pathways of formation of 2,3-butanedione have been identified during pyrolysis (Scheme 4.2). Interestingly, similar label distribution as in 2,3-butanedione was also found in 3-hydroxy-2-butanone indicating direct reduction of 2,3-butanedione into 3-hydroxy-2-butanone, possibly through disproportionation with α-hydroxycarbonyl compounds (Huyghues-Despointes and Yaylayan, 1996). Similar label distribution patterns were also found in the aqueous system (Scheme 4.2 and Table 4.3).

Table 4.3 Position[1] and Percent Label Distribution of D-glucose and Glycine Carbon Atoms in Acyclic Derivatives

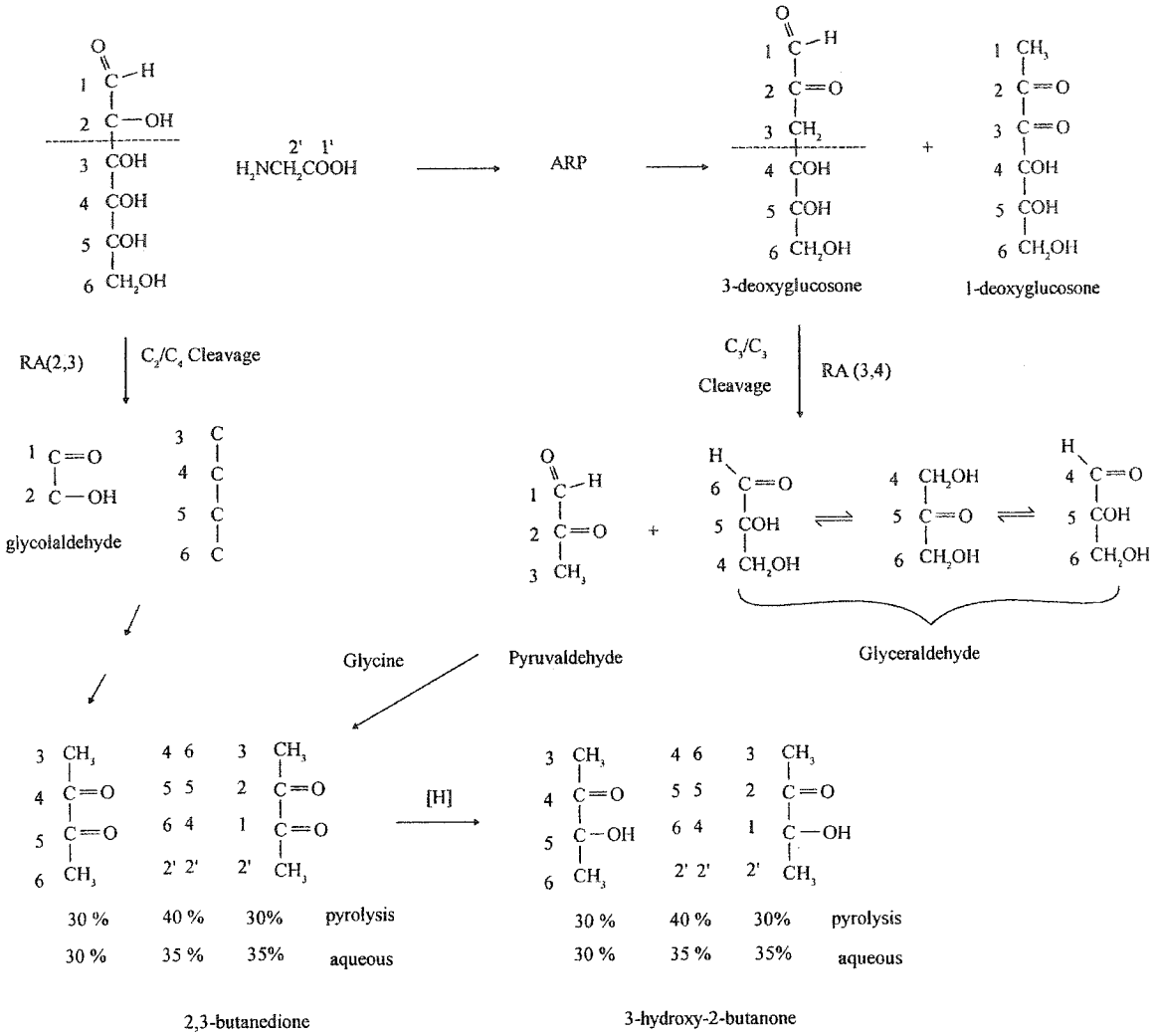
		
major pyrolysis(80%) aqueous (80%)	*minor* pyrolysis (5%) aqueous (20%)	*minor* pyrolysis (15%) aqueous (0%)
		
pyrolysis (30%) aqueous (35%)	pyrolysis (30%) aqueous (30%)	pyrolysis (40%) aqueous (35%)

[1]Numbers indicate original D-glucose and glycine (primed) carbons

Table 4.4 Position [1] and Percent Label Distribution of [^{13}C-2]glycine in Trimethylpyrazine

		
major pyrolysis(70%) aqueous (60%)	**7** *minor* pyrolysis(20%) aqueous (30%)	*minor* pyrolysis(10%) aqueous (10%)

[1]Numbers indicate original D-glucose and glycine (primed) carbons



Scheme 4.2 Proposed Fragmentation and Formation of Important Intermediates in D-Glucose/Glycine Model System [RA (x,y) = retro aldol cleavage between C-x and C-y atoms of D-glucose; aldol condensation between C-x and C-y atoms of d-glucose; [H] = reduction; numbers indicate original D-glucose carbon positions]

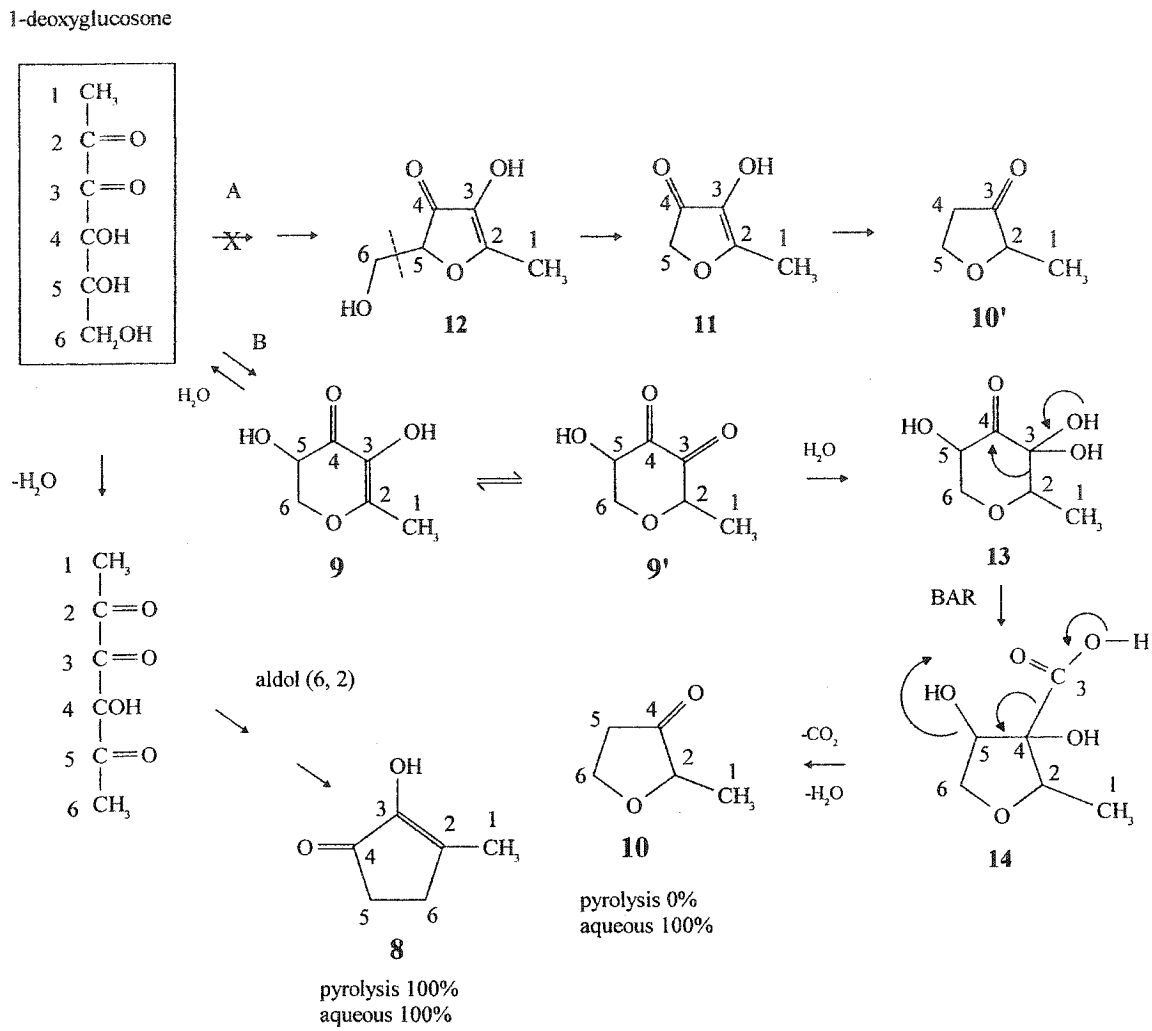
4.3.4 Formation of Trimethylpyrazine

Comparison of the incorporation of C-2' glycine atom into trimethylpyrazine (7 in Table 4.4) in both aqueous and pyrolytic systems, indicated that the major pathway (60-70%) in both systems produced singly labeled pyrazine and the two minor pathways produced unlabeled (20-30%) and doubly labeled (10%) trimethylpyrazines (Table 4.4). The label

distribution is consistent with the incorporation pattern of C-2' atoms of glycine in 2,3-butanedione (Scheme 4.2). Strecker reaction of glycine with pyruvaldehyde and 2,3-butanedione (Keyhani and Yaylayan, 1996) to eventually produce trimethylpyrazine can justify the C-2' label distribution observed in **7**, based on the origin of 2,3-butanedione and pyruvaldehyde (Scheme 4.2).

4.3.5 Formation of 2-hydroxy-3-methyl-2-cyclopenten-1-one (8, cyclotene)

In both model systems, all the six carbon atoms of D-glucose were 100% incorporated into cyclotene structure (Scheme 4.3), indicating involvement of intact hexose chain in its formation. Tressl et al. (1993b) also observed 100 % incorporation of labeled [^{13}C-1]glucose atom into cyclotene generated by autoclaving proline and hydroxyproline containing model systems. However, the position of the label varied with the amino acid. Proline generated cyclotene with label incorporation at the methyl carbon, similar to the cyclotene (**8**) generated by both pyrolysis and aqueous model systems. Whereas, in the case of hydroxyproline, [^{13}C-1]glucose label was incorporated at the position C-4 of cyclotene. Tressl et al. (1993b) proposed 1-deoxyglucosone as a precursor of cyclotene (Scheme 4.3) and Kim and Baltes (1996) identified cyclotene in the degradation mixture of pure 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (**9** in Scheme 4.3). The fact that hydrolysis of **9** generates back 1-deoxyglucosone, is a further confirmation of the above proposition.



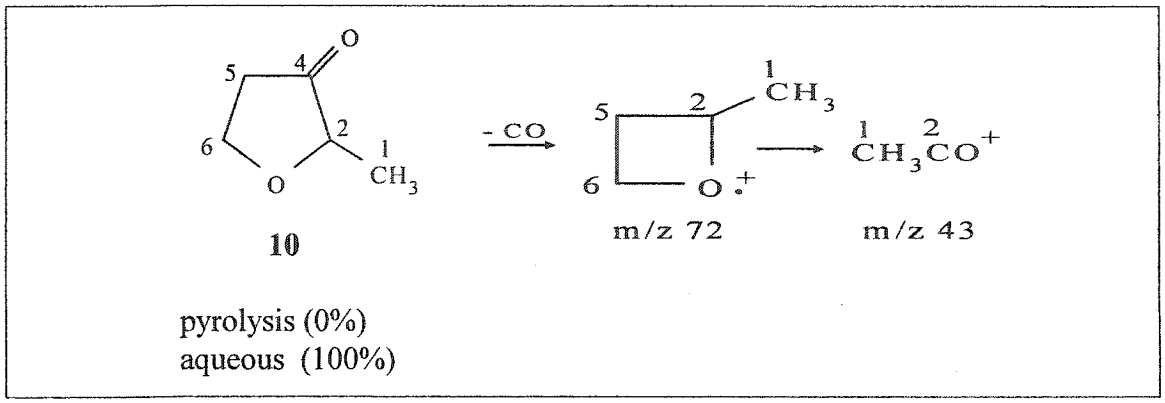
Scheme 4.3 Proposed Mechanism of Formation of Cyclotene (8) and 2-Methyl-4,5-dihydro-3(2H)-furanone (10). [Aldol (x,y) = aldol condensation between C-x and C-y atoms of D-glucose; BAR = benzilic acid rearrangement; numbers indicate original D-glucose carbon positions]

4.3.6 Formation of 2-methyl-4,5-dihydro-3(2H)-furanone (10)

Labeled studies of all glucose carbons allowed to elucidate a new formation mechanism of **10**, common to both analytical conditions. In previous investigation, with labeled studies using only [^{13}C-1]glucose, Rewicki et al. (1994) suggested the formation of **10** (Table 4.5 and Scheme 4.3) from 4-hydroxy-5-methyl-3(2H)-furanone (**11**) via 4-

hydroxy-2-(hydroxymethyl)-5-methyl-3(2H)-furanone (**12**) through cleavage of terminal hydroxymethyl group (Scheme 4.3, pathway A). Mass spectral analysis also indicated the incorporation of the [^{13}C-1]glucose label at the methyl carbons in both **11** and **10'**. According to the authors proposed pathway, C-6 atom of D-glucose should not be incorporated into the 2-methyl-4,5-dihydro-3(2H)-furanone (**10**) structure. However, studies performed with variously labeled D-glucoses (Tables 4.5 & 4.6) indicated 100% incorporation of the C-6 atom of D-glucose and no incorporation of C-3 atom of D-glucose in **10**. Furthermore, analysis of mass spectral fragmentation pattern of **10** (Tables 4.5 & 4.6) indicated the C-4 atom of D-glucose as the carbonyl carbon and the presence of two intact hexose fragments: C-1, C-2 and C-4, C-5, C-6. This fact and the lack of label scrambling in **10** strongly suggest a rearrangement mechanism for the formation of **10** starting from an intact hexose chain or ring. According to Kim and Baltes (1996), 2-methyl-4,5-dihydro-3(2H)-furanone (**10**) was one of the main products formed when pure 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (**9** in Scheme 4.3) was degraded at 150°C in aqueous solution. Sugars containing α-dicarbonyl moiety are known to undergo benzilic acid rearrangement under basic conditions to form α-hydroxy carboxylic acid derivatives (saccharinic acids) through alkyl group migration (Sowden, 1957). Similar rearrangement is also known to occur with cyclic α-diketones such as **9'** (Scheme 4.3) through ring carbon migration that leads to ring contraction and formation of geminal hydroxy carboxylic acids such as **14** (Vollhardt and Schore, 1994). Decarboxylative dehydration of **14** can yield structure **10** that is consistent with the observed label incorporation pattern.

Table 4.5 Mass Spectral Fragmentation and Percent Label Distribution in 2-methyl-dihydro-3(2H)-furanone (**10**)



10

pyrolysis (0%)
aqueous (100%)

Table 4.6. Percent Label Distribution[1] in Selected Mass Spectral Fragments of 2-methyl-dihydro-3(2H)-furanone (**10**)

Model system	**m/z 100**	**m/z 101**	**m/z 72**	**m/z 73**	**m/z 43**	**m/z 44**
	M	**M+1**	**M**	**M+1**	**M**	**M+1**
D-Glucose/Glycine	100	0	100	0	100	0
D- [1-^{13}C]Glucose/Glycine	0	100	0	100	0	100
D- [2-^{13}C]Glucose/Glycine	0	100	0	100	0	100
D- [3-^{13}C]Glucose/Glycine	100	0	100	0	100	0
D- [4-^{13}C]Glucose/Glycine	0	100	100	0	100	0
D- [5-^{13}C]Glucose/Glycine	0	100	0	100	100	0
D- [6-^{13}C]Glucose/Glycine	0	100	0	100	100	0
D-Glucose/L-[^{13}C-2]Glycine	100	0	100	0	100	0

[1] Percentages are corrected for natural abundance and for less than 100 % enrichment

4.4 Conclusion

Comparison of the label incorporation patterns of the common products formed in aqueous and pyrolytic Maillard model systems have indicated that the major pathways of formation of variety of important Maillard products follow the same mechanisms and that mechanistic information gained through pyrolysis can be applied to aqueous reactions. The presence of polar solvents, such as water, can promote some reactions or inhibit others by the solvent function as a dilutant and heat dissipating agent, or a reactant.

CONNECTING PARAGRAPH

In chapter 4, comparison of the formation pathways of the common products generated under pyrolytic and aqueous conditions has confirmed the similarity of their mechanisms of formation. This study has confirmed that mechanistic information acquired through pyrolysis is relevant to aqueous reactions occurring in food systems. In the following chapter, an application of Py-GC/MS system is demonstrated through investigation of the role of β-hydroxy amino acids in the Maillard reaction and its consequences for food systems.

CHAPTER 5

APPLICATION OF PY-GC/MS TO THE STUDY OF MECHANISM OF MAILLARD REACTION: β-HYDROXY AMINO ACID MODEL SYSTEMS

5.1 General Introduction

Carbohydrate degradations during Maillard reaction play a crucial role in determining product distribution of various sugar-derived products. Maillard reaction pathways occurring in the so-called "sugar fragmentation pool" (Yaylayan, 1997) provide the reactive α-dicarbonyl compounds and carbon skeletons for various precursors essential for the formation of different heterocyclic aroma compounds. The main feature of the degradative reactions in the "sugar fragmentation pool" is the formation of C_2, C_3, C_4, C_5 and C_6 sugar-derived building blocks of Maillard products. Recently, a new pathway was identified that also contributes to the enrichment of the "sugar fragmentation pool". This pathway involves the amino acid participation in the chain elongation process of smaller sugar derived reactive fragments (Keyhani and Yaylayan, 1996). In this chapter using variously ^{13}C-labeled intermediates, we demonstrate the unique role played by L-serine and L-threonine in generating not only sugar specific reactive intermediates that enrich the "sugar fragmentation pool" in the absence of a sugar source, but also of generating 2-keto acids and α-amino alcohols. These intermediates display the remarkable ability to undergo Amadori rearrangement, a process that has so far been thought to occur only between reducing sugars and amino acids.

5.2 Elucidation of the Role of β-hydroxy Amino Acids in the Generation of Sugar Specific Intermediates

5.2.1 Introduction

During Maillard reaction, the formation of most heterocyclic compounds requires the presence, in the reaction mixture, of both sugars and amino acids. However, the unique and interesting case of β-hydroxy amino acids such as L-serine and L-threonine is the exception (Reese and Baltes, 1992). The two amino acids can generate, during thermal decomposition, numerous heterocyclic compounds in the absence of a carbohydrate source, indicating their ability to form sugar-specific reactive intermediates such as dicarbonyls. Thermal degradation of L-serine alone (180 - 360°C) has been reported to produce a wide range of heterocyclic compounds such as methyl and ethyl substituted pyrazines and pyrroles, pyrrolylalkanols and some fused heterocyclic compounds (Kato et al., 1970; Wang and Odell, 1973; and Reese and Baltes, 1992). Kato et al. (1970) pyrolyzed L-serine at 280°C and identified several methyl and ethyl substituted pyrazines and pyrroles in addition to ethylamine, ammonia, aminoethanol, acetaldehyde and 2,5-diketo-3,6-dimethylpiperazine. Wang and Odell (1973) similarly, identified various pyrazines when L-serine was pyrolyzed at 200°C. More detailed studies on the degradation products of L-serine was carried out by Reese and Baltes (1992), they autoclaved L-serine at 120, 150 and 180°C for one hour, however, only at 150 and 180°C degradation products could be detected. The number of products increased from 58 to 70 at the higher temperature. In addition to methyl and ethyl substituted pyrazines and pyrroles, they also detected 2-(1-pyrrolyl)-ethanol, pyrrolo[1,2-a]pyrazine and various

carbonyl, dicarbonyl and α-hydroxycarbonyl compounds including 2,3-butanedione and 1-hydroxy-2-propanone. The origin of such reactive intermediates formed upon thermal degradation of amino acids and sugars is relatively difficult to determine without ^{13}C-labeling studies, due to the multiple origin of these components. By utilizing Py/GC/MS to generate and identify stable end-products formed by the incorporation of these reactive intermediates into their molecular structure using properly ^{13}C-labeled reactants, identification of their origin becomes possible (Yaylayan and Keyhani, 1998; Yaylayan et al., 1998, Yaylayan et al., 2000).

5.2.2 Materials and Methods

5.2.2.1 Reagents and Chemicals

All reagents and chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI). DL-[2-^{13}C]alanine (92 %), [2-^{13}C]glycine (98 %) L-[1-^{13}C]serine (99 %), L-[2-^{13}C]serine (99 %), L-[3-^{13}C]serine (98 %) were purchased from Cambridge Isotope Laboratories (Andover, MA), and L-threonine (98%) from Aldrich Chemical Company (Milwaukee, WI).

5.2.2.2. 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. Solid samples (1-4 mg) of L-serine, L-serine/glycine, L-serine/alanine, glycolaldehyde/phenylhydrazine or L-serine/phenylhydrazine were introduced inside a quartz tube (0.3 mm thickness) which

was plugged with quartz wool and inserted inside the coil probe. The Pyroprobe was set at the desired temperature (250°C) and with a THT (total heating time) of 20 sec. The pyroprobe interface temperature was set 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. Capillary direct MS interface temperature was 280°C; ion source temperature was 180°C. The ionization voltage was 70 eV, and the electron multiplier was 1682 V. The mass range analyzed was 30-300 amu. The column was a fused silica DB-5 column (60 m length x 0.25 mm i.d. x 25 um film thickness; Supelco, Inc.). The column initial temperature (-5°C) was increased to 260°C at a rate of 10°C/min. and held at 260°C for 15 minutes.

5.2.3 Results and Discussion

5.2.3.1 Initial Degradation Products of L-serine and L-threonine

Pyrolysis of L-serine and L-threonine at 250°C produced around seventy different products (see Figure 5.1 for serine). Similar number of products was observed during autoclaving of L-serine at 180°C (Reese and Baltes, 1992). Most of the heterocyclic compounds identified were methyl and ethyl-substituted pyrazines and pyrroles in addition to 2-(1-pyrrolyl)-ethanol. The substitution pattern of the observed pyrazines and pyrroles indicated the formation of different α- and δ-dicarbonyl compounds with three, four, five and six carbon atoms. Obviously, these dicarbonyl compounds were formed through condensation and chain elongation of shorter carbonyl intermediates formed from the initial degradation of the amino acids. Scheme 5.1 summarizes the initial degradation pathways of both amino acids, consistent with the observed products in the

decomposition mixture. The results reveal that there are three major degradation pathways: decarboxylation followed by deamination, a retro-aldol cleavage, and dehydration.

