Application of mass spectrometry and isotope labelling technique to study the role of various derivatives of α-dicarbonyl moiety in the generation of Strecker and Maillard reaction products

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Study of α-dicarbonyl moiety in the generation of Strecker and Maillard reaction products

This thesis is dedicated to my parents, especially to my mother who allowed me to reach my dreams... gracias madre por dejar tus sueños a un lado para que yo alcanzara los míos, este logro es de las dos.

ABSTRACT

The interaction of carbonyl moieties with free amino groups initiates one of the most important chemical transformations in food, the Maillard reaction. This non-enzymatic browning reaction occurring in food and its sub-reactions are part of an intricate matrix of complex chemical transformations. Among the most reactive structures produced in this reaction, the 1,2-dicarbonyl moieties have a special role of interacting with amino acids through the Strecker reaction to generate α-amino carbonyls, aldehydes and heterocyclic compounds, many of them aroma-active. Numerous studies have shown the interaction of a single amino acid with a 1,2-dicarbonyl moiety, however no systematic studies have been reported on the ability of these α-dicarbonyls to undergo multiple addition reactions with amino acids, the type of products generated, or alternative routes for their generation. The identification of other pathways generating similar products could be considered valuable methods of introducing them into the heated foods, while the understanding of multiple addition reactions may provide a better insight into detailed chemical transformations occurring in food. Furthermore, 1,2-dicarbonyl moiety can be found as part of other chemical structures such as cyclic lactones, α-keto acids and *o*-quinones. Hence a comprehensive understanding of the potential of these 1,2-dicarbonyl moieties to participate in Strecker-type reactions was also considered. For this purpose, appropriate model systems were evaluated using isotope labelling technique with various precursors in conjunction with pyrolysis gas chromatography mass spectrometry or high resolution liquid chromatography mass spectrometry of thermally reacted model systems. Initially, a model system composed of glycine and 2,3-butanedione was evaluated under dry pyrolytic conditions at 250 °C, as well as in water and under pressurized conditions at 120 °C to evaluate the potential of these dicarbonyls to undergo oxidation and form pyrazines. These studies indicated the unexpected formation of 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazine in addition to the expected tetramethylpyrazine. Isotope labelling studies using [^{13}C-1]-, [^{13}C-2]- and [^{15}N]-glycine showed the incorporation of none, one or two ^{13}C-2 atoms from glycine in the ring system of the 2,3-dimethylpyrazine structure. The formation of doubly labelled 2,3-dimethylpyrazine was rationalized through the double addition of glycine to 2,3-butanedione, and the formation of singly labelled isotopomer was justified by sequential Schiff base formation of 2-amino-butan-3-one, first with the Strecker aldehyde and then followed by glycine. The proposed pathways were also consistent with the

observed label distribution patterns of 2,3,5-trimethylpyrazine. The formation of the previously reported 4,5-dimethyl-1,2-benzoquinone from the 2,3-butanedione/glycine model system was also examined. Additional studies were carried out by replacing the glycine with sodium glycinate or glycine hydrochloride as amino acids able to modulate acid/base catalytic activity of the reaction medium. The analysis of the data indicated that replacing glycine with its corresponding salts promoted significantly the generation of 2,3,6,7-tetramethylquinoxaline relative to tetramethylpyrazine. The origin of the 2,3,6,7-tetramethylquinoxaline was traced back to the formation of 4,5-dimethyl-1,2-benzoquinone and its conversion into 4,5-dimethyl-1,2-phenylenediamine through Strecker-type transformations. Based on these observations and given that ring-B of catechins is susceptible to oxidation and formation of such *o*-benzoquinone moieties under thermal treatment, the potential of ring-B of catechins to undergo similar interactions to form adducts with amino acids, as it was observed with the 4,5-dimethyl-1,2-benzoquinone was evaluated. For this purpose high resolution ESI-TOF mass spectrometry, MS/MS analysis and isotope labelling technique were used to assess the reactivity of glycine with (+)-catechin heated under oxidative conditions at 120^{o}C for 70 min. Isotope labelling incorporation pattern of [^{13}C-1]-, [^{13}C-2]- and [^{15}N]-glycine indicated that (+)-catechin formed various adducts with glycine, two of them incorporated a single amino acid and three adducts incorporated two amino acid moieties. The fragmentation pattern of these adducts confirmed the addition of amino acid moieties to the oxidized B-ring of (+)-catechin through formation of Schiff bases. Further evaluation of the double addition and double Schiff base formation principle was carried out through the reaction of glucose and two amino acids with different side chains, glycine and cysteine. Both its volatile and non-volatile products were studied. A non-volatile adduct that was observed to form, demonstrated the occurrence of this principle for the first time through sequential Amadori rearrangements first between glucose and glycine and then followed by cysteine. The reactive dicarbonyl moiety present in α-keto acid structures was also investigated as precursors of Strecker or Maillard reaction products. A model system consisting of glyoxylic acid and glycine was utilized due to its ability to generate azomethine ylides. Although these ylides have been implicated as reactive intermediates in the Maillard reaction, their role as precursors of pyrazines remained unexplored. The model system when reacted under pyrolytic conditions at 200 °C generated most of the theoretically expected pyrazines as major products, and the isotope labelling analysis with [^{13}C-1]-, [^{13}C-2]-, and [^{15}N]-glycine confirmed

the hypothesized pathways through the formation of piperazine-2,5-dicarboxylic acid, which can undergo oxidative decarboxylation to generate dihydropyrazine moieties similar to those of the dimerization product of the α-amino carbonyl compounds generated through the Strecker reaction and consequently generate pyrazines. This mechanism differs from the Strecker degradation by leading to the incorporation of carbon atoms of the amino acids into the ring system of pyrazine. Finally, when studying the role of α-keto acids, 3-amino-4,5-dimethyl-2(5H)-furanone, a known precursor of sotolone, was observed for the first time under thermal conditions from two model systems with different precursors, pyruvic acid and glycine and from glyoxylic acid and alanine model systems. Isotope labelling studies have indicated the formation of 3-amino-4,5-dimethyl-2(5H)-furanone from 4,5-dimethylfuran-2,3-dione after its reaction with an amino acid through a variant of the Strecker reaction. The results above suggests that the use of model systems with 1,2-dicarbonyl type precursors serves as an effective approach for unravelling new pathways of formation of aroma related products. Identification of alternate pathways to important aroma precursors in food can provide food processors with flexible procedures of imparting aroma characteristics to food.

RÉSUMÉ

L'interaction entre des groupes carbonyles et amines déclenchent l'une des transformations chimiques les plus importantes dans les aliments, la réaction de Maillard. Cette réaction de brunissement non-enzymatique qui a lieu dans les aliments ainsi que ses sous-réactions font partie d'une matrice de transformations chimiques complexes. Parmi les structures les plus réactives produites par cette réaction, les groupes 1,2-dicarbonyle jouent un rôle particulier dans l'interaction avec les acides aminés par l'entremise de la réaction de Strecker pour générer des α-amino carbonyles, des aldéhydes et des composantes hétérocycliques, plusieurs desquelles ont des propriétés aromatiques. Diverses auteurs ont étudié l'interaction d'un seul acide aminé avec un groupe 1,2-dicarbonyle, cependant, aucune étude systématique a traité de la capacité de ces α-dicarbonyles à subir des réactions d'additions multiples avec des acides aminés, les types de produits générés ou les routes alternatives pour générer les produits de la réaction de Strecker mentionnés. L'identification d'autres voies générant des produits similaires pourrait être considérée comme une méthode utile pour les introduire dans les aliments chauffés tandis que la compréhension des multiples réactions d'addition peut donner un meilleur aperçu des transformations chimiques qui se produisent dans les aliments décrits. De plus, le groupe 1,2-dicarbonyl peut aussi faire partie d'autres structures chimiques telles que les lactones cycliques, les α-cétoacide et les *o*-quinones. Conséquemment, une compréhension globale du potentiel de ces groupes fonctionnels de 1,2-dicarbonyl à participer à des réactions du type Strecker fut également visée. À cet effet, des systèmes modèles adéquats ont été évalués en utilisant la technique des traceurs isotopiques avec différents précurseurs conjointement avec pyrolyse couplée à la chromatographie en phase gazeuse et la spectrométrie de masse ou la chromatographie liquide à haute performance couplée à la spectrométrie de masse des réactions thermiques des systèmes modèles. Initialement, un système modèle composé de glycine et 2,3-butanedione a été étudié sous conditions pyrolytiques à 250 ºC, ainsi que sous des conditions de haute température sous pression à 120 ºC, pour évaluer le potentiel de ces dicarbonyles à subir une oxidation et former des pyrazines. Ces études ont indiqué la formation inattendue de 2,3-diméthylpyrazine et 2,3,5-triméthylpyrazine en plus de la tetraméthylpyrazine attendue. Des études de traceurs isotopique utilisant [^{13}C-1]-, [^{13}C-2]- et [^{15}N]-glycine ont montré l'incorporation de zéro, un ou deux atomes ^{13}C-2 de la glycine dans la portion cyclique de la 2,3-

diméthylpirazine. La formation de 2,3-diméthylpyrazine doublement marquée a été rationalisée par l'entremise de la double addition de glycine à la 2,3-butanedione et la formation d'isotopomères marqués isotopiquement à un seul atome fut justifiée par la formation séquentielle de la base de Schiff de 2-amino-butan-3-one, d'abord avec l'aldéhyde de Strecker et puis suivie par la glycine. Les voies proposées étaient également compatibles avec les modèles de distribution des traceurs isotopiques observés dans la 2,3,4-triméthylpyrazine. La formation du 4,5-diméthyl-1,2-benzoquinone provenant du système modèle de 2,3-butanedione/glycine a également été investiguée. Des études supplémentaires ont été réalisées en remplaçant la glycine avec du glycinate de sodium ou de la glycine hydrochloride en tant qu'acides aminés capables de moduler l'activité catalytique acide et basique du milieu de réaction. L'analyse des données a indiqué que le remplacement de la glycine et ses sels correspondants a promu significativement la formation de 2,3,6,7-tetraméthylquinoxaline par rapport à la tetraméthylpyrazine. L'origine de la 2,3,6,7-tetraméthylquinoxaline a été retracée à la formation de 4,5-diméthyl-1,2-benzoquinone et sa conversion en 4,5-diméthyl-1,2-phénylènediamine par l'entremise de transformations du type Strecker. Suite à ces observations et étant donné que l'anneau-B de catéchines est sensible à l'oxydation et la formation de tels groupements *o*-benzoquinone sous traitement thermique, le potentiel de l'anneau-B des catéchines de participer à des interactions similaires pour former des adduits avec des acides aminés, comme il était observé avec la 4,5-diméthyl-1,2-benzoquinone a été évaluée. À cet effet, la spectrométrie de masse ESI-TOF en haute performance, l'analyse MS/MS et la technique de traceurs isotopiques ont été utilisés pour évaluer la réactivité de la glycine avec la (+)-catéchine chauffée dans des conditions d'oxydation à 120 ^{o}C pendant 70 min. Les modèles d'incorporation des traceurs isotopiques de [^{13}C-1]-, [^{13}C-2]- et [^{15}N]-glycine ont indiqué que la (+)-catéchine a formé divers produits d'addition avec la glycine, deux d'entre eux ont incorporé un seul acide aminé et trois adduits ont incorporé deux groupes fonctionnels d'acides aminés. Les profils de fragmentation de ces produits d'addition ont confirmé l'addition de fragments d'acides aminés à l'anneau B oxydé de (+)-catéchine à travers la formation de bases de Schiff. Une évaluation supplémentaire de la double addition et du principe de formation de double base de Schiff a été réalisée par la réaction du glucose et de deux acides aminés différents avec des chaînes latérales, la glycine et la cystéine. Les deux types des produits, volatils et non-volatils, ont été étudiés. Un adduit non-volatile dont la formation a été observée a également démontré, pour la première fois, que ce principe se produit à travers des réarrangements

d'Amadori successifs, d'abord entre le glucose et la cystéine et ensuite suivi par la glycine. Le groupe fonctionnel dicarbonyle réactif présent dans les structures de α-cétoacide a également été étudié comme précurseurs des produits des réactions de Strecker ou de Maillard. Un système modèle constitué d'acide glyoxylique et de glycine a été utilisé en raison de sa capacité à générer des ylures azométhines. Bien que ces ylures ont été impliqués en tant qu'intermédiaires réactionnels dans la réaction de Maillard, leur rôle de précurseurs des pyrazines demeure inexploré. Lorsque le système modèle réagit dans des conditions de pyrolyse à 200 °C il génère la plupart des pyrazines théoriquement attendues en tant que produits principaux, et l'analyse de marquage isotopique avec la [^{13}C-1]-, [^{13}C-2]-, et [^{15}N]- glycine a confirmé les voies proposées à travers de la formation de pipérazine acide 2,5-dicarboxylique, qui peut subir une décarboxylation oxydative pour produire des groupements de dihydropyrazine similaires à ceux produits par la dimérisation des composés carbonylés α-aminés générés par la réaction de Strecker générant conséquemment des pyrazines. Ce mécanisme diffère de la dégradation de Strecker en conduisant à l'incorporation d'atomes de carbone des acides aminés dans le système d'anneau de pyrazine. Enfin, en étudiant le rôle des α-cétoacides, 3-amino-4,5-diméthyl-2(5H)-furanone, un précurseur connu de sotolone, a été observée pour la première fois à partir des conditions thermiques de deux systèmes modèles avec différents précurseurs, l'acide pyruvique et la glycine, et aussi de l'acide glyoxylique et de l'alanine. Les études de marquage isotopique ont indiqué la formation de 3-amino-4,5-diméthyl-2(5H)-furanone à partir de 4,5-diméthylfurane-2,3-dione après sa réaction avec un acide aminé par l'entremise d'une variante de la réaction de Strecker. L'approche décrite ci-dessus suggère que l'utilisation de systèmes modèles avec des précurseurs de type 1,2-dicarbonyle est un moyen efficace pour établir de nouvelles voies de formation des produits aromatiques. L'identification des voies alternatives de précurseurs d'arômes importants dans l'alimentation peut fournir aux fabricants de produits alimentaires des procédures flexibles pour conférer des caractéristiques d'arôme aux aliments.

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

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CONTRIBUTION OF AUTHORS

This thesis is presented in manuscript format and consists of nine chapters. Chapter 1 introduces the concept of the participation of 1,2-dicarbonyls within the Maillard reaction and the relevance of further understanding the extent to which 1,2-dicarbonyls moieties can take part in the generation of Strecker-type products. Additionally, it presents the rational and objectives of the research and the general methodology required to achieve them. Chapter 2 presents an up-to-date literature review regarding the 1,2-dicarbonyls compounds and their participation in the Maillard reaction and the Strecker degradation, the mechanisms of detection and analysis, as well as an overview of the previously reported Strecker-type interactions. Chapters 3, 4, 5, 6, 7 and 8 are the main body of the thesis and, except for Chapter 6, all are based on published manuscripts. The copyright permissions were obtained from the journal publishers to use the articles content in the present thesis. The content of Chapter 6 will be submitted for publication. A general conclusion and a summary of the contributions to knowledge are presented in Chapter 9. Connecting paragraphs are additional text used to connect the chapters as a bridge that provides a coherent sequence to the manuscripts. This dissertation is in accordance with guidelines for thesis preparation as published by the Graduate and Postdoctoral Studies of McGill University.

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

PUBLICATIONS

Guerra, P. V., & Yaylayan, V. A. (In preparation). Synergistic role of multiple amino acids in the generation of Maillard reaction products from cysteine and glycine.

Guerra, P. V., & Yaylayan, V. A. (2014). Interaction of flavanols with amino acids: Post-oxidative reactivity of B-ring of catechin with glycine. *Journal of Agricultural and Food Chemistry*, *62 (17), 3831-3836.*

Guerra, P. V., & Yaylayan, V. A. (2013). Cyclocondensation of 2,3-butanedione in the presence of amino acids and formation of 4,5-dimethyl-1,2-phenylendiamine. *Food Chemistry*, *141*(4), 4391-4396.

Guerra, P. V., & Yaylayan, V. A. (2012). Double Schiff Base Adducts of 2,3-Butanedione with Glycine: Formation of Pyrazine Rings with the Participation of Amino Acid Carbon Atoms. *Journal of Agricultural and Food Chemistry*, *60*(45), 11440-11445.

Guerra, P. V., & Yaylayan, V. A. (2011). Thermal Generation of 3-Amino-4,5-dimethylfuran-2(5H)-one, the Postulated Precursor of Sotolone, from Amino Acid Model Systems Containing Glyoxylic and Pyruvic Acids. *Journal of Agricultural and Food Chemistry*, *59*, 4699-4704.

Guerra, P. V., & Yaylayan, V. A. (2010). Dimerization of Azomethine Ylides: An Alternate Route to Pyrazine Formation in the Maillard Reaction. *Journal of Agricultural and Food Chemistry*, *58*(23), 12523-12529.

CONFERENCE ORAL PRESENTATION

Guerra, P. and Yaylayan, V. (2013) Application of mass spectrometry and isotope labelling technique to the characterization of thermally generated compounds from targeted precursors. Presented at the 3rd MS Food Days, held in Trento, Italy, October 9-11, 2013.

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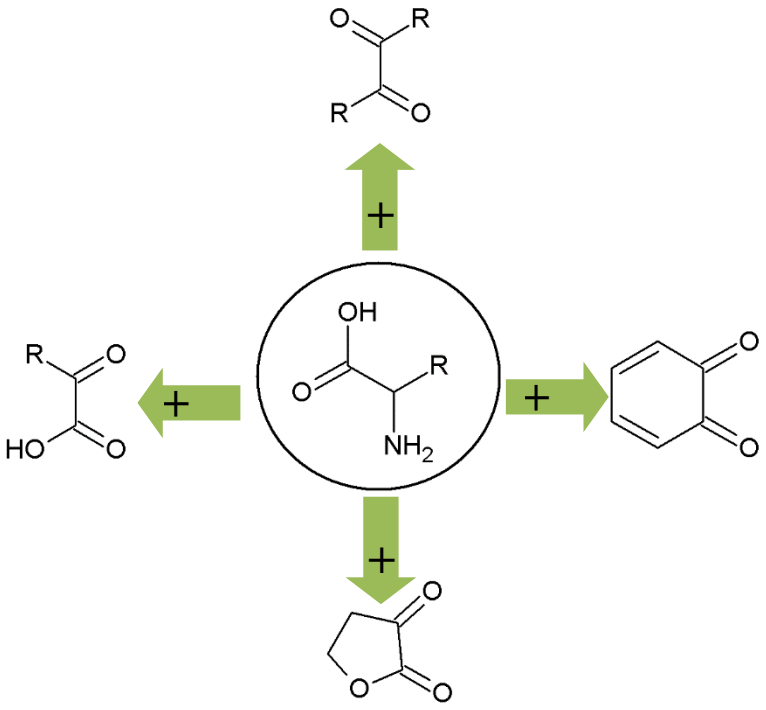
LIST OF ABBREVIATIONS

AGEs	Advance glycation end products
amu	Atomic mass unit
ESI	Electrospray ionization
GC	Gas chromatography
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
LC	Liquid chromatography
MRPs	Maillard reaction products
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Py	Pyrolysis
t_R	Retention time
TOF	Time of flight

CHAPTER 1. INTRODUCTION



1.1 General introduction

The 1,2-dicarbonyl moiety is one of the earliest identified and most reactive structures capable of converting amino acids into aroma-active heterocyclic compounds through various chemical transformations including the Strecker reaction. This sub-reaction of the Maillard reaction is well studied for the interaction of a single amino acid to the 1,2-dicarbonyl moiety to generate Strecker aldehydes, pyrazines, as well as α-amino carbonyls that are considered precursors of many heterocyclic compounds including the aforementioned pyrazines (Rizzi, 2008; Yaylayan & Haffenden, 2003a). Although the interaction of a single amino acid to the 1,2-dicarbonyl difunctional moiety is well studied as a part of the Strecker reaction, no systematic studies have been reported on the ability of the 1,2-dicarbonyls to undergo multiple addition reactions and the type of products generated. In addition, no systematic studies have been conducted on the alternative routes to the generation of Strecker reaction products such as pyrazines, amino-carbonyl or the so-called Strecker aldehyde. In 1998, Shu observed the formation of pyrazines from a model system lacking 1,2-dicarbonyl compounds. He proposed that pyrazines and aroma-active aldehydes could also be formed from the interaction of amino acids and acetoin, an α-hydroxy carbonyl compound, emphasizing that both products can be formed by a pathway different from standard Strecker degradation. Later, Hofmann and Schieberle (2000) also showed an alternative route to form Strecker aldehydes, through the oxidative degradation of Amadori compounds in the absence of α-dicarbonyls, and in 2008, Rizzi reported the reaction of amino acids with α,β-unsaturated carbonyl compounds that produced Strecker aldehydes and related transamination products, such as α-amino carbonyls. Nevertheless, due to the importance of such products, a more detailed investigation to identify alternate routes to their formation is required. Furthermore, since the reactive α-dicarbonyl moiety can also be found as part of other structures such as cyclic lactones, keto-acids and *o*-quinone, such potentially reactive precursors could play a significant role in understanding the scope of this reaction and in enhancing our ability to control the formation of specific products.

1.2 Rational and Research Objectives

In the past, studies with model systems have generated a wealth of information regarding the role of individual amino acids and carbohydrates within the Maillard reaction and its sub-reactions, demonstrating how certain reactants can give rise to specific compounds, which has served as the foundation for the current knowledge of the reaction. However, more recently some studies have demonstrated the existence of alternative routes of reaction for the formation of known Maillard reaction products. Hence, a detailed understanding of the potential of different 1,2-dicarbonyl moieties to participate in Strecker-type reactions can significantly contribute to the successful development of desirable food products with, for example the desired aroma composition.

1.2.1. General objective

The main goal of this work is to identify new pathways that could generate Strecker degradation products, such as aroma-active aldehydes, α-amino carbonyls or pyrazines, through the use of Maillard reaction model systems, constituted of selected reactive intermediates containing 1,2-dicarbonyl moieties within their structure, and isotope labelling technique in conjunction with pyrolysis gas chromatography mass spectrometry (Py-GC/MS) and high resolution liquid chromatography mass spectrometry (HRLC-MS).

1.2.2. Specific objectives

1. Study the potential of a simple α-dicarbonyl compound, 2,3-butendione, to undergo double-addition reaction pathway with amino acids and form pyrazines, as an alternative route to the Strecker degradation.
2. Explore the ability of 2,3-butendione to form *o*-quinones when in the presence of amino acids, as well as study the reactions that this newly formed chemical with an α-dicarbonyl moiety within its structure can undergo with amino acids.
3. Evaluate the extent of the *o*-quinones interaction with amino acids, by studying the potential of ring-B of catechins to undergo similar interactions and form adducts with amino acids.
4. Verify the extension of the double addition reaction pathways by evaluating the volatile and non-volatile products of a model system of a single sugar and two different amino acids, as

an attempt to develop a more accurate understanding of the interactions of the amino acids within real food models.

5. Study the potential of keto acids, as α-dicarbonyl moieties, as precursors of Strecker or Maillard reaction products, by the evaluation of a model system of glyoxylic acid/glycine that is unable to generate α-dicarbonyl compounds.
6. Evaluate the role of keto acids in the formation of a lactone, a chemical with α-dicarbonyl moiety in their structure, and further reactions to generate an aroma chemical precursor.

1.3 General Experimental Approach

Isotope labelling studies with the aid of Py-GC/MS or HRLC-MS were used to study volatile and non-volatile products generated from various model systems. These techniques combined together can serve to trace back selected atoms of a reaction product to their origin in the starting material, if appropriate labelled precursors are available or could be synthesized. In particular isotope labelling analysis of the samples by Py-GC/MS represents an advantage over other similar techniques as an integrated reaction, separation and identification system, where the pyrolysis interface serves as a micro-reactor. Hence, it is possible to introduce expensive isotopically labelled compounds into the reaction mixture for mechanistic studies, without being limited by the number of analysis, since these analyses require only sub-milligram quantities of the expensive labelled precursors, thus reducing drastically the cost of such experiments (Yaylayan & Keyhani, 2000). Pyrolytic reactions are also known to mimic chemical processes happening during roasting or baking through the Maillard reaction and its sub-reactions. Even though the reactions carried out under pyrolytic conditions can generate higher number of products, however, when they are compared to traditional reactions in aqueous systems, similar products can also be found (Yaylayan et al., 2000).

1.4 Significance of the Proposed Research

Although 1,2-dicarbonyls are well known reactants involved within the Maillard reaction and its sub-reaction the Strecker degradation, there is a lack of systematic studies reporting their ability to undergo double addition reactions, or their involvement in alternative pathways for the

generation of Strecker degradation products, such as pyrazines, aroma-active aldehydes and 1,2-amino carbonyls. Additionally, there is a gap in the literature regarding the involvement of 1,2-dicarbonyl compounds within other structures, such as 2-keto acids and 2-keto-lactones, in the Strecker type reactions. A deeper understanding of the behaviour of various derivatives of α-dicarbonyl moieties and discovery of alternative pathways of their reactions provide an additional hold to control the formation of some flavor compounds, such as sotolone and some pyrazines, as well as a clue on how antioxidant properties of *o*-quinonoid moieties could be modified by amino acids.

CHAPTER 2. LITERATURE REVIEW

2.1 General introduction

Food systems are composed of carbohydrates, lipids, proteins, minerals, vitamins and various other minor components. This natural composition becomes even more complex during thermal processing and storage due to the occurrence of chemical transformations such as the Maillard reaction. The Maillard reaction is a non-enzymatic chain of reactions initiated with the interaction of amino groups with carbonyl compounds and terminate with the formation of brown polymers called melanoidins, in addition to aroma and taste active compounds, antioxidants and thermally generated toxins (Finot, Aeschbacher, Hurrell & Liardon, 1990; Ledl & Schleicher, 1990). It was named after Luis-Camille Maillard, who first reported this sugar-amino acid reaction in 1912 (Kawamura, 1983). Although the reaction was first observed in food systems (Ling, 1908), its role in biology and medicine has been also of particular importance due to its influence on aging, neurodegeneration, atherosclerosis and diabetes complications, among others (Reddy & Beyaz, 2006; Stitt, 2005). One of its most important sub-reactions is the Strecker degradation, which is responsible for the formation of key aroma-active aldehydes and pyrazines in foods from the interaction of a α-dicarbonyl compound with an amino acid (Nursten, 2005). Although both reactions, Maillard and Strecker, have been widely studied, the scope of the research on the reaction mechanism has been mainly focused on the interaction of a single amino acid with a sugar or its reactive intermediates and on the participation of α-dicarbonyl compounds. However, the role of many derivatives of α-dicarbonyl moiety such as those constituting as part of aromatic rings, or lactone or carboxylic acid has been neglected, as well as the effect of multiple addition of amino acids on the formation pathways of important Maillard and Strecker reaction products. Due to the relevance of the Maillard reaction and the complexity of its numerous routes, the identification of such related pathways could provide a better insight of the interactions occurring among its different intermediates and precursors and provide more alternatives to produce foods with desired characteristics.

2.2 General reaction pathways of the Maillard reaction

The Maillard reaction is a non-enzymatic chain reaction initiated with the formation of a Schiff base adduct between a carbonyl compound and the free amino group of an amino acid, peptide or

protein. After rearrangement, the Schiff base can form either an Amadori product, if the carbonyl group is an aldose or a Heyn's product if the reactant is a ketose. This however, is only the *initial stage* of the reaction. With the formation of reactive intermediates, the Maillard reaction evolves into a complex matrix of interactions, between the initial reactants and the reactive intermediates known as *intermediate* and *final stages* (Nursten, 2005). In the *intermediate stage* primary products are formed through sugar fragmentation and dehydration, as well as amino acid degradation. These are considered the building blocks of the Maillard reaction. In the *final stage,* reactions like aldol condensation and aldehyde-amine reactions predominate, and in particular the latter reaction is known by its involvement in the formation of highly coloured products (Nursten, 2005). In consequence, the Maillard reaction products can be the result of the degradation of amino acids, or fragmentation and/or degradation of sugars and Amadori or Heyn's products as well as the interaction between them (Nursten, 1981; Yaylayan, 1997). However, these three stages of the Maillard reaction mentioned above represent a simplified overview of the reaction. In real food systems, there are components other than sugars and amino acids or their degradation or interaction products, such as lipids, which can also affect the Maillard reaction, either by promoting or hindering the normal pathways and possibly forming new compounds. Therefore, the use of appropriate model systems that will mimic the chemical interactions occurring within foods, limiting the type and the extent of interactions happening, is a crucial step to identify the relevance of each reactive intermediate in the formation of a final product.

2.2.1. Thermal degradation products of amino acids within the Maillard Reaction

Amino acids can be classified by the structure of their side chains, depending if they are aliphatic, or aromatic or contain hydroxyl groups, sulphur atoms, acidic groups or their amines or basic groups, among others. Generally, the side-chain of amino acids is incorporated into the Maillard reaction products (from now on MRPs) in the early stages of the reaction. In consequence, numerous reaction products, specific to the amino acids are generated in food systems. One of the most side chain-dependants MRPs formed is Strecker aldehyde, which will be further described later. The chain elongation reaction of compounds containing terminal aldehydes also results into amino acid specific MRPs (Yaylayan & Keyhani, 2001). In this process the side chain of the amino acid is attached to the aldehyde moiety converting it into a

ketone. Other Maillard reaction intermediates with a structure specific to the parent amino acid are the α-keto-acids. These compounds can be generated through transamination reaction. However, oxidative decarboxylation after deamination of amino acids has also been described as another route for the formation of α-keto acids (Rössnes, Velíšek & Davídek, 2001).

2.2.2. Carbohydrates and their role in the Maillard reaction

Carbohydrates present in foods are mainly classified as mono, di or polysaccharides. The most common monosaccharides in foods are glucose, fructose and galactose. The degradation of these carbohydrates plays a crucial role within the Maillard reaction, providing the α-dicarbonyl compounds and carbon skeletons for various precursors, through the sugar fragmentation reactions during the first stages of the Maillard reaction. The main reactions to which carbohydrates are subjected under thermal conditions are carbon skeleton cleavages (such as retro-aldolization, β-dicarbonyl cleavage and α-dicarbonyl cleavage), which result in the formation of sugar fragments, isomerisation and cyclization products (Hollnagel & Kroh, 1998; Paine III, Pithawalla & Naworal, 2008; Yaylayan et al., 2000). Among all sugar fragments, α-dicarbonyls are certainly the most important reactive intermediates in food systems and are necessary for the formation of flavor compounds, especially heterocycles and their difunctional capacity can help in the study of the amino acid side chain incorporation into MRPs (Hollnagel et al., 1998).

2.3 Initial products of the Maillard reaction

The initial interaction of a free amine with a carbonyl containing compounds through a condensation reaction leads to the formation of a Schiff base. This Schiff base can cyclize into a glycosylamine, which under acidic conditions can rearrange into 1-amino-1-deoxy-2-ketose, a step known as the Amadori rearrangement (Nursten, 2005). Alternatively, under low moisture conditions it has been observed that the Schiff base can cyclize into a 5-oxazolidinone intermediate, which decarboxylates into a non-stabilized azomethine ylide (Chu & Yaylayan, 2009a; Tsuge, 1987) as shown in Figure 1. Chu and Yaylayan (2009a) also confirmed the participation of the 5-oxazolidinone as an intermediate in the Amadori rearrangement. In this case, instead of undergoing decarboxylation, the ring opens through a proton shift of the C-2

hydrogen atom of the carbonyl moiety (sugar), followed by Amadori rearrangement. In general, Amadori rearrangement products undergo similar reactions to that of reducing sugars and amino acids, such as nucleophilic additions and base-catalyze enolization, but under milder reaction conditions. The enolization reactions of Amadori compounds followed by β-elimination produces 3-deoxy-2-hexosuloses through 1,2-enolization, and 1-amino-1,4-dideoxy-2,3-hexodiulose as well as 1-deoxy-2,3-hexodiulose through 2,3-enolization (Yaylayan & Huyghues-Despointes, 1994). These 1,2-dicarbonyls can be seen as intramolecular disproportionation products of sugars and as such can undergo enolization, cyclization and loss of water and form smaller carbonyl and heterocyclic compounds (Ledl et al., 1990).