Retro aldol cleavage (pathway A, Scheme 5.1) produces formaldehyde and glycine in the case of serine and acetaldehyde and glycine in the case of threonine. Decarboxylation (pathway B) produces 1-aminoethanol in the case of L-serine and 1-amino-2-propanol in the case of L-threonine. Subsequent deaminations produce acetaldehyde or acetone. Ammonia, 1-aminoethanol and acetaldehyde have all been detected during the decomposition of L-serine (Keto et al., 1970) whereas acetone and 1-amino-2-propanol were observed during pyrolysis of threonine supporting the presence of this pathway. On the other hand, dehydration (pathway C) can lead to the formation of pyruvic acid in the case of L-serine and 2-ketobutanoic acid in the case of L-threonine. These acids can decarboxylate and form acetaldehyde and propanal, respectively. It has been shown (Yaylayan and Wnorowski, 2002) that 2-keto acids can be thermally converted into amino acids in the presence of ethanolamine. This process converts pyruvic acid into alanine and ethanolamine into glycolaldehyde (Scheme 5.2).

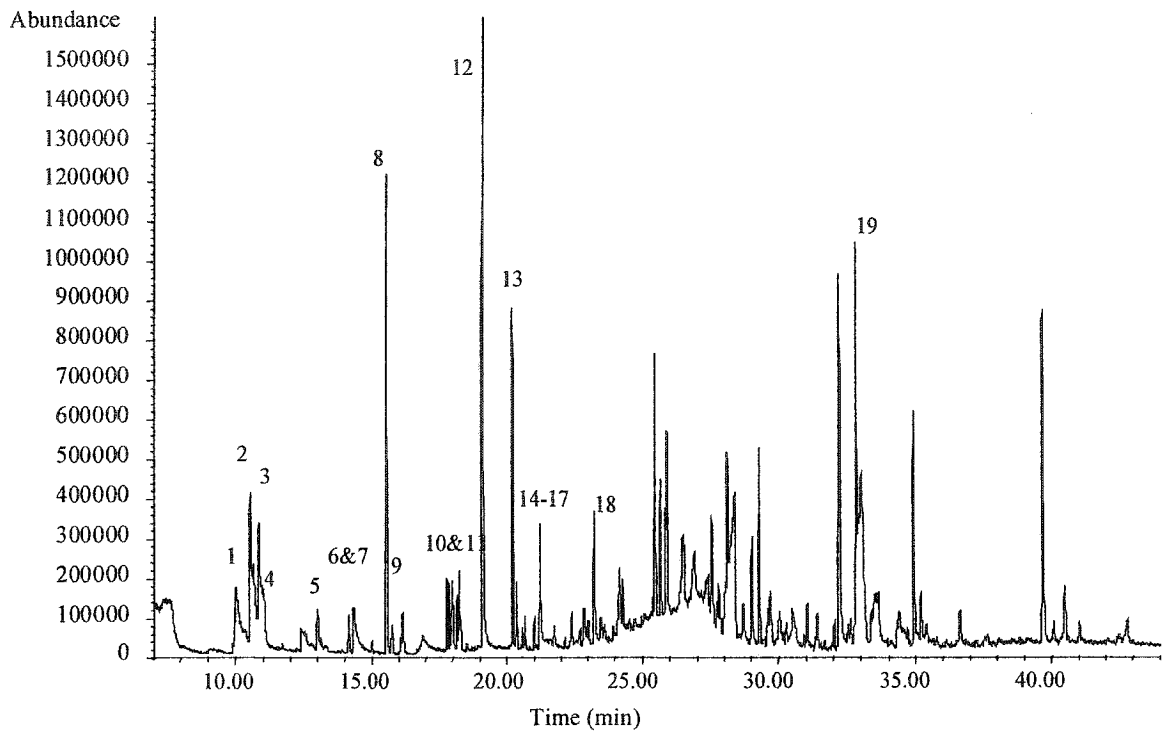
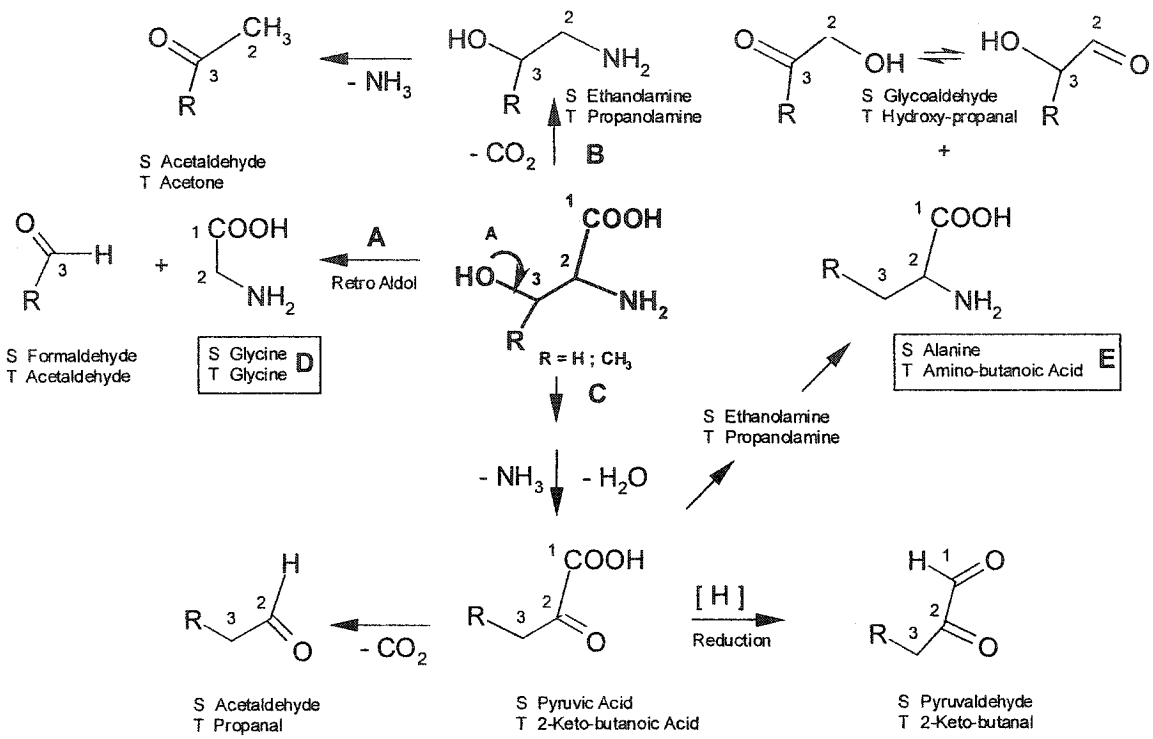
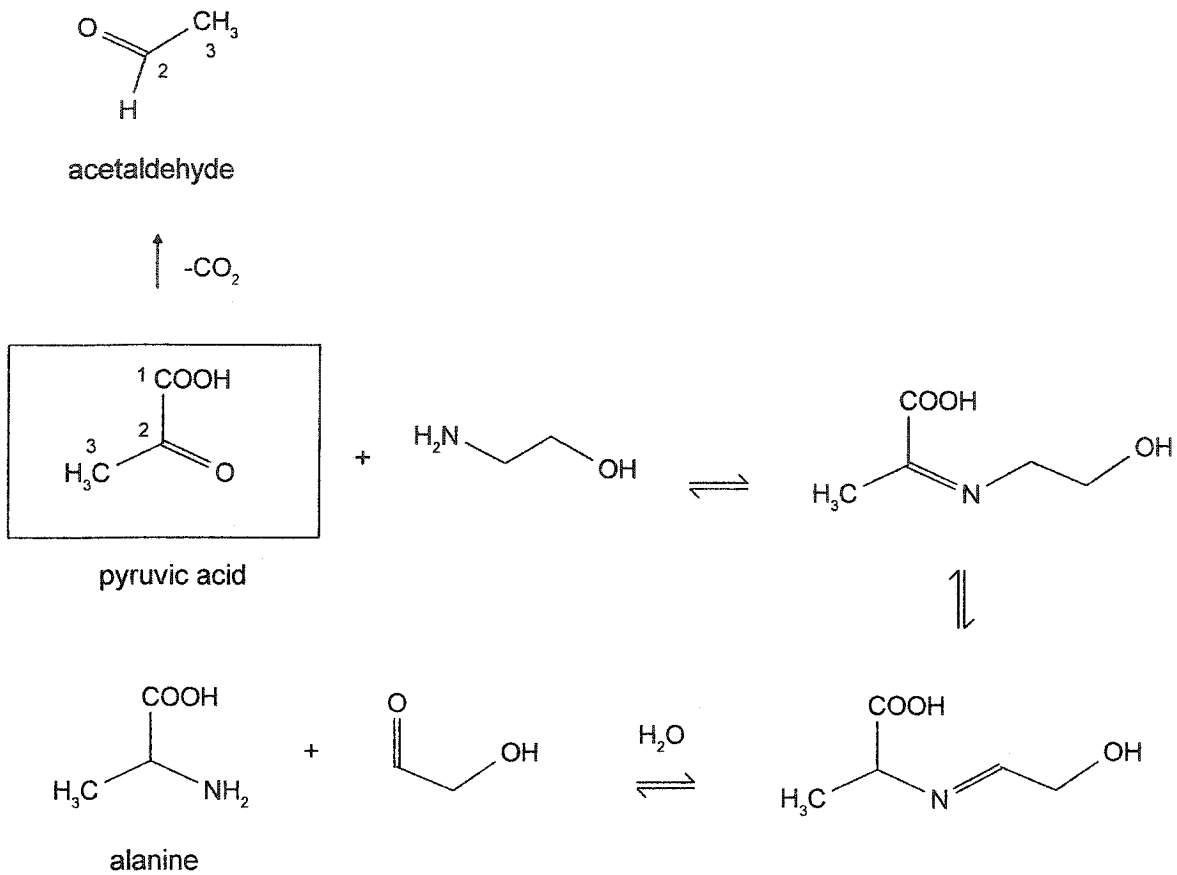


Figure 5. 1 Pyrogram of L-serine (250 °C for 20 sec): (1) 2-amino-1-ethanol; (2) pyrazine; (3) pyrrole; (4) 2-methyl-2-oxazoline; (5) methylpyrazine; (6) isoxazole; (7) propaneamide; (8) ethylpyrazine; (9) 2,3-dimethylpyrazine; (10) 2-ethyl-6-methylpyrazine; (11) 2-ethyl-3-methylpyrazine; (12) 2-(1-pyrrolyl)ethanol; (13) 2,6-diethylpyrazine; (14) 2,3-diethyl-5,6-dimethylpyrazine; (14-17) 2,3-diethylpyrazine, 2,5-diethylpyrazine, 2-ethyl-6-vinylpyrazine; 2,3-dimethyl-5-ethylpyrazine; (18) 2,3-diethyl-5,6-dimethylpyrazine; (19) cyclic dimer of alanine



Scheme 5.1 Initial Thermal Degradation Products of L-Serine (S) and L-Threonine (T)



Scheme 5.2 Proposed Formation Mechanism of Glycoaldehyde and D,L-Alanine from L-Serine, (numbers indicate original carbon atom locations of L-serine)

The formation of alanine and glycine was confirmed due to the detection of their cyclic dimers (cycloglycylalanine and 2,5-diketo-3,6-dimethylpiperazine) and because of the formation of glycine and alanine specific products such as methyl- and ethyl-substituted pyrazinones (Yaylayan, 1997). In addition, detection of unlabeled dimers in the model systems containing L-serine and labeled L-alanine or glycine indicated that these amino acids can also be formed via thermal degradation of L-serine (Table 5.1). However, the

observed percentage of unlabeled glycine in this mixture indicates that it is produced in much smaller amounts relative to alanine (Yaylayan et al., 2000). Baltes (1990) predicted the formation of similar initial degradation products from L-serine.

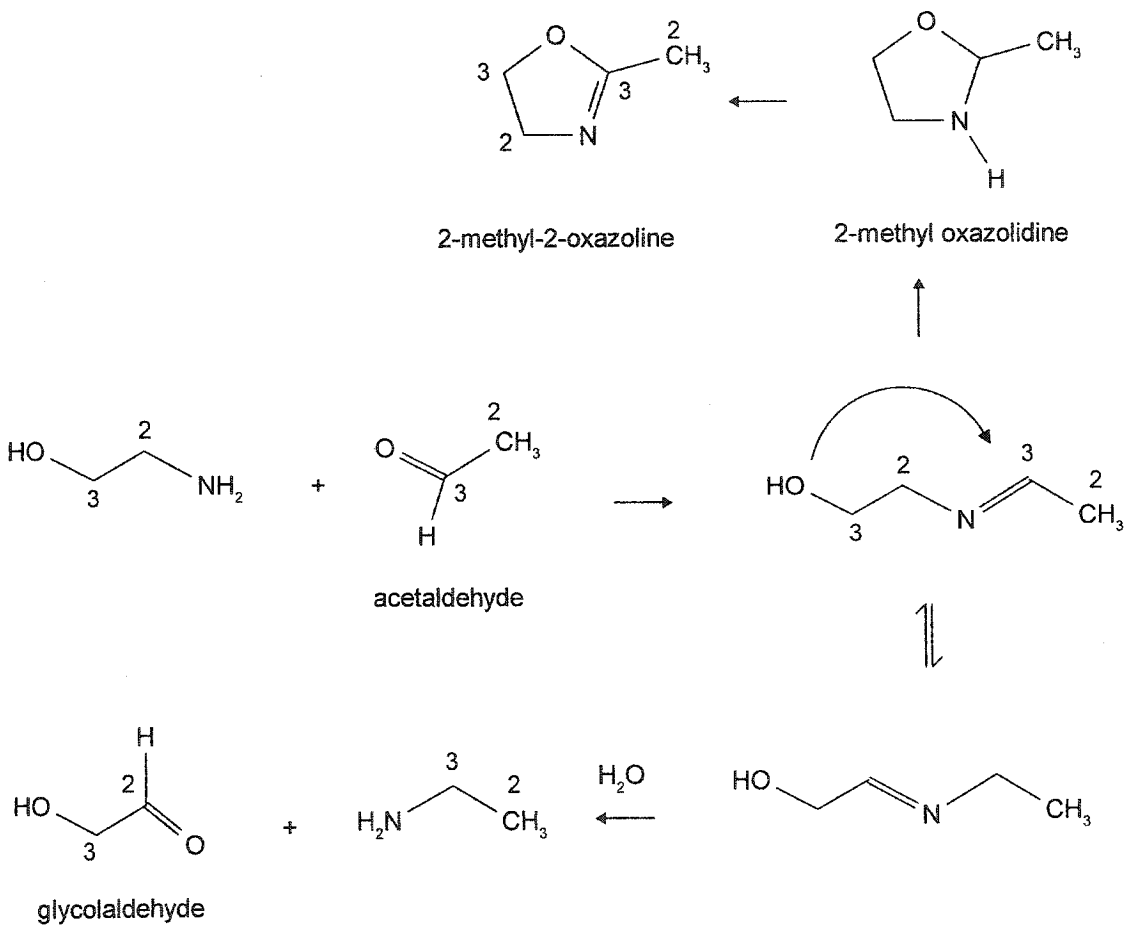
Table 5.1 Percent Incorporation of ^{13}C-labeled Carbon Atoms in Cycloglycylalanine and 2,5-diketo-3,6-dimethylpiperazine (alanine cyclic dimer)

2,5-Diketo-3,6-dimethylpiperazine	M	M +1	M+2
L-[1-^{13}C]serine	0	0	100
L-[2-^{13}C]serine	0	0	100
L-[3-^{13}C]serine	0	0	100
L-serine/[2-^{13}C]alanine (1:3)	20	50	30
Cycloglycylalanine			
L-serine/[2-^{13}C]glycine (1:3)	5	95	

5.2.3.1.1 Formation of Glycolaldehyde, Alanine and Ethylamine from Labeled Serines

The reaction of aminoethanol with carbonyl compounds is a common method of synthesis of oxazolidines (Acheson, 1976) as shown in Scheme 5.3. Detection of 2-methyl-2-oxazoline (Figure 5.1) in the pyrolyzate indicates the interaction of acetaldehyde with aminoethanol during pyrolysis of L-serine. Analysis of label incorporation in 2-methyl-2-oxazoline also indicated the presence of two C-2 and two C-3 atoms of L-serine, consistent with the proposed mechanism. Isomerization of the intermediate imine thus formed (Scheme 5.3) and its subsequent hydrolysis can generate ethylamine and glycolaldehyde. Ethylamine has been detected in the pyrolysis mixture of L-serine (Kato et al., 1970). Similarly, formaldehyde and pyruvic acid can be converted into their corresponding amines through their interaction with aminoethanol. Formaldehyde can generate methylamine containing C-3 atom of L-serine and pyruvic acid can generate D,L-

alanine (Scheme 5.2). The presence of 2,5-diketo-3,6-dimethylpiperazine (cyclic alanine dimer) in the pyrolysis mixture (Table 5.1) confirms the formation of alanine from L-serine. Kato et al. (1970), also isolated 2,5-diketo-3,6-dimethylpiperazine from thermal degradation of L-serine. Utilization of L-[3-^{13}C], L-[2-^{13}C] and L-[1-^{13}C]serines produced



Scheme 5.3 Proposed Mechanism of Formation of Glycolaldehyde and Ethylamine from Labeled Serines

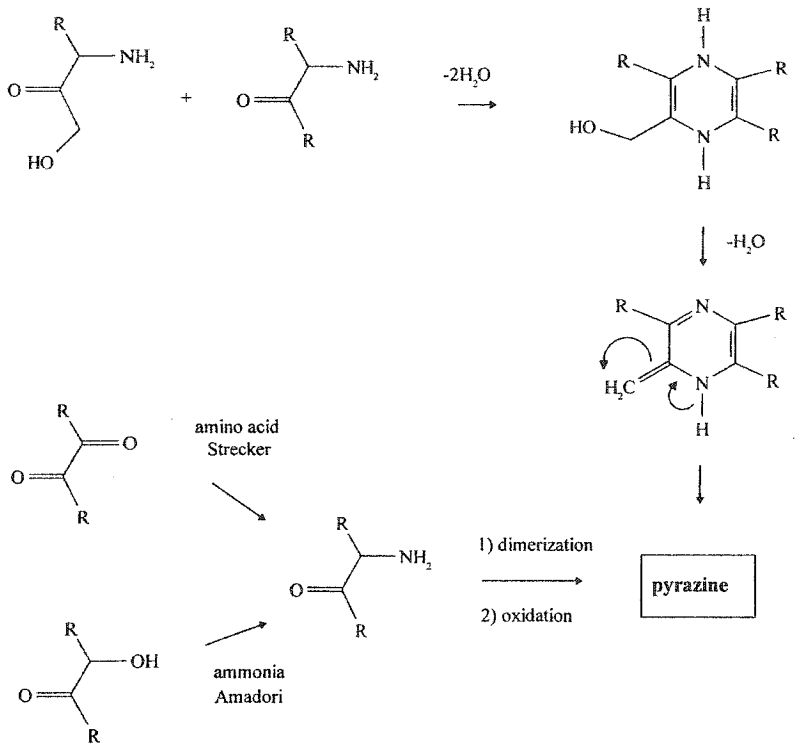
(Numbers indicate original carbon atom locations of L-serine)

100 % doubly labeled 2,5-diketo-3,6-dimethylpiperazine in all cases. A mixture of L-serine with L-[2-^{13}C]alanine produced 20 % unlabeled and 50 % singly labeled dimer indicating an abundance of an alternative source of alanine formation in the system. Therefore, under pyrolytic conditions L-serine could be considered an efficient producer of alanine. Although glycolaldehyde is not reported as a degradation product of L-serine, however evidence for its formation can be provided though trapping experiments with phenylhydrazine. N-Ethylidene-N'-phenylhydrazine was the major reaction product of phenylhydrazine in the presence of glycolaldehyde and as well as in the presence of L-serine. Interestingly, performing the pyrolysis of L-serine in the presence of phenylhdrazine prevented the formation of most heterocyclic compounds observed during pyrolysis of L-serine alone and the only major product detected was N-ethylidene-N'-phenylhydrazine. This observation indicates the important role of glycolaldehyde as the major precursor of other carbonyl compounds during the pyrolysis of L-serine. Furthermore, similar effect was observed when L-serine was pyrolyzed in the presence of excess glycine. The intensities of pyrazine and pyrrole peaks were reduced significantly and the major products were pyrazinones that are known to require glycolaldehyde as their important precursor (Keyhani and Yaylayan, 1996).

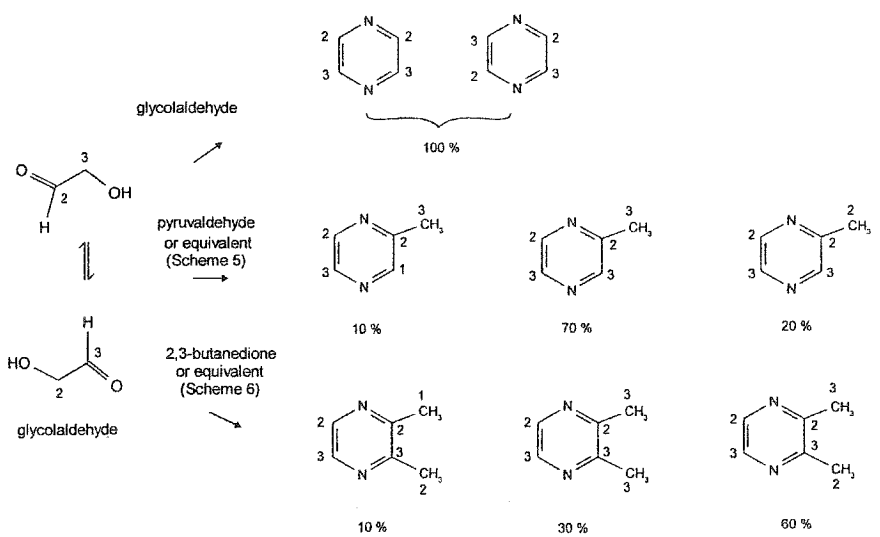
5.2.3.2 Advanced Degradation Products of L-serine

Incorporation of reactive carbonyl compounds into stable end-products has long been utilized to investigate their origin in the reaction mixture (Yaylayan et al., 1998). Useful and stable end-products that form abundantly in Maillard systems are the different pyrazines. Pyrazines can be formed by the dimerization of α-aminocarbonyl compounds

followed by oxidation. An exception is when one of the reactants is a α-amino-α'-hydroxyl derivative, then a dehydration step instead of oxidation will generate pyrazines (Scheme 5.4). α-Aminocarbonyl compounds on the other hand can be formed either by Strecker reaction or by Amadori rearrangement of a α-hydroxycarbonyl compound with ammonia (Scheme 5.4). Consequently, reactive precursors of pyrazines can also include α-hydroxy carbonyls and α,β-dihydroxy carbonyls in addition to α-dicarbonyl compounds (Scheme 5.4). Using ^{13}C-labeled L-serines at the different carbon atoms and analyzing the label incorporation in pyrazines, the origin of such precursors can be predicted (Scheme 5.5). For example, analysis of label distribution in the parent pyrazine formed in the L-serine pyrolyzate, allowed unambiguous determination of the origin of its carbon atoms. Such analysis confirmed 100 % incorporation of two C-2 and two C-3 atoms of L-serine into the pyrazine structure, indicating the reaction of glycolaldehyde with ammonia through Amadori rearrangement to form 2-aminoacetaldehyde (α-amino-carbonyl) as the precursor of parent pyrazine (Scheme 5.5). Some authors propose that upon heating, L-serine will decarboxylate and dimerize to form parent pyrazine (Baltes and Bochmann, 1987). This pathway although sound, will represent a minor route to pyrazine formation due to the fact that generated upon decarboxylation ethanolamine will rather deaminate and/or react with reactive carbonyl intermediates than dimerize which would be less thermodynamically favourable.