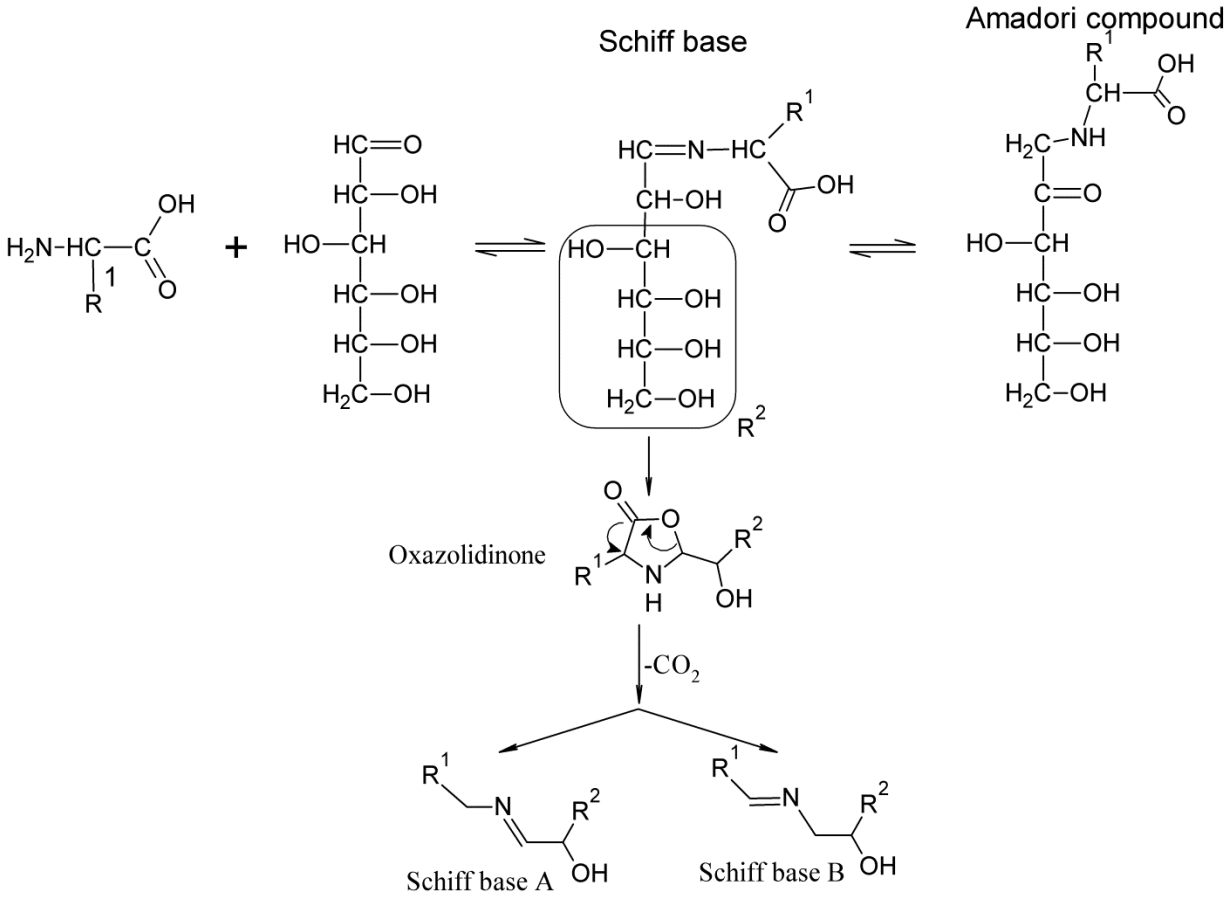


Figure 1. Initial reactions between an amino acid and a reducing sugar under Maillard reaction conditions

2.3.1. Mechanisms of formation of α-dicarbonyls compounds through retro-aldolization

The chemical formation of short chain α-dicarbonyl compounds under Maillard reaction conditions could result from the sugar degradation directly or its derivatives, such as the Amadori products. The cleavage occurs through retroaldolization favored at pH above 7 which can be followed by oxidation or dehydration steps (Ledl et al., 1990; Shibamoto, 2014; Yaylayan et al., 1994). This leads to the formation of α-hydroxycarbonyls and α-dicarbonyls of 2, 3 and 4 carbon, among others, which are more reactive than the parent compounds (Cämmerer, Wedzicha & Kroh, 1999). Among the α-dicarbonyls formed, 2,3-butendione or diacetyl has particular importance in food research, mainly because of its presence in many types of foods, such as cheese (Barbieri et al., 1994; Zeppa, Conterno & Gerbi, 2001), wine and beer (Bartowsky & Henschke, 2004; Romano & Suzzi, 1996), although in this cases is mainly produced by the action of yeast and lactic acid bacteria. It has been a common flavor chemical used by flavorists to provide butter and roast notes and specially in the formulation of margarine flavoring (Bauer, Garbe & Surburg, 2001). Its desirability in foods is dependent on the specific product, in the case of cheeses it is desirable and its formation process is exploited, but in the case of wine and beer it could actually be considered undesirable, especially at high concentrations. Additionally, diacetyl has not only been reported as a mutagenic agent, but more recently it has been associated with bronchiolitis obliterans after high exposure to diacetyl vapor (Sauler & Gulati, 2012), chronic lung disease (Sauler et al., 2012) and even Alzheimer (More, Vartak & Vince, 2012). Diacetyl is chemically formed by the C_2/C_4 cleavage (keto-enol bonding cleavage) of glucose or fructose after tautomerization (Shibamoto, 2014) or by chain elongation reaction of smaller α-dicarbonyl compounds and an amino acid (Yaylayan & Keyhani, 1999).

2.3.2. Azomethine ylides as important reactive intermediates in the Maillard reaction

Azomethine ylides are considered as 1,3-dipoles and have been identified as intermediates in the formation of pyrroles through the cycloaddition reactions (Carruthers & Coldham, 2004). Azomethine ylides can be classified into stable and unstable forms. The difference arises from the substitutions of the central nitrogen atom, if it is an hydrogen, the ylide is unstable, whereas stable ylides have an alkyl group (Padwa, 2002). It has been observed that generation of unstable azomethine ylides can occur through decarboxylative condensation of α-amino acids with carbonyl compounds and it was presumed that 5-oxazolidinones are intermediates in this reaction

(Tsuge, 1987). Thanks to the studies developed to understand the mechanism of formation of acrylamide, a thermally generated toxicant, azomethine ylides have been also recognized as intermediates in the Maillard reaction, as shown in Figure 2 first proposed by Yaylayan, Wnorowski & Perez Locas (2003b) and later by various other groups (Blank et al., 2005; Perez Locas & Yaylayan, 2008; Schieberle, Köhler & Granvogl, 2005; Yaylayan, Perez Locas, Wnorowski & O'Brien, 2005a; Yaylayan, 2009; Yaylayan, Wnorowski & Perez Locas, 2003b). Additionally, azomethine ylides are known to undergo dimerization and lead to the formation of piperazine moieties (Freeman & Govindarajoo, 1995; Tsuge & Kanemasa, 1989). The participation of these ylides in the Maillard reaction may confirm the presence of an alternative pathway in which Schiff bases undergo cyclization to oxazolidin-5-one before undergoing rearrangement into an Amadori product. The oxazolidin-5-ones can easily decarboxylate to form the azomethine ylide (stabilized by resonance) (Blank et al., 2005; Yaylayan et al., 2003b). Another example of the formation of azomethine ylide in the presence of the Maillard reaction intermediates was presented by Joucla and coworkers (1988). They showed a mechanism for the formation of azomethine ylides from formaldehyde (Strecker aldehyde of glycine) and various amino acids.

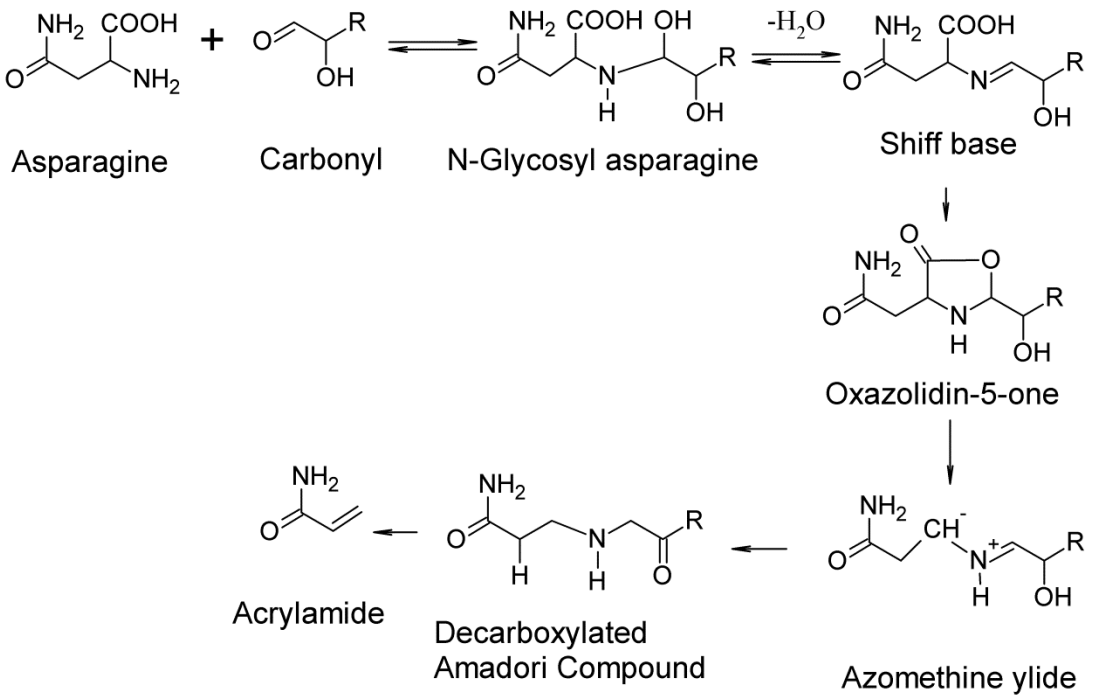


Figure 2. Formation of acrylamide from asparagine in the presence of α-hydroxycarbonyls by the azomethine ylide pathway (Blank et al., 2005).

2.4 Reactions of α-dicarbonyl compounds under Maillard reaction conditions

1,2-Dicarbonyls are involved in many reactions when in the presence of amino acids. Among them, aldol condensation, cross-linking and Strecker reaction, which are further explained in the following sections.

2.4.1. Aldol condensation of α-dicarbonyls

Different carbonyl compounds are formed under Maillard reaction conditions which can undergo aldol condensations. Among them, aldol condensation of aldehydes has been widely studied, and special attention has been placed in the development of browning reactions from aldehydes alone, such as furfural, or with the methylene group of other chemicals, such as furanone (Hodge, 1953; Nursten, 2005). It has been demonstrated that amino compounds, in particular amine salts, serve as catalysts for the aldol condensation of acetaldehyde, hence Maillard reaction conditions should be the ideal medium for this reaction to occur. There are only a few reports in the literature that focus on the aldol condensation of α-dicarbonyl compounds. Diacetyl has been observed to undergo aldol condensation and generate 2,5-dimethyl-*p*-benzoquinone (Hodge, 1953) and other authors have proposed the formation of 4,5-dimethyl-1,2-benzoquinone as a possible intermediate in their observations (Piloty & Baltes, 1979; Reese & Baltes, 1992). Even though all models included both the diacetyl and an amino acid, none of the authors have emphasised the need of the amino acid as a catalyst in this reaction. Additionally, the formation of 4,5-dimethyl-1,2-benzoquinone or its mechanism of formation has not been formally studied.

2.4.2. Involvement of α-dicarbonyl compounds in protein cross-linking under Maillard reaction conditions

Cross-linking of proteins consists in the formation of covalent bonds between polypeptide chains. It could be intramolecular, which means the bonds are within a protein, or intermolecular, in the case where the covalent bonds are between different proteins (Gerrard & Cottam, 2012). The occurrence of cross-linking among food proteins could affect their functional and/or their nutritional properties, due to structural changes. Cross-linking by Maillard reaction is known because of its participation in the formation of advance glycation end products (AGEs) or advance Maillard reaction products, which are adducts formed between reducing sugars or their

degradation products such as dicarbonyls and the side chains of proteins, specifically lysine and arginine residues. AGEs can be formed *in vivo* in the human body, or in thermally treated foods, which are also referred as dietary AGEs. They have been of particular interest in research, due to their correlation with biological disorders such as diabetes or uremia (Henle, 2003; Henle, 2005; Nagaraj, Shipanova & Faust, 1996b). Additionally, protein cross-linking has been recognized as having a profound effect on food texture. A study of the effect of three different aldehydes, of proven capacity to crosslink with a model protein (Gerrard, Brown & Fayle, 2002) such as wheat proteins shows how the presence of glutaraldehyde strengthens the gluten network in bread resulting in lower ability of the gas cells to expand, but had no perceivable effect on the properties of croissants (Gerrard, Brown & Fayle, 2003a; Gerrard, Brown & Fayle, 2003b). A similar study was also developed for soy-based foods (Gerrard et al., 2005). The α-dicarbonyls have also been identified as relevant intermediates in the cross-linking of proteins during Maillard reaction, although the research focus has mainly been oriented towards their participation *in vivo* cross-linking (Lederer & Klaiber, 1999). A comparative study of the cross-linking ability of different types of α-dicarbonyls with a model protein ribonuclease A looked at the relationship between structure and reactivity (Meade, Miller & Gerrard, 2003). Despite the inability to develop a general prediction due to the complexity of the structure-activity relationships, the researchers were able to identify a higher efficiency of methylglyoxal when compared to glyoxal or diacetyl, mainly due to the reactivity of the aldehyde group versus keto groups and steric factors. Regardless of all the knowledge developed during the years about the occurrence of cross-linking by Maillard reaction, the extent of the reaction and the exact structures of the products of cross-linking with α-dicarbonyls are still not fully understood, especially with regards to the thermally generated cross-links in foods. However, recent studies of multiple addition reactions of amino acids with α-dicarbonyls opened the door to a better understanding of the formation of cross-linked structures of this type occurring in thermally treated foods (Chu, Sleno & Yaylayan, 2011).

2.4.3. Relevance of the Strecker degradation to the Maillard reaction

The 1,2-dicarbonyl compounds are the most reactive intermediates formed in the Maillard reaction from various reducing sugars or even from fatty acids (Gobert & Glomb, 2009). The 1,2-dicarbonyl moieties can also be found as a part of the aromatic structure of different

compounds such as *o*-quinones (Rizzi, 2008). The commonly accepted interaction of amino acids with α-dicarbonyl compounds is generally referred to as the Strecker degradation (Rizzi, 2008) which represents one of the most recognizable reactions in food chemistry and a sub-reaction in the complex matrix of the Maillard reaction. It was first reported by Strecker in 1862 (Strecker, 1862), but it was only until the 1950's when this reaction was linked to food flavor formation, particularly in dairy products (Keeney & Day, 1957; Patton, 1954). The reaction involves the oxidation of α-amino acids and the transfer of the amino group into the α-dicarbonyl compound accompanied by the release of carbon dioxide. Its reaction pathway consists in the formation of α-amino carbonyls, carbon dioxide and the corresponding Strecker aldehyde through the interaction of an amino acid with 1,2-dicarbonyl compounds as shown in Figure 3 (Nursten, 2005; Rizzi, 2008).

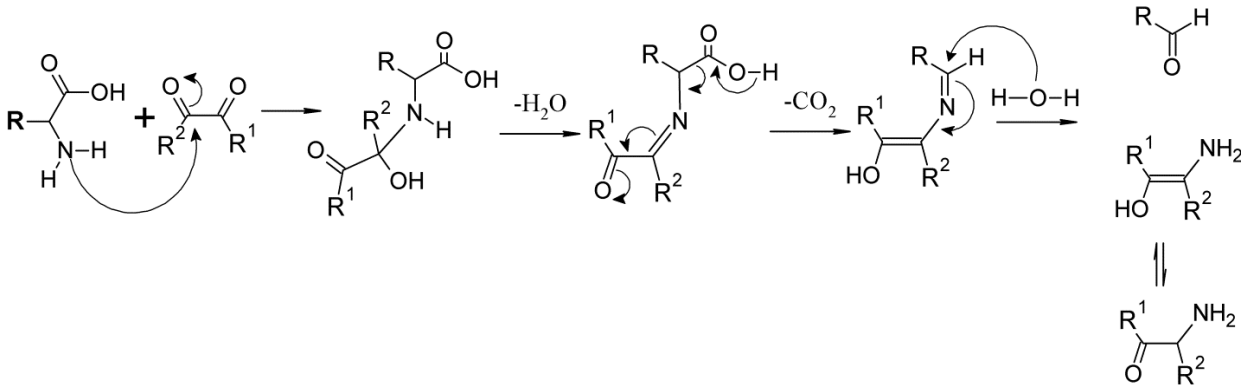


Figure 3. Strecker aldehyde formation in the presence of a carbohydrate source – Strecker degradation reaction.

The Strecker aldehydes are key aroma compounds that have been observed, both under the presence and in the absence of a carbohydrate source (carbonyl groups). In the absence of carbohydrate, the mechanism of formation of the Strecker aldehydes proceeds through the oxidative decarboxylation of the amino acid (Yaylayan et al., 2001), as shown in Figure 4.

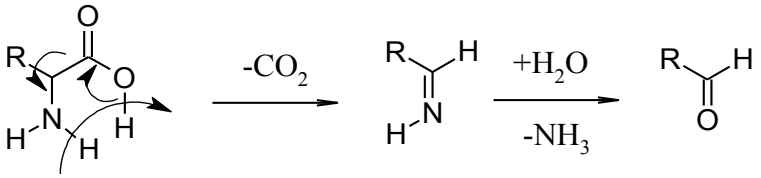


Figure 4. Strecker aldehyde formation in the absence of a carbohydrate source (Yaylayan et al., 2001).

Dimerization of α-amino carbonyl compounds formed during the Strecker reaction constitutes the major known mechanism for the formation of pyrazines during the Maillard reaction. The intermediate dihydropyrazine can either get oxidized or incorporate an aldehyde which results in pyrazines formation. Among all the aroma compounds formed during the Maillard reaction, pyrazines have been identified as the main contributors to roasted aromas in diverse foods and are one of the few classes of compounds that are mainly associated with desirable aromas (Maga & Katz, 1982; Maga & Sizer, 1973). Since the type of amino acid will define the pyrazines formed, the aroma characteristic of the products of the thermal reaction of a specific amino acid will be defined by its side chain.

2.4.3.1. Pyrazines and their relevance in food

The Maillard reaction in food is not only known for its potential of forming brown colored polymers, but also for its participation in the generation of many character impact aroma chemicals of many thermally processed foods. Flavour chemicals from the Maillard reaction have been classified according to their precursors into three groups (Nursten, 1981), the ones resulting directly from the "simple" degradation of sugars, from amino acids or from their interaction. Pyrazines fall into the third group, because the main mechanism responsible for their formation proceeds through the Strecker degradation which involves the reaction between an amino acid and a degradation product of sugars or Amadori compounds. Although pyrazines were first isolated in 1879 in beet sugar molasses, it was only until the 60s that they were widely investigated and reported (Mottram, 2007). They have been found in many thermally treated foods, from roasted cereals and nuts, to coffee, cocoa, potato and beef among others (Maga et al., 1973), as well as naturally formed in some vegetables such as bell peppers and asparagus (Murray & Whitfield, 1975). Their concentration vary between 0.001 and 40 ppm (Müller & Rappert, 2010) with recognition thresholds as low as under 1ppm (Maga et al., 1982) and

detection thresholds in water as low as 0.00001 ppm (Shibamoto, 1986). Although their main aroma descriptor is roasted and nutty, some even provide floral or green notes (Maga et al., 1982).

2.4.4. Strecker-type interactions

Reactions that can produce α-amino carbonyls, aroma-active aldehydes or pyrazines from the interaction of sugars, amino acids, Amadori or Heyn's products or any of their degradation or interaction products, but in the absence of at least one of the traditional Strecker precursors can be classified as a Strecker-type interaction. In 1998, Shu observed the formation of pyrazines from a model system lacking of 1,2-dicarbonyls. He proposed that pyrazines and aroma-active aldehydes could also result from the interaction of amino acids and acetoin, emphasizing that both products can be formed by a pathway different from standard Strecker degradation (Shu, 1998). Hofmann and Schieberle (2000) also showed an alternative route to the formation of Strecker aldehydes through the oxidative degradation of Amadori compounds without α-dicarbonyls present. Alternatively, α,β-unsaturated carbonyls and quinones have also been reported to participate in Strecker-type reactions (Rizzi, 2008).

2.5 Role of flavanols in the Maillard reaction

Flavanols are present in many food products and are particularly recognized for their antioxidant activity. Catechin, epicatechin and epigallocatechin are some of the chemicals that belong to this group. Their presence in foods is associated with fruits and vegetables, but tea and wine have shown particularly higher concentrations (Arts, van de Putte & Hollman, 2000; Pietta, 2000). In food and in Maillard model systems, epicatechin and related phenolic compounds have been shown to cause a reduction in the intensity of some aroma compounds (Colahan-Sederstrom & Peterson, 2004; Totlani & Peterson, 2005; Totlani & Peterson, 2006) by acting as trapping agents for key Maillard reaction intermediates such as 3-deoxy-2-hexosulose. In some other cases, like in beer, antioxidants provide protection against the development of undesirable aroma notes, such as the "papery" note imparted by the presence of *trans*-2-nonenal which results from the oxidation of linoleic acid (Maillard, Soum, Boivin & Berset, 1996). These results allowed to identify a way of controlling the antioxidant activity of beer, and consequently the formation of

undesirable aroma chemicals, by selection of appropriate barley varieties due to their differences in total phenolic content and controlling the malting stage due to increased antioxidant activity during malting as a result of formation of Maillard reaction products. In this case both flavanols and MRPs together provide the total antioxidant activity of malt. The interaction of MRPs with catechins have also been studied to determine their role in food color change (Es-Safi, Cheynier & Moutounet, 2000). The fate of furfural compounds normally used to evaluate non-enzymatic browning was evaluated in the presence of (+)-catechins. The results indicated the formation of adducts between (+)-catechin and both furfural and 5-(hydroxymethyl)furfural. The bond among both structures was proposed to occur in ring A of (+)-catechin (Es-Safi et al., 2000). Further studies with Maillard reaction model systems have also indicated the interaction of sugar fragments with ring A of epicatechin (Totlani et al., 2005). Additionally, studies of the participation of flavanols in the inhibition of AGE have been performed which revealed that catechin does not seem to have significant effect in inhibiting AGEs from methylglyoxal and proteins (Wu & Yen, 2005).

2.6 Analytical techniques for mechanistic studies of the Maillard reaction

Due to the relevance of the Maillard reaction to the formulation of high quality processed foods, the identification of alternative pathways taking place under Maillard reaction conditions can provide additional approaches to produce foods with desirable characteristics. There are many strategies that can be used to identify mechanisms of reaction, such as the identification of the products, intermediates and catalysts, isotopic labelling and kinetic studies (Smith & March, 2006). Usually, more than one strategy is needed to identify the mechanism, since many reactions can proceed by different mechanisms under the same conditions.

2.6.1. Importance of model system approach for the study of the Maillard reaction

Most of the current knowledge regarding the Maillard reaction has been generated through the use of model systems. They are used to mimic the chemistry of foods by simplifying the complexity of the reaction and to study systematically the influence of different sugars, amino acids and other food matrix components on the final outcome of the reaction (Fayle, Gerrard & Belton, 2002). The reliability of model systems as a tool to study foods is still under debate due

to the fact that such models can oversimplify the real foods and overlook factors that might occur only in more complex systems. In Maillard model systems, the use of amino acids with smaller substituents, such as glycine and alanine reduces the complexity of the reactions taking place while enabling the researcher to identify the important mechanistic details. Additionally, as a general rule the smaller the substituents, the lesser is the steric hindrance, and higher the feasibility of the reactions to occur, hence a broader overview of the possible reactions to take place can be achieved. On the other hand, using amino acids with side chains containing sulphur atoms serves as a marker when using certain techniques of analysis such as high resolution mass spectrometry. In the case of the source of carbonyl groups, glucose is the most commonly used in standard model systems. This monosaccharide is the most abundant in food systems. In addition to sugar and amino acids, reactive intermediates from amino acids, sugar degradations or their interaction products, such as Strecker aldehydes, 1,2-dicarbonyl compounds and 2-keto-acids can be useful to obtain a clearer insight into the details of the reaction mechanism. The use of reactive intermediates can serve two purposes, to confirm a mechanism by the addition of a suspected intermediate, or as starting material in the study of a sub-reaction of the Maillard reaction, such as the Strecker reaction.

2.6.2. Applications and limitations of pyrolysis in the study of the Maillard reaction

The term pyrolysis usually refers to a reaction that occurs purely by thermal energy at high temperatures in the absence of air and solvent, causing decomposition and elimination reactions that generally result in the formation of smaller molecules through the free radical reactions that start by the bond breaking in the molecules (Sobeih, Baron & Gonzalez-Rodriguez, 2008). Pyrolysis is usually performed at temperatures above 250°C, and it is classified according to the temperature range into *mild pyrolysis,* when it is carried out between 300-500°C, *common pyrolysis* for reactions carried out between 500 and 800°C, and those carried out above 800°C are classified as *vigorous pyrolysis*. Although reactions that occur at temperatures below 250°C are usually classified as simple thermal reactions, when in the absence of air, it is still considered *pyrolysis* (Huyghues-Despointes, Yaylayan & Keyhani, 1994). In food research, pyrolysis has traditionally been used for fast characterization of complex molecules such as lipids, proteins and polysaccharides (Challinor, 1996; Raghavan, Ho & Daun, 1986). Pyrolysis coupled to gas chromatography/mass spectrometry (Py-GC/MS) at temperatures between 150 and 350ºC has

been used to study Maillard reaction products and their mechanisms of formation (Huyghues-Despointes et al., 1994; Nikolov & Yaylayan, 2012; Yaylayan et al., 2003b). At these temperatures, Maillard reaction precursors are usually able to melt which allows them to interact in the absence of a solvent. Additionally, pyrolytic reactions occurring at around 200^{o}C are known to mimic the events happening during roasting or baking processes through the Maillard reaction (Wnorowski & Yaylayan, 2000). The technique of Py-GC/MS serves as an integrated reaction, separation and identification system that allows the study of a reaction as complex as the Maillard reaction by exposing small quantities of different reactants to a set of temperatures in a model system under controlled conditions. Hence, it is possible to introduce isotopically labelled compounds into the reaction mixture for mechanistic studies, because such analyses requires only sub-milligram quantities of the expensive labelled precursors, thus reducing drastically the cost of such experiments (Yaylayan et al., 2000). Therefore, all the atoms of a reaction product can be traced back to their origin in the starting material if appropriate labelled precursors are used. This technique however limits the study only to the volatiles formed from reactions in the absence of oxygen and solvent. Studying the Maillard reaction in the presence of oxygen or water, as well as the study of both volatile and non-volatile products provides additional information regarding the behaviour of compounds in real foods. It has been demonstrated that reactions carried out under pyrolytic conditions can generate a much higher number of products when compared to identical reactions in aqueous systems, nevertheless similar products can be found following comparable mechanistic pathways (Yaylayan et al., 2000). On the other hand, oxidation has been identified as a determining stage in the formation of final Maillard reaction products, such as the formation of different aroma notes than the non-oxidized counterpart (Tai & Ho, 1997). Alternative Py-GC/MS-based methodologies have been developed and reported to overcome these limitations by the use of post-pyrolytic derivatization to study the non-volatile residues and by having a sample pre-concentration trap that allows the study of samples thermally treated under air or in the presence of moisture but with the advantages of using Py-GC/MS system (Chu & Yaylayan, 2008a; Yaylayan, Haffenden, Chu & Wnorowski, 2005b).

2.6.3. The use of gas or liquid chromatography coupled with mass spectrometry to the study of Maillard reaction products

Mass spectrometry has been the key technique in the study of food MRPs, both with or without application of chromatography (Fay & Brevard, 2005). In particular, gas chromatography coupled to mass spectrometry has been widely used in the separation and identification of Maillard reaction products, especially in the study of flavor compounds, and in mechanistic studies, due to the availability of large mass spectral libraries. The use of GC-MS has allowed the generation of substantial amount of literature particularly in the area of volatile chemicals or low and medium molecular weight products. Although some non-volatile products can also be identified through GC-MS, they usually need a derivatization step. In this case, LC-MS or mass spectrometry without the separation stage is mostly used. Although GC-MS has been previously used to study both volatile and non-volatile Maillard products generated by pyrolysis (Yaylayan et al., 2005b), the non-volatile portion was evaluated through the derivatization with hexamethyldisilazane of the pyrolyzed residue of the reaction mixture. Additionally, Xu and colleagues (2013) studied the volatile and non-volatile compounds from the Maillard reaction of peptide, cysteine, thiamine and xylose. This study demonstrated the potential of using LC-MS/MS and isotope labelling for proposing a probable structure and the fragmentation route of non-volatiles formed from peptides in the Maillard reaction, by the analysis of aqueous model systems with an adjusted pH of 4.8 and heated at 140^{o}C for 90 min. Additionally, the gas chromatography-olfactometry-mass spectrometry analysis of the volatile compounds allowed the identification of a possible, but not conclusive, correlation between the type of amino acid sequence in the peptides and the formation of sulfur containing compounds, which opens the door to possibilities in controlling the formation of meaty flavor compounds. The first reports of aroma formation from the Maillard reaction were reported nearly 100 years ago, although it was only until the 1960's that more elaborate studies were performed using specific food categories, which together with the synthesis of the identified chemicals and subsequent confirmation of their structure allowed the development of a broad collection of MS, IR, NMR, and UV spectral databases (Fay et al., 2005; Weenen, Kerler & van der Ven, 1997). Mechanistic studies on the formation of volatiles in model systems composed of amino acids and sugars started only in the mid 80's using GC-MS (Shu, Hagedorn, Mookherjee & Ho, 1985; Tressl, Helak, Koppler & Rewicki, 1985). The focus of these studies was the identification of amino-acid specific

products, such as thiazoles and sulfides from sulphur containing amino acids such as cystine or 2-acethyl- and 2-(2-furfuryl)piperidine for proline. Stable isotope labelling in these type of studies was only incorporated in the 90's (Tressl, Helak, Kersten & Rewicki, 1993), although radioisotopic labelling was used earlier (Koehler, Mason & Newell, 1969). Although the latter method offers higher sensitivity in the detection, however, it suffers from serious drawbacks such as the need for specific detectors and the need for the sample to undergo chemical degradation (retrosynthetic analysis) in order to determine the number of labelled atoms (Schieberle, 2005). Tandem mass spectrometry was later introduced into the GC analysis (GC-MS-MS) as a means to discriminate between co-eluting isotopomers or to accurately locate the labelling site in the molecule under study (Amrani-Hemaimi, Cerny & Fay, 1995) which served to confirm the formation route of many alkylpyrazines, such as diethyl-methylpyrazine isomers, from a sugar and an amino acid and through the reaction of dihydropyrazines and aldehydes formed from the Strecker degradation. Additionally, a comparison of the labelled and unlabelled pyrazines formed from the reaction of [^{13}C-3]alanine and glucose allowed the researchers to identify the location of the labelled carbon coming from the amino acid, for example in the case of the ethyl substituted pyrazines, where the ^{13}C was located in the C2 of the ethyl group, which was corroborated by the study of all the MS/MS fragments.

The non-volatile Maillard reaction products correspond mainly to intermediate and high molecular weight products. The major focus of the studies has been the colored or brown compounds, such as the melanoidins, while a few have focused on the non-volatile sensory active compounds associated with taste descriptors (Ames, 1998; Hofmann, 2005). Among the taste compounds generated in foods during thermal treatment, the Maillard reaction is responsible for the bitter tasting compounds present in foods, like roasted coffee and sugar derived caramel (Frank & Hofmann, 2002; Hofmann, 2005). Regardless of all the research dedicated until now to the study of non-volatile MRPs, when compared to volatile Maillard reaction products, the gaps in knowledge of the non-volatile MRPs is still bigger. This problem has been attributed to the similarities between the structures of the compounds, the complexity of the products mixtures, or the length of analysis (Ames, 1998). Traditionally, analysis of these non-volatile reaction products requires an extraction step. Bailey and colleagues (1996) attempted to reduce the time of the non-volatile Maillard reaction products analysis by developing a rapid method for the direct analysis of the products by HPLC-diode array detection

from aqueous systems. Nevertheless, this study had the drawback of only being able to identify types of molecules, i.e. by allowing the authors to characterize the products as furan-like, pyrole-like, among others. More recent studies have used HPLC-diode array detection to assess the non-volatile profile of the Maillard reaction products (Shen, Tseng & Wu, 2007), but a step of extraction is still required in order to be able to reach a better identification. Additionally, LC-MS and LC-MS/MS analysis, coupled to isotopic labelling analysis has also been used for non-volatile MRPs studies. In an attempt to better understand the relationship between the degree of glycation and the solubility of melanoidins in honeys, Brudzynski and Miotto (2011) analyzed an isolated melanoidin fraction by LC-ESI-MS to study the carbohydrate composition. They identified the presence of bound hexose oligomers in the melanoidin fraction. Taste related compounds have also been the focus of non-volatile MRPs analysis, particularly bitter taste related compounds. For this purpose, isotopic labelling, LC-MS and LC-MS/MS were used to verify the chemical structure of two important bitter-taste compounds previously identified: quinizolate and homoquinizolate (Frank et al., 2002). Through this analysis, both chemicals were not only identified, but also their formation mechanism was elucidated, which differs only towards the end by a Retro-Aldol cleavage of formaldehyde and eventually generate quinizolate, instead of the simple formation of an enamine.

2.6.4. Application of isotope labelling technique to study the mechanism of the Maillard reaction

The use of stable isotopes in Maillard reaction model systems was first introduced in the 90's for the study of proline- and hydroxyproline-specific products arising from their interaction with [^{13}C-1]glucose (Tressl et al., 1993). The use of such labelled compounds can assist in the elucidation of complex pathways of the Maillard reaction and the assignment of the structures of mass spectral fragments, by tracing back the incorporation of label atoms to their starting material. Most commonly used isotopes for labelling purposes are ^{13}C, ^{15}N, ^{17}O, ^{18}O and ^{2}H (Hanson, 2011; Johnstone, 1972; Schieberle, 2005). Depending on the type of product under investigation, the labelled precursors used can be either singly-, multi- or fully-labelled. Singly-labelled and multi-labelled precursors were used in the study of origin of the carbon atoms in various sugar-derived carbonyl compounds, including 2,3-butanedione and 2,3-pentanedione, from L-alanine/D-glucose model systems (Yaylayan et al., 2000). In this case, the use of both

types of labelled precursors allowed to discriminate among the different mechanistic alternatives and to identify if there are more than one pathway, as it was demonstrated in the formation of 2,3-pentanedione. Haffenden and Yaylayan (2005) demonstrated how the use of singly- and fully-labelled glucose (and singly-labelled glycine) can help in the elucidation of the mechanism of formation of 2,5-dihydropyrazine from a glucose/glycine model system. In this case, no label incorporation of glucose carbon atoms was observed but only the incorporation of four carbon atoms and two nitrogen atoms from glycine, that has led to the proposal of a mechanism of formation of 2,5-dihydropyrazine, detected as its silylated derivative as shown in Figure 5. The specific mass spectral fragment at m/z 113 in the case of the silylated 2,5-dihydropyrazine, helps to further confirm the proposed structure. In this case the ion at m/z 113 showed the increment by one when either C-1 and C-2 labelled glycine were used (Haffenden et al., 2005).