Scheme 5.4 Carbonyl Precursors and Proposed Pathways of Formation of Pyrazine



Scheme 5.5 Carbonyl Precursors and Percent Isotopomers of Pyrazine and Methyl- and 2,3-Dimethylpyrazines (see Tables 5.2 and 5.3). Similar to parent pyrazine, the positions of the C-2 and C-3 atoms originating from glycolaldehyde in methyl- and 2,3-dimethylpyrazines can be interchanged. Numbers indicate original carbon atom locations of L-serine.

5.2.3.2.1 Origin of Pyruvaldehyde and Three-carbon Precursors of 2-Methylpyrazine

Methylpyrazine can be used to determine the origin of pyruvaldehyde or an equivalent of three-carbon precursor unit. Labeled studies have indicated the incorporation of all the three carbon atoms of L-serine in different ratios, into the methylpyrazine (Table 5.2 and Scheme 5.5). Analysis of the labeled data in Table 5.2 confirmed the existence of three pathways (designated as A, B and C in Scheme 5.6) of formation of carbonyl precursors of methylpyrazine. According to the labeled data in Table 5.2, the major pathway (A in Scheme 5.6, 70%) involves aldol addition of formaldehyde to glycolaldehyde to generate glyceraldehyde with two C-3 and one C-2 atoms of L-serine. Reaction of ammonia with glyceraldehyde can generate the Amadori rearrangement product able to form methylpyrazine by reaction with 2-amino-acetaldehyde. Pathway B (20%) shows the interaction of glycine with glycolaldehyde (Yaylayan and Keyhani, 1998) to form 1-aminoacetone, incorporating two C-2 and one C-3 atoms of L-serine. The minor pathway (C, 10%) proposes reduction of pyruvic acid to pyruvaldehyde to account for the incorporation of C-1, C-2 and C-3 atoms of L-serine into methylpyrazine.

Table 5.2 Percent Incorporation of ^{13}C-labeled Carbon Atoms in Methylpyrazine

Model	Percent label incorporation				
	M	**M+1**	**M+2**	**M+3**	**M+4**
L-[1-^{13}C]serine	90	10	0	0	0
L-[2-^{13}C]serine	0	0	80	20	0
L-[3-^{13}C]serine	0	0	30	70	0
L-serine/[2-^{13}C]glycine (1:3)	25	75			

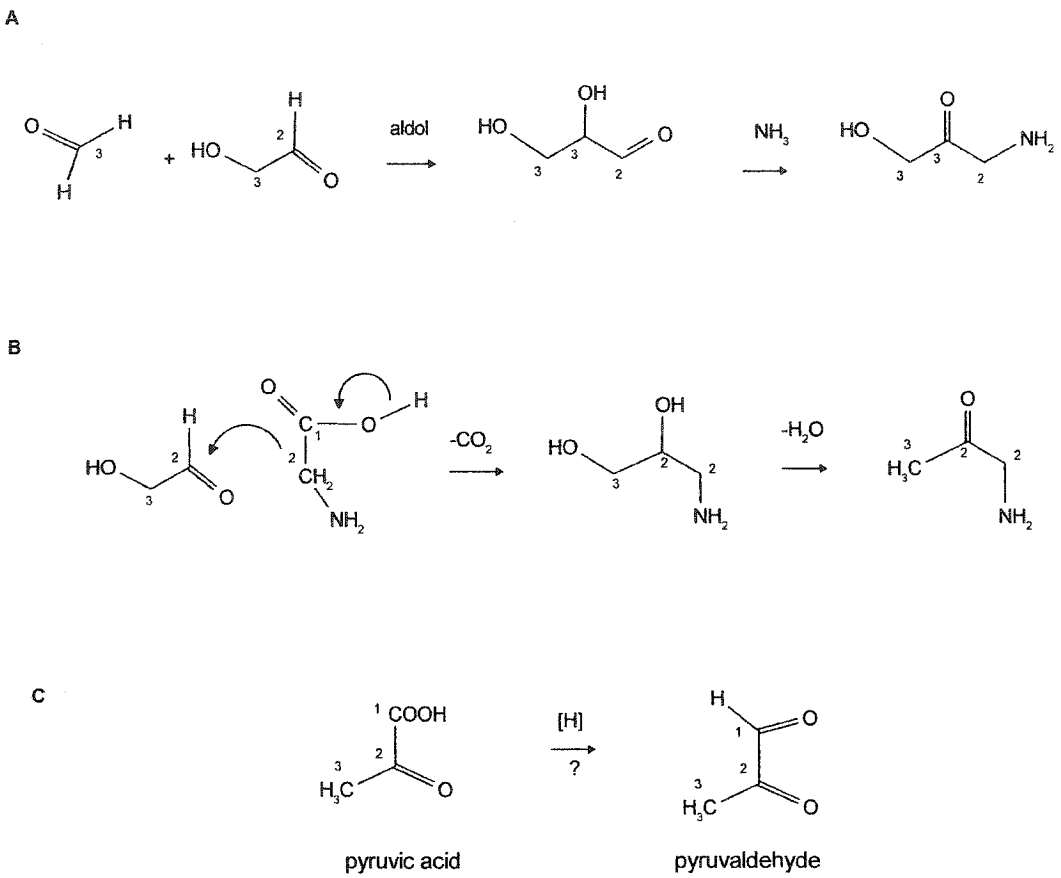
Table 5.3 Percent Incorporation of ^{13}C-labeled Carbon Atoms in 2,3-dimethylpyrazine

Model	Percent label incorporation				
	M	**M+1**	**M+2**	**M+3**	**M+4**
L-[1-^{13}C]serine	90	10	0	0	0
L-[2-^{13}C]serine	0	0	30	70	0
L-[3-^{13}C]serine	0	0	10	60	30
L-serine/[2-^{13}C]glycine (1:3)	10	30	60		
L-serine/[2-^{13}C]alanine (1:3)	60	40			

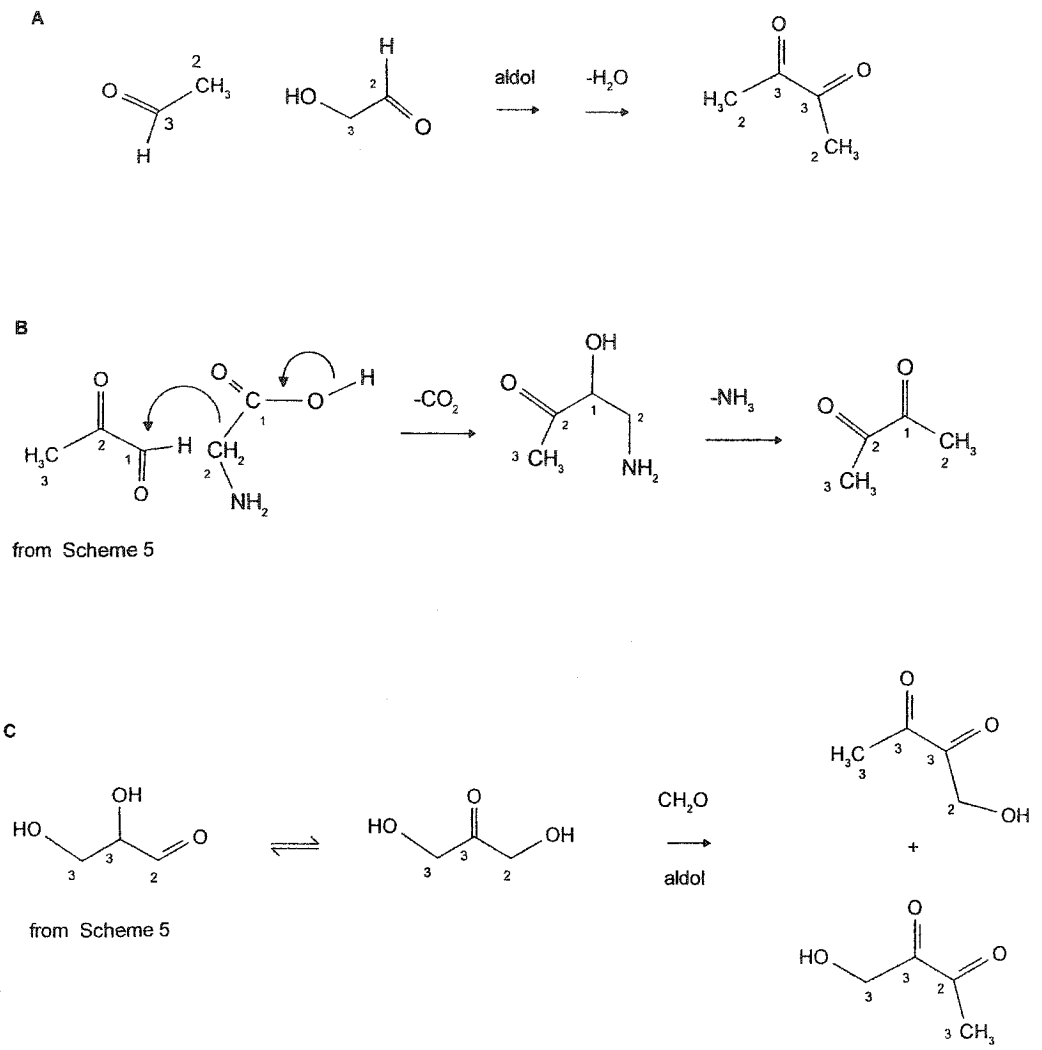
5.2.3.2.2 Origin of 2,3-butanedione and other Four-carbon Precursors of 2,3-Dimethylpyrazine

Similar to methylpyrazine generation from pyruvaldehyde, 2,3-dimethylpyrazine can be used to determine the origin of 2,3-butanedione formed in the pyrolysis products of L-serine. Again, labeled studies have indicated the incorporation of all the three carbon atoms of L-serine, in different ratios, into 2,3-dimethylpyrazine structure (Table 5.3 and Scheme 5.5). Analysis of the data in Table 5.3 indicated incorporation of four C-3 and only three C-2 atoms of L-serine into 2,3-dimethylpyrazine. In addition, 10 % of 2,3-dimethylpyrazine detected was formed from 2,3-butanedione or its equivalent, incorporating one C-1, one C-3 and two C-2 atoms of L-serine. On the other hand, 30 % was formed by the incorporation of one C-2 and three C-3 atoms of L-serine and 60% by the incorporation of two C-2 and two C-3 atoms (Scheme 5.5). Scheme 5.7 summarizes the possible mechanistic routes of formation of 2,3-butanedione or its equivalent, consistent with label incorporation patterns of 2,3-dimethylpyrazine. Aldol condensation between glycolaldehyde and acetaldehyde can generate 2,3-butanedione incorporating two C-2 and two C-3 atoms (Scheme 5.7 mechanism A). On the other hand, pyruvaldehyde, produced as shown in Scheme 5.6, can undergo chain elongation through

glycine reaction (Yaylayan and Keyhani, 1998) to produce 2,3-butanedione having two C-2, one C-1 and one C-3 L-serine atoms (Scheme 5.7 mechanism B). Glycine formed *in situ* through retro aldol reduction of serine or threonine (Scheme 5.1. D) can thus elongate α-hydroxy carbonyls chains by one methyl group, C-2. On the other hand, alanine, formed *in situ* from serine (Scheme 5.1. E), can donate C_2 bloc of C-2 and C-3 as confirmed by isotope distribution, and amino-propanoic acid, formed *in situ* from threonine (Scheme 5.1. E), can donate C_3 bloc of C-2 and C-3 and C-4. This fact can explain observed higher substitution of formed heterocyclic compounds generated from threonine compared to serine (Baltes and Bochmann, 1987; Chen and Ho, 1999). Finally, aldol condensation of glyceraldehyde with formaldehyde produces 1-hydroxy-2,3-butanedione incorporating one C-2 and three C-3 atoms of L-serine (Scheme 5.7 mechanism C). Unfortunately, the mass spectral fragmentation patterns of the above pyrazines could not be used to extract information related to the sequence of the serine atoms incorporated into the pyrazines to confirm the exact locations of the labeled atoms to further support the proposed pathways.



Scheme 5. 6 Proposed Formation Pathways of Pyruvaldehyde and its Equivalents from L-Serine, (numbers indicate original carbon atom locations of l-serine)



Scheme 5.7 Proposed Formation Pathways of 2,3-Butanedione or its Equivalents (Numbers indicate original carbon atom locations of L-serine)

5.2.3.3 Confirmation of the Proposed Pathways

The proposed mechanisms shown in Schemes 5.6 and 5.7 are based on the assumption that the main source of formaldehyde is the C-3 atom of L-serine as shown in Scheme 5.1. This assumption can be justified based on the limited amount of glycine produced from L-serine degradation thus preventing an extensive Strecker reaction to occur to produce significant amounts of formaldehyde (having the C-2 atom of L-serine) from glycine. Furthermore, the preference of glycine to undergo double addition rather than Strecker reaction with α-dicarbonyl compounds has been verified (Keyhani and Yaylayan, 1996). However, to confirm the participation of glycine in the formation of precursors of pyrazines as depicted in Schemes 5.6 and 5.7, L-serine was reacted with excess [^{13}C-2]glycine. The presence of excess [^{13}C-2]glycine in the mixture can lead to the formation of formaldehyde as Strecker aldehyde (incorporating the labeled C-2 atom) and consequently, generate according to Scheme 5.6, unlabeled and singly labeled methylpyrazines. Unlabeled methylpyrazine can be produced through pathway C where there is no glycine involvement and singly labeled methylpyrazine can be formed through pathways A and B. On the other hand, according to Scheme 5.7, unlabeled, singly and doubly labeled 2,3-dimethylpyrazines should be formed in the reaction mixture containing [^{13}C-2]glycine. Pathway A should produce unlabeled isotopomer since there is no glycine or formaldehyde involvement, pathway B should produce singly labeled pyrazine and pathway C should produce singly and doubly labeled pyrazines depending on the origin of formaldehyde. Inspection of Tables 5.2 and 5.3 shows consistent data with the above predictions. Furthermore, to confirm the major pathway (A in Scheme 5.7) of formation of 2,3-butanedione which involves aldol condensation of acetaldehyde, the

Strecker aldehyde of L-alanine, L-serine was also pyrolyzed in the presence of L-[^{13}C-2]alanine. L-Alanine is known to efficiently produce acetaldehyde in the presence of dicarbonyls. Consequently, if acetaldehyde is indeed involved in the formation of 2,3-butanedione, then the mixture should produce singly labeled 2,3-dimethylpyrazine through pathway A and unlabeled 2,3-dimethylpyrazine through pathways B and C. The latter two pathways do not involve acetaldehyde. Again, the data presented in Table 5.3 confirms the above predictions.

5.2.4 Conclusion

Unlike other amino acids reported in the literature, degradation of L-serine or L-threonine in the absence of sugars can lead to the formation of a multitude of heterocyclic compounds, thus it allows to amplify β-hydroxy amino acids importance, especially in systems lacking carbohydrates, if generation of flavours is required. In addition, they can also produce two other amino acids, glycine and alanine. Analysis of the label distribution in pyrazine, methylpyrazine and 2,3-dimethylpyrazine generated from the various model systems, indicated the importance of glycolaldehyde in the formation of longer chain reactive dicarbonyl compounds. The study has shown a crucial role of hydroxy group attached to C-3 of β-hydroxy amino acids in the formation of sugar-specific α-dicarbonyls and α-hydroxy carbonyls generated through glycoaldehyde or 2-hydroxy propanal formed from reactions of shorter carbonyl intermediates of L-serine and L-threonine degradation. L-Threonine compared to L-serine, generated more substituted compounds as expected.

5.3 Formation of α-keto Acids and α-amino Alcohols from β-hydroxy Amino Acids: Discovery of an Alternate Pathway to Amadori Products

5.3.1 Introduction

The widely accepted pathway for the formation of Amadori product involves the well established carbonyl-amine reaction between reducing sugars and amino acids. The first step of this interaction leads to the formation of an imine known as the Schiff's base. The possibility of this imine to be converted into its isomeric imine during the Maillard reaction has been proposed by Høltermand (1966) and has been referred to as transamination reaction, since its hydrolysis can generate the corresponding α-keto acid and an amino-sugar. Høltermand (1966) isolated, for example, trace amounts of pyruvic acid from alanine/glucose model system without being able to detect the presence of the corresponding amino-sugar. Similar isomerization of the imine, formed this time between α-keto acids and amino acids has been observed by Herbst and Engel (1934) and its mechanism characterized by Cram and Guthrie (1966) as base catalyzed methylene-azomethine rearrangement. Since its first proposition by Høltermand (1966) no evidence for the occurrence of this type of transamination reaction during Maillard reaction has been provided in the literature. However, the conversion of amino acids into 2-ketoacids in the presence of glyoxal has been also suggested by Davidek et al. (1990) to occur during Maillard reaction. In order to provide evidence for the occurrence of isomerization of imines under Maillard reaction conditions, model systems of pyruvic acid/aminoethanol and alanine/glycolaldehyde were studied by Pyrolysis-Gas Chromatography/Mass spectrometry (Py-GC/MS) (Yaylayan and Wnorowski, 2002)

using variously [^{13}C]-labeled pyruvic acids. Comparison of the pyrolysis products generated at 250°C and separated on a PLOT column indicated that ~ 30% of the peaks between the two model systems were common to both systems. Furthermore, the results of this labeling studies have also confirmed the formation of pyruvic acid in the reaction mixture of alanine/glycolaldehyde and the presence of alanine and glycolaldehyde in pyruvic acid/ethanolamine mixture, consistent with the occurrence of imine isomerization under the experimental conditions. In this study, detailed investigation of the carbonyl/amine reaction between pyruvic acid and selected amino alcohols, was carried out at different temperatures using Fourier-Transform Infrared Spectroscopy (FTIR) and GC-MS/MS to monitor the formation and isomerization of the initial imine and its subsequent rearrangement into Amadori product.

5.3.2 Materials and Methods

All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) with the HPLC grade purity, without further purification.

5.3.2.1 Model Systems

Components of the model systems were mixed at 20°C at the molar ratio 3:2 of amine source to carbonyl, vortexed for 30 sec, and placed either on IR window for scanning or was introduced inside the quartz tube for Py-GC/MS analysis.

5.3.2.2 FTIR Analysis

Infrared spectra were recorded on a Bomem MB-Series spectrometer (Bomem, Quebec, Canada), equipped with DTGS detector and purged with dry air. The spectra were acquired on CaF_2 IR windows with no spacer at various temperatures at a rate of 20 scans per min. A total of 64 scans at 4 cm^{-1} resolution were co-added for temperature studies, and one scan at 4 cm^{-1} resolution was applied for fast initial reaction monitoring using Kinetics Scanning. Processing of the FTIR data was performed using GRAMS/32 AI version 6.01.

5.3.2.3 Temperature and pH Effect Studies

For IR monitored temperature study, alkaline sample solutions were placed in a CaF_2 IR temperature-controlled cell with no spacer. Temperature was raised from 20°C to desired with a rate of 30°C/min controlled with an OMEGA CN8500 Controller. The sample was scanned the moment the desired temperature was reached, then once again after a 30 min incubation. For confirmation of the reversibility of the reaction, after the incubation at a high temperature, the IR cell was cooled to 20°C with dry air, scanned and after holding for 30 min scanned again, before heating again to the next desired temperature. Each sample was scanned at the following temperatures 20, 40, 60, 80, 100, 120, 140 and 150°C. For GC/MS identification of pH and temperature effect on Shift's base isomerization into Amadori product, mixtures of methyl pyruvate/2-amino-2,3-propanediol under basic conditions (1:3 molar ratio) or after adding of 0.01M HCl were pyrolyzed at 150, 200, 250, and 300°C for 120 sec and quantitatively compared based on the generation of Amadori product m/z 175.

5.3.2.4 Py-GC/MS and GC-MS/MS Conditions

A Varian CP-3800 gas chromatograph equipped with a sample pre-concentration trap (SPT) and coupled to a Saturn 2000 mass selective detector and interfaced to a CDS Pyroprobe 2000 unit (CDS Analytical Inc., Oxford, PA), through a valved interface (CDS 1500) was used for Py-GC/MS analysis. The column used was a fused silica DB-5MS (50m length x 0.2 mm i.d. x 33 μm film thickness; J&W Scientific, ON). Semi-liquid samples of the size of 0.2mg were mixed with silica gel (Merck, grade 60) and was inserted inside the quartz tubes (0.3mm thickness) and desorbed at 150°C for 120 sec, then concentrated on SPT at 50°C (Tenax GR) and subsequently directed to GC column for separation and MS/MS analysis. The GC column flow rate was regulated by Electronic Pressure Controller and set at pressure pulse of 60 psi for 4 min, then maintained at constant flow of 1.5mL/min. Capillary direct MS interface temperature was set at 250°C, manifold at 50°C and ion trap temperature at 175°C. The ionization voltage of 70 eV was used and EMV was set at 1750 V. The GC oven initial temperature was set at -5°C for 5 min and then increased to 50°C at a rate of 50°C/min; after that the temperature was increased to 250°C at a rate of 8°C/min and kept at 250°C for 5 min. The MS fragmentation and peaks identification was estimated with the use of Varian software SatView (ver. 5.52) and NIST Library (ver.1.7). MS/MS dissociation of selected Parent Ions was optimized to the conditions listed in Table 5.4.

Table 5.4 MS-MS Optimization Conditions

Segment Set points	Ionization Mode	Ion Preparation
Scanned mass range 33-250 m/z	Target TIC 25,000 c	Parent Ion Mass: 175, 161, 160, 144, 143, 129, 101, 100 m/z
Scan time 0.3 sec/scan	Max Ion. Time 25,000 μ sec	Isolation Window 3.0 m/z
Emission Current 80 μA	Pre-scan Ion. Time 1,500 μ sec	Resonant CID Waveform
Filament Delay 17.2 min	Background Mass 44 m/z	Excitation Storage Level Calculated as Parent Ion divided by 1.4
	RF Dump Value 250 m/z	Excitation Amplitude: range 0.0 to 2.0 volts, increase 0.2

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. Solid samples (1-4 mg) were introduced inside a quartz tube (0.3 mm thickness) which was plugged with quartz wool and inserted inside the coil probe. The Pyroprobe was set at the desired temperature (250°C) with a THT (total heating time) of 20 sec. The pyroprobe interface temperature was set at 250°C. The GC column flow rate was regulated by Electronic Pressure Controller and set at pressure pulse of 60 psi for 2 min, then maintained at constant flow of 1.5mL/min. Universal injection port operated under splitless conditions with septum purge of 3mL/min. Capillary direct MS interface temperature was 250°C; ion source temperature was 180°C. The ionization voltage was 70 eV, and the electron multiplier was 2400 V. The mass range analyzed was 30-300 amu. The column was either a fused silica DB-5 column (60 m length x 0.25 mm i.d. x 25 μm film thickness; Supelco, Inc.) or a PLOT-Q column (30 m length x 0.32 mm id x 0.20 μm film thickness; Hewlett-

Packard). The column initial temperature (-5°C) was increased to 260°C at a rate of 10°C/min. and held at 260°C for 15 min.