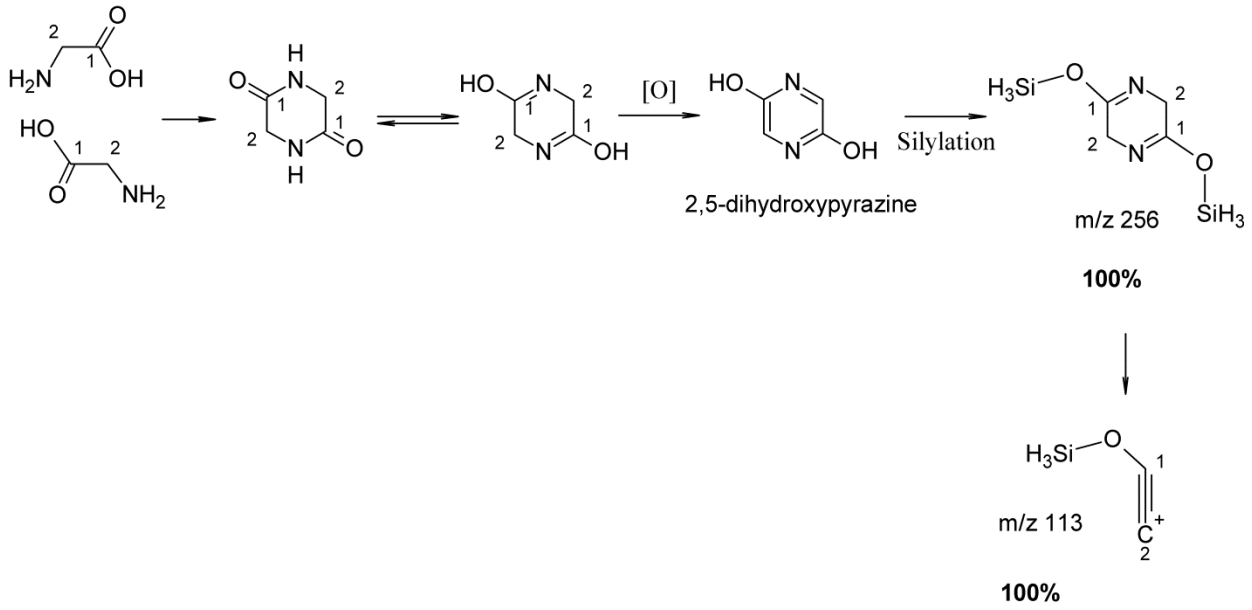


Figure 5. Proposed mechanism of formation of silylated 2,5-dihydropyrazine from glycine. The origin of carbon and nitrogen atoms incorporated into m/z 256 where 100% originated from glycine (Haffenden et al., 2005).

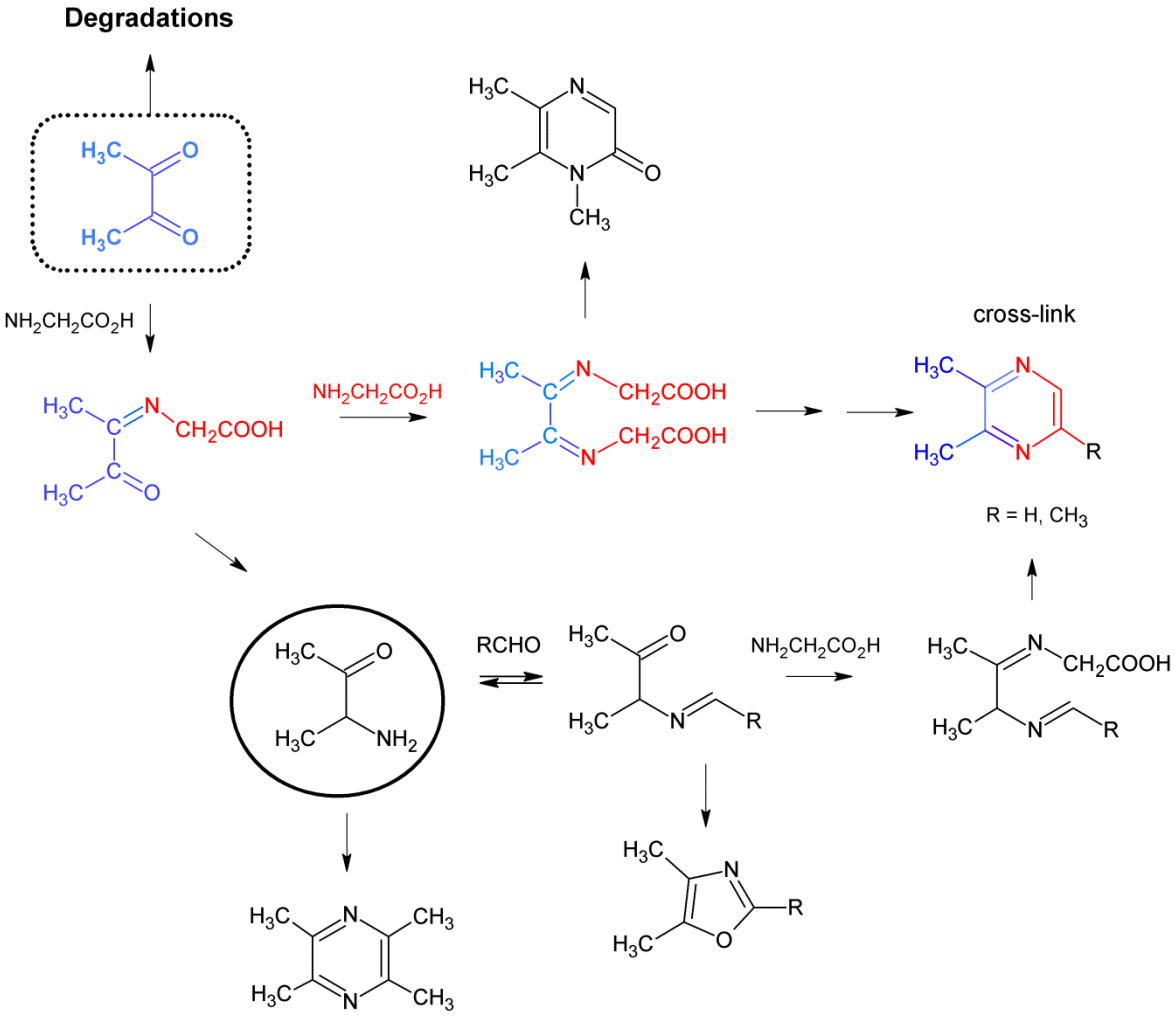
Alternatively, CAMOLA or Carbon Module Labelling Approach proposes the use of a 50% mixture of unlabelled and fully-labelled precursors and analyzing the data based on the expected label incorporation pattern and the mass spectrometric data (Schieberle, 2005). This technique is especially valued in the rationalization of mechanisms that involves scrambling of labels indicating complex fragmentations of the sugar backbone. The technique provides the advantage

of the reduction by half of the amount of labelled precursors used and as a result, a reduction in the associated cost. CAMOLA has been used for the analysis of volatile compounds, such as the aroma chemicals resulting from the interaction of [^{13}C-5]ribose and cysteine (Cerny & Davidek, 2003), as well as non-volatile MRPs such as bitter-tasting chemicals (Frank et al., 2002). In both cases, the use of the CAMOLA is very helpful when most of the label atoms of the sugar are observed in the products under study, but if less labelled atoms are incorporated in the product, the results obtained with the CAMOLA approach are challenging to interpret and sometimes impossible for the researchers to be able to rationalize a possible route of formation or the chemical structure of the molecule, for instance the study of the formation pathway for 4-hydroxy-2,5-dimethyl-3(2H)-furanone (4-HDF) from glucose and proline, which provided different label results depending on the thermal treatment and the water content (Schieberle, 2005). When the 1 to 1 mixture of unlabelled and fully labelled glucose and proline was subjected to thermal treatment in an autoclave in the presence of water, the main isotopomer formed was [^{13}C-3]4-HDF, different to what was observed under roasting conditions which yielded a 1 to 1 mixture of the unlabelled and C-6 labelled 4-HDF. These results suggested that under aqueous conditions, the main route involved the participation of two C-3 fragments from glucose. To overcome this type of difficulty many authors evaluate the CAMOLA model system by incorporating the suspected reaction intermediates and in some cases a quantification step is required. Similar strategy was followed by Cerny and Davidek (2003) for the identification of the different pathways responsible for the aroma chemicals formed from cysteine and ribose. In this case, 4-hydroxy-5-methyl-3(2H)-furanone and 2-furaldehyde were incorporated as intermediates in the model system of cysteine/[^{13}C5]ribose. To analyze models in which the product has a mixed or complicated labelled incorporation, such as only a single atom labelled, a better approach is the use of individually labelled chemicals. Nevertheless, the cost of such analysis is elevated. The use of Py-GC/MS, as an integrated reaction, separation and analysis technique, provides a solution by a decrease in the amount of reactant to be used, hence singly-, double- and/or multi-labelled molecules could be used, eliminating the inconvenience of complicated statistical analysis.

CONNECTING PARAGRAPH

Chapter 2 provides the background regarding the generation of Maillard and Strecker reaction products and the current literature on the involvement of derivatives of α-dicarbonyl moieties within these reactions. In chapter 3, the potential of 2,3-butanedione to undergo double-addition reaction with amino acids is evaluated through the study of a model system of glycine with 2,3-butanedione, as an alternative route to the Strecker reaction to form pyrazines. This chapter was published in the Journal of Agricultural and Food Chemistry. Reprinted with permission from Guerra, P. V., Yaylayan, V. A. (2012) Double Schiff Base Adducts of 2,3-Butanedione with Glycine: Formation of Pyrazine Rings with the Participation of Amino Acid Carbon Atoms. *J. Agric. Food Chem.*, *60* (45), 11440-11445.

CHAPTER 3. DOUBLE SCHIFF BASE ADDUCTS OF 2,3-BUTANEDIONE WITH GLYCINE: FORMATION OF PYRAZINE RINGS WITH THE PARTICIPATION OF AMINO ACID CARBON ATOMS



3.1 Abstract

The 1,2-dicarbonyl compounds are well-known for their ability to undergo one-to-one interaction with amino acids and generate aroma-active pyrazines through the Strecker reaction. An earlier publication reported the generation of tetrahydropyrazine moiety from double addition of amino acids to 1,2-dicarbonyl compounds. To evaluate the potential of this intermediate to undergo oxidation and form pyrazines, a model system composed of glycine and 2,3-butanedione was evaluated under pyrolytic conditions at 250ºC, as well as under pressurized high temperature conditions at 120ºC. These studies have indicated the unexpected formation of 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazine in addition to the expected tetramethylpyrazine. Isotope labelling studies using [^{13}C-1]glycine (98%), [^{13}C-2]glycine (99%), and [^{15}N]glycine (98%) have shown that as expected, tetramethylpyrazine was completely unlabelled whereas, 51% of 2,3-dimethylpyrazine incorporated two ^{13}C-2 atoms from glycine, 20% incorporated one atom and 29% was unlabelled. Furthermore, label incorporation pattern in the major mass spectral fragment at m/z 67 indicated that the C-2 atoms originating from glycine reside in the ring system of the 2,3-dimethylpyrazine. The formation of doubly labelled 2,3-dimethylpyrazine was rationalized through proposition of double addition of glycine to 2,3-butanedione, the formation of singly-labelled isotopomer was justified by sequential Schiff base formation of 2-amino-butan-3-one first with the Strecker aldehyde then followed by the glycine. This pathway can also generate the double labelled pyrazine. Finally, the unlabelled pyrazine was proposed to form through the Strecker reaction of 2,3-butanedione and its degradation product glyoxal with glycine. The proposed pathways were also consistent with the observed label distribution patterns of 2,3,5-trimethylpyrazine.

3.2 Introduction

The chemical transformations of 1,2-dicarbonyl compounds in the presence of amino acids are known to generate diverse compounds through the Strecker reaction, cross-linking, as well as through quinoxaline formation. Pyrazines derived from such interactions are known to be character impact compounds in foods such as coffee (Blank, Sen & Grosch, 1992; Czerny,

Mayer & Grosch, 1999) and chocolate (Schnermann & Schieberle, 1997), and are important in many baked products (Cho & Peterson, 2010; Coleman, Ho & Chang, 1981; Pozo-Bayón, Guichard & Cayot, 2006), showing low detection threshold values. As such understanding their various formation pathways represents an important aspect of improving the quality of processed foods. Shibamoto and Bernhard (1977) presented detailed schemes on different pathways of formation of α-aminocarbonyls the main precursors of pyrazines in food. Although the Strecker degradation is the predominant pathway of pyrazine formation during the Maillard reaction (Adams, Polizzi, van Boekel & De Kimpe, 2008; Rizzi, 2008), there is evidence from the literature that other mechanisms of pyrazine formation may exist (Adams et al., 2008). Recently (Guerra & Yaylayan, 2010), dimerization of azomethine ylides has been identified as a novel route to pyrazines in the absence of 1,2-dicarbonyl compounds. Furthermore, Chu et al. (2011) observed the occurrence of a multiple addition reaction of amino acids with 1,2-dicarbonyl compounds such as 3-deoxyglucosone and the formation of a tetrahydropyrazine moiety, a known precursor of pyrazines. The double addition reaction has also been previously proposed as a mechanism of formation of pyrazinones and quinoxalinones from 3-deoxyglucosone or cyclotene in the presence of glycine or alanine. (Keyhani & Yaylayan, 1997) To investigate the potential of double addition pathway to generate pyrazines, the 2,3-butanedione/glycine model was studied using isotope labelling technique to confirm the anticipated incorporation of C-2 carbon atoms of glycine into the ring structure of the pyrazines.

3.3 Materials and Methods

3.3.1. Reagents and Chemicals

Glycine (99%), glyoxal trimeric dihydrate (95%), pyruvic aldehyde dimethylacetal, 2,3-butanedione (97%), 3,4-hexanedione, 2,3,5-trimethylpyrazine (99%), tetramethylpyrazine (98%) and silica gel (grade 60, 230-400 mesh) were obtained from Sigma-Aldrich Chemical Co (Oakville, ON, Canada). The labelled [^{13}C-1]glycine (98%), [^{13}C-2]glycine (99%) and [^{15}N]glycine (98%) were obtained from Cambridge Isotope Laboratories (Andover, MI).

3.3.2. Pyrolysis-Gas Chromatography-Mass Spectrometry (Py-GC/MS)

The Py-GC/MS analysis was performed according to procedure described by Chu and Yaylayan (Chu & Yaylayan, 2009b) with some modifications. A Varian CP-3800 GC equipped with a sample pre-concentration trap filled with Tenax GR was coupled to a Varian Saturn 2000 Mass Spectrometry detector (Varian, Walnut Creek, CA, USA). The pyrolysis unit included a valved interface (CDS 1500), which was installed onto the GC injection port and connected to a CDS Pyroprobe 2000 unit (CDS Analytical, Oxford, PA, USA). The model systems evaluated consisted of 1:1:3, 2:1:3, 3:1:3 and 7:1:3 ratios of glycine to 2,3-butanedione to silica gel. Between 0.9 to 1.5 mg of sample mixtures were packed inside a quartz tube (0.3mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed at 250ºC for 20 seconds under Helium atmosphere. Isotope labelling studies were performed using a 1:1:3 glycine to 2,3-butanedione to silica gel model system with labelled glycine. Additional confirmations of the mechanism proposed were done through the evaluation of the 1:1:3 model system spiked with either glyoxal trimeric dehydrate or pyruvic aldehyde dimethylacetal. After pyrolysis, the volatiles were concentrated on the sample pre-concentration trap, at 50ºC and subsequently directed towards the GC column for separation. The column used was a DB-5MS column (5% diphenyl, 95% dimethyl-polysiloxane) with dimensions of 50m length x 0.2mm internal diameter x 33µm film thickness (J&W Scientific, ON, Canada). The carrier gas employed was helium. Its flow rate was regulated by an Electronic Flow Controller (EFC) and set at a delayed (30 s) pressure pulse of 70 PSI during the first 4 minutes and maintained with a constant flow of 1.5ml/minute for the rest of the run. The GC oven temperature was set to –5ºC during the first 5 minutes using CO_2 as the cryogenic cooling source and then was increased to 50ºC at a rate of 50ºC/minute. Then, the oven temperature was again increased to 270º C at a rate of 8ºC/minute and kept at 270ºC for 5 minutes. The mass spectrometry detector used was an ion-trap mass spectrometer. The MS transfer-line temperature was set to 250ºC, manifold temperature was set at 50ºC and the ion trap temperature was set to 175ºC. The ionization voltage of 70eV was used and EMV was set to 1500V.

3.3.3. Pressurized High Temperature Reaction with Q-tube™ (PHTR)

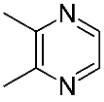
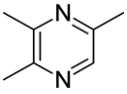
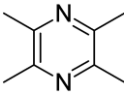
Samples of glycine/2,3-butanedione/silica gel in a proportion of 3:1:3 were also analyzed using a 12 mL Q-tube™ reactor from Q Labtech LLC. A vial with 0.5g of sample was placed inside the

pressure tube reactor. The sample was then heated for 30 minutes at 110°C and then the temperature was raised to 140°C and kept for 10 minutes. A part of the sample was analyzed by Py-GC/MS while the rest was directly analysed by GC/MS without pyrolysis.

3.3.4. Identification of pyrazines

Pyrazines were identified by comparison of their retention times with commercial standards and through NIST library matches. The data reported in Table 1 to Table 4 are based on at least two replicate analyses with a percent standard deviation of < 15%.

Table 1. Intensities of pyrazines identified in glycine/2,3-butanedione/silica gel model in area/mole of glycine.

			Area/mol glycine (x10^{10})		
Compound	**Structure**	**Time**	**Pyrolysis[a]** 250°C	**Dry PHTR[c]**	**Aq. PHTR[c]**
2,3-dimethylpyrazine		11.9	1.8 [b] 0.7 [c]	1.6	0.7
2,3,5-trimethylpyrazine		13.1	4.4 [b] 2.0 [c]	13.6	5.2
Tetramethylpyrazine		14.6	69.4 [b] 53.6 [c]	774.9	220.6

[a] Values are calculated based on duplicates with a percent standard deviation of <15%.
Model systems were evaluated in a 1:1:3 [b] or a 3:1:3 [c] proportion.
PHTR Pressurized high temperature reaction with Q-Tube™.

3.4 Results and Discussion

The sugar-derived 1,2-dicarbonyl compounds are well known intermediates that can undergo one-to-one interaction with amino acids during the Maillard reaction generating reactive precursors to various pyrazines through the Strecker reaction (Figure 6 pathway A). The carbon skeleton of such pyrazines is entirely derived from sugar atoms. On the other hand, double Schiff

base formation of amino acids with 1,2-dicarbonyl compounds has been shown (Figure 6 pathway B) to generate tetrahydropyrazine moiety that in theory can be oxidized into pyrazines incorporating carbon atoms from the amino acids in its ring structure (Chu et al., 2011). To investigate the formation of pyrazines through the double addition pathway, isotope labelling experiments were performed using a simple 1,2-dicarbonyl compound and an amino acid. The model system was evaluated under pyrolytic conditions at 250^{o}C and in pressurized high temperature reactors (PHTR) at 120^{o}C at different ratios and dilutions with silica gel. The reaction of 2,3-butanedione with glycine is expected to generate only tetramethylpyrazine (**1** in Figure 6), however as shown in Table 1, two additional pyrazines 2,3-dimethyl- and 2,3,5-trimethylpyrazines were also detected in addition to trace amounts of 2,5-dimethylpyrazine (not shown) indicating the statistical preference of the major α-amino carbonyl precursor in the reaction pool, the 3-amino-2-butanone to interact with itself or with other minor α-amino carbonyls to generate the observed pattern of pyrazines (Figure 7). To investigate the unexpected formation of 2,3-dimethyl- and 2,3,5-trimethylpyrazine, isotope labelling studies were conducted using variously labelled glycine with unlabelled 2,3-butanedione. Such studies indicated the complete absence of glycine carbon atoms from the tetramethylpyrazine structure, however, 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazines showed various percentages of incorporation of C-2 atoms and 100% incorporation of two nitrogen atoms from glycine (see Table 2) indicating the occurrence of both Strecker and an unknown non-Strecker pathway to generate pyrazines.

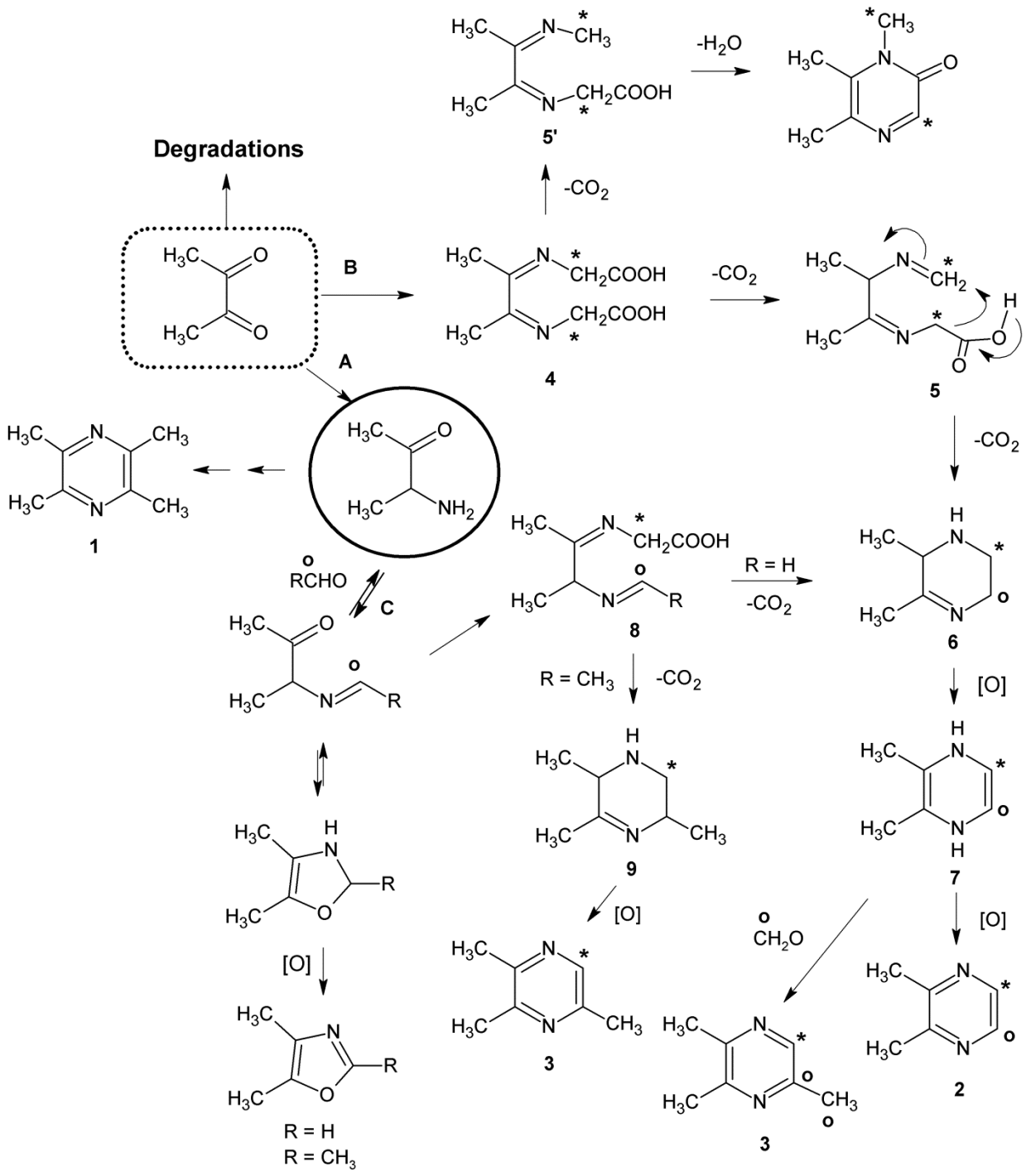


Figure 6. Proposed mechanism of formation of pyrazines through pathways A, B and C in the model system of 2,3-butanedione in the presence of [^{13}C-2]-glycine. * = Presence of C-2 atom of glycine; o = Presence of C-2 atom of glycine depending on the pathway, [O] = oxidation.

Table 2. Percent incorporation of [^{13}C-2]glycine into pyrazines formed in a glycine/2,3-butanedione/silica (1:1:3) model system.

			[^{13}C-2]glycine			
Compound	**Time**	**MW**	**M**	**M+1**	**M+2**	**M+3**
2,3-dimethylpyrazine[a]	11.9	108.14	29	20	51	0
2,3,5-trimethylpyrazine[a]	13.1	122.16	47	27	8	18
Tetramethylpyrazine[a]	14.6	136.19	100	0	0	0

[a]No incorporation of ^{13}C-1from glycine and 100% incorporation of 2 x^{15}N in all listed compounds

3.4.1. Formation of unlabelled pyrazines in [^{13}C-2]glycine/2,3-butanedione model system

In the above model system studied, the only expected pyrazine is the completely unlabelled tetramethylpyrazine since the only α-dicarbonyl present in the model system was unlabelled 2,3-butanedione. As mentioned above, not only other pyrazines were detected but they also exhibited various percentages of ^{13}C-2 atom incorporation patterns as presented in Table 2. The formation of completely unlabelled pyrazines can be only explained by the degradation of 2,3-butanedione into glyoxal and pyruvaldehyde, the two unlabelled α-dicarbonyls needed for their formation (see Figure 7). To our knowledge, there are no published reports that document the formation of glyoxal or pyruvaldehyde from 2,3-butanedione, although generation of acetaldehyde and formaldehyde has been reported (Yaylayan et al., 2003a). Replacing 2,3-butanedione with 3,4-hexanedione in the glycine model system also generated pyrazines that could only be justified if the degradation of 3,4-hexanedione into shorter chain α-dicarbonyl is assumed, similar to the 2,3-butanedione case. Additional confirmation of the occurrence of glyoxal and pyruvaldehyde was provided by spiking the model system with glyoxal and pyruvaldehyde which as expected resulted in the increased levels of all the pyrazines (see Table 3). Although the above results demonstrate that glyoxal and pyruvaldehyde are formed from the pyrolytic degradation of 2,3-butanedione, however, the mechanism of this reaction still remains unknown.

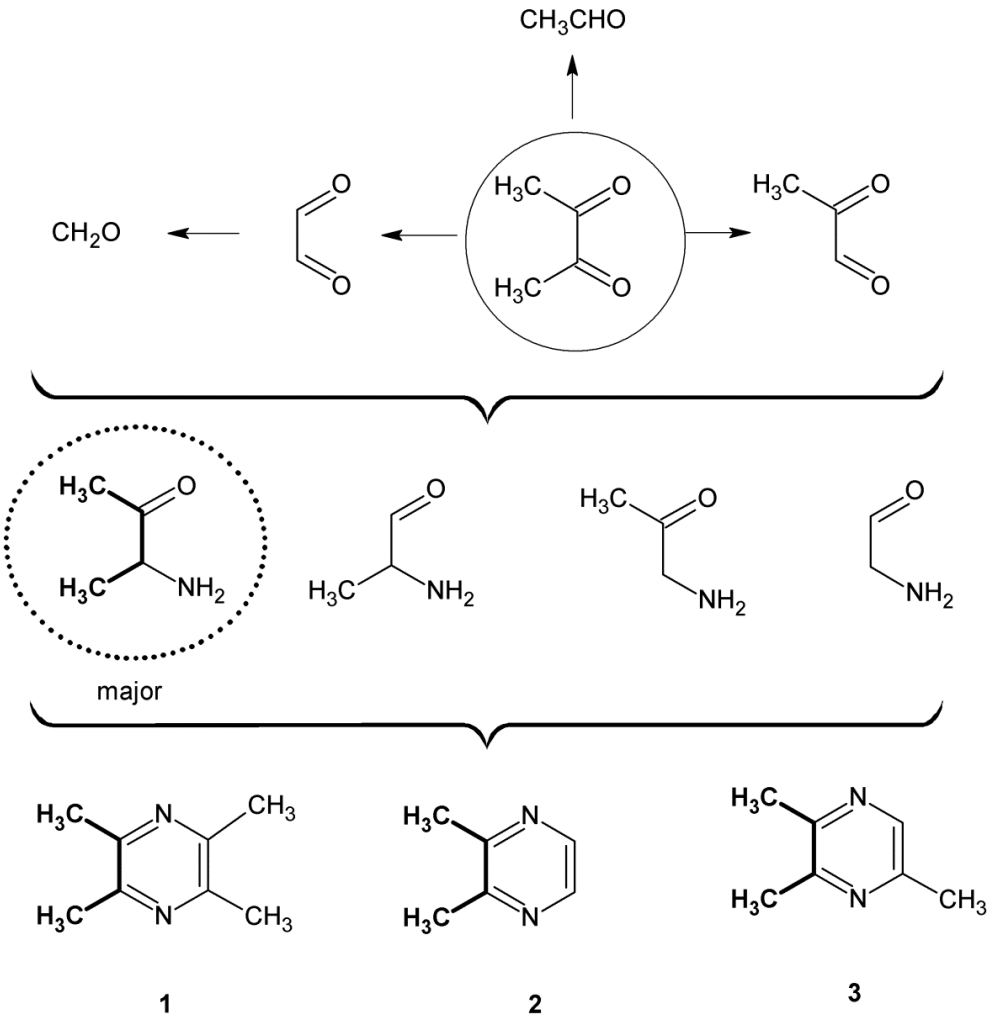


Figure 7. Schematic presentation of thermal degradation products of 2,3-butanedione followed by their Strecker degradation and formation of unlabelled pyrazines.

Table 3. Intensities of pyrazines after spiking a model system of glycine/2,3-butanedione/silica (1:1:3) with glyoxal trimeric dihydrate and pyruvic aldehyde dimethylacetal.

		Area/mol glycine (x10^{10})		
Pyrazine	**Time**	**Parent model**	**Spiked with glyoxal**	**Spiked with pyruvaldehyde**
2,3-dimethyl-	11.9	1.8	42.0	23.6
2,3,5-trimethyl-	13.1	4.4	82.0	57.3
Tetramethyl-	14.6	69.4	127.9	197.7

3.4.2. Formation of labelled pyrazines in [^{13}C-2]glycine/2,3-butanedione model system

The 2,3-dimethylpyrazine which was detected in the above model system, is an important aroma contributor in Parmigiano-Reggiano cheese with nutty and coffee aroma qualities (Qian & Reineccius, 2002), as well as in other foods. Therefore, it is important to understand the different pathways that can lead to its formation in addition to the Strecker reaction and dimerization of the azomethine ylides (Guerra et al., 2010). As was mentioned above, this pyrazine was formed in the 2,3-butanedione/glycine model system with the contribution of C-2 atoms from glycine where isotope labelling studies have indicated the incorporation of one (20%) and two (50%) such carbon atoms from ^{13}C-2 glycine, similarly, 53% of trimethylpyrazine was also found to be formed from the contribution of multiple C-2 atoms from glycine (Table 2). The Strecker mechanism cannot justify the incorporation of ^{13}C-2 atoms into the ring system of the pyrazines. There is evidence from the mass spectral data of the 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazine that the label incorporation occurred at the ring carbon atoms of the pyrazines (see Figure 8). Under the electron impact conditions, methyl-substituted pyrazines undergo a well-established fragmentation through the loss of acetonitrile molecule (Porter & Baldas, 1971) generating an ion at m/z 67 in the case of 2,3-dimethylpyrazine and the ion at m/z 81 in the case of 2,3,5-trimethylpyrazine. Due to the symmetrical nature of 2,3-dimethylpyrazine, this loss can occur from both methyl groups equally, any label incorporation in the methyl groups should register a 50% loss in the label incorporation in m/z 67. However as shown in Figure 8, the m/z 67 retained the percent distribution of labels in the original parent ion, indicating the

incorporation of the amino acid carbons into the pyrazine ring structure and not as methyl substituents. On the other hand, in the case of 2,3,5-trimethylpyrazine, the label incorporation pattern should slightly deviate from the distribution of the parent ion due to the possibility of a loss of acetonitrile molecule and formation of ion F1 at m/z 81 that partially carries the label as a side chain. As a result it is expected to observe a slight variation in the label incorporation pattern particularly in the intensity of ion M+3 due to the simultaneous loss of two labels.

3.4.3. Double Schiff base formation of amino acids with 1,2-dicarbonyl compounds – pathway B

As mentioned above, the evidence from label incorporation into the mass spectral fragments at m/z 67 and m/z 81 arising from the loss of acetonitrile moieties from both pyrazines indicates the involvement of C-2 carbon atoms from glycine in the process of pyrazine ring formation consistent with the proposed mechanism of tetrahydropyrazine formation by Chu et al., (2011) (see Figure 6 pathway B). According to this mechanism one of the double Schiff base adduct (**4**) can undergo decarboxylation through 5-oxazolidinone intermediate (Hidalgo, Delgado, Navarro & Zamora, 2010) to generate two isomeric Schiff bases (Chu et al., 2009a) **5** and **5'**. Intermediate **5** can cyclize as shown in Figure 6 to generate 2,3-dimethyl-tetrahydropyrazine structure **6,** incorporating two C-2 carbon atoms. The structure **6** can get oxidized into dihydropyrazine (**7**) and finally into 2,3-dimethylpyrazine (**2**). The formation of double Schiff base adduct **4** can also be inferred from the fact that 1,5,6-trimethylpyrazin-2(1H)-one was detected in the same model system, the latter has been stipulated to arise from **4** through intra-molecular cyclic amide formation (Keyhani & Yaylayan, 1996; Keyhani et al., 1997) as shown in Figure 6. Although the double addition pathway B can explain the formation of doubly labeled 2,3-dimethylpyrazine, however it cannot explain the formation of neither singly labelled 2,3-dimethylpyrazine nor any of the labelled 2,3,4-trimethylpyrazines which is considered an important aroma compound found in many food products such as popcorn (Buttery, Ling & Stern, 1997) and cocoa beans (Van Praag, Stein & Tibbetts, 1968).