5.3.3 Results and Discussion

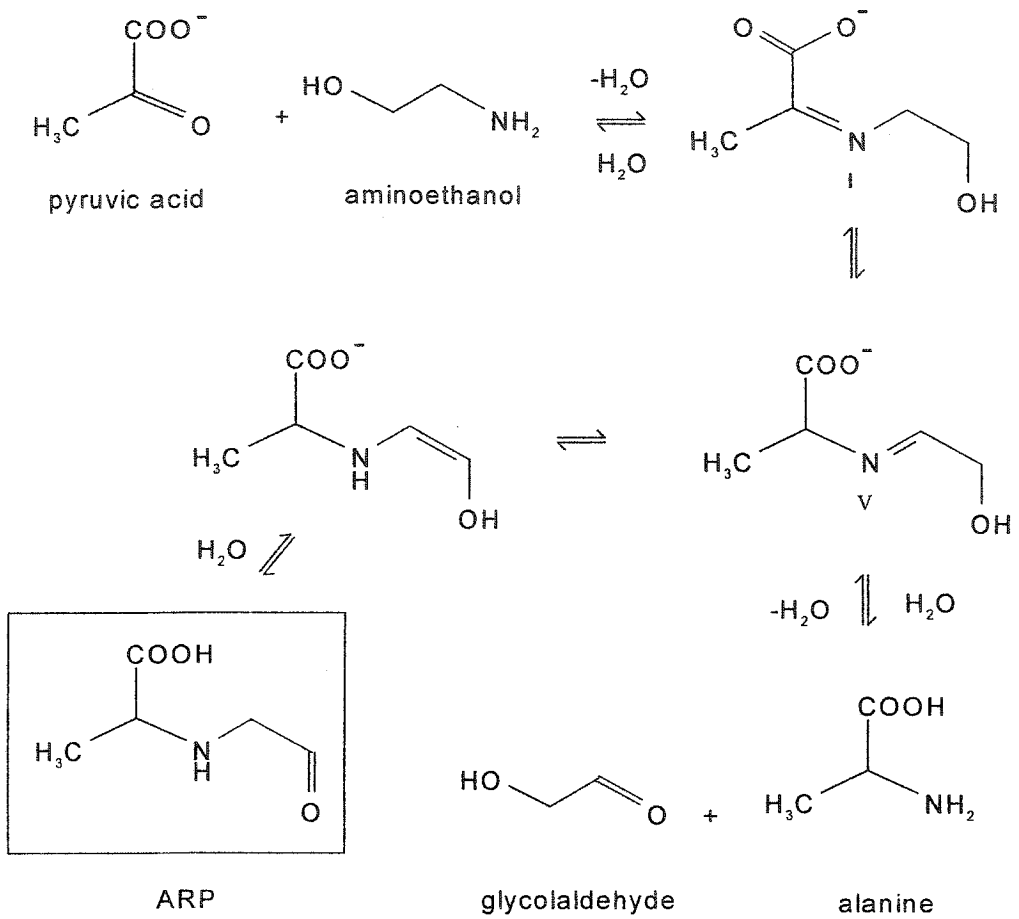
The mechanistic studies on interconversion of keto acids with amino alcohols and amino acids with hydroxy carbonyls and subsequent identical Amadori product formation were conducted from a dual prospective. Application of PYROLYSIS-GC/MS allowed to thermally treat labeled model systems consisting of either pyruvic acid/ethanolamine or alanine/ glycolaldehyde in order to mechanistically study the formation similarity of their common stable end-products. Resemblance of label distribution in common products formed in both model systems provides then the evidence for their interconversion.

FTIR monitoring and GC/CI-MS, GC/MS-MS analysis, on the other hand, allowed to identify the transamination steps of keto acid into corresponding amino acid, to monitor in detail the keto acid/amino alcohols interaction through isomerization and hydrolysis of formed imines, to identify the reaction conditions, such as temperature and pH effects, and to confirm identity of common to both systems Amadori product.

5.3.3.1 Py-GC/MS Mechanistic Studies of Interconversion of 2-keto Acids/2-Amino Alcohols and Alanine/Glycoaldehyde Labeled Model Systems

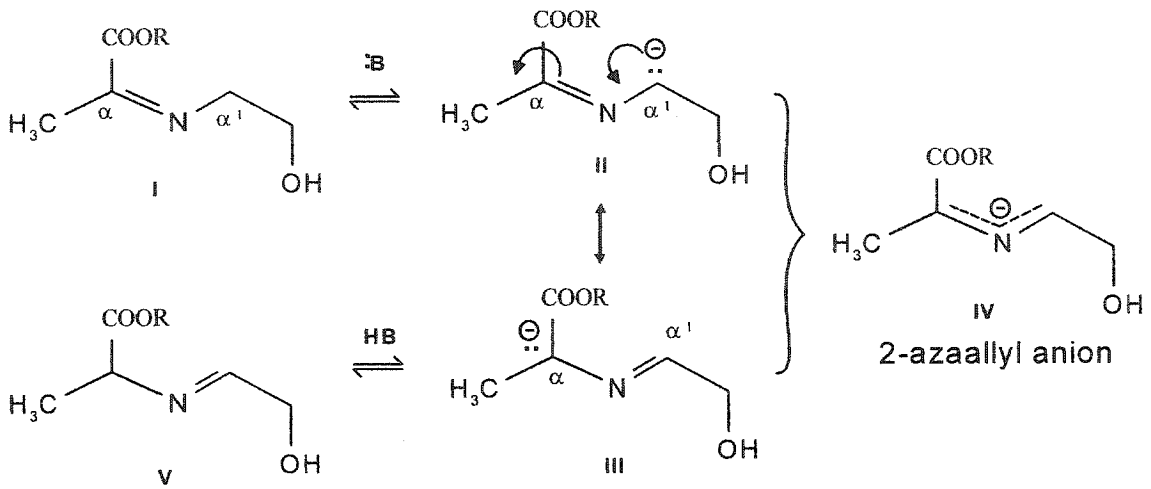
Maillard reaction of L-serine alone leading to the formation of L-alanine and glycoaldehyde requires two major steps: dehydration, to generate pyruvaldehyde and

decarboxylation, to generate ethanolamine as depicted in Scheme 5.1. These two intermediates will consequently condense to generate L-alanine and glycolaldehyde. Formation of alanine was confirmed through the detection of 2,5-diketo-3,6-dimethylpiperazine, cycloglycylalanine, and amino acid-specific pyrazinones (Yaylayan et al., 2000; Yaylayan and Wnorowski, 2001). Consequently, glycolaldehyde could undergo various chain elongation reactions with formaldehyde and the Strecker aldehydes of alanine and glycine to produce various dicarbonyls as precursors to heterocyclic compounds. Glycoaldehyde presence was also detected indirectly through trapping with phenylhydrazine and detection of parent pyrazine as a major product (Yaylayan et al., 2000). The outcome of the L-serine thermal generation of glycine, L-alanine, formaldehyde, acetaldehyde, glycolaldehyde, pyruvaldehyde, and ethanolamine and aldol carbonyl chain elongation is formation of multitude of alkylated pyrazines, pyrroles, pyrrolylalkanols and fused heterocyclic compounds, uncommon to any other amino acid besides β-hydroxy amino acids. Moreover, mechanistic studies indicated conversion of pyruvic acid into alanine. The observed conversion of pyruvic acid (Scheme 5.1), a 2-ketoacid, into amino acid, alanine, deemed further attention due to its implications for the Maillard reaction. Literature data reports that the conversion of amino acids into 2-ketoacids in the presence of glyoxal has been previously suggested to occur during Maillard reaction (Davidek et al., 1990). The proposed mechanism of this conversion is shown in Scheme 5.8. According to this scheme, the initial imine (I) undergoes an isomerization reaction followed by hydrolysis of the isomerized imine (V) to form alanine and glycolaldehyde.

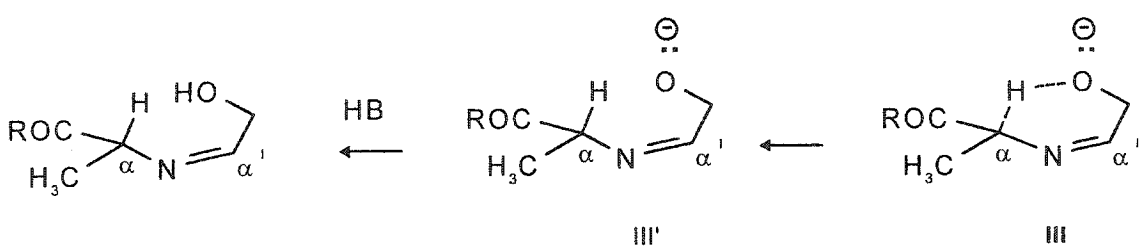


Scheme 5.8 Proposed Transamination Reaction Between Pyruvic Acid and Ethanolamine

Base-catalyzed tautomerization of imines such as I in Scheme 5.9 involves the formation of a delocalized 2-azaallyl anion (Cainelli et al., 1996) (structure IV in Scheme 5.9). This anion can be protonated by the conjugate acid (HB) formed in the reaction mixture at both α (see structure III) and α' (see structure II) positions with the establishment of an equilibrium between imines I and V (Scheme 5.9). This type of imine isomerization has been recognized as a prototype of the biochemical transamination between amino acids and pyridoxal (Cainelli et al., 1996). Recently these intermediates have been generated



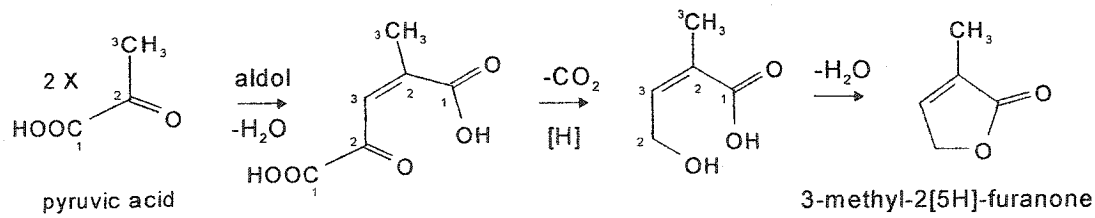
Scheme 5.9 Proposed Mechanism of 1,3-Prototropic Shift and Formation of 2-azaallyl Anion from Imines



Scheme 5.10 Proposed Mechanism of Intramolecular Proton Transfer in Imine

under mild conditions at room temperature through deprotonation of imines using potassium *tert*-butoxide (Cainelli et al., 1996). A concerted mechanism was proposed (Cram and Guthrie, 1996) for this isomerization process in which the base removes a proton from one carbon (α or α') synchronously with the donation of a proton to the other carbon by its conjugate acid. In the specific case of the imines generated from keto esters and amino ethanols such as I (Scheme 5.9 & 5.10), the β-hydroxy group can act as a proton donor to the α position through the formation of a H-bond in a stable six-member ring intermediate as shown in Scheme 5.10 (structure III). This process will protonate the α-position preferentially due to the entropy factor and thus lead the reaction to completion rather than to equilibrium.

On the other hand the isomerization of the imine V (Schemes 5.8 & 5.9) generated from alanine and glycolaldehyde is favoured due to the acidity of the α-H relative to α'-H. According to Scheme 5.9, therefore the imines I and V could exists in a tautomeric equilibrium under basic conditions regardless whether the initial mixture contains only amino acid/sugar or keto acid/aminoalcohol. In order to provide evidence for the occurrence of transamination reaction under Maillard reaction conditions, model systems of sodium pyruvate/aminoethanol and alanine/glycolaldehyde were studied. Comparison of the pyrolysis products generated at 250°C and separated on a PLOT column indicated a significant number of common peaks (~ 30%) between the two model systems.



Scheme 5.11 Proposed Mechanism of Dimerization Pyruvic Acid and Formation of 3-Methyl-2-[5H]-furanone

5.3.3.1.1 Evidence for the Formation of Pyruvic Acid and Ethanolamine in Alanine/glycolaldehyde Model System

The most direct evidence for the formation of pyruvic acid in alanine/glycolaldehyde model system was obtained from the detection of 3-methyl-2[5H]-furanone in their pyrolysis mixture. This indicator compound was the major product identified during pyrolysis of pyruvic acid alone. Studies with labeled pyruvic acids have indicated the presence of one C-1 , two C-2 and two C-3 atoms in this product. Scheme 5.11 shows a plausible mechanism of aldol condensation between two molecules of pyruvic acid to produce 3-methyl-2[5H]-furanone, consistent with the label incorporation pattern. Furthermore, labeled studies with alanine/glycolaldehyde model system also indicated that all the carbon atoms in 3-methyl-2[5H]-furanone identified in the model system, originated from alanine alone and the pattern of incorporation of alanine carbon atoms was identical to that in pyruvic acid (one C-1, two C-2 and two C-3 atoms of alanine). On the other hand, acetaldehyde was the major degradation product of aminoethanol and as such could be used as an indicator compound for the presence of aminoethanol in the

alanine/glycolaldehyde model system. Analysis of the pyrolysis mixture generated from labeled alanines and glycolaldehyde, on a PLOT column have indicated that only 70% of acetaldehyde have originated from Strecker reaction of alanine as evidenced by the label incorporation, indicating the remaining 30% must have originated from glycolaldehyde carbon atoms through the proposed ethanolamine transamination reaction (Scheme 5.8).

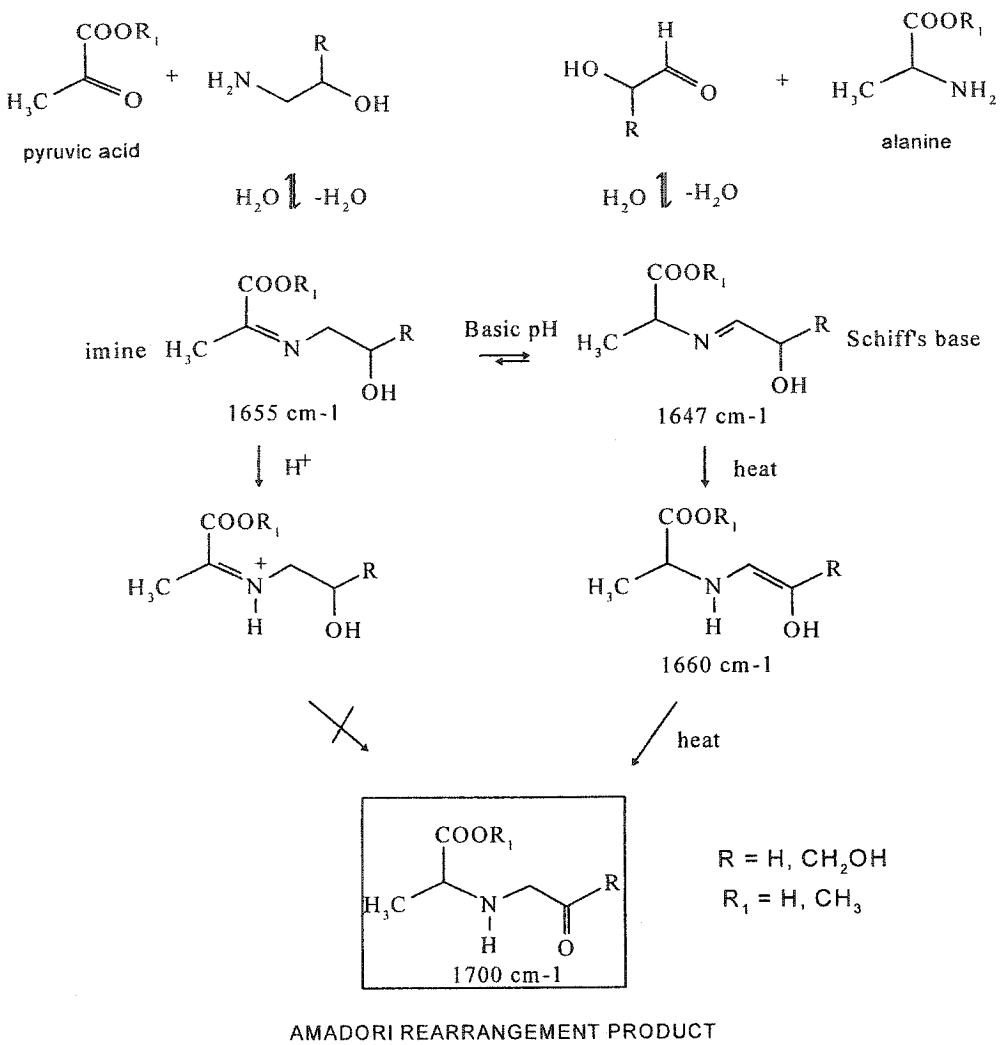
5.3.3.1.2 Evidence for the Formation of Alanine and Glycolaldehyde in Pyruvic Acid/ethanolamine Model System

Similar to serine system, the formation of alanine was confirmed due to the detection of trace amounts of 2,5-diketo-3,6-dimethylpiperazine, the cyclic dimer of alanine on DB-5 column. Glycolaldehyde formation was indirectly verified by detection of parent pyrazine incorporating no carbon atoms from pyruvic acid rather only from ethanolamine.

5.3.3.2 FTIR Monitoring of Carbonyl/Amine Isomerization and Hydrolysis into Imines followed by MS-MS and CI-MS Identification of Formed Amadori Product

Preliminary analysis using selected model systems containing pyruvic acid and amino alcohols have indicated the presence of strong spectral interference when solvents or inorganic acids and bases were used to conduct the FTIR experiments and to control the pH of the samples. To overcome these limitations only neat liquid reactants were selected for further studies, such as methyl pyruvate, pyruvic acid, amino ethanol and 1-amino-2,3-propanediol. In addition, excess amino alcohol or pyruvic acid were used to control the pH of the reaction mixtures. Furthermore, the preliminary studies also indicated that the initial reaction rate at room temperature, between the pyruvic acid and the amino

alcohols (Scheme 5.12) was very fast, reaching completion within 12 minutes of mixing of the reactants. As a result two types of experiments were carried out, one in which the reactants were mixed directly on the CaF_2 window at 20°C (room temperature) and immediately scanned using a rate of either one scan per second such that each spectrum represented a passage of three seconds to monitor in detail imine formation and isomerization into the Schiff's base (Scheme 5.12), or using a rate of 10 scans per second such that each spectrum represented a passage of 30 sec to monitor the completion of the reaction. The second type of FTIR experiments involved high temperatures (up to 100°C) and long times incubations (up to 24 h) to monitor the conversion of the Schiff's base into Amadori rearrangement product as illustrated in Scheme 5.12.



Scheme 5.12 Proposed Mechanism of Formation of Amadori Product from both Pyruvate/Amino Alcohol and from Alanine/Glycoaldehyde Systems

5.3.3.2.1 Monitoring Imine Formation and its Isomerization into the Schiff's Base at Room Temperature under Basic Conditions

To prevent salt formation, methyl pyruvate (MP) instead of pyruvic acid was used to monitor the reaction with excess (1:1.5) 1-amino-2,3-propanediol (APD). Excess reagent was used to ensure basic conditions. Each reactant was applied separately on each of the CaF_2 window without spacer and immediately assembled into the cell and scanned at room temperature using a rate of one scan per second such that each spectrum represented the passage of three seconds. Figure 5.2 shows time-dependant spectral changes between the nominal time zero and 150 sec in the 1850 - 1500 cm^{-1} range. Inspection of Figure 5.2 indicates a dramatic and simultaneous decrease in the intensities of the carbonyl and of the ester bands of MP centered at 1724 cm^{-1} and at 1754 cm^{-1} respectively. Almost 90 % of the intensity of the bands decreased within 2.5 minutes. The disappearance of the carbonyl band was accompanied by the appearance of an imine band centred at 1655 cm^{-1}, and the disappearance of the ester band was accompanied by the simultaneous appearance of amide (amide I at 1670 cm^{-1}, amide II at 1537 cm^{-1}) and carboxylate bands (1602 cm^{-1}). These observations can be explained by the simultaneous carbonyl-amine reaction and formation of the imine intermediate (Scheme 5.12) and under the presence of excess basic APD, conversion of the ester into an amide. Water released from carbonyl-amine reaction also initiated some hydrolysis of the ester as indicated by the presence of a carboxylate band centered at 1602 cm^{-1}. A closer look at the 1700-1630 cm^{-1} spectral region (Figure 5.3) indicates that the initial imine (spectrum at t = 0 s) was formed very fast, even during the preparation and application of the sample on to the CaF_2 windows and the start of spectral acquisition.

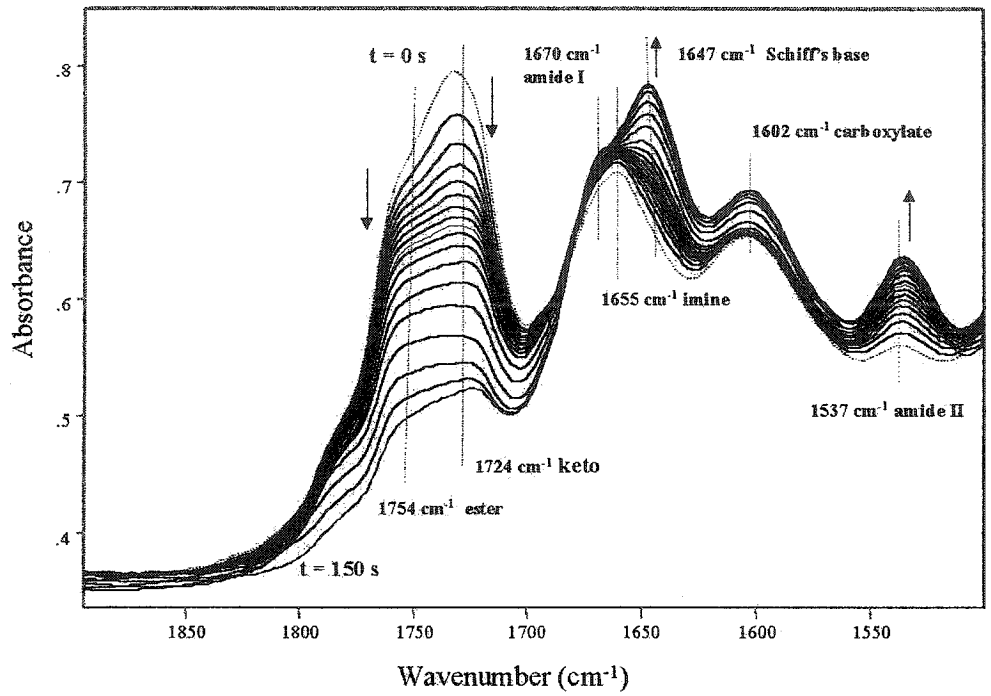


Figure 5.2 Time-dependant FTIR Spectra of a Mixture of Methyl Pyruvate and Amino-Propanediol at Room Temperature between 1850 – 1500 cm^{-1}

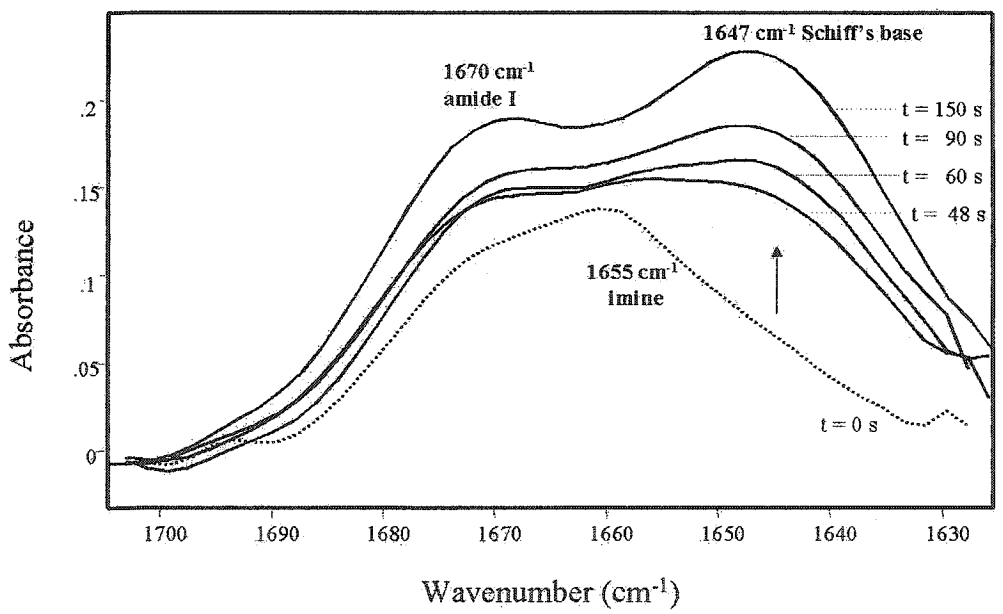


Figure 5.3 Time-dependant FTIR Spectra of a Mixture of Methyl Pyruvate and Amino-Propanediol at Room Temperature between 1700 - 1630 cm^{-1}

Thus at nominal time zero, substantial amount of imine (1655 cm^{-1}) has already accumulated as shown in Figure 5.2, however, over time, this band disappears with the appearance of another band at 1647 cm^{-1} which was assigned to the formation of the Schiff base. Furthermore, Figure 5.3 also indicates that during the first 60 sec, the rate of formation of the imine was faster than the rate of its isomerization, however, after 60 sec at room temperature, the rate of isomerization becomes faster than the rate of its formation and continues to be so until the reaction reaches completion after 12 minutes. Cainelli et al., (1996) also observed a very fast (20 minutes) room temperature imine isomerization using N-diphenylmethanimines in the presence of potassium *tert*-butoxide and THF as solvent.