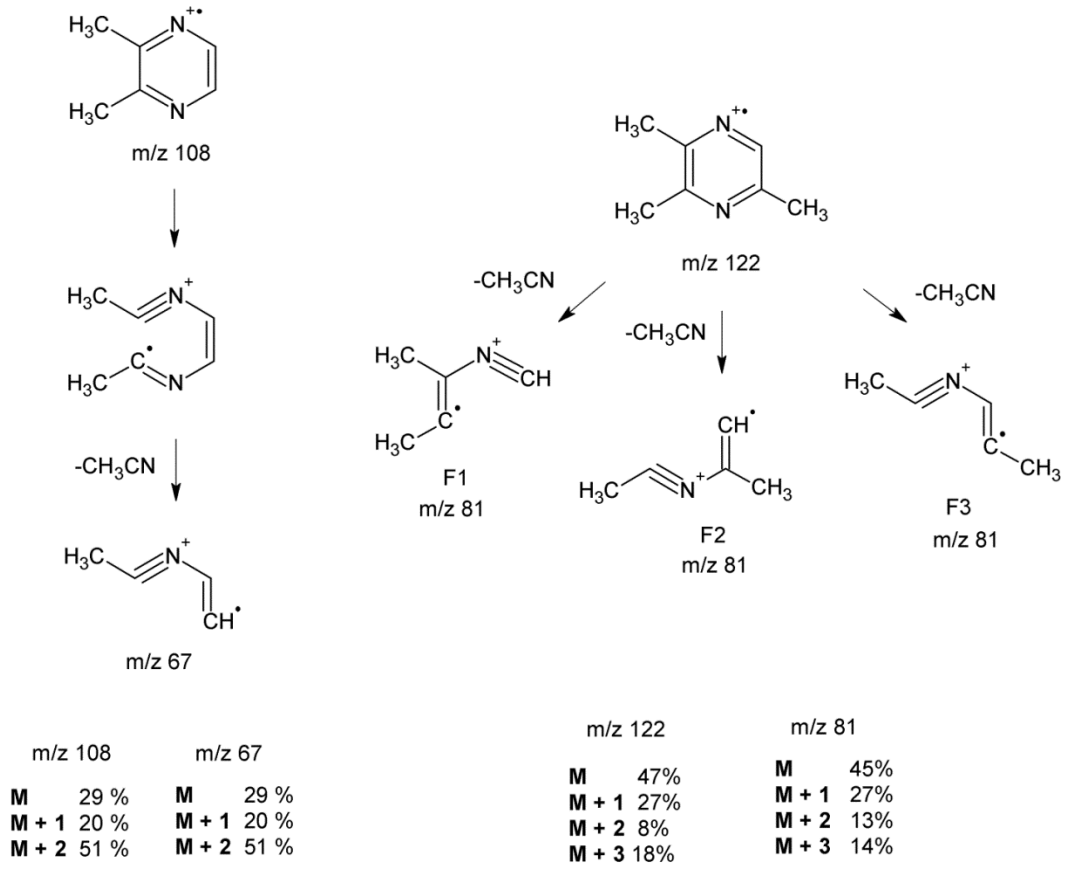


Figure 8. Mass spectral fragmentation of 2,3-dimethyl- and 2,3,5-trimethylpyrazines.

3.4.4. Schiff base formation of α-amino carbonyl intermediates with aldehydes - pathway C

The formation of singly labelled 2,3-dimethylpyrazine and all the labelled isotopomers of 2,3,5-trimethylpyrazine listed in Table 2 can be justified by assuming the ability of reactive aldehydes such as Strecker aldehyde to form Schiff base adducts (Figure 6, pathway C) with 3-amino-butan-2-one the precursor of tetramethylpyrazine generated through the Strecker reaction (pathway A). In fact, the formation of Schiff base adducts with formaldehyde and acetaldehyde is verified in this model system through the detection of their corresponding cyclization products 4,5-dimethyl-1,3-oxazole and 2,4,5-trimethyl-1,3-oxazole as shown in Figure 6 and as reported earlier (Yaylayan et al., 2003a). The oxazolines, the precursor of oxazoles, have also been observed as products from the reaction of 2,3-butanedione with amino acids (Ho & Hartman, 1982). Oxazoles have been identified as important aroma compounds in baked potatoes (Ho &

Coleman, 1980) and roasted peanuts (Lee, Ho & Chang, 1981). Oxazoles therefore can be utilised as chemical markers not only for the presence of α-amino carbonyl intermediates in the Maillard reaction, but also for the presence of small reactive aldehydes trapped by them. The two detected oxazoles therefore indicate the formation of formaldehyde and acetaldehyde in the model system studied and according to Table 4 the 70% of formaldehyde generated in the system is labelled and 30% unlabelled. These percentages depend on the reaction conditions used such as the presence of silica. Acetaldehyde generated from the degradation of 2,3-butanedione on the other hand, should be unlabelled generating unlabelled trimethyloxazole. Labelled formaldehyde can be formed through the Strecker degradation of labelled glycine, resulting in a singly labelled 4,5-dimethyl-1,3-oxazole, the unlabelled formaldehyde and acetaldehyde can arise from the degradation of the only source of unlabelled carbons that is 2,3-butanedione as proposed in Figure 7. The oxidative α-dicarbonyl cleavage could also result in the formation of formic acid or acetic acid according to the mechanism proposed by Davídek et al. (2006) Recently, Granvogl et al. (2012) demonstrated the ability of oxazolines to release back the Strecker aldehydes under hydrolytic conditions.

Table 4. Percent incorporation of [^{15}N]-, [^{13}C-1]-, and [^{13}C-2]glycine into 4,5-dimethyl-1,3-oxazole and 2,4,5-trimethyl-1,3-oxazole in a glycine/2,3-butanedione/silica (1:1:3) model system.

	4,5-dimethyl-1,3-oxazole			**2,4,5-trimethyl-1,3-oxazole**		
Label	**M**	**M+1**	**M+2**	**M**	**M+1**	**M+2**
[^{13}C-1]-	100	0	0	100	0	0
[^{13}C-2]-	30	70	0	100	0	0
[^{15}N]-	0	100	0	0	100	0

3.4.5. Formation of pyrazines through pathway C

As shown above, the 3-amino-butan-2-one not only can dimerize into tetramethylpyrazine (**1**) but also it can form Schiff base adducts with simple aldehydes (Figure 6 pathway C). These intermediates can cyclize to generate oxazoles or react further with amino acids to form a second

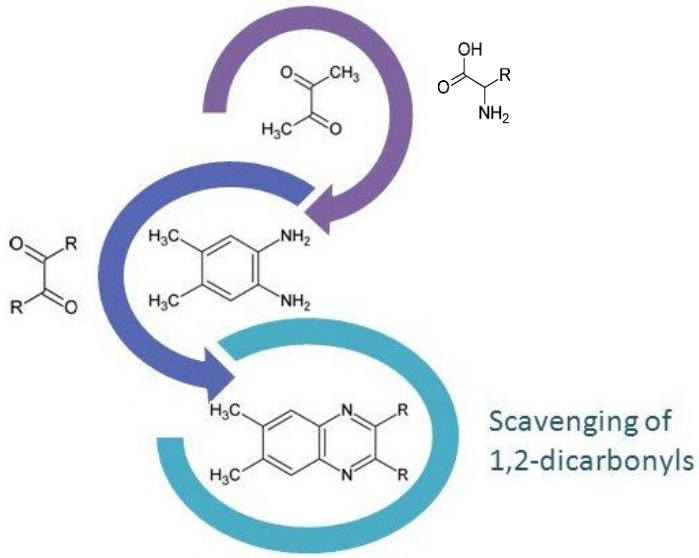
Schiff base adduct **8** which is chemically identical to the intermediate **5** (if R = H) and can undergo similar cyclization and form **6** if the initial aldehyde was formaldehyde and generate **9** if the initial aldehyde was acetaldehyde. Tetrahydropyrazine structures similar to **6** or **9** generated from alanine and 3-deoxyglucosone have been also identified earlier (Chu et al., 2011). Intermediates **6** and **9** can undergo further oxidation steps to generate dihydropyrazines and eventually pyrazines **2** and **3** with various degrees of label incorporation depending upon the origin of formaldehyde. Structure **6** originating from **5** will always be doubly labelled in the [^{13}C-2]glycine models whereas structure **6** originating from **8** could be singly or doubly labelled depending on the origin of formaldehyde. Structure **6** can therefore generate singly labelled or doubly labelled pyrazine **2.** Finally, addition of formaldehyde to either singly labelled or doubly labelled dihydropyrazine **7** can justify the formation of doubly labelled or triply labelled pyrazine **3**.

The isotope labelling approach allowed the identification of two distinct pathways for the generation of aroma active pyrazines, one initiated through double Schiff base formation between the 1,2-dicarbonyl compounds and the amino acids and the other similar to Strecker reaction is initiated by the formation of α-amino carbonyls followed by sequential Schiff base formation first at the amino group with a Strecker aldehyde then followed at the carbonyl group by an amino acid. The consequences of such pathways could be important in food where the diversity of pyrazine formation can be rationalized not only by the type of α-dicarbonyl compounds formed from sugar but also by the type and number of amino acids present. Furthermore, the process of double Schiff base formation can be viewed as a model for cross-linking reactions occurring in food during processing and pyrazines themselves could be viewed as cross-linked structures.

CONNECTING PARAGRAPH

Chapter 3 presented a comprehensive study on an alternative route to the Strecker reaction that leads to the generation of pyrazines through double addition of glycine to 2,3-butanedione. In addition to pyrazines, benzopyrazines or quinoxalines were also observed to be formed in the above reaction. Quinoxalines are usually formed through the scavenging of 1,2-dicarbonyls by 1,2-benzenediamine derivatives. Given that 1,2-benzenediamine derivatives have not been previously observed under Maillard reaction conditions, Chapter 4 presents evidence for their generation for the first time. This pathway involves the initial formation of *o*-quinone followed by Strecker degradation generating an amino carbonyl moiety and a Strecker aldehyde. This chapter was published in Food Chemistry. Reprinted from *Food Chemistry*, 141/4, Guerra, P. V., Yaylayan, V. A., Cyclocondensation of 2,3-butanedione in the presence of amino acids and formation of 4,5-dimethyl-1,2-phenylendiamine, pp 4391-4396,

CHAPTER 4. CYCLOCONDENSATION OF 2,3-BUTANEDIONE IN THE PRESENCE OF AMINO ACIDS AND FORMATION OF 4,5-DIMETHYL-1,2-PHENYLENDIAMINE



4.1 Abstract

The chemical interaction of 2,3-butanedione with amino acids through Strecker reaction has been studied extensively, however, the formation of previously reported 4,5-dimethyl-1,2-benzoquinone from 2,3-butanedione/amino acid model systems is not investigated in detail. In this study such model systems containing 2,3-butanedione were investigated under pyrolytic conditions using glycine, sodium glycinate and glycine hydrochloride as amino acids able to modulate acid/base catalytic activity of the reaction medium. The analysis of the data indicated that replacing glycine with its corresponding salts promoted significantly the generation of 2,3,6,7-tetramethylquinoxaline relative to tetramethylpyrazine, the indicator compound for the Strecker reaction. The origin of the 2,3,6,7-tetramethylquinoxaline was traced back to the formation of 4,5-dimethyl-1,2-benzoquinone through isotope labelling studies. Furthermore, these studies have also indicated the ability of glycine not only to catalyze the cyclocondensation of butanedione into 4,5-dimethyl-1,2-benzoquinone but also its conversion into 4,5-dimethyl-1,2-phenylenediamine through Strecker-type transformations. The trapping of 2,3-butanedione by this *in-situ* generated 4,5-dimethyl-1,2-phenylenediamine gave rise to the observed 2,3,6,7-tetramethylquinoxaline.

4.2 Introduction

The 1,2-dicarbonyl compounds have been mainly associated with the Strecker reaction (Rizzi, 2008), cross-linking (Nagaraj, Shipanova & Faust, 1996a) and formation of various heterocyclic compounds during the Maillard reaction (Rizzi, 2008), however, their conversion into aromatic compounds or benzene derivatives has not been studied in detail, although such conversions from intact sugars into hydroxylated benzene derivatives have been documented in the literature (Haffenden et al., 2005; Lang, Mueller & Hofmann, 2006). Piloty & Baltes (1979) and Reese & Baltes (1992) identified the formation of benzoquinone derivatives such as 2,5-dimethyl-1,4-benzoquinone and 4,5-dimethyl-1,2-benzoquinone in model systems of 2,3-butanedione with selected amino acids. Once generated, such benzoquinones similar to their open chain counterparts can also undergo Michael and Strecker type reactions (Bittner, 2006; Nikolantonaki

& Waterhouse, 2012; Rizzi, 2006) or reduction into hydroxylated benzene derivatives that are considered important chemical moieties in food due to their redox activity. Such moieties can be found in various phenolic compounds and flavonoids in food and as such are expected to influence their redox potentials during processing. The study of the different mechanisms involved in the formation of benzoquinones and/or hydroxylated benzene derivatives under Maillard reaction conditions can provide further insight into the important precursors involved in their generation during the Maillard reaction. Among the different 1,2-dicarbonyl compounds formed from sugar, 2,3-butanedione, is one of the most important since it is widely present in many types of foods such as thermally processed and fermented. There are two main mechanisms proposed for the formation of 2,3-butanedione under Maillard reaction conditions one through C_2/C_4 cleavage of glucose and the other through a chain elongation of smaller 1,2-dicarbonyl compounds mediated by amino acids (Keyhani & Yaylayan, 1999; Keyhani et al., 1996), on the other hand fermented food products such as cheeses (Barbieri et al., 1994), wine and beer (Romano et al., 1996) also contain significant amounts of 2,3-butanedione generated through the action of yeast and lactic acid bacteria and the concentration range can vary from 0.01 to 4 ppm among the fermented foods (Langler, Libbey & Day, 1967; Martineau, Acree & Henick-Kling, 1994). In this study we explore the mechanism of formation and further reactions of benzoquinones in 2,3-butanedione/amino acid model systems.

4.3 Materials and Methods

4.3.1. Reagents and Chemicals

Glycine (99%), alanine (>98%), serine (>99%), glycylglycine (>99%), 2,3-butanedione (97%), 2,3-pentanedione (97%), glycine hydrochloride (>99%), glycine sodium salt hydrate (98%), 4,5-dimethyl-1,2-phenylendiamine (98%), 2-amino-4,5-dimethylphenol, acetaldehyde (>99.5%), paraformaldehyde (95%), ammonium chloride (>99.5%), silica gel (grade 60, 230-400 mesh) and potassium hydroxide (powder) were obtained from Sigma-Aldrich Chemical Co (Oakville, ON, Canada). The labelled [^{13}C-1]glycine (98%), [^{13}C-2]glycine (99%) and [^{15}N]glycine (98%) were obtained from Cambridge Isotope Laboratories (Andover, MI).

4.3.2. Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS)

A Varian CP-3800 GC equipped with a sample pre-concentration trap (SPT) filled with Tenax GR was coupled to a Varian Saturn 2000 Ion Trap Mass Spectrometer (Varian, Walnut Creek, CA, USA). The pyrolysis unit included a valved interface (CDS 1500), which was installed onto the GC injection port and connected to a CDS Pyroprobe 2000 unit (CDS Analytical, Oxford, PA, USA). Between 0.9 to 1.5 mg portions of the model systems consisting of 1:1:3; 1:2:3 and 1:3:3 ratios of amino acid to 2,3-butanedione to silica gel and those listed in Table 5 were packed inside a quartz tube (0.33µm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed at 250ºC for 20 seconds under helium atmosphere. The samples were analyzed on a DB-5MS column (5% diphenyl, 95% dimethyl-polysiloxane) with column dimensions of 50m length x 0.2mm internal diameter x 33µm film thickness (J&W Scientific, ON, Canada) using helium as the carrier gas. The volatiles after pyrolysis were either concentrated using the SPT at 50ºC or bypassed the SPT step. The carrier gas flow rate was regulated by an Electronic Flow Controller (EFC) and set at a delayed (30 s) pressure pulse of 70 psi during the first 4 minutes and maintained with a constant flow of 1.5 mL/minute for the rest of the run. The GC oven temperature was set to –5ºC during the first 5 minutes using CO_2 as the cryogenic cooling source and then was increased to 50ºC at a rate of 50ºC/minute. The oven temperature was again increased to 270º C at a rate of 8ºC/minute and kept at 270ºC for 5 minutes. When samples bypassed the SPT the helium pressure was increased at a rate of 400 psi/min from 5 to 70 psi and held for 6 minutes. The pressure was then decreased at the same rate until it reached 33 psi and held for 5 minutes, and finally increased again to 50 psi at a rate of 3.5 psi/min. The GC oven temperature was set to the same conditions except the final temperature of 270º C was held for 5.40 minutes instead of 5 minutes. The MS transfer-line temperature was set to 250ºC, manifold temperature was set to 50ºC and the ion trap temperature was set to 175ºC. The ionization voltage of 70eV was used and EMV was set to 1500V. **Structural identification** was performed using AMDIS (ver. 2.65) and NIST Standard Reference Databases (data version 05 and software ver. 2.0d) and when possible by comparison of their retention times (see Table 6) and mass spectra to that of commercially available standards or generated pyrolytically through the use of appropriate precursors (see Table 5) in addition to isotope labelling data. The reported percent label incorporation values (corrected for natural abundance and for percent enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of

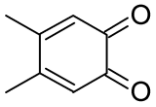
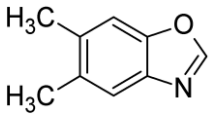
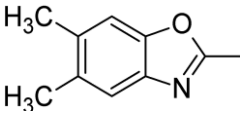
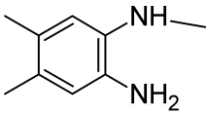
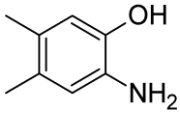
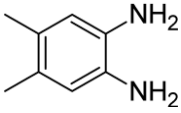
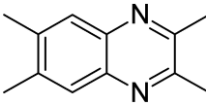
5%. **Isotope labelling studies** were performed through replacing glycine with [^{13}C-1]glycine, or [^{13}C-2]glycine (99%) or [^{15}N]glycine (98%) in the model systems mentioned above.

Table 5. Composition of all model systems analyzed by Py-GC/MS

Model System[a]	Target compound
2,3-butanedione + KOH [b] glycine sodium salt + 2,3-butandione glycine sodium salt +glycine hydrochloride+2,3-butandione	4,5-dimethyl-1,2-benzoquinone (**3**)
2-amino-4,5-dimethylphenol (5) + formaldehyde [b] glycine + 2,3-butanedione + formaldehyde (spike) glycine + 2,3-butanedione	5,6-dimethyl-1,3-benzoxazole (**6**)
2-amino-4,5-dimethylphenol (5) + acetaldehyde [b] glycine + 2,3-butanedione + acetaldehyde glycine +2,3-butanedione	2,5,6-trimethyl-1,3-benzoxazole (**7**)
glycine+2,3-butanedione	N-methyl-4,5-imethylbenzene-1,2-diamine (**8**)
2-amino-4,5-dimethylphenol (5)	2-amino-4,5-dimethylphenol (**5**)
glycine + 2,3-butanedione [c] alanine + 2,3-butanedione [c] 4,5-dimethyl-1,2-phenylendiamine (2)	4,5-dimethyl-1,2-phenylendiamine (**2**)
glycine + 2,3-butanedione glycine sodium salt + 2,3-butanedione glycine hydrochloride + 2,3-butanedione glycine sodium salt + glycine hydrochloride + 2,3-butandione glycine + 2,3-butanedion + glyoxal glycine + 2,3-butanedione + ammonium chloride glycine + 2,3-butanedione + 2-amino-4,5-dimethylphenol alanine + 2,3-butanedione serine + 2,3-butanedione phenylalanine + 2,3-butanedione glycylglycine + 2,3-butandione 4,5-dimethyl-1,2-phenylendiamine (2) + 2,3-butanedione	2,3,6,7-tetramethylquinoxaline (**1**)
glycine + 2,3-butanedione + glyoxal	6,7-dimethylquinoxaline
2,3-butanedione + ammonium chloride + KOH 2,3-butanedione + ammonium chloride glycine sodium salt + glycine hydrochloride+ glyoxal + 2,3-butanedione glycine sodium salt + glycine hydrochloride + 2,3-pentanedione + 2,3-butanedione	

[a] All model systems were diluted with silica gel except for 2,3-butanedione + KOH mixture and analyzed using various molar ratios.
[b]Model systems prepared using only 2:1 molar ratio.
[c] Model systems analysed by GC-MS using the SPT trap.

Table 6. Products and intermediates observed in glycine/2,3-butanedione/silica gel model systems.

Compound	Time (min)	MW	Structure
4,5-dimethyl-1,2-benzoquinone (**3**)	18.6	136.15	
5,6-dimethyl-1,3-benzoxazole (**6**)	21.1 [a,b]	147.17	
2,5,6-trimethyl-1,3-benzoxazole (**7**)	23.5 [a,b]	161.20	
N-methyl-4,5-dimethylbenzene-1,2-diamine (**8**)	23.7 [a]	150.22	
2-amino-4,5-dimethylphenol (**5**)[d]	24.6	137.17	
4,5-dimethyl-1,2-phenylendiamine (**2**)	24.7 [a, c]	136.19	
2,3,6,7-tetramethylquinoxaline (**1**)	28.8 [a,b]	186.25	

[a] NIST library positive match.
[b] Retention times confirmed with in-situ generation from known precursors (see Table 1).
[c] Retention times confirmed with commercial standards.
[d] Not observed under the experimental conditions, retention time based on the commercial standard.

4.4 Results and Discussion

The well-known interaction of amino acids with 1,2-dicarbonyl compounds generates the extensively documented formation of pyrazine derivatives through the Strecker reaction. However, Strecker reaction is not the only reaction that 1,2-dicarbonyls can undergo in the presence of amino acids (see Figure 9).

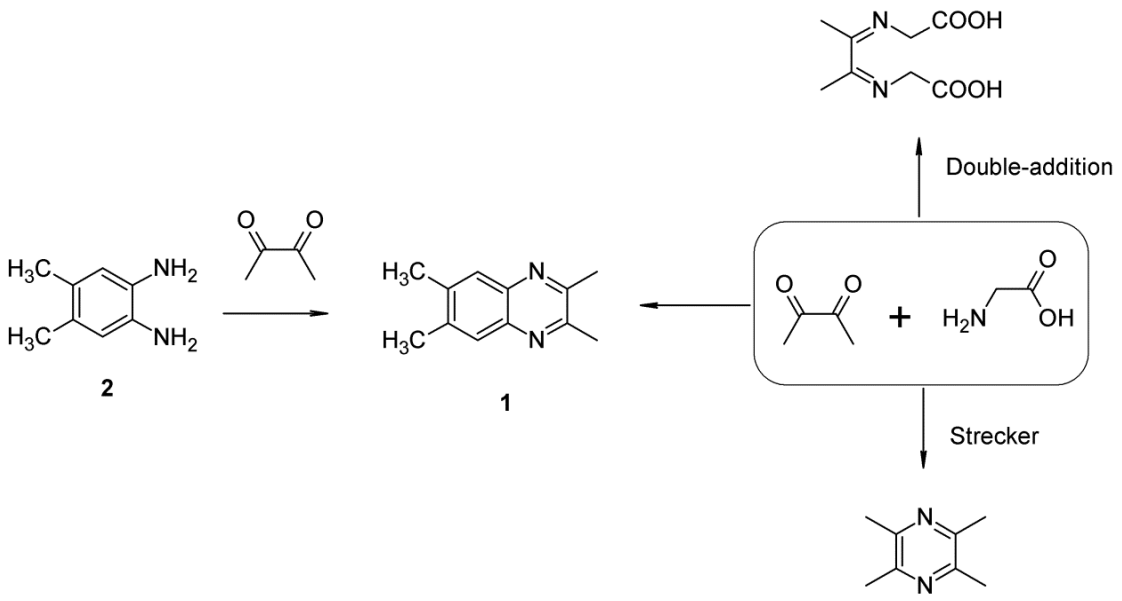


Figure 9. Possible interaction pathways of 2,3-butanedione with amino acids

In an effort to understand the impact of the ratio of amino acids to that of the 1,2-dicarbonyl compounds in undergoing the Strecker reaction versus other reactions such as double addition (Chu et al., 2011; Guerra & Yaylayan, 2012), various ratios of 2,3-butanedione to that of glycine were studied, as well as the effect of the substitution of glycine with sodium glycinate and glycine hydrochloride as pH modulators. These studies have indicated that not only the ratio of the reactants play an important role in determining the amount of pyrazine formation but also the presence of glycine salts (see Table 7). An equimolar amount of glycinate and glycine hydrochloride generated for example the highest amount of the pyrazine. Furthermore, what seemed to be a minor product at the retention time of 28.8 min when glycine was used increased significantly when it was replaced with an equimolar ratio of sodium glycinate and glycine hydrochloride or with sodium glycinate alone (Table 7).

Table 7. Ratios of tetramethylpyrazine to tetramethylquinoxaline in 2,3-butanedione/glycine/ /silica gel model systems as a function of reaction conditions.

	Area/mol glycine (x10^{10})				
Model system / **Compound**	1:1:3 Glycine	1:2:3 Glycine	1:3:3 Glycine	1:1:3 NaGlycinate	0.5:0.5:1:3 Mixture[a]
2,3,5,6-tetramethylpyrazine[b]	40.0	494.5	156.3	332	686.7
2,3,6,7-tetramethylquinoxaline	1.2	12.7	4.3	88.1	310.7
Ratio	34:1	39:1	37:1	4:1	2:1

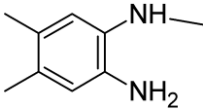
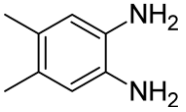
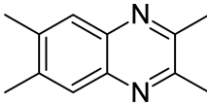
[a] Equimolar mixture of sodium glycinate and hydrochloride salt of glycine, when only glycine.HCl was used none of the products were observed. [b] Indicator compound for the Strecker reaction

The peak at the retention time of 28.8 min was found to represent 2,3,6,7-tetramethylquinoxaline (**1** in Figure 9) based on NIST library search and based on the comparison of its retention time to that of pyrolytically generated standard from 4,5-dimethyl-1,2-phenylendiamine (**2**) and 2,3-butanedione reaction (see Figure 9). Furthermore, 2,3,6,7-tetramethylquinoxaline has been reported earlier (Hartman & Ho, 1984; Piloty et al., 1979), to occur in various model systems of 2,3-butanedione. It appears that the ability of 2,3-butanedione to undergo reactions other than Strecker reaction is mainly dependent on the ability of the reaction medium for acid/base catalysis. Table 7 indicates that the pathway responsible for the formation of the quinoxaline derivative becomes as important as the Strecker reaction (estimated from the relative intensity of tetramethylpyrazine, the indicator compound for Strecker reaction) when sodium glycinate or a mixture of glycinate and the hydrochloride salt are used. Isotope labelling experiments with [^{13}C-1], [^{13}C-2] and [^{14}N]glycine indicated that no carbon atoms were incorporated into the quinoxaline structure from glycine except two nitrogen atoms (see Table 8) confirming the 2,3-butanedione as the sole origin of all carbon atoms in its structure.

4.4.1. Significance of 2,3,6,7-tetramethylquinoxaline (1) formation

The detection of 2,3,6,7-tetramethylquinoxaline (**1**) in the model system of glycine and 2,3-butanedione indicates the *in-situ* formation of 4,5-dimethyl-1,2-phenylendiamine (**2**) and its subsequent entrapment by 2,3-butanedione as quinoxaline derivative (see Figure 9). When an equimolar amount of glyoxal was added to the glycine/2,3-butanedione model system, in addition to compound **1** the 6,7-dimethylquinoxaline was also detected confirming the above hypothesis of formation of **2** in the reaction mixture. Although such aromatic *o*-diamines have been used extensively in the literature to trap 1,2-dicarbonyls in the Maillard reaction, however, they were not reported so far to be formed in the Maillard reaction itself. The free 4,5-dimethyl-1,2-phenylendiamine was detected only when sample pre-concentration trap was utilized during the analysis (Table 5).

Table 8. Percent incorporation of [^{15}N]-glycine into products generated from glycine/2,3-butanedione/silica model system.

			[^{15}N]glycine		
Compound	**Structure**	**Area/mol glycine (x10^{11})**	**M**	**M+1**	**M+2**
N-methyl-4,5-dimethylbenzene-1,2-diamine (**8**) [a]		6.5 [c]	0	0	100
4,5-dimethyl-1,2-phenylendiamine (**2**) [b]		1.1 [d]	0	0	100
2,3,6,7-tetramethylquinoxaline (**1**) [b]		1.3 [c] 1.7 [d]	0	0	100

[a] 100 % incorporation of ^{13}C-2-glycine was observed
[b] No incorporation of ^{13}C-1 or ^{13}C-2 from glycine was observed
[c] Observed in model system of glycine/2,3-butanedione/silica gel (1:3:3) and analysed bypassing the SPT trap
[d] Observed in model system of glycine/2,3-butanedione/silica gel (1:1:3)

4.4.2. Proposed mechanism of formation of 4,5-dimethyl-1,2-phenylendiamine (2)

Based on the isotope labelling data (Table 8) the formation of the diamine (**2**) could be rationalized through the initial aldol cyclocondensation of two molecules of 2,3-butanedione to generate 4,5-dimethyl-1,2-benzoquinone intermediate **3** as proposed in Figure 10. This *o*-benzoquinone moiety, as well as its isomer 2,5-dimethyl-1,4-benzoquinone (**4**), have been previously reported by Piloty & Baltes (1979) and Reese & Baltes (1992) as intermediates in the formation of redox active 4,5-dimethyl-1,2-benzenediol and 2,5-dimethyl-1,4-benzendiol moieties respectively when 2,3-butanedione was heated at 180ºC in the presence of various amino acids.

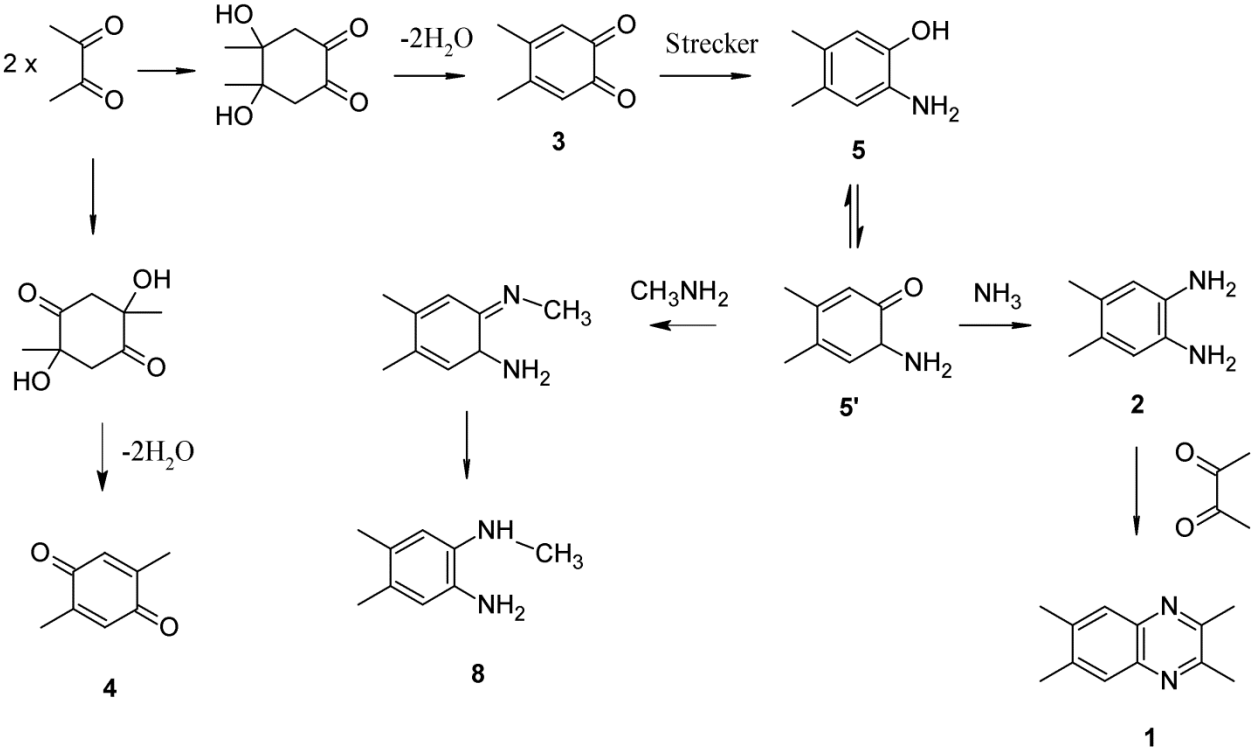


Figure 10. Proposed mechanism of formation of 2,3,6,7-tetramethylquinoxaline from the interaction of 2,3-butanedione with any amino acid.

The formation of benzoquinones through cyclocondensation most probably proceeds through acid/base catalysis since in the absence of amino acids it was not detected and in the presence of sodium glycinate and a mixture of glycinate and glycine hydrochloride the intensity of the 2,3,6,7-tetramethylquinoxaline (**1**) was significantly increased (Table 7). In addition, the major

peak arising from pyrolysis of 2,3-butanedione in the presence of KOH was 4,5-dimethyl-1,2-benzoquinone (**3**). Pyrolysis of butanedione alone did not generate **3**. This intermediate that we could not detect in the glycine/2,3-butanedione model systems, due to its reactivity, can be converted through Strecker degradation (Nikolantonaki et al., 2012; Rizzi, 2006) into structure **5'** (Figure 10) in equilibrium with 2-amino-4,5-dimethylphenol (**5**). Although the structure **5** was not observed under the experimental conditions, however, indirect evidence for its formation was obtained through detection of two of its derivatives; 5,6-dimethyl-1,3-benzoxazole and 2,5,6-trimethyl-1,3-benzoxazole (**6** and **7** in Figure 11). Aldehydes are known to trap 2-aminophenols as benzoxazole derivatives at high temperatures (Maddila & Jonnalagadda, 2012; Terashima, 1982). Formaldehyde and acetaldehyde can be generated from the degradation of 2,3-butanedione in this model system as previously observed (Guerra et al., 2012) and react with **5** to generate the adducts shown in Figure 11. Furthermore, reacting commercially available 2-amino-4,5-dimethylphenol (**5**) with formaldehyde and acetaldehyde generated products with identical retention times and mass spectra to those peaks observed in the model systems. Spiking experiments with the appropriate aldehydes using glycine/2,3-butanedione model system enhanced the corresponding peaks. The reaction of ammonia with 2-amino-4,5-dimethylphenol can affect its conversion into 4,5-dimethyl-1,2-phenylendiamine as shown in Figure 10. Interestingly, N-methyl-4,5-dimethylbenzene-1,2-diamine (**8** in Figure 10) was also detected in the reaction mixture indicating a similar reaction with methylamine generated from the decarboxylation of glycine. When the model system of glycine/2,3-butanedione was spiked with 2-amino-4,5-dimethylphenol or when equimolar amount of ammonium chloride was added, the intensity of **1** was enhanced by 11 and 8 fold respectively relative to the amount found in the parent glycine/2,3-butanedione model system, providing further evidence for the involvement of **5** and ammonia in the formation of **1** as shown in Figure 10. On the other hand spiking the model system with 1,2-diamino-4,5-dimethylphenol increased the intensity of **1** by more than 1000 fold.