When 1-amino-2,3-propanediol (APD) was replaced with excess 1-amino-ethanol (AE), similar spectral changes were observed, except the conversion of the pyruvate ester into amide occurred very fast, even before imine formation and isomerization. The effect of early amide formation on the absorption frequency of the neighbouring carbonyl carbon was observed by a shift from 1724 cm^{-1} to 1737 cm^{-1}.

When methyl pyruvate (MP) was replaced with excess pyruvic acid (PA) and the reaction was similarly monitored in the presence of either 1-amino-2,3-propanediol (APD) or with 1-amino-ethanol (AE) the spectral changes indicated the formation of pyruvate ion (1602 cm^{-1}) and formation of some imminum ion centered at 1632 cm^{-1} confirming requirement for base catalysis for its isomerization.

5.3.3.2.2 Effect of Temperature on the Stability of the Schiff's Base under Basic and Acidic Conditions - Rearrangement into Amadori Product

Incubation of methyl pyruvate (MP) with excess 1-amino-2,3-propanediol (APD) or with 1-amino-ethanol (AE) at 20°C, has been demonstrated above to effect the formation of imine (1655 cm^{-1}) and its subsequent base-catalyzed isomerization into the Schiff base (1647 cm^{-1}) within 12 minutes. Under acidic conditions, Schiff bases are known to undergo facile rearrangement into Amadori product and/or hydrolyze depending on the reaction conditions. In order to study the temperature stability of the Schiff base under alkaline conditions, the reaction mixture of methyl pyruvate (MP) with excess 1-amino-2,3-propanediol (APD) was IR monitored at different temperatures and times. Figure 5.4 indicates the formation of the Schiff base at 20°C as was demonstrated above, however, as the temperature was raised to 40°C and subsequently to 60°C the absorption peak centered 1647 cm^{-1} was shifted to 1660 cm^{-1}. This was attributed to the isomerization of the Schiff base and formation of enaminol moiety (Yaylayan et al., 1999b) as indicated in Scheme 5.12. Formation of enaminol moiety was accompanied to a small extent by an intramolecular esterification as was evidenced by the peak centered at 1776 cm^{-1}. Further heating and incubation at 100°C indicated a decrease in the intensity of the enediol band and emergence of a shoulder centered at 1700 cm^{-1} (Figure 5.5). The new band was assigned to the formation of Amadori product as shown in Scheme 5.12.

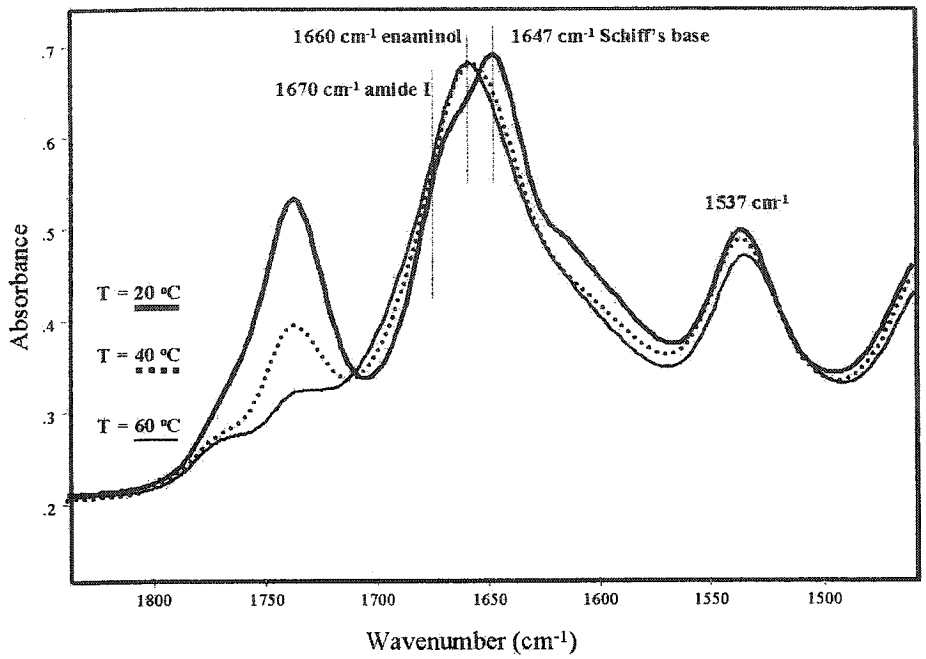


Figure 5.4 Temperature-dependent FTIR Spectra of a Mixture of Methyl Pyruvate and Amino-Propanediol between 1800 – 1500 cm^{-1}, at temperatures 20, 40 and 60°C

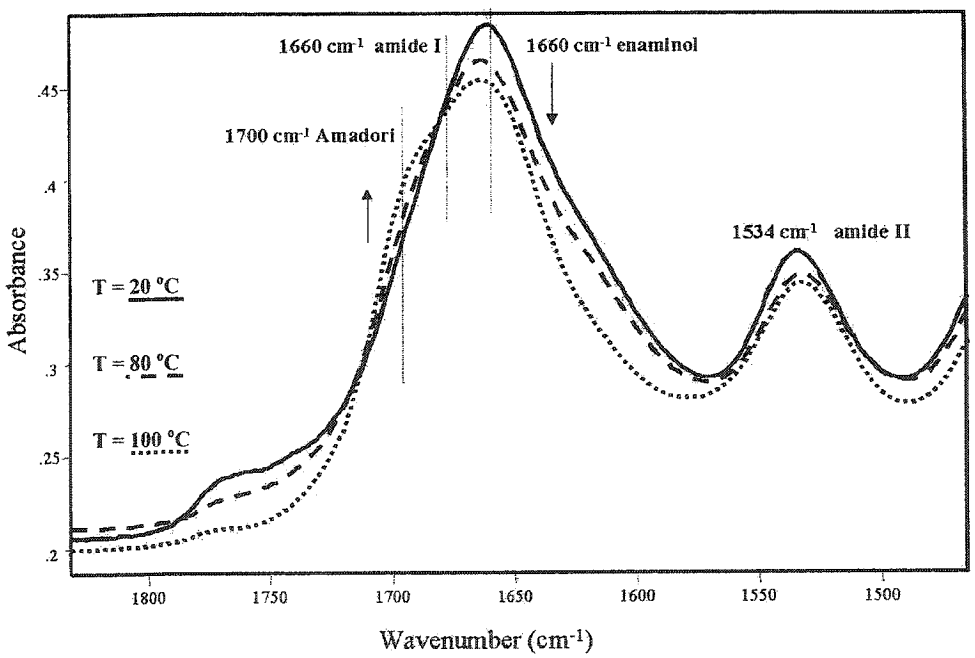


Figure 5.5 Temperature-dependent FTIR Spectra of a Mixture of Methyl Pyruvate and Amino-Propanediol between 1800 – 1500 cm^{-1}, at temperatures 20, 80 and 100°C

Alternatively, if pyruvic acid were used to react with 1-amino-2,3-propanediol, it would also form imine I (Scheme 5.9), however, consequent imine isomerization and formation of Schiff base is possible only under alkaline conditions. Formation of α-conjugated enaminol cation will not allow isomerization into carbonyl bond, thus will not generate Amadori product (Scheme 5.12). However, addition of 0.01M HCl to the methyl pyruvate/1-amino-2,3-propanediol Schiff base, accelerated the imine isomerization into Amadori product in similar manner as temperature effect under mild heating.

Comparison of pH and incubation temperature on Schiff base stability and isomerization into Amadori product can be seen in Figure 5.6. The GC/MS chromatograms show clearly that Shift's base acidification in conjunction with temperature elevation accelerates the conversion process. Moreover, under basic conditions temperature seems to be accelerating retro aldol fragmentation and hydrolysis of Schiff base leading to carbonyls and amino-carbonyls generation instead (Davidek et al., 2002, Yaylayan, 2003).

5.3.3.2.3 Detection of Amadori intermediate by Py-GC/MS, Py-GC/CI-MS & GC/MS-MS

In order to provide direct evidence for the ability of pyruvic acid pathway to generate Amadori product, alanine methyl ester/glyceraldehyde model system was analyzed under the same experimental conditions as that of methyl pyruvate/1-amino-2,3-propanediol model by Py/GC-MS. Both systems are expected to produce a common Amadori product as shown in Scheme 5.12. Spectral comparison of both chromatograms has lead to

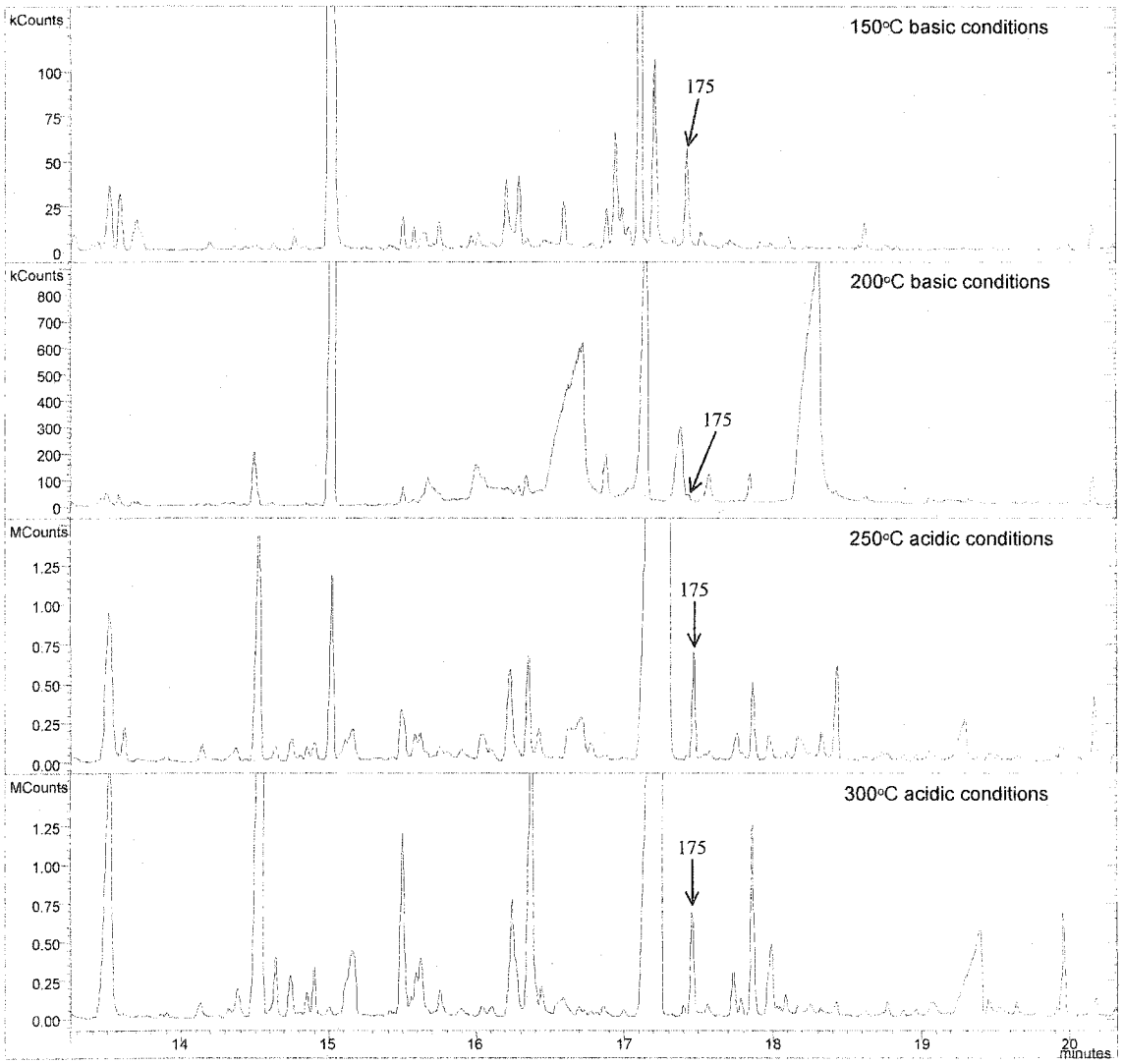


Figure 5.6 Temperature- and pH-dependant GC-MS Chromatograms of a Mixture of Methyl Pyruvate/Amino Propanediol

identification of a common peak at retention time 17.5 min having identical electron impact fragmentation pattern and the expected molecular mass of m/z 175 (Figure 5.7) as confirmed by chemical ionization. Furthermore, tandem mass spectrometry was used to provide evidence that the electron impact mass spectral fragmentation pattern shown in Figure 5.7 is consistent with the proposed structure of the methyl ester of the Amadori product. Data obtained from optimized EI-MS/MS of m/z 175 are shown in Table 5.5.

Table 5.5: Daughter Ions observed in the mass spectrum of peak at m/z 175

Parent Ion	Daughter Ions [1]
175	**161, 160,** 144, 143, **129**
161	**129**, 128, 100
160	144, 143, **129**, 101, 59
144	**129**, 69
129	116, **101, 100, 59**
101	**85**, 59
100	**85**

[1] Bold characters represent major daughter ions

According to the EI-MS spectrum shown in Figure 5.7, it seems that ion at m/z 129 is the most stable ion and can be envisaged to be formed through three pathways as shown in Scheme 5.13. One through a single step cyclization from the parent ion or through stepwise formation from either demethylated parent ion at m/z 160 or from dehydroxymethylated parent ion at m/z 144. The ion m/z 129, will in turn lose CO to give two isomeric ions at m/z 101, which consequently after losing of a proton can generate isomeric ions at m/z 100. Finally, ion m/z 59 can be formed directly from m/z 175 by cleavage of carbon atom in alpha position to nitrogen atom.

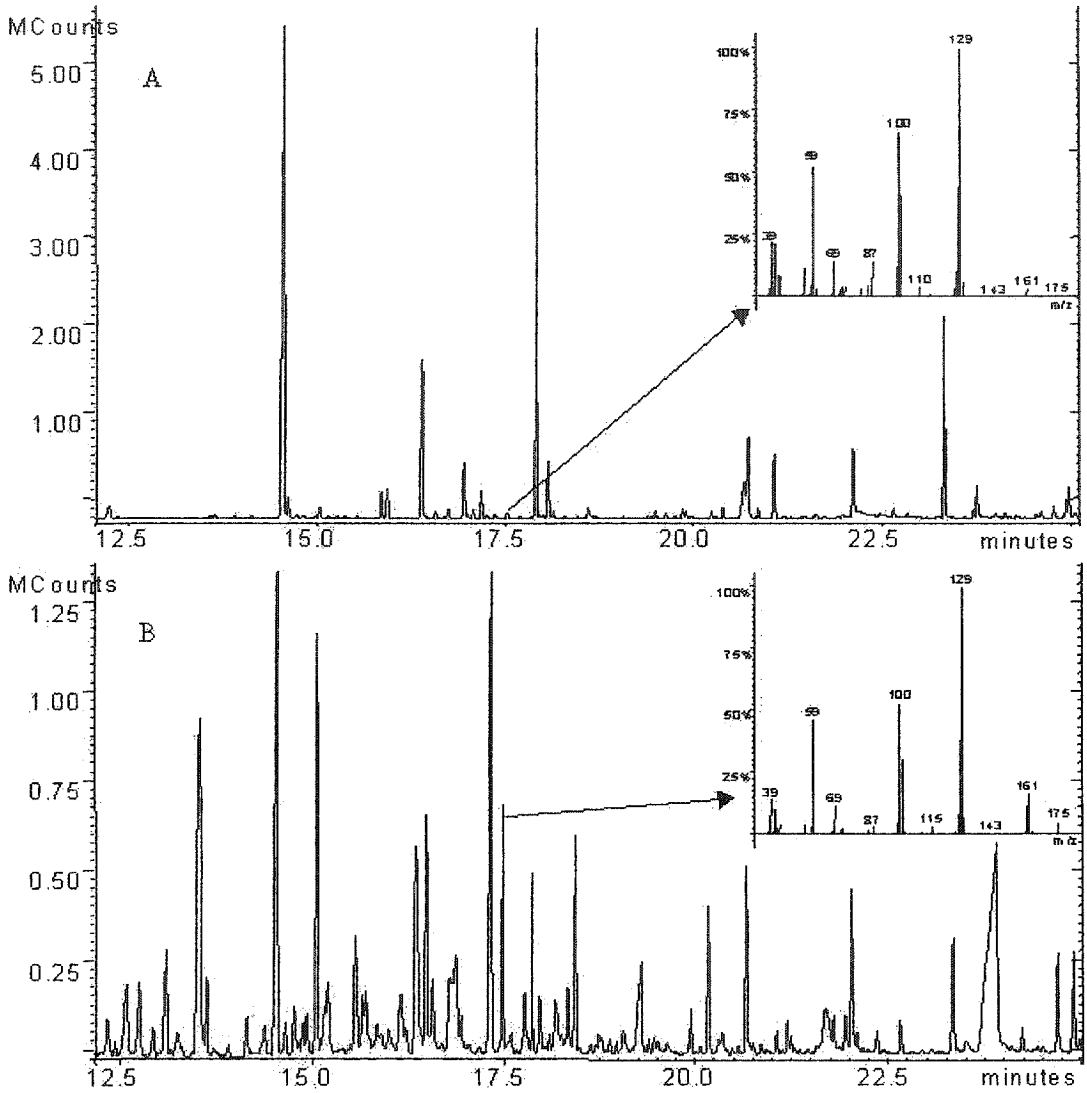
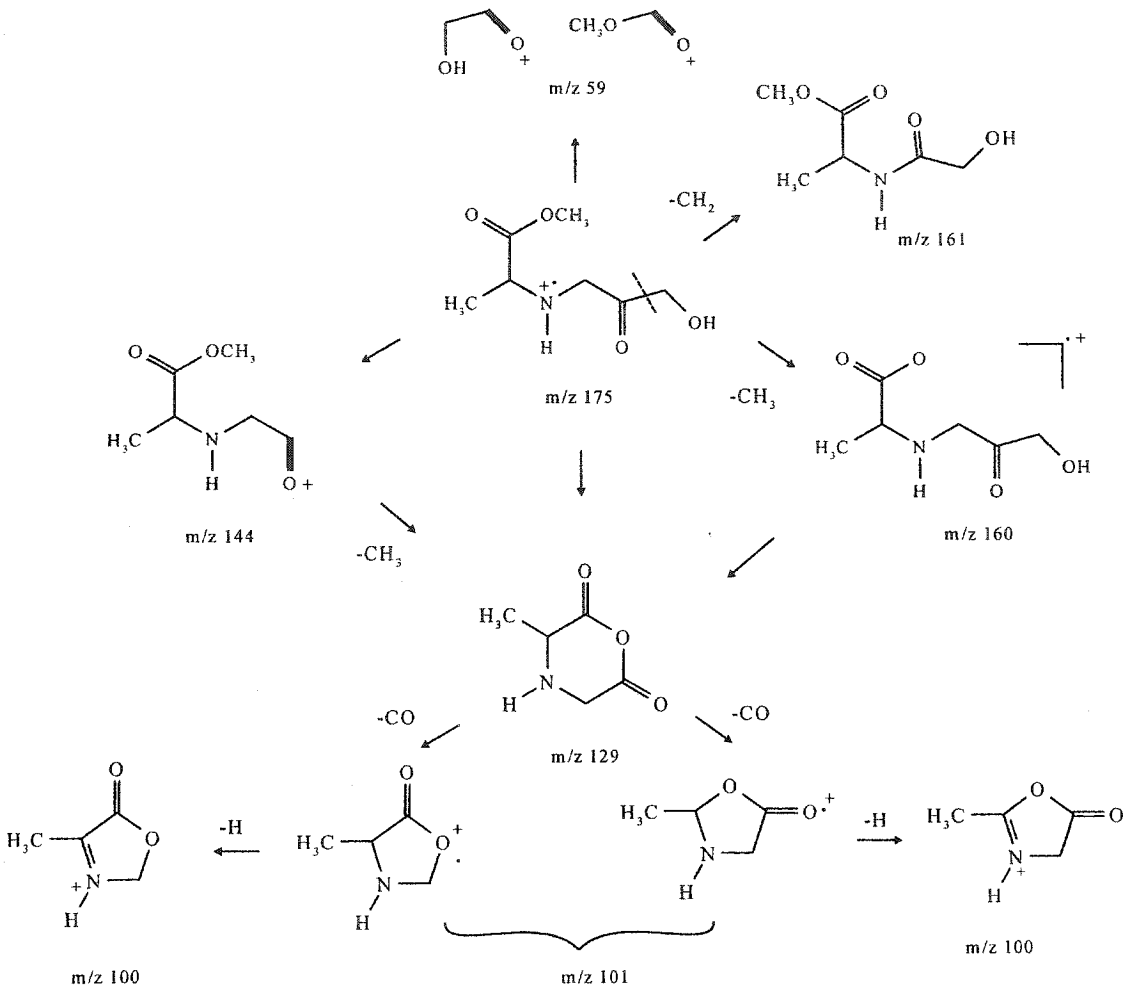


Figure 5.7 Pyrograms of Mixtures of (A) Methyl Pyruvate/1-amino-2,3-propanediol and (B) Alanine Methyl Ester/Glyceraldehyde. Inserts are the EI-MS Fragmentation Patterns of the Peak at 17.5 min



Scheme 5.13 Proposed Electron Impact Fragmentation Patterns of Amadori Product of Methyl Alanine with Glyceraldehyde

5.3.4 Conclusion

FTIR monitoring and Py-GC/MS-MS studies of isotopically labeled methyl pyruvate and amino-propanediol and alanine methyl ester with glyceraldehyde model systems, have indicated formation of identical Amadori product from both systems. FTIR monitoring of the course of the reaction under basic pH condition and room temperature has indicated formation of imine and subsequent isomerization through 1,3-prototropic shift a common to both systems Schiff base. Acidification of the Schiff base or subsequent heating induce further isomerization and formation of Amadori product. This mechanism provides a novel route to Maillard reaction products initiated not as generally recognized from a carbonyl and amine condensation, but rather of keto acid and amino alcohol.