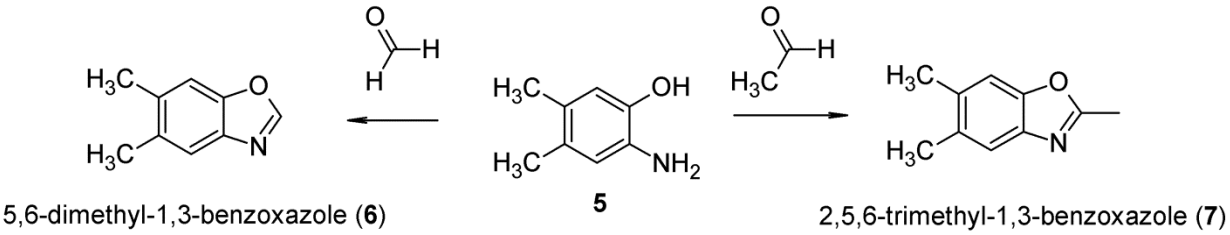


Figure 11. Reaction of 2-amino-4,5-dimethylphenol with formaldehyde and acetaldehyde to form benzoxazole derivatives.

4.4.3. Generality of cyclocondensation of 1,2-dicarbonyl compounds

As has been observed in the literature (Piloty et al., 1979), the cyclocondensation of 2,3-butanedione was independent of the type of amino acid used as confirmed by performing the reaction with glycine, alanine, serine, phenylalanine and glycylglycine. Every model system produced the 2,3,6,7-tetramethylquinoxaline (**1**) independently of the amino source but in different amounts. However, when other dicarbonyls such as glyoxal and 2,3-pentanedione were used, the former, in the presence of 2,3-butanedione did not generate any detectable cyclocondensation products incorporating glyoxal, most probably due to its enhanced reactivity with nitrogen nucleophiles relative to its ability to undergo aldol condensation, whereas the 2,3-pentanedione generated peaks that were consistent with the structure of isomeric quinoxaline derivatives expected from its cyclocondensation products. Recently, Miyazato et al. (2013) also reported formation of 2,6-dimethyl-1,4-cyclohexanedione moiety from cyclocondensation of 2-hydroxy-3-pentanone and hydroxyacetone.

4.5 Conclusion

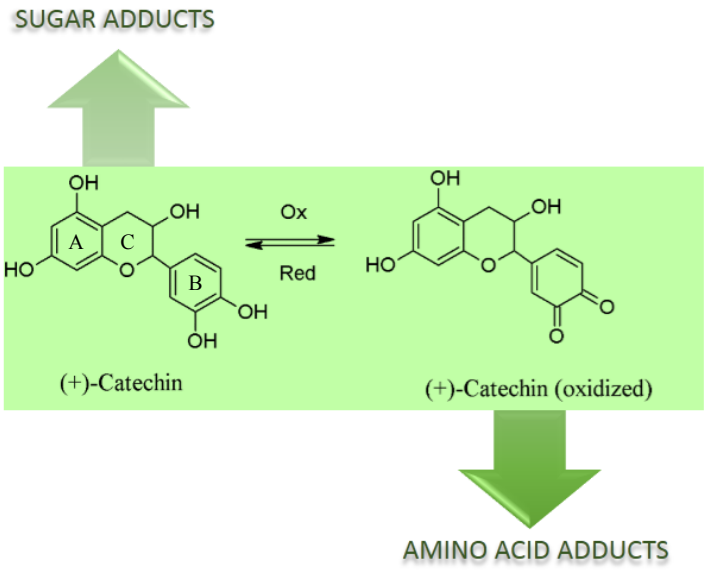
In addition to initiating Strecker type reactions, the 2,3-butanedione can undergo cyclocondensation through aldol type reaction to generate 4,5-dimethyl-1,2-benzoquinone intermediate and/or its isomers under acid/base catalysis. These redox active moieties could play an important role in the degree of browning associated with the Maillard reaction. Furthermore their formation could be monitored through their ability to undergo nucleophilic reactions with amino acids (Nikolantonaki et al., 2012) and eventual formation of aminophenols and

benzenediamine derivatives that could scavenge 1,2-dicarbonyls present in the mixture through formation of quinoxaline derivatives as observed in this study.

CONNECTING PARAGRAPH

The previous chapter provided evidence for the conversion of a 1,2-benzoquinone moiety into 4,5-dimethyl-1,2-phenylenediamine through Strecker type transformations and the release of the corresponding Strecker aldehyde. Based on this observations, the flavonoid (+) catechin - which can generate a similar 1,2-quinone moiety after oxidation - was evaluated to confirm its ability to undergo similar interactions with glycine and form amino acid adducts. For this purpose high resolution LC/MS was used to assess the formation of non-volatile catechin-amino acid adducts through the interaction with oxidized ring B. This chapter is published in the Journal of Agricultural and Food Chemistry. Reprinted with permission from Guerra, P. V., & Yaylayan, V. A. (2014). Interaction of flavanols with amino acids: Post-oxidative reactivity of B-ring of catechin with glycine. *Journal of Agricultural and Food Chemistry*, *62 (17), 3831-3836.*

CHAPTER 5. INTERACTION OF FLAVANOLS WITH AMINO ACIDS: POST-OXIDATIVE REACTIVITY OF B-RING OF CATECHIN WITH GLYCINE



5.1 Abstract

Flavanol-related structures such as epicatechin and catechins have been associated with potential antioxidant activity in food and are known to interfere with the Maillard reaction through scavenging of reactive dicarbonyl compounds. High resolution ESI-TOF mass spectrometry and isotope labelling technique were used to assess the reactivity of glycine with (+)-catechin heated under oxidative conditions at 120ºC for 70 min. Evidence based on accurate mass analysis of the products obtained and the isotope incorporation pattern of [^{13}C-1]glycine, [^{13}C-2]glycine and [^{15}N]glycine experiments indicated that (+)-catechin formed various adducts with glycine, two of them incorporated a single amino acid and three adducts incorporated two amino acid moieties. Some of these adducts underwent dehydration reaction at ring C and in some the C-ring remained intact. Detailed MS/MS analysis of the fragmentation patterns of these adducts have confirmed the addition of amino acid moieties to the oxidized B-ring of (+)-catechin through formation of Schiff bases. Formation of such non-volatile (+)-catechin/amino acid adducts provides insight into how amino acid can have the potential of modifying the antioxidant properties of (+)-catechin and how catechin in turn has the potential of modifying the profile of the Maillard reaction.

5.2 Introduction

Epicatechin and related phenolic compounds are known to alter the pathways of the Maillard reaction and reduce the intensity of related aroma chemicals as observed in aqueous and low moisture model systems (Totlani et al., 2005; Totlani et al., 2006), as well as in food matrixes such as milk, granola bars and cocoa models (Colahan-Sederstrom et al., 2004). The mechanism behind this interference has been related to the ability of ring A in epicatechin to sequester the reactive dicarbonyl compounds needed for the generation of aroma chemicals, through electrophilic aromatic substitution reactions, thanks to the presence of two highly activating electron donating groups in the *meta* position, in contrast to the *ortho* configuration (Noda & Peterson, 2007; Totlani et al., 2006). Additionally, experiments involving Maillard model systems constituted by hexoses, glycine and epicatechin, showed the reactivity of this flavan-3-ol

with the sugar, but not with glycine, showing no incorporation of glycine carbons into the adducts observed (Totlani et al., 2005; Totlani et al., 2006). Recently, catechins have also been reported as trapping agents for the reactive imine intermediates linked to the non-enzymatic browning (Bin, Peterson & Elias, 2012). As catechins influence the Maillard reaction and flavor formation in food, their antioxidant activity (Sang et al., 2003; Zhu, Huang, Yu, LaVoie, Yang & Ho, 2000) can be equally influenced by the same reaction. The studies performed on the interaction of epicatechin with Maillard reaction precursors have indicated that unlike dicarbonyls the amino acids did not produce any specific effect under the reaction conditions. Considering the widespread occurrence of oxidation reactions in food, information regarding the participation of the oxidized form of catechins in the Maillard reaction could provide tools for better understanding of their chemical behavior under oxidative or non-oxidative conditions. Recently, it has been shown that 4,5-dimethyl-1,2-benzoquinone, a redox active moiety could undergo nucleophilic addition reactions with amino acids and eventually form aminophenols and benzenediamine derivatives (Guerra & Yaylayan, 2013). Given that ring-B is susceptible to oxidation and formation of such *o*-benzoquinone moieties under thermal treatment, the purpose of this study was to evaluate the potential of ring-B of catechins to undergo similar interactions under oxidative conditions, to form adducts with amino acids, as it was observed with the 4,5-dimethyl-1,2-benzoquinone (Guerra et al., 2013).

5.3 Materials and Methods

5.3.1. Reagents and Chemicals

(+)-Catechin hydrate (98%), glycine (99%) and methanol HPLC grade (>99.9%) were obtained from Sigma-Aldrich Chemical Co (Oakville, ON, Canada). The labelled [^{13}C-1]glycine (98%), [^{13}C-2]glycine (99%) and [^{15}N]glycine (98%) were obtained from Cambridge Isotope Laboratories (Andover, MI).

5.3.2. Sample Preparation

(+)-Catechin hydrate (7.6 mg) in distilled water (1000 μL) was heated in an oven alone or in the presence of glycine (2.8 mg) or its labelled analogous (total weight 10.4 mg) in a 1:1.5 molar ratio, using an open vial at 120^{o}C (oven temperature) for 70 min until dry. The dry residue was

diluted in 2 mL of 50 % methanol/water mixture with the help of sonication. Finally, the samples were centrifuged before LC/MS analysis.

5.3.3. High resolution LC/MS

LC-MS experiments were performed on a UPLC coupled to a quadrupole time-of-flight instrument (Synapt G2-S, Waters). Data was acquired in positive electrospray mode. The liquid chromatography was performed on a Hypersil Gold PFP column, particle size 3 µm, 100 L x 2.1 mm (ThermoFisher) using a reverse phase gradient from 5 % to 90 % acetonitrile and a flow rate of 0.3 mL/min. The desolvation gas flow rate was set to 1000 L/h at temperature of 500 °C, the source temperature was 150 °C. The capillary and cone voltages were set at 3 kV and 40 V, respectively. The scan time was 1.0s and the inter-scan delay was 0.010 s. The MS/MS acquisition was performed in fastDDA mode, using a collision energy from 20 V to 35V from QTOF. Structural identification was performed by accurate mass analysis with the aid of ChemCalc.(Patiny & Borel, 2013) in addition to isotope labelling data. Isotope labelling studies were performed through replacing glycine with its labelled analogous [^{13}C-1]glycine, or [^{13}C-2]glycine (99%) or [^{15}N]glycine (98%) in the model systems mentioned above. Furthermore, the MS/MS data were compared with previously reported (Horai et al., 2010) mass spectra of (+)-catechin. The correspondence of the peaks to those generated through labelling experiments was verified through matching their retention times on Hypersil Gold PFP liquid chromatography column.

5.4 Results and Discussion

Although the interaction of ring A of catechin with the Maillard reaction intermediates such as 1,2-dicarbonyl compounds is well established (Totlani et al., 2005; Totlani et al., 2006), however, the interaction of catechins with amino acids has not been observed or reported yet mainly due to the fact that such studies have been conducted in the absence of air or under nitrogen and in theory, an oxidation step is necessary to generate reactive *o*-quinonoidal moieties (Guerra et al., 2013) for their interaction with amino acids. In this study, (+)-catechin hydrate was heated alone or in the presence of glycine under oxidative conditions (open flask) and the reaction mixtures were analyzed by high resolution ESI TOF/MS. Comparison of the reaction

profiles of the two systems have indicated the formation of new chromatographic peaks in the (+)-catechin hydrate/glycine model system having masses higher than that of (+)-catechin. Furthermore, isotope labelling studies with [^{13}C-1]glycine, [^{13}C-2]glycine and [^{15}N]glycine indicated the incorporation of one or two glycine moieties in five of the adducts identified, as shown in Figure 12 and Table 9 (structures **1** to **5**). Two of these adducts (**1** and **5**) underwent dehydration of ring-C of (+)-catechin and in the remaining three adducts (**2**, **3** and **4**) ring C remained intact.

Table 9. Percent incorporation of labelled glycine atoms into structures **1** to **5** generated from glycine/(+)-catechin model system.

		[^{13}C-1]-glycine				**[^{13}C-2]-glycine**				**[^{15}N]-glycine**			
Structure[a]	***m/z***	**M**	**M+1**	**M+2**	**Error[a] (ppm)**	**M**	**M+1**	**M+2**	**Error[a] (ppm)**	**M**	**M+1**	**M+2**	**Error[a] (ppm)**
1	328	0	100	0	12	0	100	0	12	0	100	0	33
2	348	0	100	0	12	0	100	0	12	0	100	0	32
3	304	100	0	0	20	0	100	0	20	0	100	0	34
4	405	0	0	100	25	0	0	100	26	0	0	100	6.6
5	341	0	100	0	12	0	0	100	25	0	0	100	64

[a] Error in the calculation of molecular formulas with label incorporation

5.4.1. Interaction pathways of glycine with oxidized B-ring of catechin

The ability of glycine to undergo either single or double addition reactions with 1,2-dicarbonyl compounds in general (Chu et al., 2011) and with *o*-quinonoidal moieties in particular has been observed previously (Guerra et al., 2012; Guerra et al., 2013). Detailed analysis of the adducts listed in Table 9 and shown in Figure 12, have indicated that the structure **1** (shown as one possible isomer) observed at *m/z* 328 corresponds to a single addition product of glycine to an oxidized (+)-catechin with subsequent dehydration of ring C.

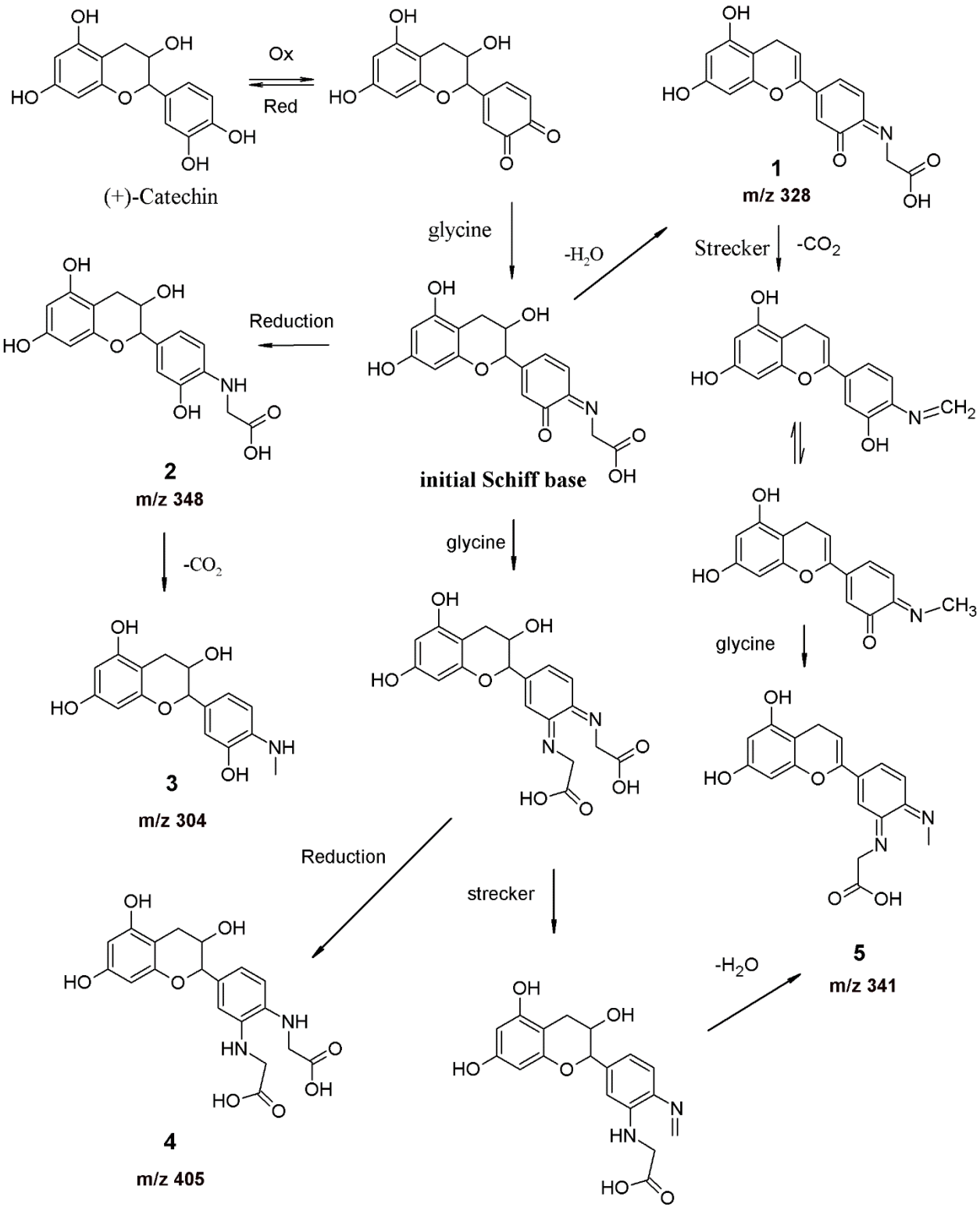


Figure 12. Proposed mechanism of (+)-catechin-glycine adducts formation. (For structures **1**, **2**, **3** and **5** only one isomer is shown out of possible two isomers).

Alternatively, the initial Schiff base adduct (see Figure 12) can either undergo reduction to generate structure **2** at *m/z* 348 or can react with a second molecule of glycine and then undergo reduction this time generating the structure **4** at *m/z* 405. In turn, structure **2** can undergo decarboxylation to generate structure **3** at *m/z* 304. Finally, the structure **1** can undergo Strecker decarboxylation followed by isomerization of ring B and addition of second molecule of glycine to generate structure **5** at *m/z* 341. The high resolution mass spectral data obtained (see Table 10) for structures **1** through **5** confirmed their elemental composition consistent with the proposed structures. In addition, the label incorporation data using [^{13}C-1]glycine, [^{13}C-2]glycine and [^{15}N]glycine further supported the proposed structures (see Table 9).

Table 10. Calculated elemental composition of (+)-catechin-glycine adducts.

Structure	*m/z*	M	Elemental composition	Error[a] (ppm)
(+)Catechin	291	291.0812	$C_{15}H_{14}O_6$	7.5
1	328	328.0755	$C_{17}H_{13}NO_6$	3.7
2	348	348.1015	$C_{17}H_{17}NO_7$	2.9
3	304	304.1123	$C_{16}H_{17}NO_5$	5.4
4	405	405.1216	$C_{19}H_{20}N_2O_8$	0.91
5	341	341.1072	$C_{18}H_{16}N_2O_5$	3.7

[a] Error in the calculation of elemental composition

5.4.2. MS/MS analysis of (+)-catechin-glycine adducts

To provide additional detailed evidence for the proposed structures, the characteristic ion fragmentation patterns previously reported for catechin (Cren-Olivé, Déprez, Lebrun, Coddeville & Rolando, 2000) and for flavones and flavonols (Ma, Li, Van den Heuvel & Claeys, 1997) were compared with those observed for structures **1** to **5**. In general, catechins undergo MS/MS

fragmentations when exposed to collision energies higher than 10 V through bond cleavages of the central ring C and generating fragments that retain either ring A or ring B moieties (see Figure 13). According to the accepted nomenclature rules, a fragment generated under positive ion mode and retaining the ring B moiety through cleavage of bonds 0 and 2 of the ring C could be designated as fragment $^{0,2}B^+$ (see Figure 13). Such B^+ fragments could be used as diagnostic ions for the formation of amino acid adducts at ring B (in this case it was termed as B_i^+ where i = 1, 2, 3 etc) and A^+ fragments could be used to confirm the nature of adducts as originating from catechin moiety.

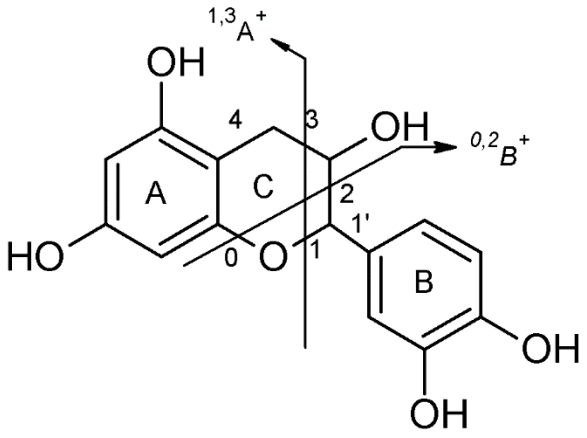


Figure 13. Nomenclature and C-ring bond cleavages in catechin.

Among the five structures reported in Table 9 the adducts with non-dehydrated C-rings (structures **2** to **4)** showed the characteristic A^+ and B^+ fragments of catechin and on the other hand, the structures that underwent dehydration at C-ring (**1** and **5**) showed only fragmentations originating from amino acid moiety such as decarboxylation and Strecker type fragmentations (see Table 11).

Table 11. Characteristic MS/MS fragmentations observed in structures **1** to **5**.

Structure	Fragmenting moiety	Fragmentation type	Daughter ions[a]
1	Amino acid	Decarboxylation Strecker degradation	283 (7.5) 269 (60), 270 (10), 329 (20)
2	Ring C	A^+ B^+	123 (30) 226 (8), 180 (15), 196 (100), 150 (90)
3	Ring C	A^+ B^+	123 (10) 152 (100), 137 (25), 138 (75)
4	Ring C	B^+	253 (100), 207 (10), 162 (10), 147 (15)
5	Amino acid	Decarboxylation Strecker degradation	297 (65) 282 (100), 283 (15), 342 (15)

[a]Relative intensities are reported in parenthesis

These observations are consistent with the fact that A and B series ions result from bond cleavages of the intact ring C. Table 12 to Table 16 show the major MS/MS fragments for structures **1** to **5** and their isotope incorporation patterns and Figure 14 to Figure 18 show the proposed fragmentation pathways (accurate mass analysis showed errors less than 26 ppm for all proposed structures). It is interesting to note that none of the structures exhibited the known *retro-Dields-Alder* reaction route of flavonoid fragmentation (Ma et al., 1997) to generate $^{1,3}A^+$ or $^{1,3}B^+$ fragments. In addition, as expected the structures with dehydrated C-ring, **1** and **5** did not show any of the characteristic fragmentations of catechins but exhibited fragmentations characteristic of amino acid derivatives (see Table 12 and Table 16, as well as Figure 14 and Figure 18).

Table 12. MS/MS data of m/z 328.0755 (structure **1**[a]).

m/z	Relative intensity (%)	Elemental composition[b]	Error (ppm)	Label incorporation from glycine
328.0859	100	$C_{17}H_{14}NO_6$	12	1 x C-1; 1 x C-2; 1 x N
283.0839	7.5	$C_{16}H_{13}NO_4$	1.9	1 x C-2; 1 x N
270.0739	10	$C_{15}H_{12}NO_4$	10	1 x N
269.0710	60	$C_{15}H_{11}NO_4$	1.8	1 x N

[a]see Figure 1; [b]see Figure 3 for corresponding proposed structures.

Table 13. MS/MS data of *m/z* 348.1015 (structure **2**[a]).

m/z	Relative intensity (%)	Elemental composition[b]	Error (ppm)	Label incorporation from glycine
348.1091	5	$C_{17}H_{18}NO_7$	2.2	1 x C-1; 1 x C-2; 1 x N
226.0725	8	$C_{10}H_{12}NO_5$	4.2	1 x C-1; 1 x C-2; 1 x N
196.0633	100	$C_9H_{10}NO_4$	12	1 x C-1; 1 x C-2; 1 x N
180.0665	15	$C_9H_{10}NO_3$	2.4	1 x C-2; 1 x N
150.0562	90	$C_8H_8NO_2$	4.6	1 x C-2; 1 x N
147.0462	55	$C_9H_7O_2$	11	-
123.0457	30	$C_7H_7O_2$	8.9	-

[a]see Figure 1; [b]see Figure 4 for corresponding proposed structures of selected ions.

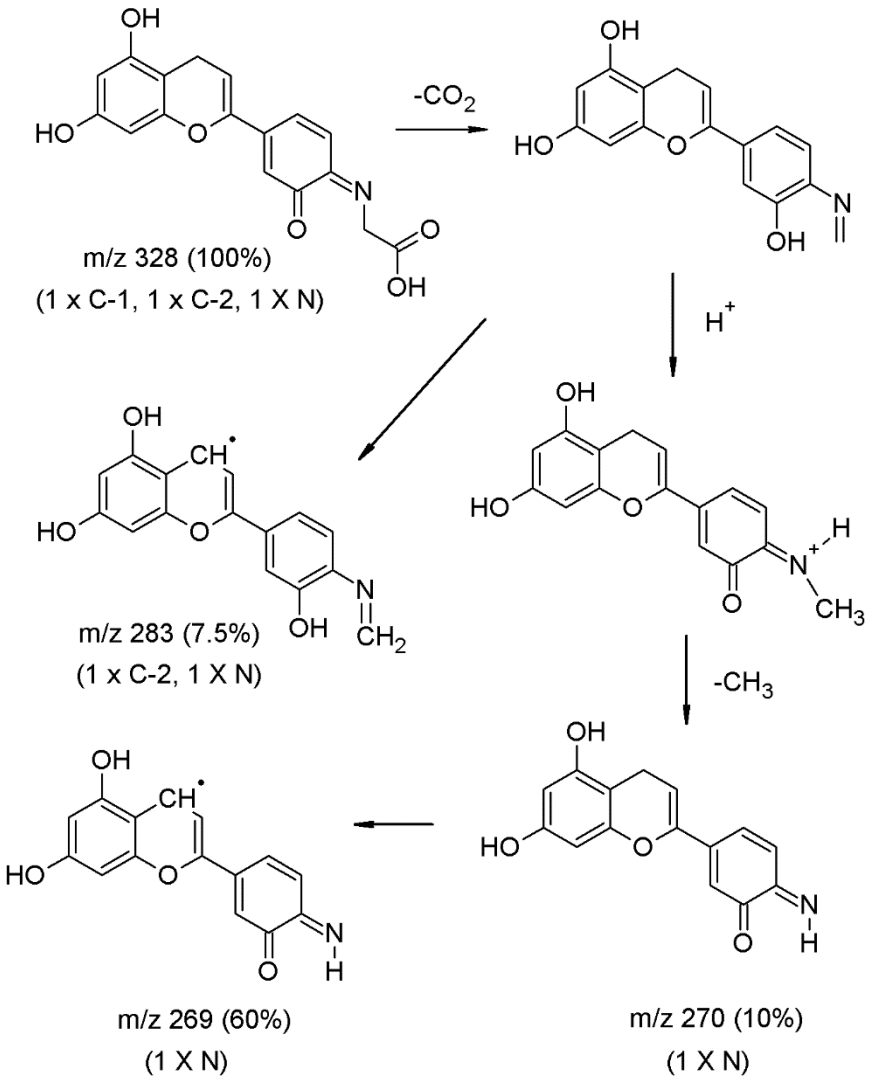


Figure 14. Proposed mass spectral fragments for structure **1.** Values in parenthesis indicate the labelled atoms incorporated from glycine.

5.4.3. Characteristic MS/MS fragmentations of structures 2, 3 and 4

Due to the presence of intact ring C in structures **2**, **3** and **4** it was not surprising to observe the characteristic fragmentations of catechin in these structures (see Figure 15 toFigure 17 and Table 13 toTable 15). The fragmentations initiated through the cleavage of C-ring bonds can generate fragment ions retaining A and B ring moieties. In structure **2**, **3** and **4** the $^{0,2}B^{+}$ series fragmentations generated the base peaks at *m/z* 196, *m/z* 152 and *m/z* 253 respectively and in general B series ions were found to predominate, allowing direct confirmation of the incorporation of glycine into the ring B through isotope labelling studies. Structures **2** and **3** also

exhibited the A series fragmentations further confirming the flavonoid origin of these structures. It is worth mentioning that the absence of previously observed retro-Diels-Alder fragmentation pathway (Ma et al., 1997) could be due to the modifications in ring B structure induced by glycine.

Table 14. MS/MS data of *m/z* 304.1123 (structure **3**[a]).

m/z	Relative intensity (%)	Elemental composition[b]	Error (ppm)	Label incorporation from glycine
304.1192	15	$C_{16}H_{18}NO_5$	2.3	1 x C-2; 1 x N
182.0804	5	$C_9H_{12}NO_3$	7.2	1 x C-2; 1 x N
152.0726	100	$C_8H_{10}NO_2$	9.5	1 x C-2; 1 x N
147.0462	15	$C_9H_7O_2$	11	-
138.0556	75	$C_7H_8NO_2$	0.69	1 x N
137.0475	25	$C_7H_7NO_2^{\bullet}$	1.3	1 x N
123.0457	10	$C_7H_7O_2$	8.9	-
109.0542	20	$C_6H_7NO^{\bullet}$	13	1 x N

[a]see Figure 1, [b]see Figure 5 for corresponding proposed structures of selected ions.

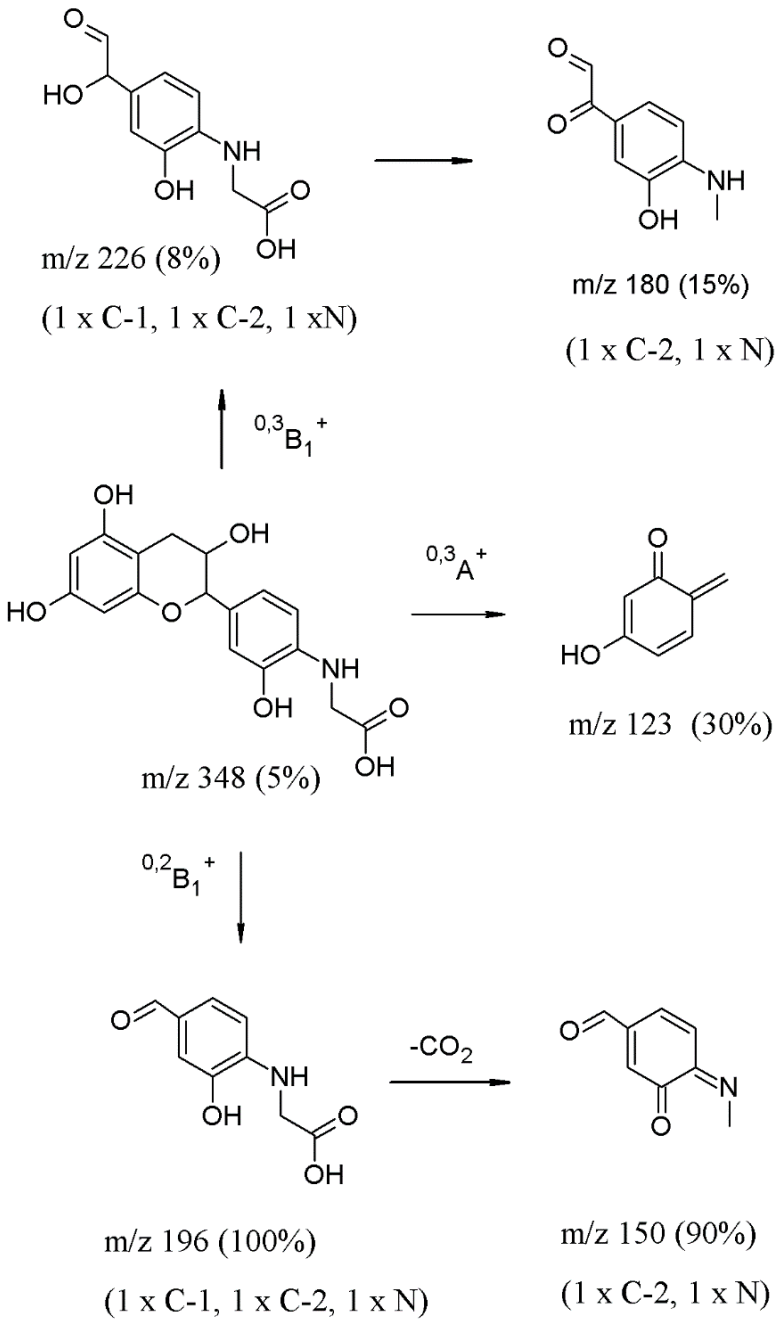


Figure 15. Proposed mass spectral fragments for structure **2**. Values in parenthesis indicate the labelled atoms incorporated from glycine.