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CONNECTING PARAGRAPH

In chapter 4, comparison of the formation pathways of the common products generated under pyrolytic and aqueous conditions has confirmed the similarity of their mechanisms of formation. This study has confirmed that mechanistic information acquired through pyrolysis is relevant to aqueous reactions occurring in food systems. In chapter 5, an application of Py-GC/MS system was demonstrated through investigation of the mechanism of Maillard reaction using a model system. In the following chapter further applications of the analytical system will be demonstrated in the area of Maillard-related quality aspects of food.

CHAPTER 6

APPLICATION OF PYROLYSI-GC/MS TO THE STUDY OF MAILLARD-RELATED QUALITY ASPECTS OF FOOD: CARCINOGENS AND CHEMICAL MARKERS

6.1 General Introduction

Pyrolysis coupled with Gas Chromatograph/Mass Spectrometer (Py-GC/MS) has been used for decades as a convenient tool for a wide range of analytical applications. One of the very first uses of pyrolysis was to thermally break long polymers of polyisoprene for analysis of its constituting material (Williams, 1862). Pyrolysis has found wider applicability after 1950's in chemistry, biosciences, forensic analyses and environmental, textile, synthetic, automobile and tobacco industries with the advent of gas chromatography, as a sample introduction device through which generated volatiles could be sent directly to analytical column for separation and MS identification extending the sample range for GC analysis. Moreover, Py-GC/MS has been used for the analysis of flavour and colour compounds (Hardt and Baltes, 1989), food contaminants, food adulteration and authenticity (Goodacre et al., 1997; Hor et al., 2001), for identification of food additives and constituents (Sjoberg and Pyysalo, 1985), investigation of outbreaks of food poisoning and non-gastrointestinal infection (Sisson et al., 1992), or even studying archaeological food residues (Pastorova et al., 1993). Capillary GC/MS coupled with pyrolysis, therefore, has been widely used by analytical chemists and, as summarized in the previous chapters, its use in solving mechanistic problems in food chemistry was first pioneered in our group, specifically the use of labelled reactants to monitor and study detailed chemistry of different pathways of aroma generation during Maillard reaction (Huyghues-Despointes et al., 1994). Other research groups followed suit and utilized similar approach (Coleman III and Chung, 2002) in their analysis of Maillard reaction. In this chapter, further applications of Py-GC/MS is explored in the area of monitoring Maillared-generated carcinogens and quantitative aspects of chemical marker generation.

6.2 Formation Kinetics of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one and its Application in Food Quality Control

6.2.1 Introduction

Recent developments in new processing technologies, such as continuous sterilization, aseptic processing, ohmic and microwave heating, have called for an increased demand for new methods of demonstration of process lethality at the center of particulate foods. A direct measurement of time-temperature history is often impractical and sometimes impossible to establish. An alternative approach for assessing time-temperature exposure of particulate foods is to monitor the formation of a chemical marker within the food and relate its concentration to time-temperature exposure. The concept of using chemical markers for monitoring food processing is based on the fact that measured concentration of thermally generated compounds can reflect the time-temperature exposure of a heated food system. This relationship can be established using calibration curves that relate marker yields to spore survivor data and lethality values. The formation of a selected chemical marker should be linearly correlated with time of heating and the marker should be thermally and chemically stable under processing conditions. Chemical markers could be formed either through thermal degradation or chemical transformations occurring naturally in foods due to heating, such as the Maillard reaction. However, not every compound generated during food processing can provide useful information. Suitable chemical markers should be nonvolatile, stable, be easily extractable from an aqueous medium and, preferably, should follow microbial destruction of first-order or pseudo first-order rates. Using chemical markers to assess time-temperature exposure of foods

can be of a great interest for ensuring commercial sterility of low-acid foods containing particles, since direct measurement of the time-temperature profile of such products is difficult to achieve. Chemical marker approaches can also be applied to non-conventional types of processing, such as ohmic heating and microwave sterilization (Kim and others 1996a,b). Currently, foods that are difficult to estimate their lethality are overheated to ensure sterility. Development of methods that allow such estimations may greatly reduce energy costs associated with such overheating in addition to applications in quality control. The use of chemical markers in thermally processed foods has been studied extensively (Mulley and others 1975; Rao and others 1981; Kim and Taub 1993; Ramaswamy and others 1996; Kim and Choi 1998). These studies have confirmed the potential use of markers in monitoring the time-temperature exposure of foods. The mathematical basis (Labuza 1979; Ross 1993) and practical studies of correlation between marker formation and destruction of microorganisms during food processing have been confirmed (Kim and Ball, 1994; Ramaswamy and others 1996; Kim and Choi 1998). In the present study a novel approach in the application of chemical markers, such as 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, is introduced to estimate lethality of processed food samples with an unknown history of sterilization.

6.2.2 Materials and Methods

6.2.2.1 Chemicals and Reagents

All chemicals and reagents were obtained from Sigma-Aldrich (Milwaukee, WI) with the HPLC grade purity.

6.2.2.2 Kinetics Experiments

Aqueous solutions (2.5 mL, pH 6.5) consisting of equimolar concentrations (0.1 M each) of α-D-glucose, D-ribose and L-glutamic acid monosodium salt were sealed in glass ampoules and heated at 110, 120 and 130°C for various time intervals (from 5 to 100 min) in an oil bath (Fisher Scientific, Montreal, Qc). After heating, samples were quickly placed in an ice bath to stop further reactions. The samples were subsequently extracted with ethyl ether and dichloromethane mixtures (3 x 100 μL, 1:1). The solvents were then evaporated under nitrogen and the residues were dissolved in 20 μL of ethyl ether - dichloromethane mixture (1:1). A 5 μL aliquot of each sample was injected using on-column technique and analyzed using GC/MS (see below). Triple triplicates were analyzed for each sample. The data obtained were used for the determination of the kinetics of marker formation (Figure 6.1).

6.2.2.3 GC-MS Analysis

The analysis was performed using a Hewlett-Packard Model 5890 Series II gas chromatograph coupled to a Series 5971 mass selective detector. A DB-5 capillary column (60 m x 0.25 mm x 0.33 μm) was used. The 1 mL/min constant flow of helium

was maintained by a Electronic Pressure Controller (Hewlett-Packard). Oven initial temperature was set at -10°C for 2 min, then increased to 50°C at a rate of 30°C/min, then to 250°C at a rate of 8°C/min, and held for 5 min. The samples were introduced using on-column injection in splitless mode. The marker eluted at 17.7 min under these conditions. The marker peak was identified through mass spectral NIST Library (ver.1.7) search.

6.2.2.4 Kinetic and Statistical Analyses

The temperature-dependence of the marker formation was determined by Arrhenius relationship (ln k vs 1/T). From the relation of lnk vs T°C, Q_{10} value was also established. F_o values for marker formation were calculated based on the following equation: $Fo = 10^{\frac{T-121.1}{10}} x \text{ time}$. ANOVA analysis was performed using the Microsoft Excel 97 statistical package. Error in estimating the intercept of regression line with the x-axis was calculated based on the method of Miller and Miller (1993).

6.2.2.5 Preparation of "Process-simulated" Model System

The above model system (10 mL) was heated for 10 min at 120°C, cooled immediately in an ice bath and stored at refrigeration temperatures. This simulated processing imparted to the model system a lethality value of (F_o) 7.76 min and produced an area count of 1.44 x 10^7 ± 3.3 % (n=6) for the marker (termed M_o).

5.2.2.6 Prediction of Accumulated Lethality in "Process-simulated" Model System

The "process-simulated" samples were subjected to "secondary heating" at 120°C for various time intervals (10, 15, 20, 25 and 30 min) in an oil bath and subsequently

analyzed for the formation of the marker as described above. The process (simulated) lethality was determined by plotting marker yield against calculated lethality (from secondary heating) and extrapolating the linear curve to the x-axis. The intercept of this curve with the x-axis determined the predicted lethality and the intercept of y-axis determined the initial marker yield (M_o) accumulated at the end of the simulated processing (Figure 6.2A).

6.2.2.7 Prediction of Process Lethality in Canned Carrots Using Py-GC/MS

Canned carrots (Ideal Whole Style Carrots, Nabisco, Etobicoke, Canada, 19 oz liquid) were obtained from a local supplier. According to the information obtained from the manufacturer, the cans were processed at 122°C for 17 min in an FMC rotary continuous cooker. The carrots were drained, sliced and the central cores were removed. The combined cores were manually homogenized and dried at room temperature in a fume hood. The powdered samples (17 mg) were introduced inside a quartz tube (0.3 mm thickness) and inserted inside the coil probe (Wnorowski and Yaylayan 1999) and heated at 120°C for 13, 14.5, 16, 17.5, 19 and 22 min and analyzed by the Py-GC/MS system for the presence of the marker. The same separation conditions were used as described above under GC/MS analysis, except the helium flow rate was set at 1.5 mL/min. The lethality was predicted by plotting marker yield against calculated lethality (Figure 6.2B).

6.2.3 Results and Discussion

Although the concept of using chemical markers has been introduced in the past, problems associated with correlation of the marker yield to useful information regarding the process history limited its applications for foods that have been already processed. According to the results of other researchers (Kim and Choi, 1998; Kim et al.,1996; Ramaswamy et al., 1996), a significant correlation exists between the experimental lethality and calculated lethality obtained from the marker concentration and microbial count reduction. Thus, predicted lethality based on marker yield is a good indication of product sterility. Based on correlating marker yields to process lethality, we have developed a novel approach to predict accumulated lethality in an a food sample with unknown process history and composition. This can be achieved through additional controlled heating of a processed food sample, at various time intervals (called "secondary heating") to further induce marker formation. Furthermore, during this controlled "secondary heating" process, where detailed information regarding time and temperature exposure of the food sample can be known, it allows the calculation of lethality values during the "secondary heating". When the secondary heating data are plotted as marker yield against calculated lethality and, subsequently, the data points are extrapolated, the x- and y-axes intercepts yield information regarding the original process lethality and the associated marker content, respectively, provided "secondary heating" is performed under identical conditions as of the original processing of the sample. In addition, for solid food samples, the "secondary heating" could be conveniently conducted inside the pyrolysis probe of a Py-GC/MS system. This technique can improve

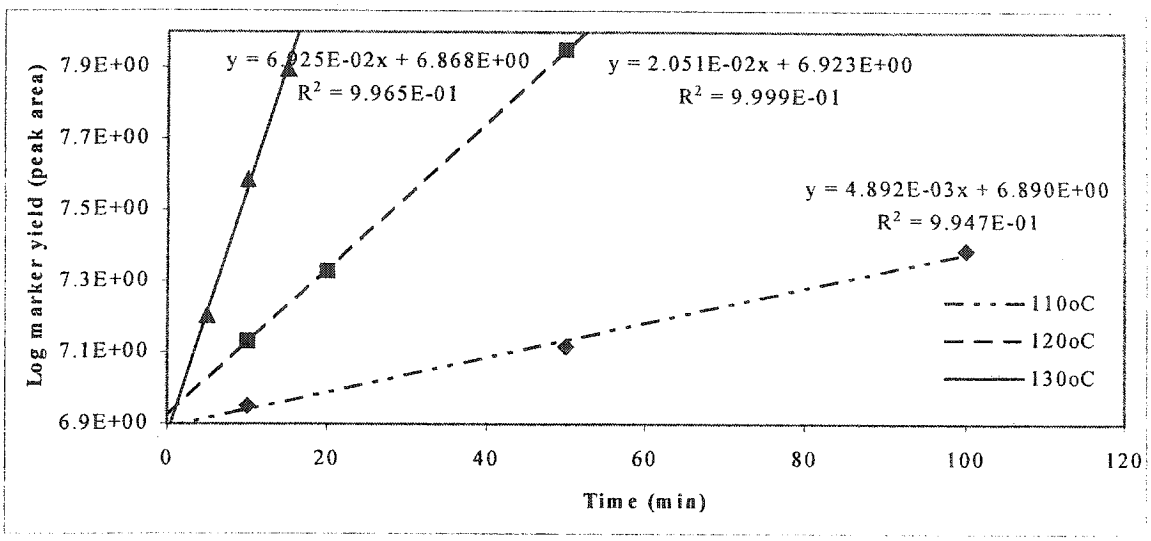


Figure 6.1 Formation Kinetics of M-1 as a Function of Log Peak Area Compared to Heating Time at Various Temperatures

the accuracy of quantification, since the marker is simultaneously formed and analyzed without the need for an extraction step.

Among several potential markers that are generated during the Maillard reaction, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (referred to in literature as M-1) was selected as a suitable compound to monitor thermal processing history. This marker has already been investigated by many researchers (Kim and Taub 1993; Kim and Ball 1994; Kim and Baltes 1996). Results of their studies have confirmed the ease of quantitative determination by several techniques such as TSP-LC-MS and GC-MS. In addition, Yaylayan and Keyhani (1996) have demonstrated that Pyrolysis-GC/MS could be used for simultaneous generation and quantification of this compound from various sources. The compound 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one can be considered as a

"universal" marker, because it can be formed during heating of any hexose alone or in the presence of amino acids, indicating its universal presence in any heated food.

6.2.3.1 Kinetic Properties of the Marker Formation in a Model System

The data obtained from the analysis of the marker formation in the model system at different temperatures were plotted as log (peak areas) against time (Figure 6.1). The results indicated that the formation of the marker followed first-order kinetics. The rate constants were determined from the first-order linear fit of log (average peak area of M-1) against time plots for the 3 temperatures 110, 120 and 130°C as $1.02E^{-03}$, $2.05E^{-03}$ and $6.92E^{-03}$ min^{-1}, respectively. The calculated rate constants were consistent with those reported in the literature (Kim and Choi, 1998; Kim and Taub 1993). The activation energy was also determined, based on the Arrhenius equation by plotting ln k vs 1/T and found to be 29.26 kcal/mol. The fact that E_a for microbial destruction ranges from 50 to 150 kcal/mole, the suitability of the use of the marker (M-1) for monitoring microbial destruction was confirmed. In addition, the Q_{10} value was also calculated to be 2.60 using the equation $Q_{10} = e^{10b}$, where b is the absolute value of the slope of ln k vs. T (°C) plot. In addition, plotting the concentration of M-1 as a function of time (Figure 6.1) indicated the existence of a linear correlation (R^2=0.994 at 110 °C, R^2=0.999 at 120°C and R^2=0.996 at 130°C) at all temperatures studied. Therefore, M-1could be used as a marker between 110-130°C and up to 100 min of processing time (linear range). Statistical analysis of the data with ANOVA confirmed the existence of a high correlation between the predicted and observed process lethalities (99.2% accuracy). In addition, statistical

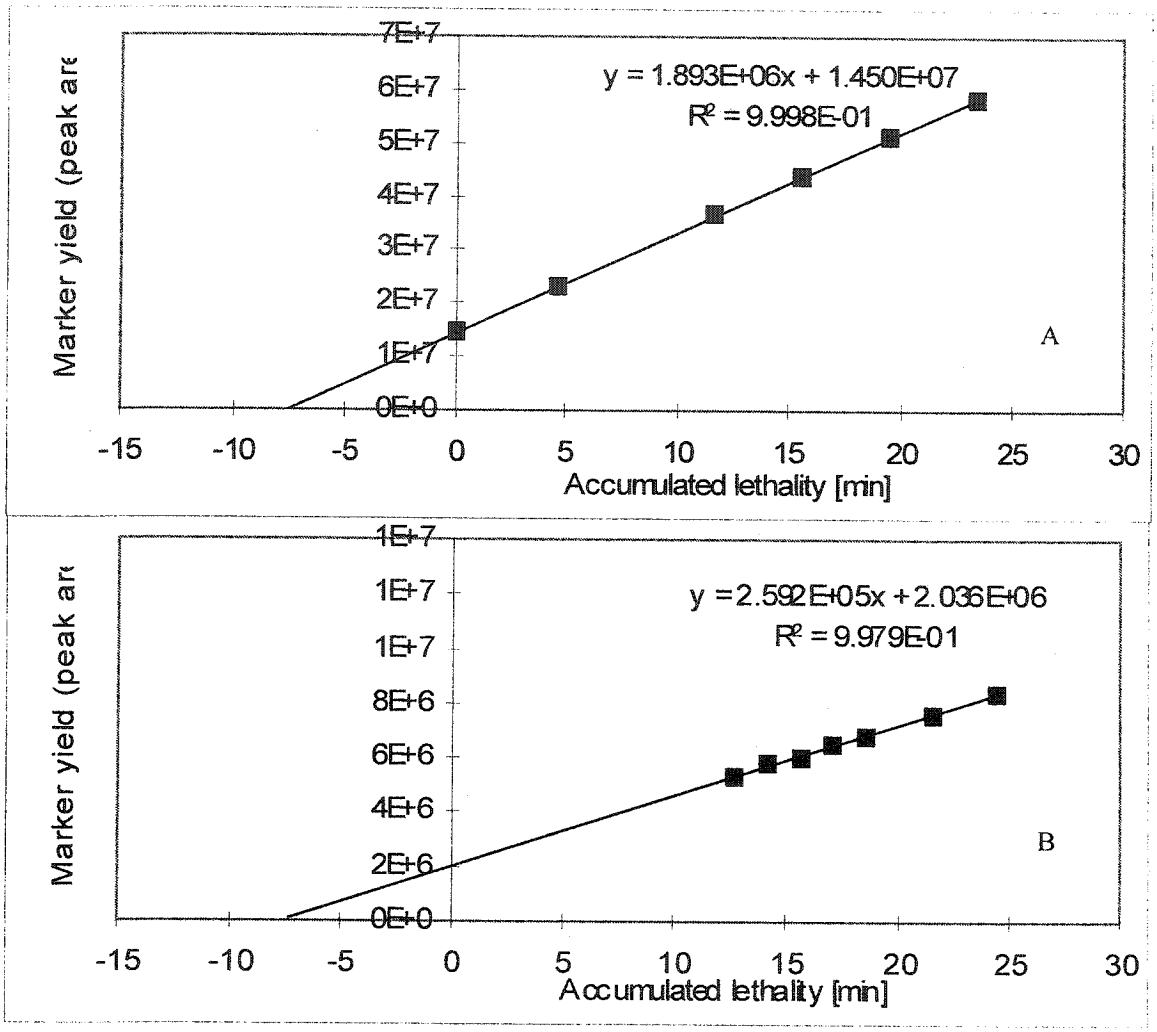


Figure 6. 2. Prediction Through Extrapolation of Accumulated Process Lethality in (A) Model System and (B) Canned Carrots

analysis of the data with ANOVA Two-Factor Without Replication indicated the existence of a significant difference in marker formation as a function of time and temperature and that as the heating time or temperature increases, the variance in estimation of marker concentration also increases.

6.2.3.2. Prediction of Process Lethality of a "Process-simulated" Model System

To confirm the hypothesis that process lethality can be predicted through what we termed as "secondary heating" and subsequent extrapolation of the obtained data, a "process-simulated" model system was prepared according to the procedure described under Materials and Methods. The simulated model system was subjected to "secondary heating" under identical conditions to that of the original process, and the data were analyzed as described above. The plot of peak area of M-1 as a function of lethality (Figure 6.2A) indicated the existence of a linear correlation (R^2=0.99). In addition, when the data points were extrapolated, the linear regression line intercepted the y-axis at a value of 1.45 x 10^7 (representing M_o-1 in peak area counts) and x-axis at a value of x = | 7.66 | ± 5.64% min (95% confidence limits), which represents the "simulated process" lethality. Compared to the expected lethality of 7.76 min (98.69% accuracy), the value obtained by the "secondary heating" method can be considered a good prediction. Furthermore, the accuracy (99.3%) of prediction of M_o-1 (expected value 1.44 x 10^7) gives further credibility to this approach. Experimental data, therefore, confirm the usefulness of this method to predict the accumulated lethality in model systems. When the "process-simulated" sample was stored for 12 months at 24°C, the analysis indicated that there was no additional marker formation, indicating that food stored at room temperature should contain marker formed only during the thermal processing.

6.2.3.3 Prediction of Minimum Process Lethality Inside the Core of Commercially Canned Carrots

As demonstrated above in the case of a model system with known history, if the "secondary heating" is performed under identical conditions to that of actual processing, extrapolation approach will predict the process lethality with high accuracy. However, in the case of a sample with unknown history to mimic its kinetics, the "secondary heating" can be performed under conditions to ensure producing larger slopes for the extrapolation curves generated from the plots of marker yield against lethality, such that the method will always predict the minimum process lethality the food sample has been exposed to in the can. This can be achieved by performing the "secondary heating" at 120°C or lower on samples taken from the centre of the food, since heat penetration studies (Kim and Taub, 1993) have indicated that the concentration of M-1, the same marker as in this study, in the centre of 2.5 cm cubes of both ham and chicken samples, was 70% lower than that on the surface of the samples. Accordingly, the predicted lethality by this approach ensures that the least heat treated part of the food received the minimum acceptable F_o.

When, commercially obtained canned carrots were analyzed through the "secondary heating" procedure described under Materials and Methods, using the convenient Py-GC/MS system, and the measured peak areas of the marker M-1 formed in the central core of the carrots were plotted against lethality, a linear correlation was found (R^2=0.99) (Figure 6.2B). In addition, when the data points were extrapolated, the regression line intercepted the x-axis at a value of x = | 7.86 | ±17% min (95% confidence limits) which

represents the predicted minimum process lethality (F_o) in the centre of the carrots. Compared to the information provided by the manufacturer, the actual lethality applied to the whole can was 22.01 min. Considering the minimum F_o required for sterility is around 2.51 min, this finding underlines the importance of estimating the real time-temperature exposure of particulate foods. Probably, for this particular can, the thermal processing time could have been reduced by 50% (F_o=22.01/2) and still achieve the needed sterility (minimum F_o=7.86/2=3.93) at the centre of the carrot. Similar prediction of process lethality of a smaller can containing corn (Whole Kernel Corn, 7 fl oz, Generation Zel, Montreal, Canada - processing conditions unknown) showed a minimum accumulated process lethality of 5.39 ± 13% min (95% confidence limits). The percent error on estimating the intercept (F_o) could be improved significantly by mechanical homogenization of the food matrix after the drying stage.

6.2.4 Conclusion

Through the documented rationale of using M-1 as a chemical marker to map heat penetration within food particulates, the results of this study confirm usefulness of using formation kinetics of selected Maillard reaction products generated with the application of Py-GC/MS in relation to food quality. The proposed technique can help to optimise the processing time, to prevent overheating in order to preserve nutritional characteristics of the food, as well as save energy. It can also serve to critically evaluate sterilization efficacy and quality of foods processed through novel non-conventional technologies, when other process validity means cannot be applied. In addition, it provides a convenient tool, which requires no standards to be prepared nor need for microbial

destruction data knowledge, for various regulatory agencies to assess the minimum accumulated lethality in different parts of a given food sample, including the centre, whether heated conventionally or non-conventionally and hence determine the adequacy of heat treatment.

6.3 Formation Mechanism of Acrylamide and its Application in Food Safety

6.3.1 Introduction

Thermal processing may induce different chemical changes in food, some are considered desirable and some deleterious. Maillard-induced carcinogen formation is one such example (Friedman, 2003). Recent findings correlating acrylamide concentrations in animal subjects to the intake of fried feed (Tareke et al., 2000) have led to the investigation of its presence in food (Tareke et al., 2002). These studies have indicated that acrylamide formation in laboratory-heated foods is temperature dependent and that only moderate levels were detected in protein-rich foods whereas, carbohydrate-rich foods exhibited much higher levels of acrylamide. Recently, two reports (Stadler et al., 2002; Motram et al., 2002), confirmed asparagine to be the amino acid precursor of acrylamide. Furthermore, Stadler et al. (2002), have provided evidence that sugar asparagine adduct; the N-glycosyl asparagine, is a direct precursor of acrylamide, indicating the involvement of Maillard reaction. To control the formation of this potential carcinogen in food, detailed knowledge of its mechanism of formation is of critical importance. In this study, using pyrolysis/gas chromatography/mass spectrometry (Py-GC/MS) (Yaylayan, 1999) and FTIR analysis an evidence is provided that the ability of

the open-chain form of the N-glycosyl asparagine (the Schiff base) to undergo intramolecular cyclization and formation of oxazolidin-5-one is the key step that allows decarboxylation of asparagine and subsequent formation of acrylamide. Py-GC/MS is also a cost effective and convenient method to perform experiments with labeled reactants (Yaylayan, 1999). Although under pyrolytic conditions a higher number of products are formed compared with aqueous reactions, however, most of the products identified in aqueous systems are also formed under pyrolytic conditions albeit in different amounts. In addition, experimental evidence was provided that the position and label distribution in the common products observed in the same model systems, between aqueous and pyrolytic reactions, are identical (Wnorowski and Yaylayan, 2000). This indicates the similarity of mechanisms of formation of the common products under both conditions. Consequently, mechanistic conclusions derived from label incorporation in the products observed under pyrolytic conditions, that are common to both systems, have relevance to the aqueous reactions. Using variously [^{13}C]-labeled glucoses we have also provided evidence for the origin of niacinamide - an indicator for the formation of decarboxylated Amadori compound in the glucose/asparagine model system.

6.3.2 Materials and Methods

6.3.2.1 Chemicals and Reagents

All reagents and chemicals were purchased from Aldrich Chemical company (Milwaukee, WI) with the HPLC grade purity and used without further purification. The labeled sugars [^{13}C-1]glucose (99%), [^{13}C-3]glucose (99%), [^{13}C-4]glucose (98%) and [^{13}C-6]glucose (98%) were purchased from Cambridge Isotope Laboratories (Andover, MA).

6.3.2.2 Pyrolysis-GC/MS Analysis

A Varian CP-3800 gas chromatograph coupled to a Saturn 2000 Ion Trap detector interfaced to a CDS Pyroprobe 2000 unit, through a valved interface (CDS 1500), was used for Py-GC/MS analysis. In all experiments, asparagine model mixtures (2.0 mg, equimolar with sugars) were introduced inside the quartz tube (0.3 mm thickness) and plugged with quartz wool and inserted inside the coil probe. The pyroprobe was set at the desired temperature at a heating rate of 50°C/ms with a total heating time of 20 seconds. The pyroprobe interface temperature was set at 250°C. The initial temperature of the column was set at -5°C for 12 minutes, and then increased to 50°C at a rate of 50°C min^{-1}; immediately the temperature was further increased to 250°C at a rate of 8°C min^{-1} and kept at 250°C for 5 min. A constant flow of 1.5 ml min^{-1} was used during analysis. Capillary direct MS interface temperature was 250°C; ion source temperature was 180°C. The ionization voltage was 70 eV and the electron multiplier was 2047 volts. The mass range analyzed was 29 - 300 amu. The column was a fused silica DB-5 MS column

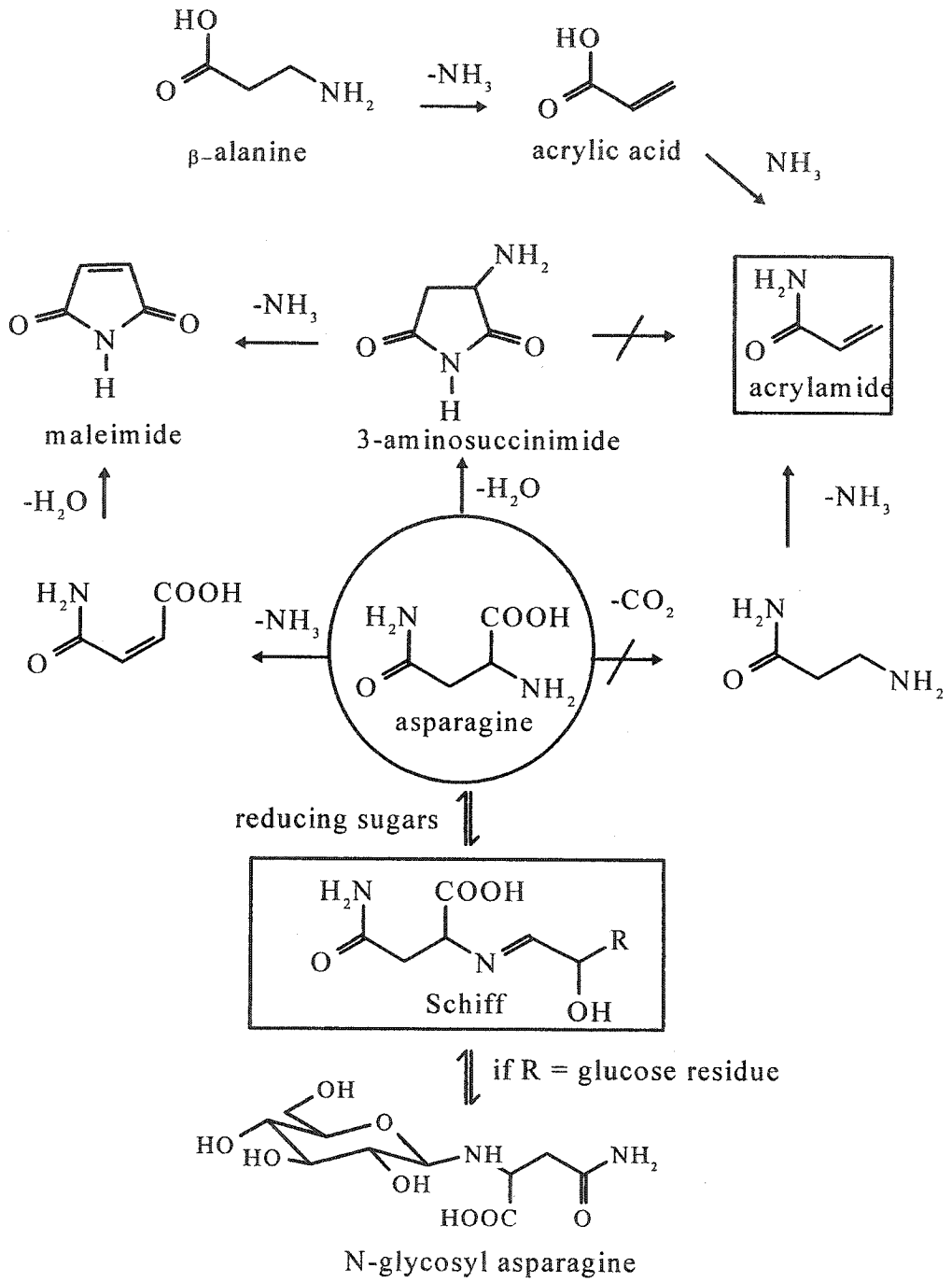
(50 m length x 0.2 mm i.d. x 0.33 μm film thickness; J&W Scientific). The identity and purity of the chromatographic peaks were determined using NIST AMDIS version 2.1 software. The reported percent label incorporation values (corrected for natural abundance and for % enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

6.3.2.3 FTIR Analysis

Saturated solutions were prepared by mixing equimolar amounts of asparagine and glyceraldehyde in methanol. Samples were analyzed after evaporation of methanol and formation of a thin film on the surface of the cell window. Infrared spectra were recorded on a MIDAC Prospect Fourier-transform infrared spectrometer purged with dry air and equipped with a deuterated triglycine sulfate (DTGS) detector. The spectra were acquired on a CaF_2 cell without spacer at room temperature unless otherwise specified. A total of 128 scans at 4 cm^{-1} resolution were co-added. Processing of the FTIR data were performed using Galactic GRAMS 32/AI.

6.3.2.4 FTIR Temperature Studies

Samples were placed in a CaF_2 cell without spacer. The temperature of the sample was regulated by placing the IR cell in a temperature-controlled cell holder. Infrared spectra were recorded as described above.



Scheme 6. 1 Thermal Decomposition Products of Asparagine and its Reaction with Reducing Sugars

6.3.3 Results And Discussion

Asparagine model systems containing either glucose, fructose, sucrose, sorbitol, glyceraldehyde, glycolaldehyde or 2,3-pentanedione were reacted using Py-GC/MS as detailed under the experimental section.

Table 6.1 Formation Efficiency of Acrylamide Expressed as Gas Chromatographic Peak Area per Mole of Starting Asparagine Generated at 350°C from Various Model Systems

Model system	Efficiency (area/mole)
Asparagine alone	0
Asparagine+sorbitol	0
Asparagine+2,3-pentanedione	trace
Asparagine+glucose	4.9×10^{11}
Asparagine+glucose [1]	1.4×10^{11}
Asparagine+glycolaldehyde [1]	2.8×10^{11}
Asparagine+fructose	6.6×10^{11}
Asparagine+glyceraldehyde	8.6×10^{11}
Asparagine+sucrose	18×10^{11}

[1] at 250°C

All model systems generated acrylamide at 250°C, however, the intensity of the acrylamide increased at higher temperatures. Interestingly, at 250°C, the asparagine/glycolaldehyde model system was more efficient then asparagine/glucose in generating acrylamide, however, at 350°C asparagine/sucrose was more efficient than asparagine/glucose model system. The least efficient system in generating acrylamide was that of asparagine/2,3-pentanedione which generated acrylamide in trace amounts at both temperatures (Table 6.1).

A cursory look at the structures of naturally occurring amino acids in food, would immediately pinpoint asparagine as a possible source of acrylamide, since decarboxylation and deamination of this amino acid in principle, could produce

acrylamide, and since such reactions are common thermally allowed reactions in food. However, the main degradation product of asparagine, identified during pyrolysis-GC/MS analysis was maleimide (Scheme 6.1 and Table 6.2). Evidently, intramolecular cyclization to form an imide is much faster compared with the decarboxylation reaction due to entropy factor (Sykes, 1975). In theory, this reaction should reduce the toxicity of asparagine in food by preventing its conversion into acrylamide. The intermediate 3-aminosuccinimide (Scheme 6.1) was detected in the pyrogram of asparagine model systems along with maleimide. On the other hand, a fast deamination reaction can also prevent intact asparagine from its conversion into acrylamide, since decarboxylation of this intermediate is not favoured due to the destabilization of the resulting negative charge at the proximity of the pi electrons of the double bond. When β-alanine was pyrolyzed under the same conditions as asparagine, only two products were detected; acrylic acid and the product of its subsequent reaction with ammonia; acrylamide, indicating the propensity of such moieties to deaminate rather then to decarboxylate.

The role of reducing sugars in enabling the conversion of asparagine into acrylamide should be considered, therefore, under the light of the above findings. The first intermediate formed when reducing sugars react with asparagine is the so called Schiff base (in equilibrium with N-glycosyl asparagine). This intermediate stabilizes itself through Amadori rearrangement process (pathway A in Scheme 6.2) which leaves the carboxylic acid free to undergo intramolecular cyclization at elevated temperatures, similar to the free asparagine and form Amadori product with N-substituted succinimide. The main residue generated from amino acid moiety identified in the pyrolyzates of

asparagine model systems was succinimide (Figure 6.3B and Table 6.2). This process, similar to that occurring in asparagine alone (Scheme 6.1) prevents the formation of acrylamide. A competing mechanism, therefore should exists that allows the Amadori product to decarboxylate before it can be trapped as inactive succinimide moiety by intramolecular cyclization.

In addition to undergoing Amadori rearrangement, the Schiff form of N-glycosyl asparagine, can also stabilize itself through intramolecular cyclization (pathway B in Scheme 6.2) initiated by the carboxylate anion (Manini et al., 2001; Yaylayan and Wnorowski, 2002) and formation of oxazolidin-5-one (Manini et al., 2001). Manini et al. (2001) have observed the formation of such an intermediate and its facile decarboxylation at room temperature in D-glucose/L-DOPA model system. This facile decarboxylation of the oxazolidin-5-one intermediate at room temperature, affords a stable azomethine ylide (Manini et al., 2001) which after tautomerization and protonation can generate decarboxylated Amadori product (Scheme 6.2). The existence of such a pathway will allow some of the Amadori rearrangement product to decarboxylate, thus preventing the formation of succinimide moiety and eventually allow the formation of acrylamide. Although the decarboxylated intermediate can be formed under mild conditions, however, it requires elevated temperatures to cleave the strong carbon-nitrogen covalent bond and produce acrylamide as shown in Scheme 6.2.

Table 6.2. Mass Spectrometric Data (ten highest peaks) of Compounds Identified in the Model Systems

Compound	***m/z* (relative intensity)**
maleimide	98(4.6), 97 (100), 70(4.3), 69(31.2), 54(48.1), 53(18.0), 52(5.2), 43(9.4), 42(5.0), 40(4.3)
maleimide [1]	98(5.1), 97 (100), 70(4.3), 69(39.3), 54(58.1), 53(29.5), 52(2.9), 43(16.5), 42(9.4), 40(6.1)
succinimide	100(4.7), 99(100), 70(9.0), 57(2.0), 56(11.0), 55(10.1), 54(4.0), 44(3.9), 43(9.8), 42(10.3)
succinimide [1]	100(4.8), 99(100), 70(5.0), 57(2.2), 56(8.0), 55(7.3), 54(2.1), 44(3.8), 43(5.8), 42(7.3)
3-aminosuccinimide	114(9.2), 86(37.8), 70(7.8), 59(5.2), 57(3.2), 56(1.1), 55(6.3), 44(6.2), 43(100), 42(66.8)
3-aminosuccinimide [1]	114(7.4), 86(32.0), 70(6.2), 59(4.1), 57(1.1), 56(1.5), 55(2.3), 44(5.8), 43(100), 42(77.1)
niacinamide	122(100), 106 (54.9), 94 (6.3), 79(8.9), 78(78.4), 77(8.0), 52(14.4), 51(33.8), 50(17.8), 44(11.6)
niacinamide [1]	122(100), 106 (57.6), 94 (4.3), 79(7.9), 78(75.6), 77(7.2), 52(17.4), 51(45.4), 50(19.9), 44(12.3)

[1] NIST Library

Table 6.3 Percent Label Distribution in Selected Mass Spectral Fragments [1] of Niacinamide Formed in Different Model Systems

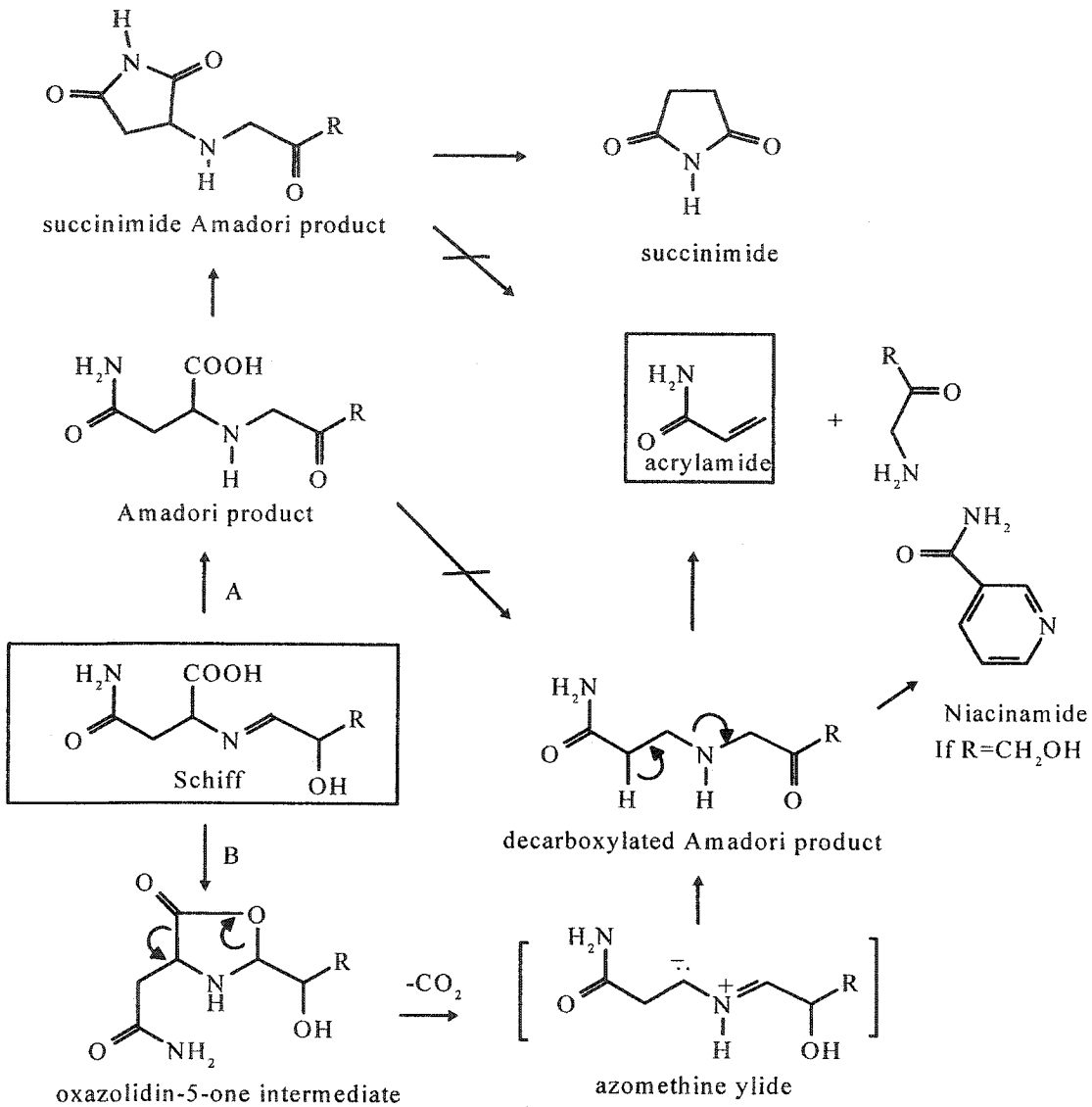
Model system	m/z 122	m/z 123	m/z 106	m/z 107	m/z 78	m/z 79
	M	M+1	M	M+1	M	M+1
D-glu/L-asn	100	0	100	0	100	0
D- [1-^{13}C]glu/L-asn	60	40	60	40	60	40
D- [3-^{13}C]glu/L-asn	60	40	60	40	60	40
D- [4-^{13}C]glu/L-asn	40	60	40	60	40	60
D- [6-^{13}C]glu/L-asn	40	60	40	60	40	60

[1] Figure 6.4 for the structures of ions at m/z 122, 106 and 78
glu = glucose; asn= asparagine

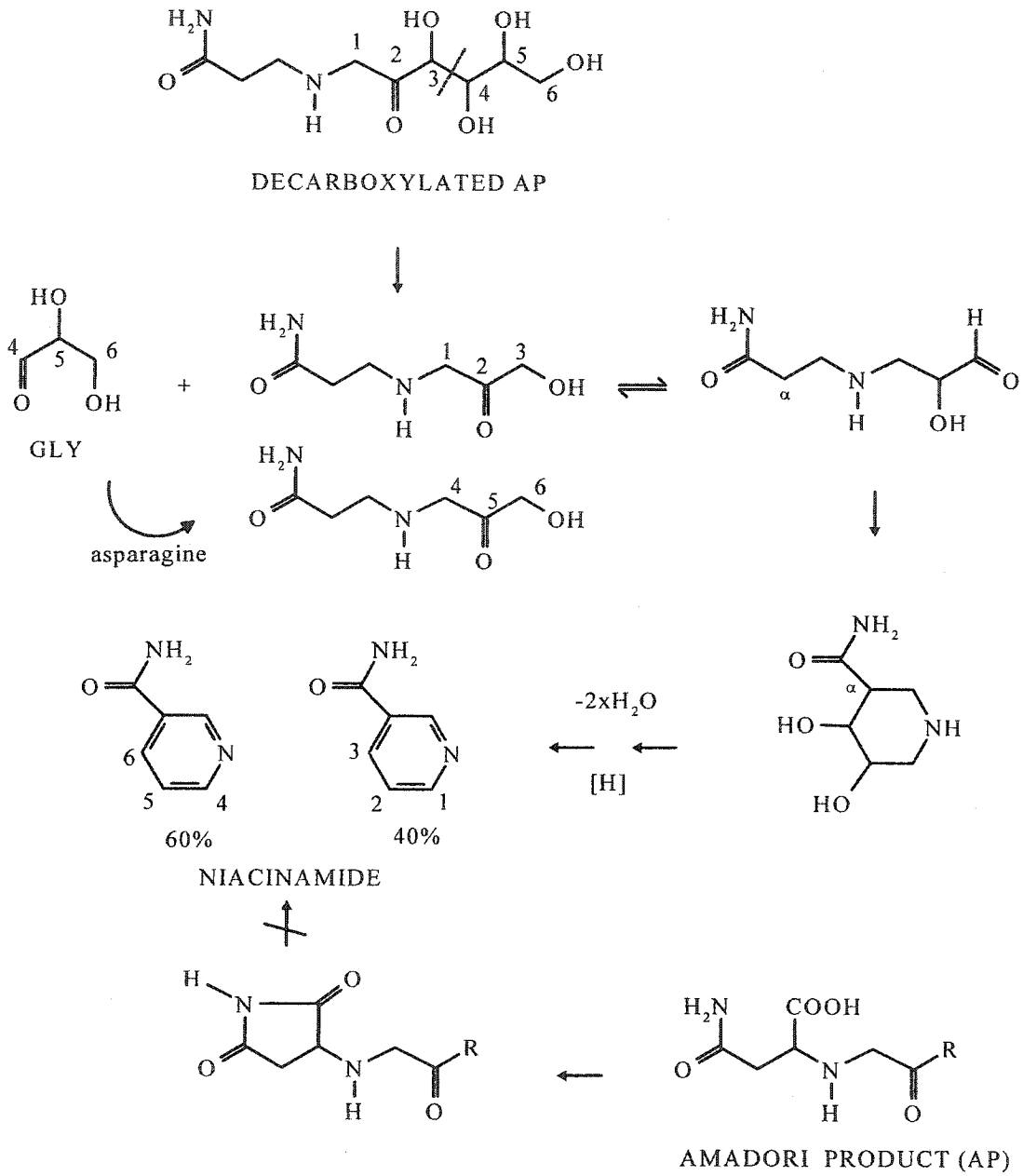
6.3.3.1 Py-GC/MS Experiments with ^{13}C-labeled Glucoses

Evidence in favour of formation of the decarboxylated Amadori intermediate comes from identification of niacinamide (Table 6.2 and Schemes 6.2 & 6.3) in the reaction mixture of asparagine/glucose model system. Niacinamide can only be formed from decarboxylated Amadori product of asparagine with glyceraldehyde through cyclization and dehydration reactions (Scheme 6.3). The Amadori product on the other hand, will quickly undergo intramolecular cyclization and form succinimide intermediate, which is unable to form niacinamide due to its altered structure, as shown in Scheme 6.3. Furthermore, Maillard model systems containing glucose are known to produce free glyceraldehyde and glyceraldehyde Amadori product (Yaylayan et al., 1999a) through retro-aldol reactions (Scheme 6.3). In fact, when glyceraldehyde was pyrolyzed in the presence of asparagine both acrylamide and niacinamide were detected in higher amounts relative to glucose model system (see Table 6.1 for acrylamide values). In addition, as illustrated in Scheme 6.3, when the glucose Amadori product undergoes retro-aldol cleavage it produces a glyceraldehyde Amadori product that retains C-1, C-2 and C-3

atoms of glucose and a free glyceraldehyde molecule with C-4, C-5 and C-6 remaining atoms of glucose. Theoretically, the niacinamide molecule should be formed containing either C-1-C-2-C-3 or C-4-C-5-C-6 unscrambled fragments as part of the aromatic ring carbon chain as shown in Scheme 6.3. The percent incorporation of these two fragments into the niacinamide structure will depend on the concentration of these two fragments in the reaction mixture. Furthermore, Scheme 6.4 indicates that these sugar carbon atoms should be retained in the following ions; *m/z* 122 (molecular ion), *m/z* 106 and *m/z* 78 during the electron impact induced fragmentation of niacinamide (Table 6.2). In order to confirm the above prediction, asparagine was reacted with variously labeled glucoses (Table 6.3) and the percent label incorporation in niacinamide was calculated and shown in Table 6.3. According to this table 40% of niacinamide was formed with C-1-C-2-C-3 fragment and 60% with C-4-C-5-C-6 fragment, confirming the proposed mechanism of niacinamide formation.