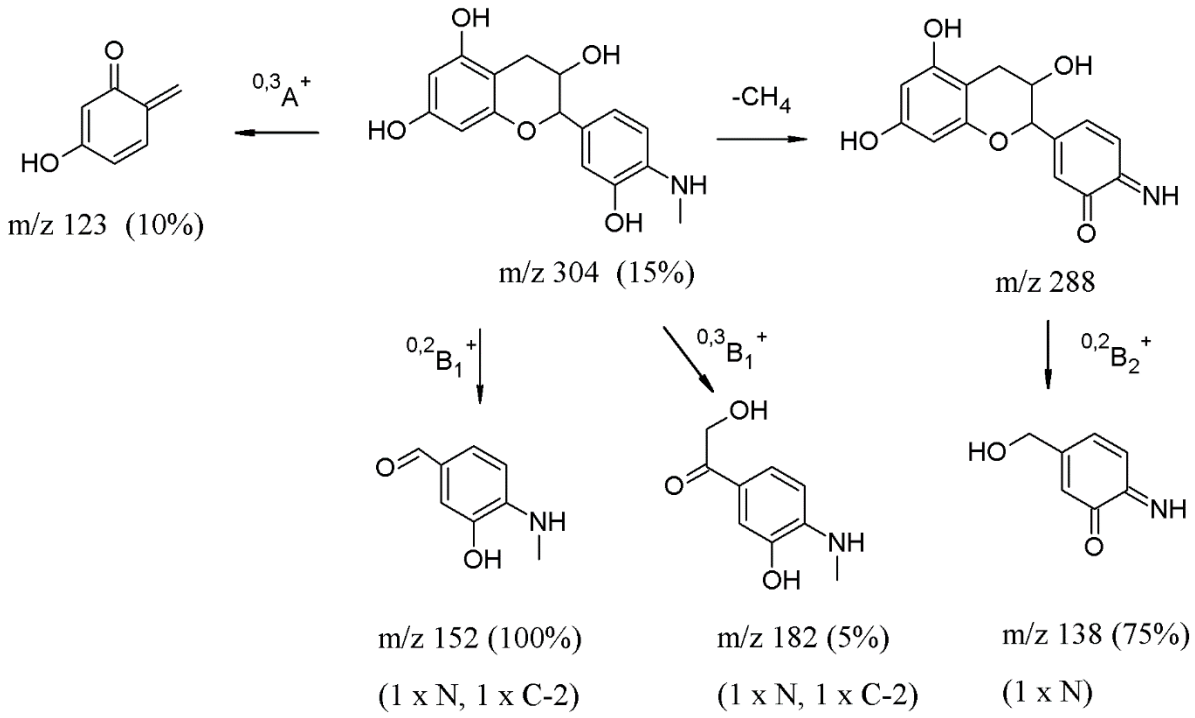


Figure 16. Proposed mass spectral fragments for structure **3**. Values in parenthesis indicate the labelled atoms incorporated from glycine.

5.4.4. Characteristic MS/MS fragmentations of structures 1 and 5

The dehydration of ring C in the structures **1** and **5** precludes them from undergoing fragmentations initiated through the cleavage of C-ring bonds and as a result they exhibit fragmentations initiated by the amino acid moiety such as decarboxylation and loss of alkyl groups of amino acids preserving in these ions the integrity of rings A, B and C (see Figure 14 and Figure 18, as well as Table 12 and Table 16). Interestingly, both structures **1** and **5** generated very stable 4*H*-chromen-4-yl radicals at m/z 269 and m/z 282 respectively as major radical ions in their MS/MS spectra. These radicals are stabilized through conjugation across both rings A and B. Formation of such radical cations under electrospray ionization conditions has been reported in the literature (Meyer & Metzger, 2003).

Table 15. MS/MS data of *m/z* 405.1216 (structure **4**[a]).

m/z	Relative intensity (%)	Elemental composition[b]	Error (ppm)	Label incorporation from glycine
405.1322	1	$C_{19}H_{21}N_2O_8$	25	2 x C-1; 2 x C-2; 2 x N
302.1061	20	$C_{16}H_{17}NO_5$	11	1 x C-2; 1 x N
254.0836	10	$C_{11}H_{13}N_2O_4O^{\bullet}$	26	2 x C-1; 2 x C-2; 2 x N
253.0831	100	$C_{11}H_{13}N_2O_5$	2.6	2 x C-1; 2 x C-2; 2 x N
207.0766	10	$C_{10}H_{11}N_2O_3$	1.7	1 x C-1; 2 x C-2; 2 x N
180.0665	10	$C_9H_{10}NO_3$	2	1 x C-2; 1 x N
162.0565	10	$C_9H_8NO_2$	6.1	1 x C-2; 1 x N
147.0462	15	$C_9H_8O_2$	11	-

[a]see Figure 1, [b]see Figure 6 for corresponding proposed structures of selected ions.

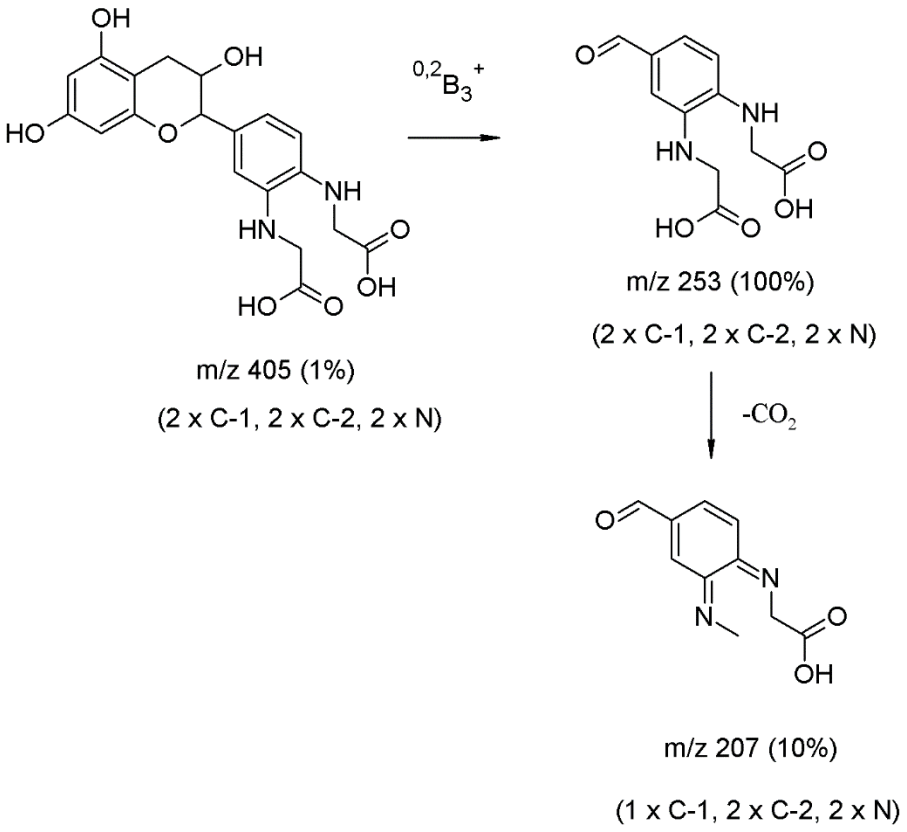


Figure 17. Proposed mass spectral fragments for structure **4**. Values in parenthesis indicate the labelled atoms incorporated from glycine.

It appears from the structural analysis of the products observed, that the reaction of glycine and the oxidized form of catechin follows a similar pattern to that of other 1,2-dicarbonyls and leads to the formation of single and double addition products such as structures **2** and **4**. It seems that the initial double Schiff base adduct on ring B is prone to reduction through disproportionation reaction coupled to catechin oxidation. Structures **2**, **3** and **4** are such examples in which the oxidized moiety of catechin is reduced back to its original oxidation state, and structures **1** and **5** represent examples of reaction products in which the reduced moiety is retained, most probably due to the additional stability gained through dehydration of the hydroxyl group in ring C. Formation of such non-volatile (+)-catechin/amino acid adducts provides insight into how amino acids can have the potential of modifying the antioxidant properties of (+)-catechin through restoration of the oxidation state of ring-B and how catechin-type structures interfere with the Maillard reaction in food through scavenging of amino acids.

Table 16. MS/MS data of *m/z* 341.1072 (structure **5**[a]).

m/z	Relative intensity (%)	Elemental composition[b]	Error (ppm)	Label incorporation from glycine
341.1109	70	$C_{18}H_{17}N_2O_5$	8.3	1 x C-1; 2 x C-2; 2 x N
297.1214	65	$C_{17}H_{16}N_2O_3$	8.5	2 x C-2; 2 x N
283.1059	15	$C_{16}H_{15}N_2O_3$	8.4	1 x C-2; 2 x N
282.1010	100	$C_{16}H_{14}N_2O_3$	1.9	1 x C-2; 2 x N

[a]see Figure 1, [b]see Figure 7 for corresponding proposed structures.

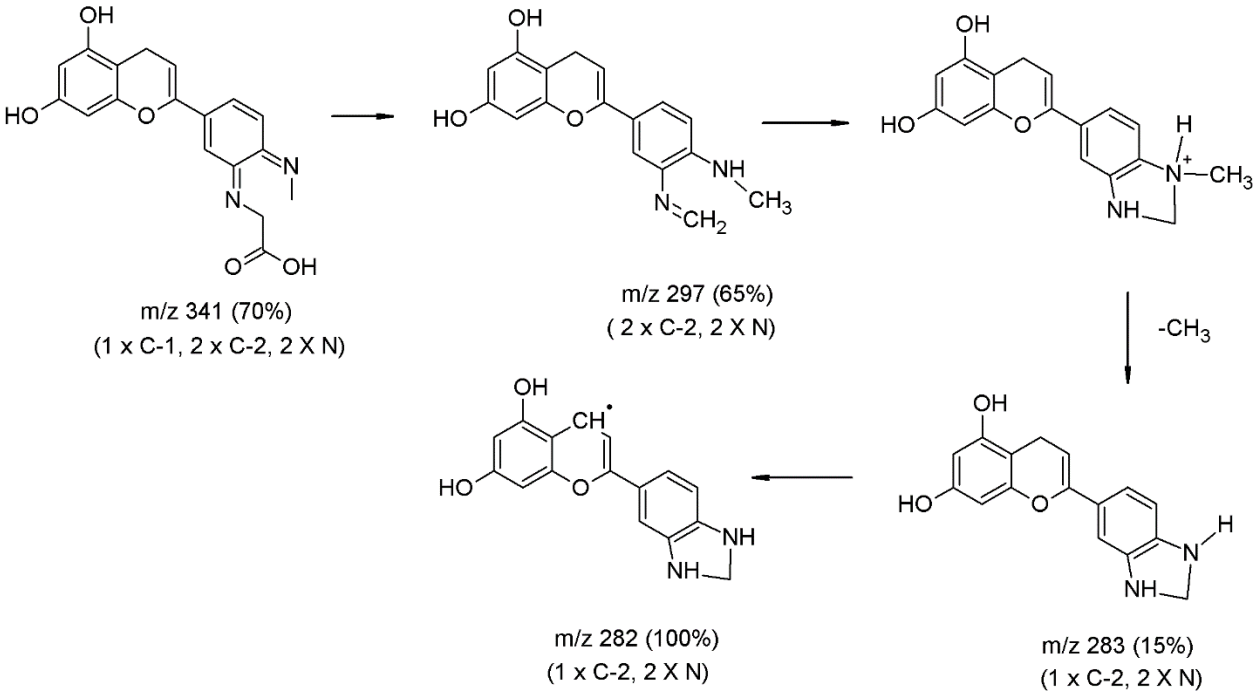
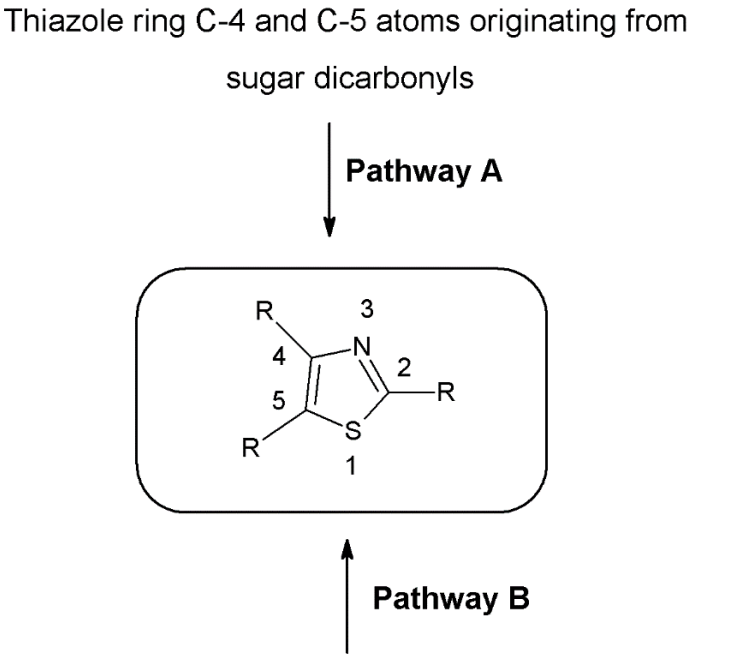


Figure 18. Proposed mass spectral fragments for structure **5**. Values in parenthesis indicate the labelled atoms incorporated from glycine.

CONNECTING PARAGRAPH

In previous chapters a detailed study of the ability of 1,2-dicarbonyl moieties to participate in double addition reactions and formation of double Schiff base adducts with a single amino acid was presented. Chapter 6 intends to provide a broader understanding of this principle and to demonstrate the ability of two different amino acids to undergo double addition reaction with sugar derived α-dicarbonyl moieties through a preliminary investigation of the volatile and non-volatile products formed in a model system of two amino acids with different side chains, glycine and cysteine, and glucose. The content of this chapter is currently in preparation to be submitted for publication.

CHAPTER 6. SYNERGISTIC ROLE OF MULTIPLE AMINO ACIDS IN THE GENERATION OF MAILLARD REACTION PRODUCTS FROM CYSTEINE AND GLYCINE



6.1 Abstract

Mechanistic studies of the Maillard reaction have been mainly focused on the evaluation of the interaction of a single sugar with a single amino acid. However, in real food systems, this reaction constitutes an intricate matrix of chemical transformations involving many amino acids, consequently, this type of simple modelling fails to extract significant amount of relevant information. Model systems with multiple amino acids consisting of labelled and unlabelled glycine, cysteine and glucose were pyrolyzed at 250^{o}C for 20 s, or heated in an aqueous solution for 90 min at 110^{o}C in an open container and the dry residue was subsequently analyzed by gas chromatography/mass spectrometry and ESI/TOF high resolution liquid chromatography. Seven thiazoles and one non-volatile products were selected for mechanistic studies. Evidence based on the mass analysis of the products obtained and the isotope incorporation pattern indicated the existence of two different pathways for the formation of thiazoles, differing in the origin of the thiazole ring carbon atoms. On the other hand, the non-volatile products observed, provided evidence for the first time of the occurrence of a double Amadori rearrangement first initiated by glycine and followed by cysteine.

6.2 Introduction

The Maillard reaction is a complex chain of reactions initiated with the interaction of amino groups with reducing sugars. It is one of the main reactions occurring in food under processing conditions and it is directly responsible for the generation of various aroma and taste active compounds, antioxidants and toxicants, in addition to brown polymers called melanoidins. The detailed knowledge of the Maillard reaction is the key element in the successful development of desirable food products with reduced amounts of process-induced toxicants or with higher levels of antioxidants and desired aromas. The use of "model systems" helps to simplify the complexity of the reaction and to study systematically the influence of different sugars, amino acids and other food matrix components on the final outcome of the reaction. In the past, studies with model systems have generated a wealth of information regarding the role of individual amino acids and demonstrated how different amino acids give rise to diverse and specific compounds.

However, only few studies have been reported using more than one amino acid to take into account its impact on the formation routes within the Maillard reaction (Hidalgo, Alcón & Zamora, 2013; Shu, 1999; Xu et al., 2013). However, none of these studies have indicated the simultaneous interaction of two free amino acids with a sugar or their derivatives. Glycine has been frequently used in Maillard reaction due to the simplicity of its side chain. On the other hand, the sulfur containing amino acid cysteine has been the focus of many meat related flavor research. Its relevance over methionine (other sulphur containing amino acid and also related to meat-like aromas), or over other amino acids, is its ability to produce hydrogen sulfide, ammonia and acetaldehyde in addition to the corresponding Strecker degradation products: aldehyde and α-aminoketone (Pripis-Nicolau, de Revel, Bertrand & Maujean, 2000). Evidence of the generation of pyruvic acid after the loss of a hydrogen sulfide molecule and acrylic acid after a dehydration step was reported by Yaylayan and colleagues (2005a), when studying the role of cysteine as a precursor of acrylamide formation. Cysteine participation in the Maillard reaction has mainly been focused on the study of the volatile reaction products, where thiophenes, thiazoles, polysulfides, pyrazines, pyroles, oxazoles and furans, among others have been observed from the reaction of cysteine and a sugar (Cho, Lee, Jun, Roh & Kim, 2010; Meynier & Mottram, 1995; Tai et al., 1997; Umano, Hagi, Nakahara, Shyoji & Shibamoto, 1995; Zhang & Ho, 1991). Pyrazines, thiazoles and oxazoles have been reported to be associated with roasted aromas in meat products. Thiazoles have been reported to have lower odor thresholds (Mottram, 1998). Although they are known to be formed from the non-enzymatic interaction of sugars and amino acids, their mechanism of formation is not fully understood to this day. Most authors have attributed their formation to the interaction of free hydrogen sulfide and ammonia with α-dicarbonyl, hydroxyketones or an aldehyde (Mottram, 1998; Yu, Tan & Wang, 2012), which was first proposed by G. Vernin in 1982. Alternatively, Mulders (1973) proposed a route for the formation of acyl thiazoles, through the reaction of α-dicarbonyl with cysteine followed by a decarboxylation and the ring closure to form a thiazolidine that can be oxidized into acyl thiazole. In addition to the volatile products of Maillard models of cysteine, there are only few publications reporting the non-volatile compounds from this model system (Cerny & Guntz-Dubini, 2013; Ota, Kohmura & Kawaguchi, 2006). Among them, Ota and colleagues (2006) characterized a product of the interaction between Amadori compounds derived from disaccharide and cysteine, which lead to the formation of known aroma compounds. The main

goal of the present research is to develop a comprehensive evaluation of the volatiles and non-volatile compounds formed from the interaction of glycine and cysteine in a multiple amino acid model system with a single sugar in an attempt to develop a more accurate understanding of the interactions of the amino acids within real food models. It is feasible that such an approach will provide more accurate means to propose alternatives for the production of specific food characteristics.

6.3 Materials and Methods

6.3.1. Reagents and Chemicals

Glycine (99%), L-cysteine (97%), D-(+)-glucose (>99.5%), glycine hydrochloride (99%), glycine sodium salt (98%), cysteine hydrochloride monohydrate (>98%), potassium hydroxide (85%), paraformaldehyde (95%), acetaldehyde (>99.5%) and methanol HPLC grade (>99.9%) were obtained from Sigma-Aldrich Chemical Co (Oakville, ON, Canada). The labelled [^{13}C-1]glycine (98%), [^{13}C-2]glycine (99%), [^{15}N]glycine (98%), [^{13}C-3]cysteine (99%), [^{15}N]cysteine (99%) and [U6]glucose (99%) were obtained from Cambridge Isotope Laboratories (Andover, MI).

6.3.2. Sample Preparation

Non-volatile products were studied by LC-MS analysis of either a pyrolysed mixture of glucose, cysteine and glycine in a 2:1:1 molar ratio, or by the oven heating of a mixture of glucose, cysteine and glycine (2:1:1 molar ratio, total weight 10 mg) heated in distilled water (1000 μL) in an open vial at 110^{o}C (oven temperature) for 90 min until dry. Both dry residues were individually diluted in 2 mL of 50% methanol/water mixture with the help of sonication. The samples were centrifuged before LC-MS analysis. The control model system consisted on a 1:1 amino acid to sugar mixture (total weight 10 mg) in distilled water (1000 μL). The GC-MS studies, for the volatile counterpart, were carried out by pyrolyzing at 250^{o}C for 20 s a 1:1:2 mixture glycine to cysteine to sugar. The control models where form of 1:1:1 of amino acid to sugar to silica gel, this last one to compensate the absence of the second amino acid. A total of 1.0 mg of sample mixtures were packed inside a quartz tube (0.3mm thickness), plugged with quartz wool. Additionally, the model system was also studied under acid/basic catalytic

conditions, with the aid of KOH diluted with silica and the substitution of cysteine for cysteine hydrochloride. In both cases, volatile and non-volatile studies, the label analysis was done with the substitution of glycine with its labelled counterpart.

6.3.3. High resolution LC/MS analysis

Experiments were performed on a UPLC system coupled to a quadrupole time-of-flight instrument Synapt G2-S (Waters, Milford, MA). Data was acquired in positive electrospray mode. The liquid chromatography was performed on a Cortecs Hilic column (2.1 x 100 mm, 1.6 μm) from Waters. The mobile phase consisted of 100 mM ammonium formate (A) and acetonitrile (B). A linear gradient was applied with initial conditions being 90 % B for 3 minutes and final conditions to 30 % B reached in 5 minutes. Final conditions were kept for 0.5 min and column was equilibrated back at initial conditions for a total run time of 10 minutes. The flow rate was 0.5 mL/min and injection volume was 1μL. The desolvation gas flow rate was set to 1000 L/h at temperature of 500 °C, the source temperature was 150 °C. The capillary and cone voltages were set at 3 kV and 40 V, respectively. The MS/MS scan time was 0.1 s and the inter-scan delay was 0.015 s. The MS was operated in Data Dependent Mode. Both survey scan and MS/MS scan were acquired from 50 to 500 Da. Collision energy was ramped from 15 to 50 eV at low mass and from 25 to 60 eV at high mass. Up to 10 functions were used for MS/MS acquisition. Structural identification was performed by accurate mass analysis with the aid of ChemCalc (Patiny et al., 2013) in addition to isotope labelling data. Isotope labelling studies were performed through replacing glycine with its labelled analogous [^{13}C-1]glycine, or [^{13}C-2]glycine or [^{15}N]glycine, or glucose with [U6-^{13}C]-glucose in the model systems mentioned above.

6.3.4. Pyrolysis-Gas Chromatography-Mass Spectrometry (Py-GC/MS)

The analysis was performed according to procedure described by Chu and Yaylayan (Chu et al., 2009b) with some modifications. A Varian CP-3800 GC was coupled to a Varian Saturn 2000 Mass Spectrometry detector (Varian, Walnut Creek, CA, USA). The pyrolysis unit included a valved interface (CDS 1500), which was installed onto the GC injection port and connected to a CDS Pyroprobe 2000 unit (CDS Analytical, Oxford, PA, USA). The sample packed in the quartz tube was inserted inside the coil probe and pyrolyzed at 250^{o}C for 20 seconds under Helium

atmosphere. The volatiles were directed towards a DB-5MS column (5 % diphenyl, 95 % dimethyl-polysiloxane) with dimensions of 50 m length x 0.2 mm internal diameter x 33μm film thickness (J&W Scientific, ON, Canada). The carrier gas employed was helium. Its flow rate was regulated by an Electronic Flow Controller (EFC) the helium pressure was increased at a rate of 400 psi/min from 5 to 70 psi and held for 6 minutes. The pressure was then decreased at the same rate until reaching 33psi and held for 5 minutes, and finally increased again to 50psi at a rate of 3.5 psi/min. The GC oven temperature was set to -5 º C during the first 5 minutes using CO_2 as the cryogenic cooling source and then was increased to 50 º C at a rate of 50 º C/min. Then, the oven temperature was increased to 270º C at a rate of 8 º C/min and held for 5.40 min. The mass spectrometry detector used was an ion-trap mass spectrometer. The MS transfer-line temperature was set to 250ºC, manifold temperature was set to 50ºC and the ion trap temperature was set to 175ºC. The ionization voltage of 70eV was used and EMV was set to 1500V. Structural identification was performed using AMDIS (v.2.65) and NIST Standard Reference Databases (data v. NIST05 and software v. 2.0d) in addition to isotope labelling data. Isotope labelling studies were performed by replacing glycine with its labelled analogous [^{13}C-1]glycine, or [^{13}C-2]glycine (99%) or [^{15}N]glycine (98%), by replacing cysteine with its labelled analogous [^{13}C-3]cysteine, or [^{15}N]cysteine, or glucose with [U6-^{13}C]-glucose in the model systems mentioned above.

6.4 Results and Discussion

The evaluation of the volatile and non-volatile products of multiple-amino acid containing model systems is expected to lead to the identification of new products or new pathways from the simultaneous interaction of two amino acids with glucose or its degradation products. In the current study of the cysteine/glycine/glucose multiple amino acid model system a comparative evaluation of both volatile and non-volatile fractions allowed the identification of seven volatiles (see Table 17) and various non-volatile compounds (in this chapter only one non-volatile example was studied in detail – see Table 20) that incorporated atoms from the two amino acids and the sugar backbone indicating the participation of both amino acids in the generation of Maillard reaction products.

Table 17. Compounds observed or enhanced in model systems with two amino acids

Compound	Molecular weight	t_R (min)	Free	Na salt	HCl salt
Thiazole (**1**)	85	11.28	+[a]	+[a]	+[a]
2-methylthiazole (**2**)	99	13.00	+[a]	+[a]	+[a,b]
4-methylthiazole (**3**)	99	13.25	+[a]	+	-
5-methylthiazole (**4**)	99	13.93	+[a]	+[a]	+
5-ethylthiazole (**5**)	113	15.72	+[a]	+	+[a]
2,4-dimethyl-thiazole (**6**)	113	20.58	+	+	-
1,5,6-trimethyl-2(1*H*)-pyrazinone (**7**)	138	22.18	+	+	+

[a] Enhanced in the model system with two amino acids when compared to single amino acid systems

[b] Observed in the cysteine.HCl hydrate/glycine, but not in cysteine/glycine.HCl

6.4.1. Volatile products of cysteine/glycine/glucose

The multiple amino acid model system was evaluated using free amino acids and their corresponding hydrochloride and sodium salts in the presence of glucose via Py-GC/MS analysis. Based on the comparison of the chromatographic peaks generated from single amino acid model systems versus those observed in the presence of both amino acids, peaks were selected for further study based on their appearance or enhancement in the multiple amino acid model systems (see Table 17). Six out of the seven volatile compounds identified were thiazole derivatives and one was a pyrazinone as shown in Table 17. Furthermore, Table 18 and Table 19 show the incorporation of various atoms from individual amino acids and sugar backbone confirming their involvement in the formation of thiazoles and the pyrazinone.

Table 18. Percent incorporation of [^{13}C-2]- and [^{15}N]Glycine atoms in the volatiles formed in cysteine/glycine/glucose model system

	[^{13}C-1]glycine			**[^{13}C-2]glycine**			**[^{15}N]glycine**	
Compound	**M**	**M+1**	**M+2**	**M**	**M+1**	**M+2**	**M**	**M+1**
Thiazole	100	0	0	14	46	40	65	30
2-methylthiazole	94	2	4	90	6	4	82	15
4-methylthiazole	95	0	5	57	38	5	67	30
5-methylthiazole	100	0	0	10	45	45	65	32
5-ethylthiazole	100	0	0	56	41	3	73	27
2,4-dimethyl-thiazole	100	0	0	15	80	5	100	0
1,5,6-trimethyl-2(1*H*)-pyrazinone	100	0	0	0	100	0	0	100

Table 19. Percent incorporation of [^{13}C-3]-, [^{15}N]cysteine and [U_6]-glucose atoms into the volatiles formed in cysteine/glycine/glucose model system

	[^{13}C-3]cysteine			**[^{15}N]cysteine**			**[U6]glucose**				
Compound	**M**	**M+1**	**M+2**	**M**	**M+1**	**M+2**	**M**	**M+1**	**M+2**	**M+3**	**M+4**
Thiazole	4	47	49	0	61	35	89	10	0	1	0
2-methylthiazole	9	49	39	18	77	0	53	5	42	0	0
4-methylthiazole	74	23	3	32	67	0	0	10	6	50	34
5-methylthiazole	16	83	0	10	90	0	45	55	0	0	0
5-ethylthiazole	0	22	76	21	79	0	55	34	7	4	0
2,4-dimethyl-thiazole	0	86	13	0	100	0	19	0	82	0	0
1,5,6-trimethyl-2(1*H*)-pyrazinone	6	95	0	0	100	0	0	0	8	91	0

In general glycine hydrochloride generated more peaks when reacted with cysteine and glucose, compared with free glycine or sodium glycinate models. The formation of substituted thiazoles shown in Table 17 could be explained by two general pathways **A** and **B** (see Figure 19 and Figure 20) based on the label substitution pattern shown in Table 18 and Table 19. Pathway **A** leads to the incorporation of sugar carbon atoms and pathway **B** leads to the incorporation of C-2' and C-3' carbon atoms of cysteine at C-4 and C-5 thiazole ring carbon positions (see Figure 19). As shown in Figure 20, both pathways need aldehydes such as Strecker aldehydes to furnish C-2 atom of the thiazole ring and the various ring substituents. In general, the interaction of aldehydes with hydrogen sulfide and ammonia has been reported as the source for thiazoles formation (Mottram, 1998; Yu & Zhang, 2010), however an alternative mechanism involving the interaction of aldehydes with cysteine was identified as pathway **B** (see Figure 20). A similar route was previously proposed by Mulders (1973) for the formation of acyl thiazoles, but no confirmation was performed and it has not been previously associated as a possible route for the formation of alkyl thiazoles. Additional tests were performed by spiking the model system of cysteine/glycine hydrochloride/glucose with paraformaldehyde to confirm the involvement of formaldehyde in the formation mechanism of 5-methylthiazole. As expected, its abundance was increased with the addition of formaldehyde.

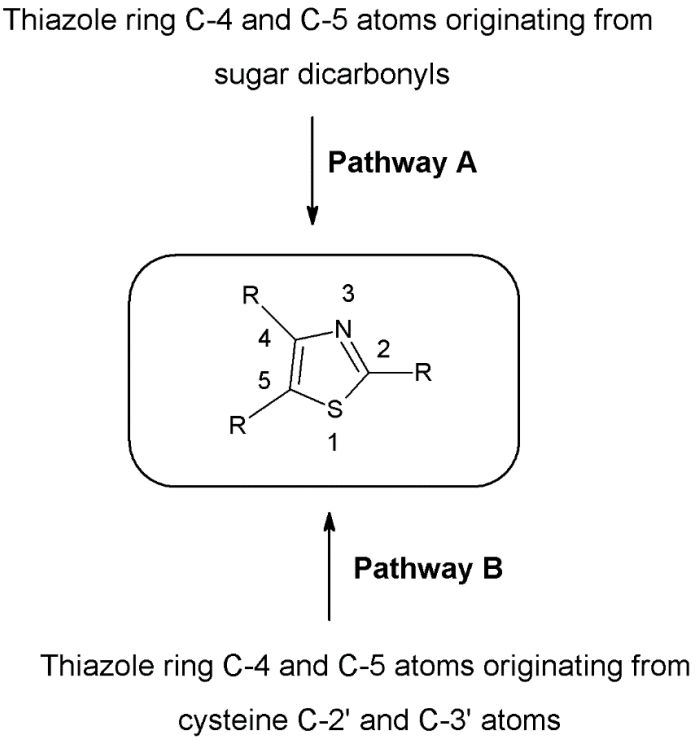


Figure 19. General pathways of formation of thiazoles

The presence of 1,5,6-trimethyl-2(1*H*)-pyrazinone has been previously reported in glycine/glucose model systems (Keyhani et al., 1996) where its formation mechanism was rationalized by the interaction of two moles of glycine with a sugar-derived α-dicarbonyl moiety. The isotope labelling experiments (Table 18 andTable 19) showed the incorporation of various atoms from the two amino acids and the sugar confirming the involvement of multiple amino acids in its formation.

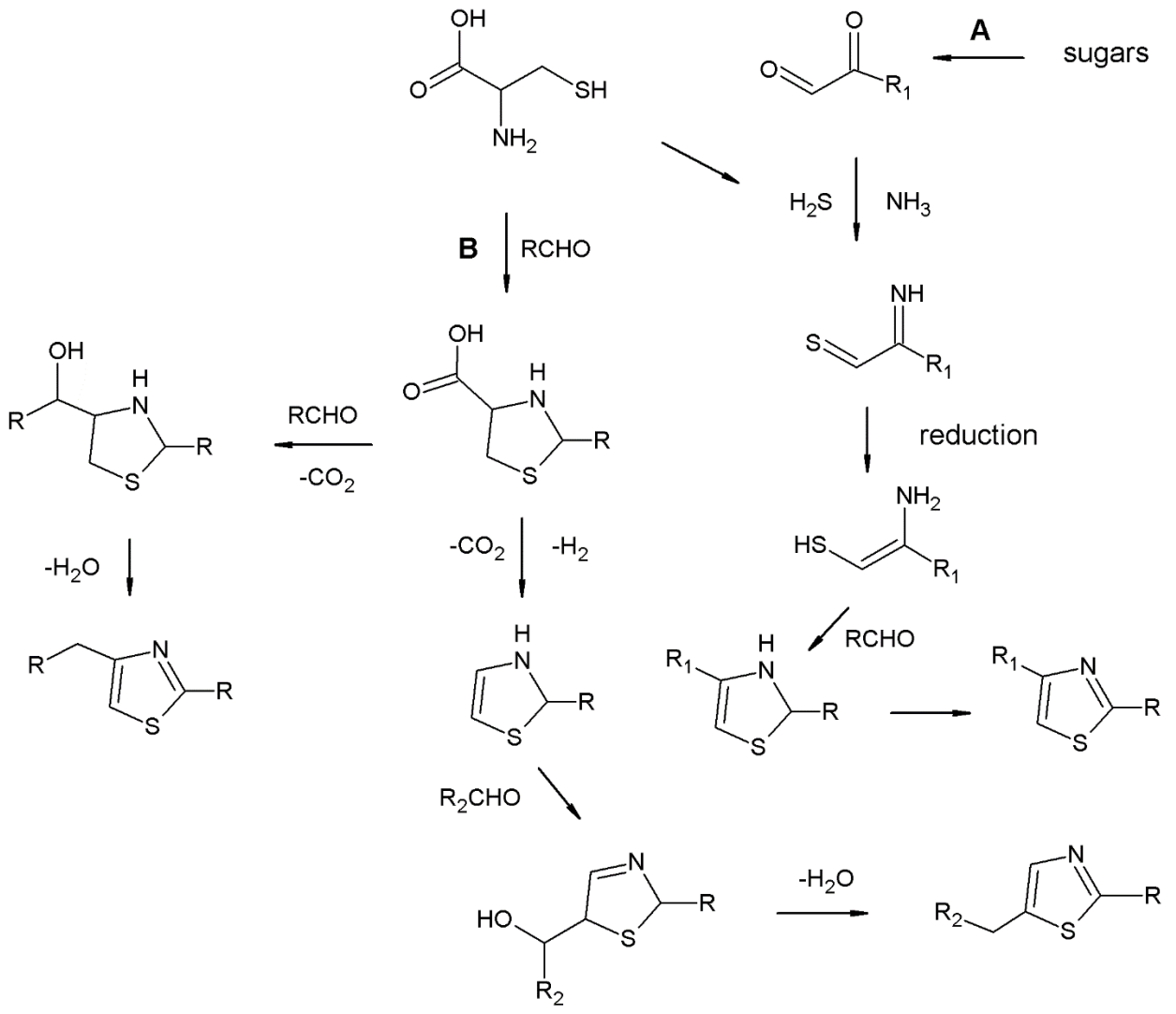


Figure 20. Proposed mechanism of formation of substituted thiazoles in cysteine/glycine/glucose model system

6.4.2. Non-volatile products from cysteine/glycine/glucose multiple amino acid model system

To identify the formation of non-volatile products from the interaction of two amino acids and a sugar, the cysteine/glycine/glucose model system was subjected to heating under both pyrolytic or in oven as described in the experimental section. The reaction residue was evaluated by high resolution LC/MS as described in the experimental section. The strategy used to identify products formed by the interaction of two amino acids and the sugar was based on first locating product peaks in the mass range of 40-400 amu that have incorporated nitrogen atoms from

glycine, sulphur atoms from cysteine in addition to carbon atoms from glucose. Various peaks were identified using the above criteria, however the ion at m/z 323 from the oven heated sample was selected for further study due to its interesting elemental composition incorporating six carbon atoms from glucose and intact glycine and cysteine moieties (see Table 20).

Table 20. Percent incorporation of labelled glycine and sugar atoms in m/z 323 generated from glycine/cysteine/glucose model system[a]

[^{15}N]-glycine				**[^{13}C-1]-glycine**			
t_R (min)	**Error (ppm)**	**% incorporation**		**t_R (min)**	**Error (ppm)**	**% incorporation**	
		M	M+1			**M**	**M+1**
5.034	24	0	100	5.024	0.38	0	100
[^{13}C-2]-glycine				**[U6-^{13}C]-glucose**			
t_R (min)	**Error (ppm)**	**% incorporation**		**t_R (min)**	**Error (ppm)**	**% incorporation**	
		M	**M+1**			**M**	**M+6**
5.036	0.38	0	100	4.915	71	0	100

[a] Values for unlabelled glycine: t_R = 4.933min; m/z = 323.0944;
Molecular Formula = $C_{11}H_{19}N_2O_7S$, error= 9.6 ppm

As shown in Figure 21 the elemental composition of the ion at m/z 323 ($C_{11}H_{19}N_2O_7S$, error = 9.6 ppm) can be justified by proposing two consecutive Amadori rearrangements one by glycine and the other by cysteine followed by a dehydration step to generate the ion at m/z 323. The structure proposed for the ion at m/z 323 is consistent with the isotope incorporation pattern shown in Table 20 in addition to the MS/MS data listed in Table 21.

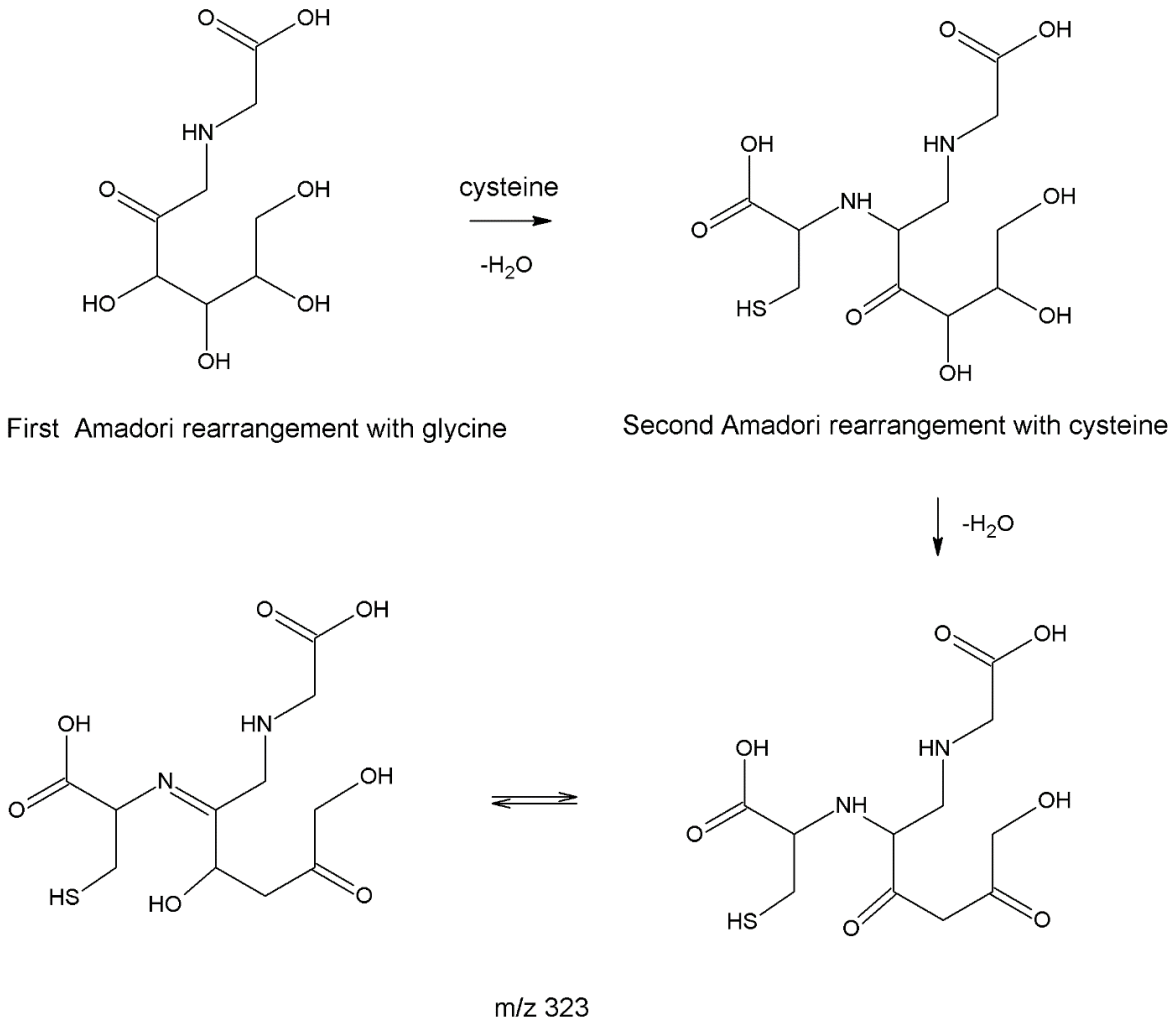


Figure 21. Proposed mechanism of formation of m/z 323 in cysteine/glycine/glucose model system.

Table 21. Major MS/MS fragment ions of peak at m/z 323.0944[a]

m/z	Relative intensity (%)	Elemental composition[b]	Error (ppm)	Label incorporation from glycine/ U6 sugar
323.1022	5	$C_{11}H_{19}N_2O_7S$	33	1 x C-1; 1 x C-2; 1 x N / 6 x C
146.0268	80	$C_5H_8NO_2S$	5.3	0 x C-1; 0 x C-2; 0 x N / 2 x C
139.0520	25	$C_6H_7N_2O_2$	8.9	1 x C-1; 1 x C-2; 1 x N / 2 x C
100.0223	100	C_4H_6NS	2.0	0 x C-1; 0 x C-2; 0 x N / 2 x C
95.0627	20	$C_5H_7N_2$	18	0 x C-1; 1 x C-2; 1 x N / 2 x C

[a]see Figure 21; [b]see Figure 22 and Figure 23 for corresponding proposed structures

As shown in Figure 22 andFigure 23, the various isomeric forms of the ion m/z 323 can be used to justify the formation of the daughter ions at m/z 146, 139, 100 and 95 through well-established chemical transformations such as retro-aldol, dehydrations and isomerization reactions. Furthermore, the proposed structures for the MS/MS data are also consistent with the isotope incorporation results listed in Table 21.

This preliminary investigation indicates that both volatile and non-volatile products are affected by the presence of multiple amino acids in synergistically generating complex Maillard reaction mixtures.

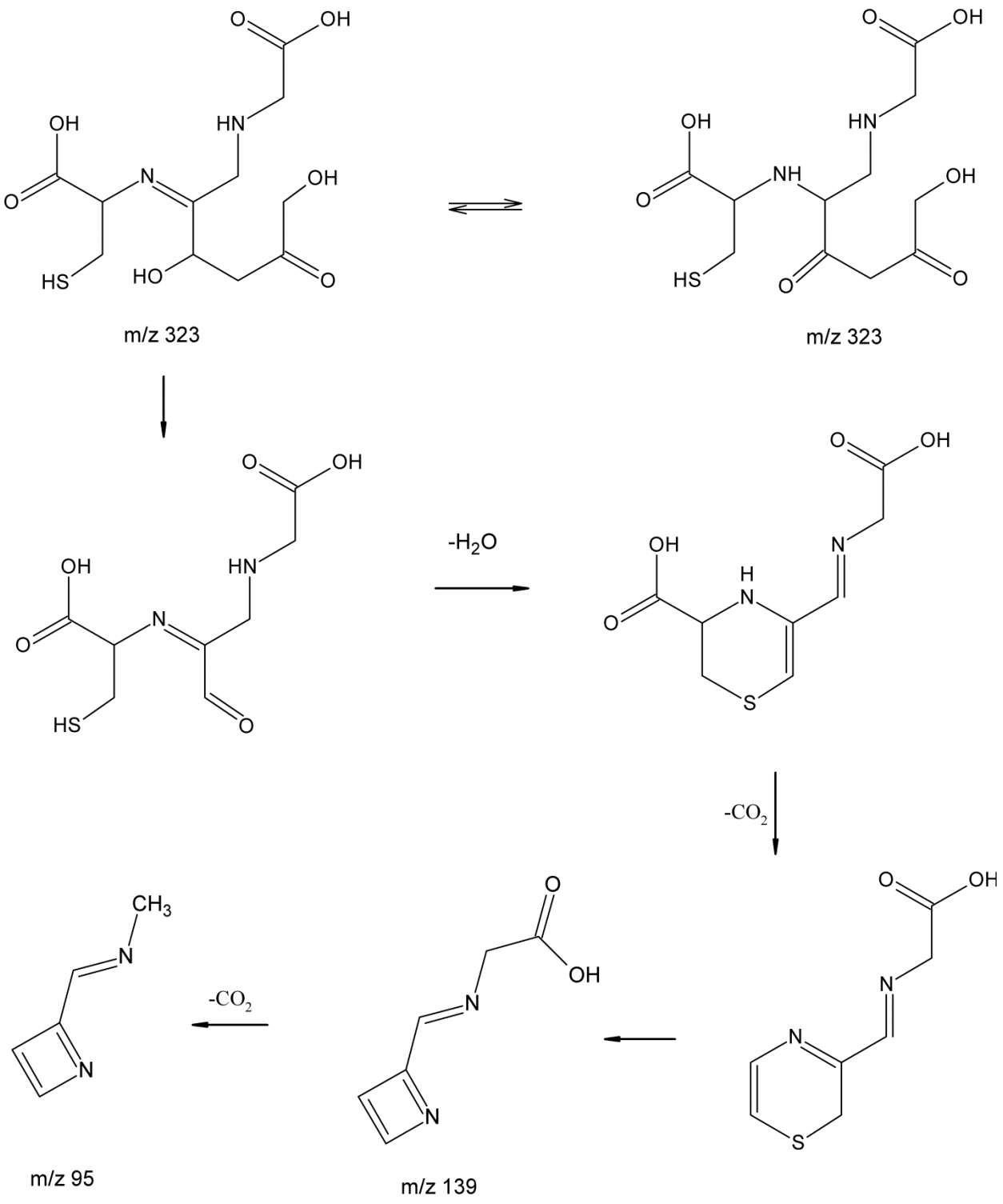


Figure 22. Proposed mechanism of formation of m/z 95 and m/z 139 from m/z 323

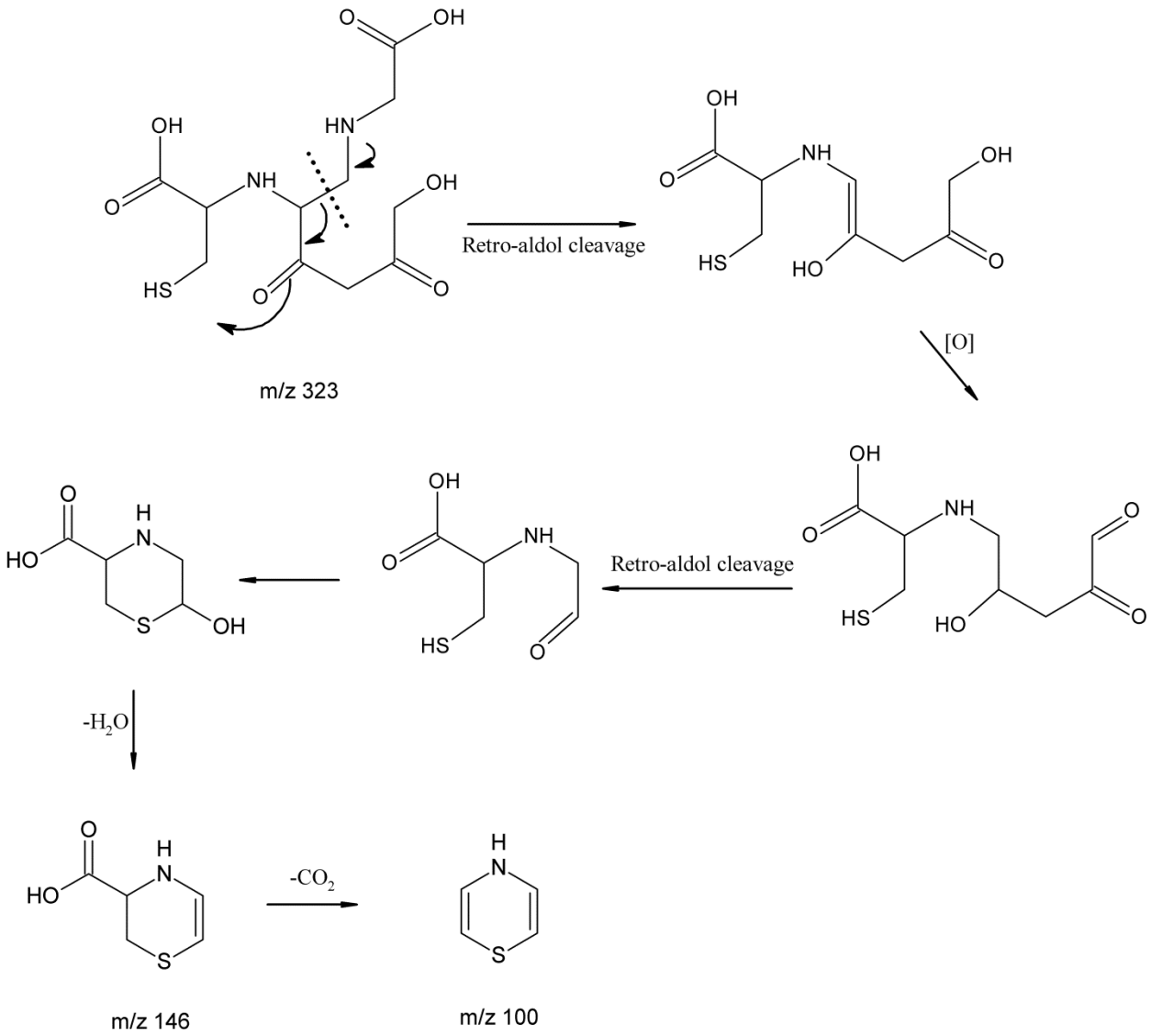
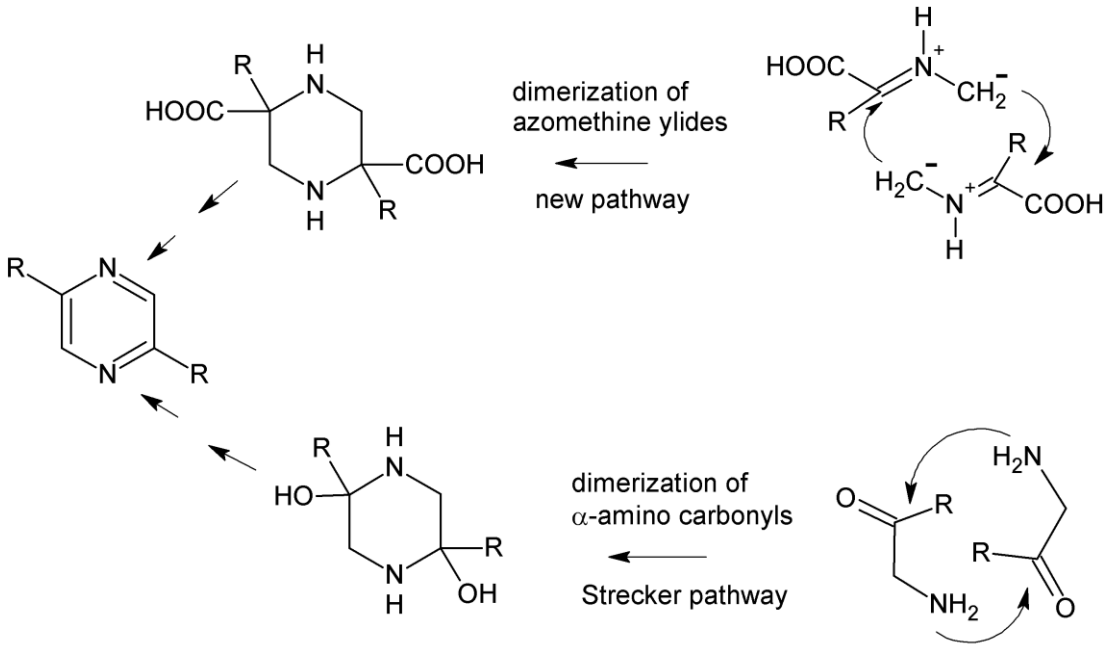


Figure 23. Proposed mechanism of formation of m/z 146 and m/z 100 from m/z 323

CONNECTING PARAGRAPH

Chapters 3 and 4 presented the potential of α-dicarbonyl moieties, such as 2,3-dicarbonyl or 4,5-dimethyl-1,2-benzoquinone to undergo Strecker type reactions with amino acids, and the underlying principle was further applied in Chapters 5 and 6. With the aim of expanding the scope and the utility of the α-dicarbonyl reactions in enhancing our ability to control the formation of specific products, additional α-dicarbonyl moieties such as α-keto acids were selected for study due to their ability to form azomethine ylides without being able to undergo classical Strecker reaction. The findings of this study are presented in Chapter 7. This chapter was published in the Journal of Agricultural and Food Chemistry. Reprinted with permission from Guerra, P. V., Yaylayan, V. A. (2010) Dimerization of Azomethine Ylides: An Alternate Route to Pyrazine Formation in the Maillard Reaction. *J. Agric. Food Chem.*, *58*(23), 12523-12529.

CHAPTER 7. DIMERIZATION OF AZOMETHINE YLIDES: AN ALTERNATE ROUTE TO PYRAZINE FORMATION IN THE MAILLARD REACTION



7.1 Abstract

Recently, azomethine ylides have been implicated as reactive intermediates in the Maillard reaction. They are known to undergo 1,3-cycloaddition reactions with dipolarophiles to form pyrroles and more importantly they can undergo dimerization reaction leading to the formation of piperazine moiety. Although the reactivity of azomethine ylides towards dipolarophiles in Maillard model systems have been studied, however, their role as precursors of pyrazines still remains unexplored. To study this possibility, a simple model system such as glyoxylic acid/glycine that is unable to generate α-dicarbonyl compounds but is able to form azomethine ylides was used to demonstrate pyrazine formation. The specific piperazine-2,5-dicarboxylic acid that is expected to form in this particular system can undergo oxidative decarboxylation to generate dihydropyrazine moieties similar to that of the dimerization product of the α-amino carbonyl compounds generated through the Strecker reaction. The model system when reacted under pyrolytic conditions at 200^{o}C indeed generated most of the theoretically expected pyrazines as major products whose structures were confirmed by comparison of their retention times with commercial standards and through NIST library matches in addition to isotope labelling data generated from labelled precursors such as [^{13}C-1]glycine, [^{13}C-2]glycine and [^{15}N]glycine.

7.2 Introduction

Pyrazine formation constitutes one of the main pathways of aroma generation during the Maillard reaction due to the characteristic sensory properties associated with pyrazines (Amrani-Hemaimi et al., 1995). The Strecker reaction plays a critical role in the transformation of α-dicarbonyl compounds that form abundantly during the Maillard reaction into pyrazine precursors, the α-amino carbonyl compounds (Rizzi, 2008). Essentially, the dimerization of such α-amino carbonyl compounds is the only known route to pyrazine moiety during the Maillard reaction. The resulting dihydropyrazines can either oxidize into pyrazine or interact with simple aldehydes to generate substituted pyrazines after a dehydration step without the need for oxidation. In addition to the above pathway, the interaction of α-dicarbonyl compounds with 1,2-

diamino moieties can also lead to the formation of dihydropyrazines without the need to be transformed into α-amino carbonyl compounds, however, 1,2-diamino moieties are not very common in food systems, although, 1,2-diaminobenzene is a well known reagent to trap α-dicarbonyl compounds as their quinoxaline derivatives in many model and food systems (Hofmann, 1999). Recently, azomethine ylides (Figure 24) have been implicated as intermediates in the Maillard reaction (Blank et al., 2005; Mottram, Low & Elmore, 2006; Rizzi, 2008; Yaylayan et al., 2003b; Zhang, Ren & Zhang, 2009) based on evidence from spectroscopic (Chu & Yaylayan, 2008b; Chu et al., 2009a) and isotope labelling studies (Chu et al., 2009a; Hidalgo et al., 2010) using different model systems at various temperatures. The azomethine ylides are known to undergo 1,3-cycloaddition reactions with dipolarophiles (Tsuge et al., 1989) to form pyrroles and more importantly they can undergo dimerization reaction leading to the formation of piperazine moiety (Freeman et al., 1995; Tsuge et al., 1989) as shown in Figure 24. Although the addition of dipolarophiles to Maillard model systems have been shown to significantly reduce the intensity of UV absorption in the region between 400-500 nm (Chu et al., 2009a) indicating the important role the azomethine ylides play as reactive intermediates, however, their role as precursors of pyrazines has not been studied yet. To explore this possibility, isotope labelling studies were performed using a simple model system that is unable to generate α-dicarbonyls but able to form azomethine ylides such as glyoxylic acid/glycine system.

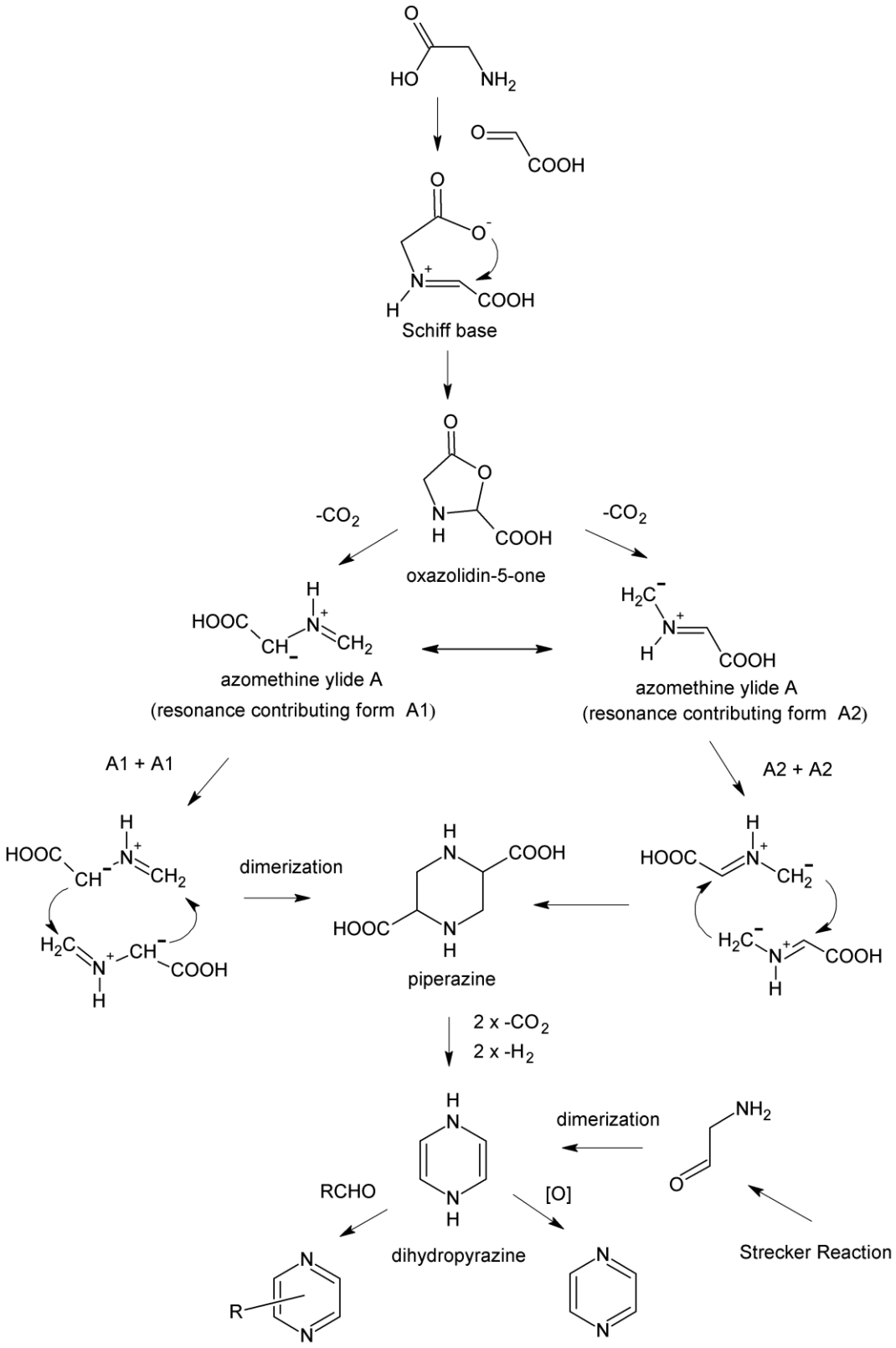


Figure 24. Generation and dimerization of azomethine ylides from glycine and glyoxylic acid.

7.3 Materials and Methods

7.3.1. Reagents and Chemicals

Glycine (99%), glyoxylic acid monohydrate (95%), pyruvic acid, 2,3-butanedione, methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3-dimethylpyrazine 2,3,5-trimethylpyrazine, 2-ethyl-3,6-dimethylpyrazine and tetramethylpyrazine were purchased from Sigma-Aldrich Chemical Co (Oakville, ON, Canada). The labelled [^{13}C-1]glycine (98%), [^{13}C-2]glycine (99%), [^{15}N]glycine (98%) were purchased from Cambridge Isotope Laboratories (Andover, MI).

7.3.2. Pyrolysis-Gas Chromatography-Mass Spectrometry (Py-GC/MS) The Py-GC/MS analysis was performed according to procedure described by Chu and Yaylayan (Chu et al., 2009b) with some modifications. A Varian CP-3800 GC equipped with a sample pre-concentration trap (SPT) filled with Tenax GR was coupled to a Varian Saturn 2000 Mass Spectrometry detector (Varian, Walnut Creek, CA, USA). The pyrolysis unit included a valved interface (CDS 1500), which was installed onto the GC injection port and connected to a CDS Pyroprobe 2000 unit (CDS Analytical, Oxford, PA, USA). The samples were analyzed on a DB-5MS column (5% diphenyl, 95% dimethyl-polysiloxane) with column dimensions of 50m length x 0.2mm internal diameter x 33μm film thickness (J&W Scientific, ON, Canada) using helium as the carrier gas. Two milligrams of sample mixtures containing 1:3 ratio of glyoxylic acid to glycine or 1:3:0.2 ratio of glyoxylic acid to glycine to pyruvic acid (spike) or 1:3:1 ratio of glyoxylic acid to glycine to 2,3-butanedione model systems were packed inside a quartz tube (0.3mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed at 200^{o}C for 20 seconds under He atmosphere. The volatiles after pyrolysis were concentrated on the sample pre-concentration trap (SPT), trapped at 50^{o}C and subsequently directed towards the GC column for separation. The GC column flow rate was regulated by an Electronic Flow Controller (EFC) and set at a delayed (30 s) pressure pulse of 70 psi for first 4 minutes and maintained with a constant flow of 1.5ml/minute for the rest of the run. The GC oven temperature was set at –5^{o}C for first 5 minutes using CO_2 as the cryogenic cooling source and then increased to 50^{o}C at a rate of 50^{o}C/minute. Then, the oven temperature was again increased to 270^{o} C at a rate of 8^{o}C/minute and kept at 270^{o}C for 5 minutes. The samples were detected by

using an ion-trap mass spectrometer. The MS transfer-line temperature was set at 250°C, manifold temperature was set at 50°C and the ion trap temperature was set at 175°C. The ionization voltage of 70eV was used and EMV was set at 1500V.

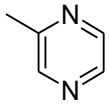
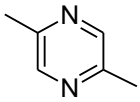
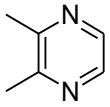
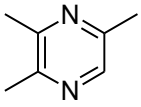
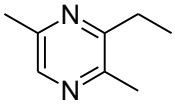
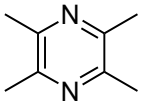
7.3.3. Identification of Pyrazines

Pyrazines were identified by comparison of their retention times with commercial standards and through NIST library matches in addition to isotope labelling data (see Table 22 andTable 23). The data reported in Table 22 andTable 23 are based on at least two replicate analyses with a percent standard deviation of < 15%.

7.4 Results and Discussion

To investigate the possible pyrazine formation through the proposed azomethine ylide dimerization pathway shown in Figure 24 as a distinct and a new route to form pyrazines, glyoxylic acid/glycine system was investigated as a model that is unable to generate α-dicarbonyl precursors needed for the Strecker reaction - the only known route to pyrazines. Glycine was reacted with glyoxylic acid under pyrolytic conditions at 200°C. Analysis of the chromatograms indicated that the above system generates mainly pyrazines in addition to few unknown structures including some pyrazinones. The intensity of the pyrazine peaks dropped when the temperature was increased to 250°C and at 150°C hardly any pyrazines were formed. The structure of all the pyrazines were confirmed through isotope labelling technique (see below) in addition to their retention times using commercially available standards and by NIST library searches as summarized in Table 22. The model system therefore generated different pyrazines as major products indicating existence of a novel pathway of pyrazine formation in the absence of α-dicarbonyl compounds. The question that is raised then is why glyoxylic acid should generate pyrazines in the presence of glycine?

Table 22. Pyrazines and their retention times identified[a] in glycine/glyoxylic acid model system.

Pyrazines	**Structure**	**Retention time (Standard) min**	**Relative amounts[b] in area per mole of glycine x 10^9**	**Origin[c]**
2-methylpyrazine		10.78 (10.80)	1.38 (trace)	A + B A + A + CHO
2,5(6)-dimethylpyrazine[d]		11.80 (11.81)	4.04 (trace)	B2 + B2 A + B + CHO
2,3-dimethylpyrazine		11.90 (11.89)	2.39 (4.8)	B1 + B2 A + B + CHO
2,3,5-trimethylpyrazine		13.22 (13.21)	3.39 (14)	B + B + CHO
2-ethyl-3,6-dimethylpyrazine		14.58 (14.60)	0.18 (0)	B2 + B2 + CH_3CHO
Tetramethylpyrazine		14.78 (14.73)	0.09 (41)	C + C C + B + CHO

[a] Structures of all pyrazines were confirmed through NIST library searches, retention times using standards, partial elemental composition and label incorporation in mass spectral fragments.
[b] Based on the average of at least two replicates with a percent standard deviation of < 15%, values in parenthesis are those produced in the presence of 2,3-butanedione.
[c] Pairs of azomethine ylides (A, B1 and B2) needed for their oxidative formation in addition to an aldehyde in case of non-oxidative pathway (see also Figures 27 and 28).
[d] Both standards have identical retention times (two isomers cannot be separated on DB-5).

Table 23. Percent incorporation of [^{13}C-2]glycine[1] into pyrazines in glyoxylic acid/glycine model.

Compound	M	M+1	M+2	M+3	M+4	M+5
2-methylpyrazine	10	30	38	22	0	0
2,5(6)-dimethylpyrazine	5	12	28	32	22	0
2,3-dimethylpyrazine	5	14	28	32	21	0
2,3,5-trimethylpyrazine	0	7	18	31	28	16

[1][^{15}N]glycine incorporated two nitrogen atoms and [^{13}C-1]glycine showed no incorporation in all the listed pyrazines

7.4.1. Formation and dimerization of azomethine ylides in glyoxylic acid/glycine system

Glyoxylic acid similar to other α-keto acids is a known Maillard reaction product (Rössnes et al., 2001) and is capable of forming Schiff bases with amino acids. However, due to the inability of this Schiff base to stabilize itself through Amadori rearrangement it prefers instead to undergo intramolecular cyclization and form oxazolidin-5-one moieties similar to other amino acid/carbonyl systems (Aurelio, Box, Brownlee, Hughes & Sleebs, 2003; Tsuge, 1987). Under Maillard reaction conditions however, oxazolidin-5-ones are prone to undergo decarboxylation (Chu et al., 2008b; Hidalgo et al., 2010) followed by formation of azomethine ylides (see Figure 24). One resonance contributing form of the ylide has the negative charge on the α-carbon of the α-keto acid (form A1) and the other on the amino acid C-2 atom (form A2). In the absence of dipolarophiles, such non-stabilized acyclic azomethine ylides can either hydrolyze in the presence of water to generate the corresponding amine or dimerize to form piperazines (Freeman et al., 1995; Tsuge et al., 1989) (see Figure 24). The specific piperazine-2,5-dicarboxylic acid that can be formed in this particular case can undergo oxidative decarboxylation (Murai, Morishita, Nakatani, Fujioka & Kita, 2008) to generate dihydropyrazine moieties similar to that of the dimerization product of the α-amino carbonyl compounds generated through the Strecker reaction (see Figure 24 andFigure 25). Such intermediates can be easily converted into pyrazines due to the oxidative and non-oxidative pathways available for their conversion, especially in the presence of simple aldehydes that can easily arise through decarboxylation of α-keto-acids such as glyoxylic acid (Figure 26). Furthermore, if the α-keto acid and the amino acids involved have

substituents, then due to the regiochemistry of dimerization two different isomers of pyrazines can result (see Figure 25).