Scheme 6. 2 Mechanism of Acrylamide Formation from a Decarboxylated Amadori Product of Asparagine; R = [CH(OH)]nCH^2(OH); n = 0 - 3



Scheme 6. 3 Mechanism of Niacinamide Formation in Asperagine/glucose Model System and Percent Label Incorporation. [Carbon numbers represent original glucose carbon atom locations; AP = Amadori product, Gly = glyceraldehyde]

6.3.3.2 Monitoring the Decarboxylation by FTIR Spectroscopy and Py-GC/MS

To confirm that the ease of decarboxylation of asparagine moiety was due to the formation of a Schiff base adduct, asparagine was reacted with glucose and sorbitol, the latter is unable to form a Schiff base with asparagine, and the resulting pyrograms were compared. Figure 6.3 clearly indicates the presence of a significant peak due to CO_2 release in the case of glucose model system and its complete absence in the case of sorbitol model system.

Additional evidence for the facile formation of decarboxylated asparagine Amadori product was obtained from infrared spectral analysis of a mixture of asparagine and glyceraldehyde in methanol. These studies have indicated that within fifteen minutes of incubation at room temperature, Amadori product has already started to form (pathway A Scheme 6.1) as indicated by the appearance of the absorption band at 1737 cm^{-1} which was assigned to the carbonyl band (Yaylayan et al., 1999a; Yaylayan et al., 1999b) of the Amadori product (Figure 6.4 b). This assignment was based on the fact that the Amadori product is a derivative of dihydroxyacetone (Yaylayan et al., 1999a). Further incubation of the sample at 40°C for six days have caused the disappearance of both symmetrical and antisymmetrical stretching frequencies of the carboxylate bands at 1493 and 1580 cm^{-1} (Figure 6.4c) indicating decarboxylation reaction and formation of decarboxylated Amadori product (pathway B in Scheme 6.2). The spectral properties of this mixture did not change upon further incubation at 40°C, however, when the temperature of the mixture was raised to 180°C and the spectrum was acquired, the data showed the

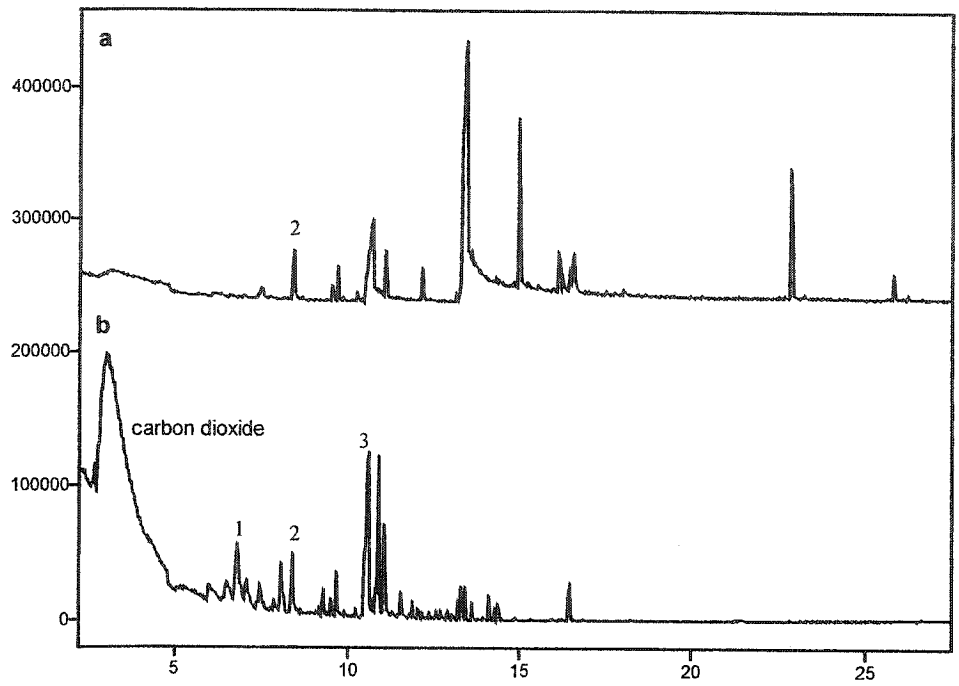


Figure 6.3 Pyrograms of (A) Sorbitol/Asparagine and (B) Glucose/Asparagine Model Systems. Peaks: 1. Acrylamide; 2. Maleimide; 3. Succinimide

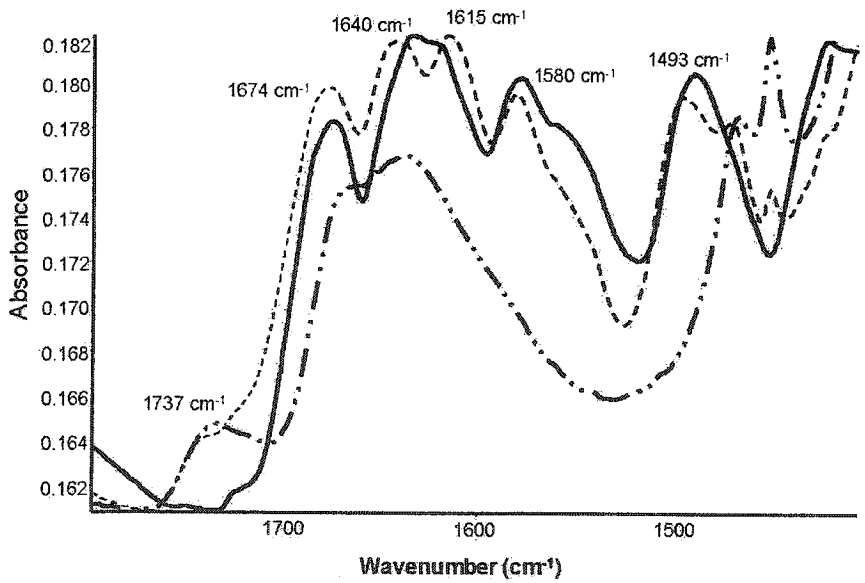
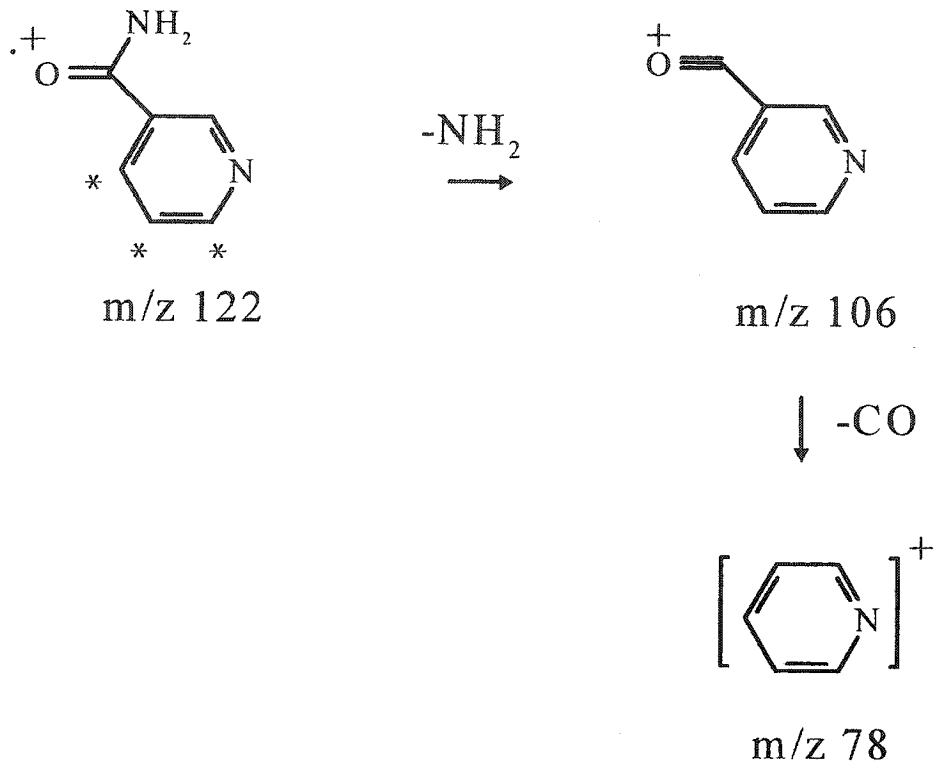


Figure 6. 4 Overlaid Infrared Spectra (1400-1800 cm^{-1})of (A) Asparagine (solid line), (B) Asparagine/Glyceraldehyde after 15 min of Incubation at Room Temperature (dotted line), and (C) Aspargine/Glyceraldehyde after 6 Days of Incubation at 40°C (dash-dot line)



Scheme 6. 4 Mass Spectral Fragmentation Pattern of Niacinamide

* Represents Glucose Carbon Atoms

disappearance of the amide I (1674 cm^{-1}) and amide II (1615 cm^{-1}) absorption bands of the amino acid, indicating cleavage and decomposition of product as shown in Scheme 6.2.

Furthermore, the cyclic form of the Schiff base, the N-glycosyl asparagine, has already been confirmed (Stadler et al., 2002) to be an intermediate in the asparagine/glucose reaction and that the acrylamide yield from the pyrolysis of synthetic N-glycosyl asparagine was significant and comparable to the yield from asparagine/glucose model

system under the same conditions (Stadler et al., 2002), providing further evidence for the intermediary of decarboxylated Amadori product as the precursor of acrylamide.

6.3.4 Conclusion

Similar to asparagine, high temperature-induced cyclization reaction to form succinimide Amadori product (Pathway A in Scheme 6.2) is highly favoured over the competing thermal decarboxylation reaction required for the formation of acrylamide. However, the low energy pathway (Pathway B in Scheme 6.2) provided for the decarboxylation of the N-glycosyl asparagine through the formation of oxazolidin-5-one, makes it possible to by-pass cyclization reaction and promote the formation of acrylamide in carbohydrate/asparagine mixtures. This mechanism has been recently confirmed (Zyzak et al., 2003) through the use of LC/MS.

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CHAPTER 7

GENERAL CONCLUSIONS AND SUMMARY OF CONTRIBUTIONS TO KNOWLEDGE

Pyrolysis-gas chromatograph/mass spectrometer, as an integrated reaction, separation and identification system, has been demonstrated to be an ideal tool to study the formation of Maillard reaction products. The use of labeled starting materials, such as reducing sugars and amino acids, provides a convenient way to elucidate the detailed mechanism of formation of not only stable end-products but also that of reactive intermediates incorporated in them. Optimization of the operating parameters of Py-GC/MS system was necessary to reduce the amount of expensive ^{13}C- or ^{15}N- labelled materials used and to prevent degradation or secondary interactions of labile intermediates during the transfer from the pyrolysis interface to the analytical column. Furthermore, Py-GC/MS system, compared to classical approach not only shortens the time of analysis form hours to minutes, but also eliminates the need for extraction since generated volatiles are directly transferred into GC column. Employment of mass spectrometer as a detector with a built-in library allows convenient and unambiguous identification of eluted compounds. Moreover, small sample sizes in the range of 0.1-3.0 mg has a great importance in analyzing rare or expensive samples, such as isotopically labeled materials. Py-GC/MS analysis is also less labour intensive compared to other analytical methodologies. In addition, high temperature and low moisture level during pyrolysis, compared to aqueous model reactions, are closer to the conditions of Maillard reactions occurring during

baking or roasting. Furthermore, the comparison of mechanistic pathways during pyrolysis has been shown to be identical to that of aqueous systems. Thus mechanistic model studies conducted with Py-GC/MS find great relevance and application for understanding complex reactions occurring in the food matrix. Lack of Maillard reaction products in microwavable and low-sugar foods can be substantially improved with the knowledge gained through the studying the thermal degradation of β-hydroxy amino acids under pyrolytic conditions. Apparently serine and threonine are able to generate sugar-specific hydroxy-carbonyls, that upon reaction with amine source can carry on Maillard reaction and generate various flavouring compounds. The labeling studies of serine with Py-GC/MS system allowed to unambiguously determine that it is the β-positioned hydroxy group formed as glycolaldehyde which is reacting with available amino sources to form Amadori product, or condensing with other carbonyls to form longer chains responsible for the formation of different heterocyclic aroma compounds.

Direct and indirect detection of 2-keto acids and 2-amino alcohols from the thermal degradation β-hydroxy amino acids suggested and later documented their participation in a transamination reaction leading to a novel route to Amadori product. This fact may modify in the future, the definition of Maillard reaction as an interaction between sugars and amino acids. Finally, the ability of Py-GC/MS system to accurately control time and temperature exposure of the reactants, facilitated also performance of kinetic studies of chemical marker formation. The information gained from kinetic studies can be used in safety and quality control of processed foods.

The contribution to knowledge is summarized below:

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

1. The fundamental basis of understanding the impact of the pyrolyzer, when used as a chemical reactor, on the quality of chromatograms obtained and the effect of pressure programming on the efficiency of sample transfer during Py-GC/MS analysis and demonstration of the utility of sample preconcentration trap in enhancing the sensitivity of the analysis.

2. Evidence through labeled studies of the similarity between mechanism of formation of common products generated under aqueous and pyrolytic conditions, thus demonstrating the relevance of mechanistic information obtained from Py-GC/MS system to processes occurring in food products.

3. Experimental evidence, through labeling studies, on the formation of β-keto acids in L-serine and L-threonine model systems and demonstrated their role as a precursor to an alternative route to the formation of Amadori product supported by monitoring with FTIR and structural studies with CI-MS and MS-MS.

4. A pathway of the detailed mechanism of formation of acrylamide from asparagine.

5. A methodology of using Maillard-generated chemical markers to predict process lethality.

APPENDIX A

Isotopic Labeling Techniques in Mechanistic Studies

Analysis of Maillard reaction is not limited merley to identification of the products formed in different model systesm. One of the most important and laborious aspects of the study also includes the elucidation of mechanism of formation of the stable end-products through labelling studies. Since the proposed mechanisms may provide the means of controlling the reaction in processed or stored foods and perhaps also in biological systems. Calculation of percent incorporation and identification of precise location of labeled atoms into end-products allows to gain information on the origin and fate of different intermediates.

The isotopic incorporation of ^{13}C or ^{15}N-atoms, into the detected products is calculated as per cent ratio of molecular mass ion M of unlabeled compound to molecular mass ion M+1 (if one ^{13}C or ^{15}N incorporated) or M+2 (if two isotopically labeled atoms are incorporated) of the labled analogs, corrected for natural abundance of carbon(s) or nitrogen(s). Labeled positions are estimated by MS data interpretation by identifying the fragment in the mass spectrum, with the incorporated isotopic atom(s), consequently providing an evidence for proposed mechanism. This type of calculations can be easily visualised using as an example acetic acid formation from glucose-glycine model system.

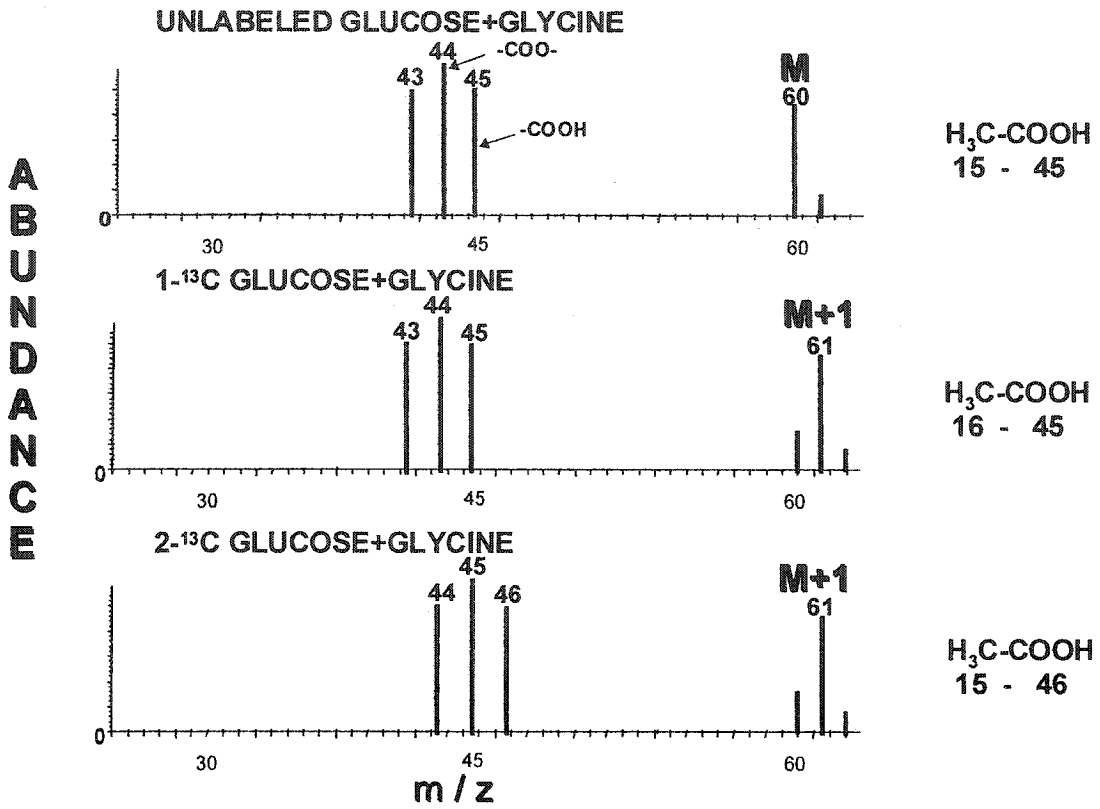


Figure A.1 Isotope Distribution in Acetic Acid generated from Glucose (unlabeled, 1-^{13}C and 2-^{13}C labeled)-glycine (unlabeled) Model Systems

Acetic acid is a two carbon compound. In order to establish how it is formed in glucose-glycine reaction mixture, ten model systems containing unlabeled and specifically labeled glucoses and glycines are needed to be studied (see Figure A.2). Isotope distribution obtained from MS data interpretation is presented in Figure A.1. Data in Figure A.2 shows that labeled acetic acid (M+1) is mostly formed from first and second carbon atoms of glucose (around 70 %) and partially from other carbon atoms including first and second carbon atoms of glycine. Although percent label incorporation suggests which glucose carbons are used to form acetic acid (Figure A.1), however, the position of the labels in the acetic acid can be identified only by the examination of fragmentation patterns under Electron Impact (EI) conditions (Figure A.1). Comparison of EI fragment

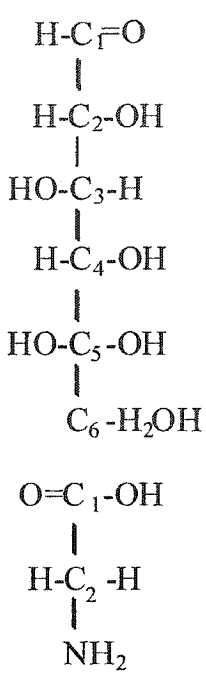
Model	M	M+1
Glycine/Glucose	100	0
 Glycine/[1-^{13}C]Glucose	30	70
Glycine/[2-^{13}C]Glucose	30	70
Glycine/[3-^{13}C]Glucose	98	2
Glycine/[4-^{13}C]Glucose	98	2
Glycine/[5-^{13}C]Glucose	93	7
Glycine/[6-^{13}C]Glucose	93	7
[1-^{13}C]Glycine/Glucose	83	17
[2-^{13}C]Glycine/Glucose	83	17
[^{15}N]Glycine/Glucose	100	0

Figure A.2 Percent Label Incorporation in Acetic Acid generated from Glucose glycine Model Systems

masses of unlabeled and labeled molecules will allow the identification of mass fragments in which the heavier isotopes are located. The molecular mass of acetic acid is 60 amu, and under EI conditions of MS the molecular ion may fragment into different masses, however, the most relevant fragments are those of masses at m/z 45 (corresponding to carboxyl group) and m/z 15, corresponding to methyl group. For the present discussion, only three cases will be considered, i.e. unlabeled and 1-^{13}C or 2-^{13}C labeled glucoses reacting respectively with unlabeled glycine. The analysis of mass spectra (Figure A.2) of acetic acid, confirms the increase of molecular mass of acetic acid by one atomic mass unit when 1-^{13}C or 2-^{13}C glucoses were used. The mass spectra however, give different fragmentation patterns when 1-^{13}C or 2-^{13}C-labelled glucoses are

used. Careful comparison of the spectra reveals the different location of the heavier isotope. The fragmentation data indicates that 1-^{13}C is used to form the methyl group in acetic acid (fragment mass increase from unlabeled m/z 15 to labeled 16), while 2-^{13}C is used to generate carboxyl group of the acid (fragment mass increase from unlabeled m/z 45 to labeled 46).

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Professor V Yaylayan

yaylayan@macdonald.mcgill.ca

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