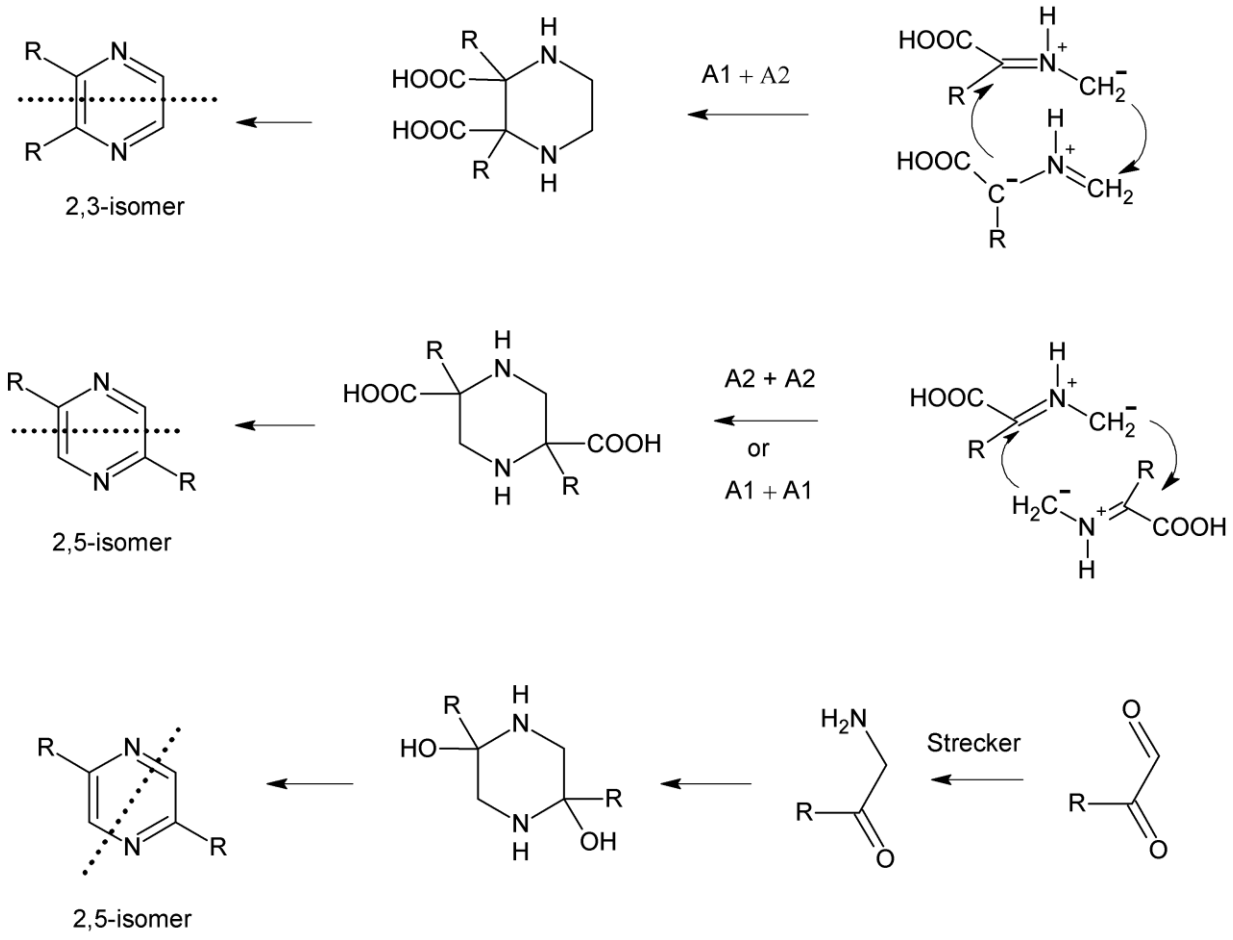


Figure 25. Dimerization of azomethine ylides and α-amino carbonyls and formation of pyrazine isomers

For example if A1 and A2 forms dimerize in the system shown in Figure 25 then 2,3-disubstituted pyrazines are formed and on the other hand, dimerization of A2 and A2 or A1 and A1 can lead to the formation of 2,5-disubstituted pyrazines. To help elucidate and confirm the proposed new pathway of pyrazine formation the reaction was studied using separately labelled glycine at N, C-1 and C-2 atoms. The structures of all the pyrazines detected in the model system were consistent with those predicted based on the proposed mechanism not only in terms of label incorporation patterns but also in the patterns of their possible alkyl substituents.

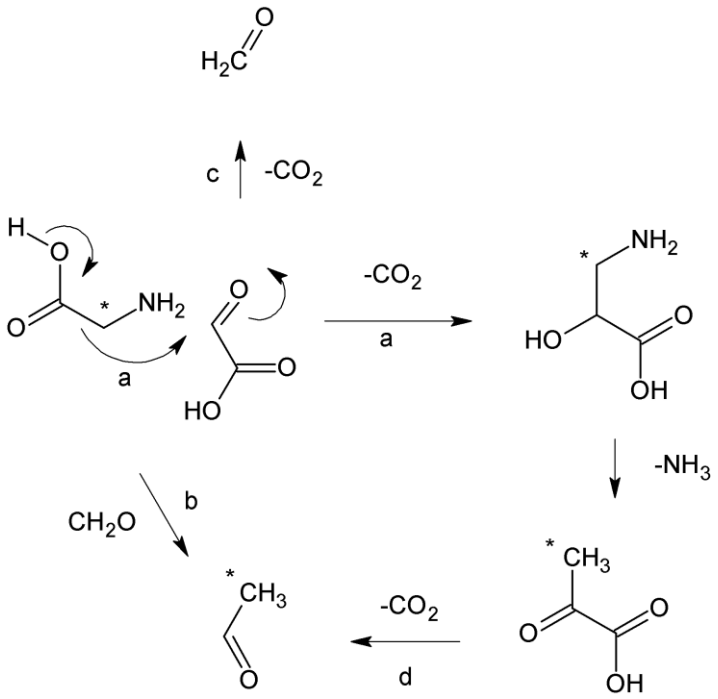


Figure 26. Formation of formaldehyde and acetaldehyde through chain elongation reactions (pathways a and b) and through decarboxylation of α-keto acids (pathways c and d) in glycine/glyoxylic acid model system

7.4.2. Pyrazines from glyoxylic acid/glycine model system - structural evidence

According to the proposed mechanism shown in Figure 27, glycine can undergo two types of interactions with glyoxylic acid; one through carbonyl-amine reaction and formation of a Schiff base and the other through aldol-type chain elongation reaction (Yaylayan et al., 1999) (see Figure 26). The former can lead to the formation of azomethine ylide A (A1↔A2) and the latter can lead to chain elongation of glyoxylic acid and subsequent formation of pyruvic acid which in turn can react further with glycine to generate new azomethine ylide B (B1↔ B2).

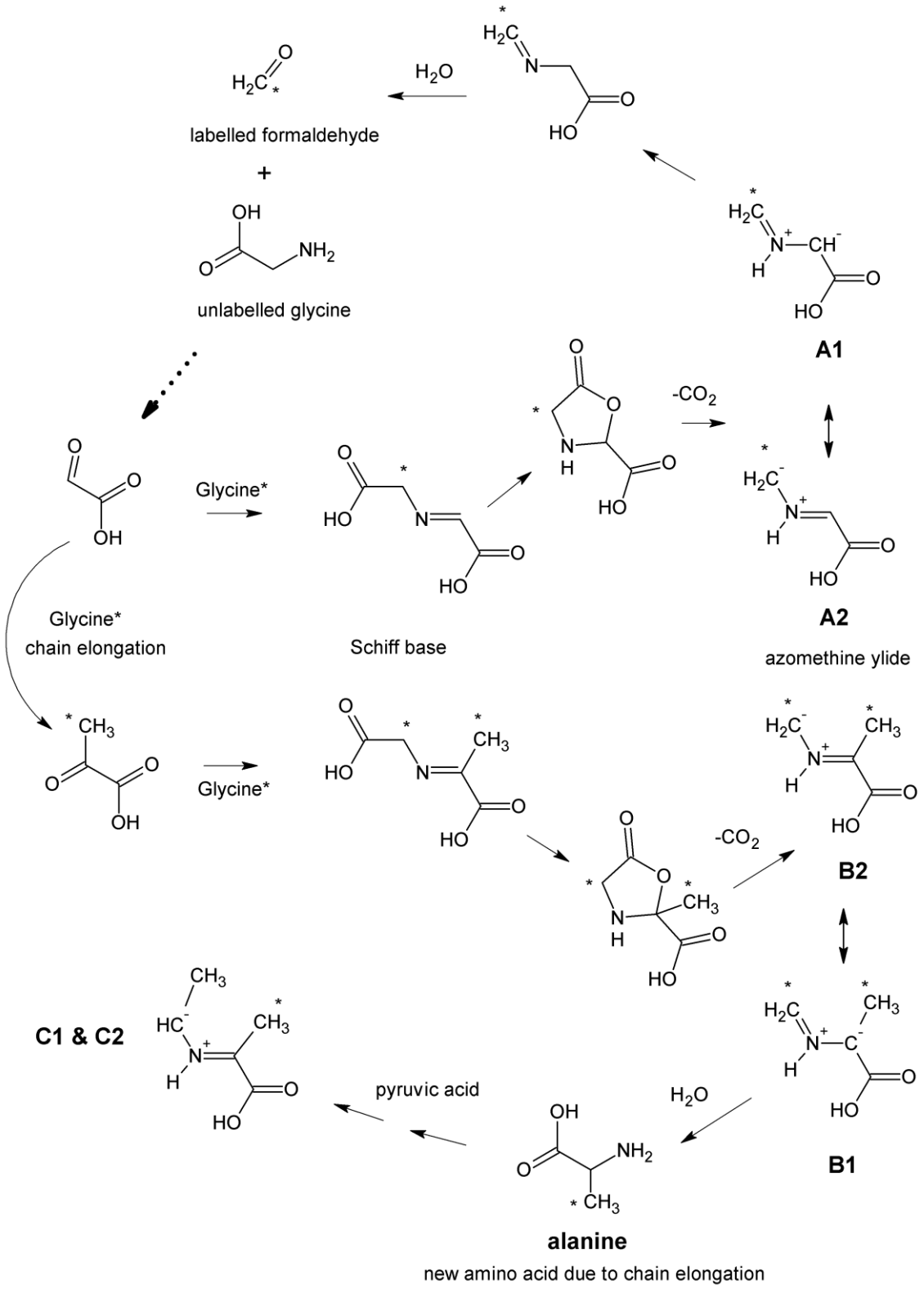


Figure 27. Proposed mechanism of interaction between [^{13}C-2]glycine and glyoxylic acid and generation of azomethine ylides A and B. Glycine* indicates [^{13}C-2]glycine

Chain elongation reaction of glyoxal in the presence of alanine for example has been already demonstrated to generate 2-oxobutanal (Chu et al., 2009b). Figure 28 shows all the four possible dihydropyrazine derivatives that theoretically could result from random dimerization of the azomethine ylides A1, B1, A2 and B2. Oxidation of these dihydropyrazines should generate the following pyrazines; the parent pyrazine, methylpyrazine, 2,5-dimethylpyrazine and 2,3-dimethylpyrazine. According to Table 22 all these pyrazines were detected except the parent pyrazine (not easily retained on DB-5) and they constituted together more than 60% of the total pyrazine peak area. However, the methylpyrazine and all the three isomers of dimethylpyrazine could also be formed through non-oxidative pathway by the reaction of either dihydropyrazine or dihydromethylpyrazines with formaldehyde. Decarboxylation of glyoxylic acid (Figure 26) and hydrolysis of ylides A and B (Figure 27) could be efficient sources of formaldehyde in the system. The fact that significant amount of 2,3,5-trimethylpyrazine was detected in the system confirms the importance of non-oxidative pathway in this model system since the oxidative pathway to generate this pyrazine requires the presence of alanine which is expected to form only in small amounts (labelling experiments below support this conclusion). The remaining two pyrazines 2-ethyl-3,6-dimethyl and tetramethylpyrazine can be formed only through non-oxidative pathway and are expected to be present in only small amounts, due to the requirement of acetaldehyde and alanine for their generation (see Figure 27)**.**

7.4.3. Evidence from labelling studies

Label incorporation patterns in the most abundant pyrazines are shown in Table 23. As expected all the listed pyrazines incorporated two nitrogen atoms of glycine and none incorporated C-1 atom consistent with the proposed mechanism shown in Figure 27. This figure also traces the fate of the ^{13}C-2 atom of glycine as it reacts with glyoxylic acid to form ylides A and B. According to the proposed mechanism ylide A should incorporate only one ^{13}C-label and ylide B should incorporate two ^{13}C-labels. Furthermore, hydrolysis of ylide A should generate unlabelled glycine from unlabelled glyoxylic acid portion and labelled formaldehyde from the labelled glycine portion of the ylide A.

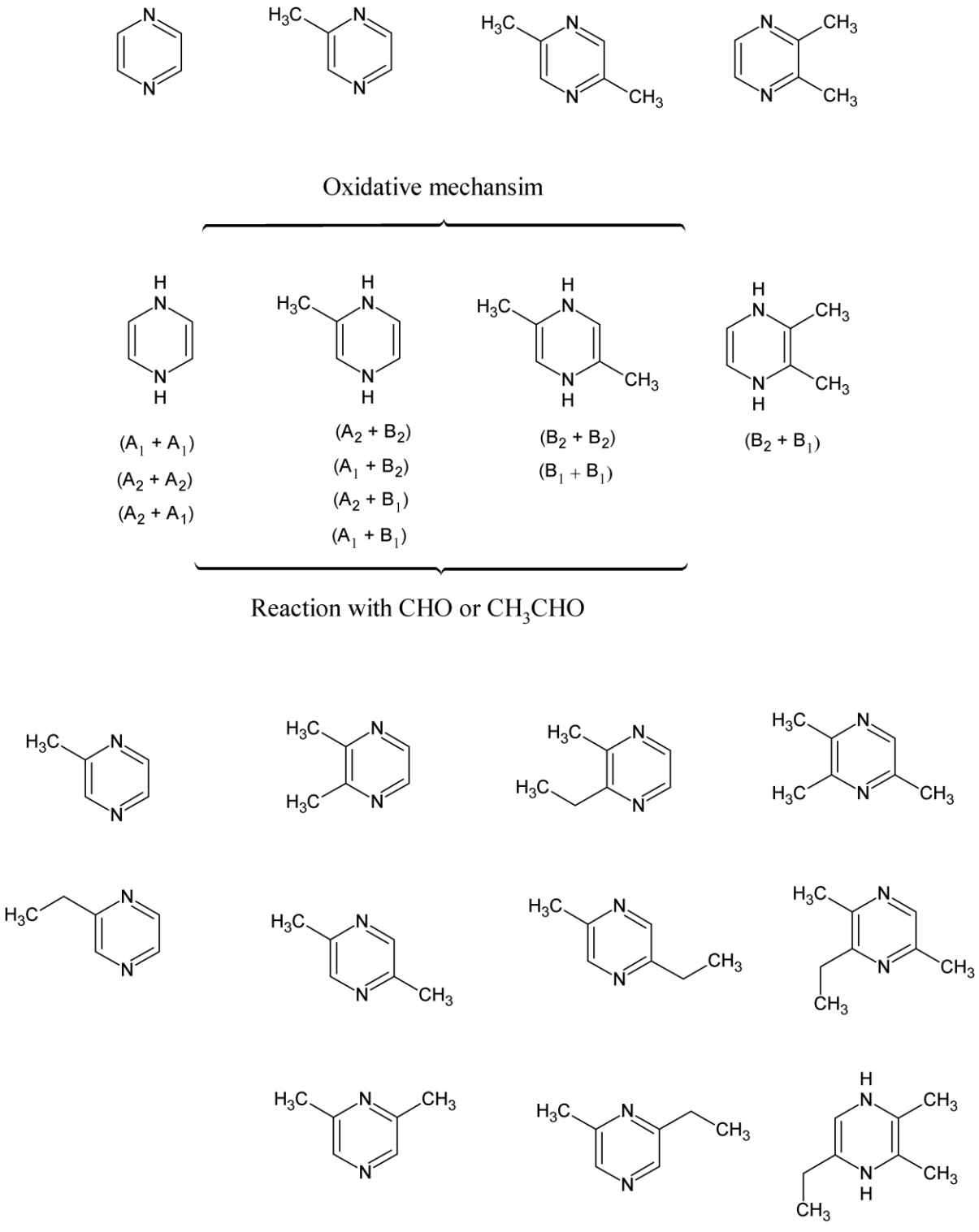


Figure 28. Possible dihydropyrazine structures that could be generated through random dimerization of azomethine ylides A and B and their subsequent conversion into various pyrazines through oxidative and non-oxidative pathways.

Similarly, hydrolysis of ylide B should generate labelled alanine and labelled formaldehyde. It is expected however, that the reaction will proceed to generate mainly ylides A and B in addition to labelled formaldehyde and unlabelled glycine. Unlabelled formaldehyde however can arise from decarboxylation of glyoxylic acid (Figure 26). Consequently, the most intense pyrazines are expected to arise from random dimerization of ylides A and B and through formaldehyde participation in the non-oxidative pathway (Figure 28). On the other hand, pyrazines arising from acetaldehyde participation through non-oxidative pathway are expected to be less intense due to the tendency of pyruvaldehyde to react with glycine rather than decarboxylate and generate acetaldehyde. Spiking glycine/glyoxylic acid model with pyruvic acid not only enhanced the intensities of the peaks requiring ylide B but also those requiring acetaldehyde (results not shown). Therefore, based on the proposed mechanism in Figure 27, out of the ten most probable pyrazine structures, the six most expected pyrazines (mainly requiring ylides A and B and formaldehyde) were detected in the system.

7.4.3.1. Label incorporation in 2-methylpyrazine

The 2-methylpyrazine can be formed through both oxidative (A + B) and non-oxidative pathways (A + A + CHO) (Table 22). According to Figure 27, the maximum number of label incorporation should not exceed three if it is formed through oxidative pathway and in the case of non-oxidative pathway, the maximum number of label incorporation again should not exceed three if formaldehyde is labelled otherwise the maximum number of label incorporation will be two. Furthermore, as described above and shown in Figure 28 the hydrolysis of ylide A can generate unlabelled glycine which in turn can recycle into the reaction system and generate unlabelled ylides A and B. As a consequence 2-methylpyrazine can be generated with no label and with one, two or three label incorporations as shown in Table 23. As expected only 10% of the isotopomers had no label due to small amount of water available in the system from glyoxylic acid monohydrate reagent.

7.4.3.2. Label incorporation in 2,5(6)-dimethylpyrazine and 2,3-dimethylpyrazine

The dimethylpyrazines can be formed through oxidative (B + B) and non-oxidative pathways (A + B + CHO). Accordingly they show very similar label incorporation patterns (see Table 23). In the oxidative pathway dimethylpyrazines are expected to incorporate maximum four labels and

in the non-oxidative pathway depending on the origin of formaldehyde they can incorporate up to four labels. Again due to the generation of unlabelled glycine in the system it is expected to observe a pattern of unlabelled and partially labelled dimethylpyrazines as shown in Table 23.

7.4.3.3. Label incorporation in 2,3,5-trimethylpyrazine.

Trimethylpyrazine can be formed only through non-oxidative pathway (B + B + CHO) and accordingly it is expected to exhibit incorporation of up to five labelled atoms depending on the origin of formaldehyde. In this particular case there is statistically less chance to generate completely unlabelled isotopomer, however other partially labelled structures were detected as shown in Table 23.

7.4.4. Glycine reaction with glyoxylic acid in the presence of 2,3-butanedione.

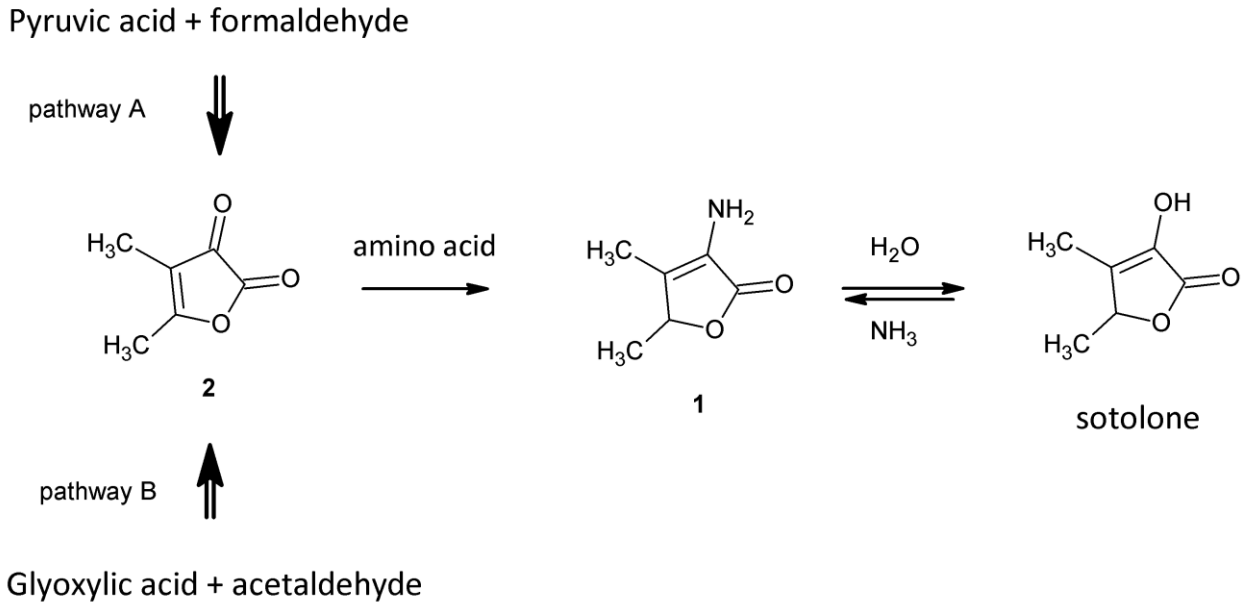
To evaluate the importance of azomethine ylide dimerization pathway relative to the Strecker reaction, glyoxylic acid/glycine reaction was performed in the presence of 2,3-butanedione in a 1:3:1 ratio and compared with glyoxylic acid/glycine model in 1:3 ratio. In this model system, glycine is in competition to undergo both reactions generating tetramethylpyrazine only through the Strecker reaction and the other pyrazines only through azomethine ylide dimerization. The results have indicated that the presence of 2,3-butanedione did not prevent pyrazine formation through dimerization of azomethine ylide since in addition to tetramethylpyrazine comparable amounts of other pyrazines were also formed (see Table 22). When the ratio of glycine in the model system was increased, the amounts of all the pyrazines were also increased.

Structural evidence and the observed isotope labelling patterns of the pyrazines identified in glycine/glyoxylic acid model system point out to a novel pathway of pyrazine generation in the Maillard reaction mixtures distinct from the Strecker reaction. Dimerization of azomethine ylides formed between the amino acid and the α-keto acid constitutes the only possible mechanism able to rationalize the formation of the observed pyrazines.

CONNECTING PARAGRAPH

In Chapter 7 the ability of α-keto acids to generate Strecker-type reaction products was studied during the thermal reaction of a glyoxylic acid/glycine model system. While evaluating this model system, 3-amino-4,5-dimethyl-2(5H)-furanone, a known precursor of sotolone, was also observed. Since sotolone is an important aroma chemical and given that this was the first time this precursor was observed to be formed thermally, Chapter 8 addresses the formation pathway of 3-amino-4,5-dimethyl-2(5H)-furanone from two sets of model systems, pyruvic acid/glycine and glyoxylic acid/alanine. Furthermore, 4,5-dimethylfuran-2,3-dione, a lactone with an α-dicarbonyl moiety in their structure was also identified as a reactive intermediate in the formation of 3-amino-4,5-dimethyl-2(5H)-furanone. This serves as an additional example of participation of α-dicarbonyl derivatives in the generation of flavor products through thermal reactions. This chapter was published in the Journal of Agricultural and Food Chemistry. Reprinted with permission from Guerra, P. V., Yaylayan, V. A. (2011) Thermal Generation of 3-Amino-4,5-dimethylfuran-2(5H)-one, the Postulated Precursor of Sotolone, from Amino Acid Model Systems Containing Glyoxylic and Pyruvic Acids. *J. Agric. Food Chem.*, *59* (9), 4699-4704.

CHAPTER 8. THERMAL GENERATION OF 3-AMINO-4,5-DIMETHYLFURAN-2(5H)-ONE THE POSTULATED PRECURSOR OF SOTOLONE, FROM AMINO ACID MODEL SYSTEMS CONTAINING GLYOXYLIC AND PYRUVIC ACIDS



8.1 Abstract

The 4,5-dimethyl-3-hydroxy-2(5H)-furanone (sotolone) a naturally occurring flavor impact compound can be isolated from various sources especially fenugreek seeds. It can also be thermally produced from intermediates generated from the Maillard reaction such as pyruvic and ketoglutaric acids, glyoxal and 2,3-butanedione. A naturally occurring precursor of sotolone the 3-amino-4,5-dimethyl-2(5H)-furanone was thermally generated for the first time from pyruvic acid and glycine or from glyoxylic acid and alanine model systems. Isotope labelling studies have implicated 4,5-dimethylfuran-2,3-dione as an intermediate that can be converted into 3-amino-4,5-dimethyl-2(5H)-furanone through Strecker-like interaction with any amino acid. Furthermore, these studies have also indicated the presence of two pathways for the formation of 4,5-dimethylfuran-2,3-dione; one requiring pyruvic acid and a formaldehyde source and the other requiring glyoxylic acid and acetaldehyde. Self-aldol condensation of pyruvic acid followed by lactonization and further aldol reaction with formaldehyde can generate the same intermediate as the self-aldol addition product of acetaldehyde with glyoxylic acid followed by lactonization. The pyruvic acid pathway was found to be more efficient route than glyoxylic acid pathway. Furthermore, the pyruvic acid/glycine model system was able to generate sotolone in the presence of moisture and in the presence of ammonia commercial sotolone was converted back into 3-amino-4,5-dimethyl-2(5H)-furanone.

8.2 Introduction

Chemical reactions occurring in food during thermal processing generating identical structures to that formed enzymatically such as during metabolic processes occurring in plants are rarely encountered. Furaneol®, Strecker aldehydes and some pyrazines (McIver & Reineccius, 1986) are few examples of important Maillard reaction products that at the same time are also known to be formed enzymatically in various fruits and vegetables. Similarly, sotolone, 4,5-dimethyl-3-hydroxy-2(5H)-furanone, was isolated from various natural sources such as fenugreek seeds (Girardon, Sauvaire, Baccou & Bessiere, 1986), mushrooms (Rapior, Fons & Bessière, 2000) and lovage (Blank & Schieberle, 1993) as well as from processed foods such as cane sugar

(Tokitomo, Kobayashi, Yamanishi & Muraki, 1980), botrytized wines (Masuda, Okawa, Nishimura & Yunome, 1984), aged sake (Takahashi, Tadenuma & Sato, 1976), aged Port wine (Silva Ferreira, Barbe & Bertrand, 2003) and coffee (Blank et al., 1992). Sotolone is an important flavour impact compound with a very low threshold value of 0.02 ng/L air (Blank et al., 1992). Its aroma characteristics changes from caramel-like at low concentrations to curry-like aroma at high concentrations (Kobayashi, 1989). Thermally, it can be formed through intermediates generated from the Maillard reaction such as pyruvic and ketoglutaric acid the latter originating from glutamic acid (Kobayashi, 1989; Pisarnitskiĭ, Bezzubov & Egorov, 1987). In aged sake, sotolone can be produced by condensation of ketobutyric acid and acetaldehyde, both being acid decomposition products of threonine (Takahashi et al., 1976). Sotolone can also be detected in aqueous solutions of glyoxal/2,3-butanedione at pH 5 (Hofmann, 1996). Sotolone, the character-impact compound of fenugreek seed (*Trigonella foenum-graecum* L.) was first reported by Rijkens and Boelens (1975), however, its metabolic origin still remains unclear. There are indications that oxidative deamination of 4-hydroxyisoleucine (HIL) the most abundant amino acid in fenugreek seed or its corresponding lactone the 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone (Blank, Lin, Fumeaux, Welti & Fay, 1996) could be possible precursors of sotolone in fenugreek (Peraza-Luna, Rodríguez-Mendiola, Arias-Castro, Bessiere & Calva-Calva, 2001) (see Figure 29). In addition, 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) was reported by Rapior et al. (2000) to be also present in the cultures of *Lactarius helvus* a mushroom from Europe producing sotolone and in fenugreek seed (*Trigonella foenum-graecum)* by Peraza-Luna et al., (2001). Although both amino compounds have been postulated as precursors of sotolone, their possible role in the metabolic pathways of sotolone formation has not been investigated. The 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) can be hydrolyzed into sotolone as shown in Figure 29. Here we report for the first time the mechanism of thermal generation of 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) from amino acid model systems containing glyoxylic and pyruvic acids. Such precursors could be used as controlled delivery systems of sotolone aroma in processed foods.

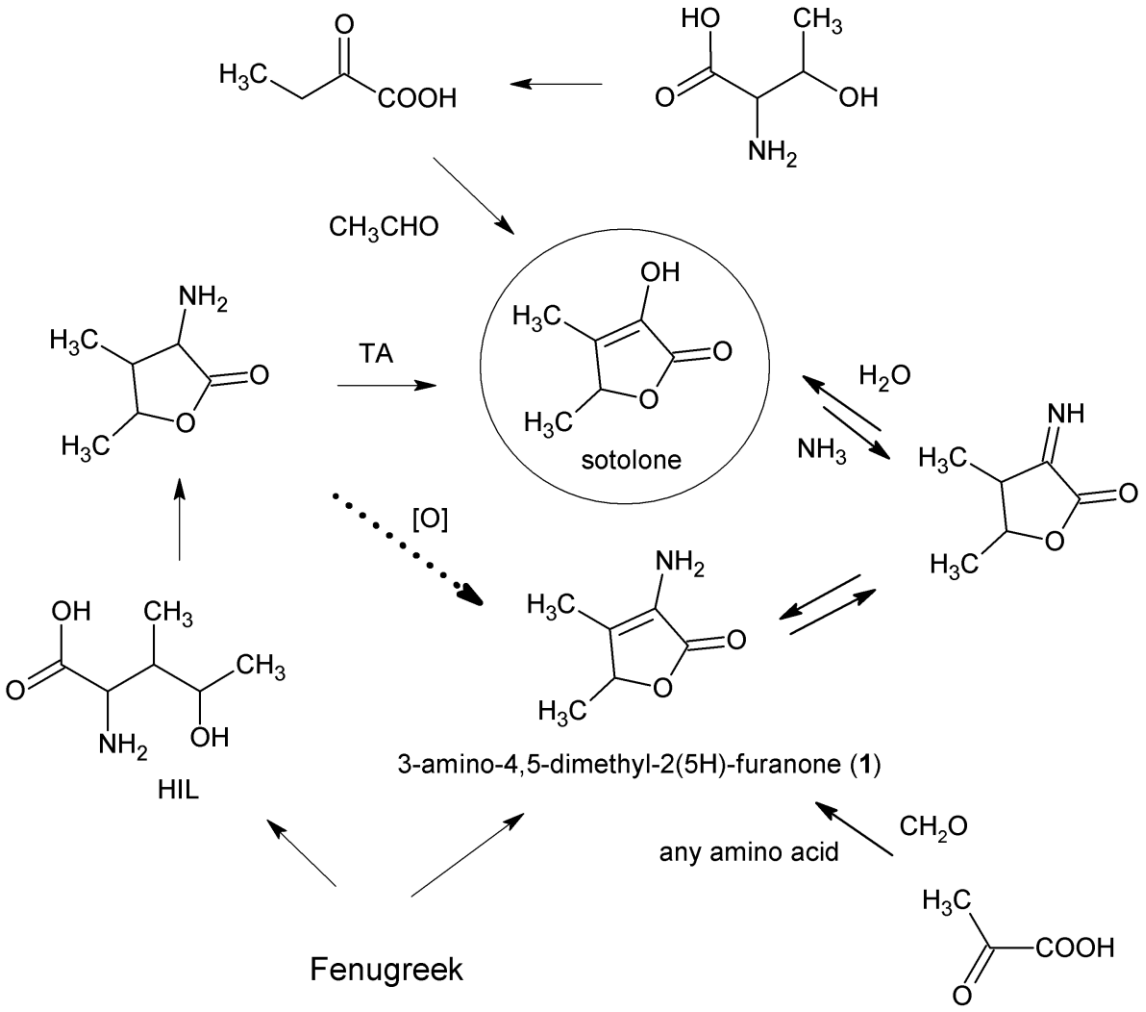


Figure 29. Known precursors of sotolone. Dotted arrows indicate proposed pathways. TA= Transaminase, HIL = 4-hydroxyisoleucine

8.3 Materials and Methods

8.3.1. Reagents & Chemicals

Glycine (99%), L-alanine (99%), pyruvic acid (98%), glyoxylic acid (98%), glycine hydrochloride (98%), pyruvic acid sodium salt (98%), sotolone (3% solution), ammonium chloride and paraformaldehyde (95%) were purchased from Sigma-Aldrich Chemical Co (Oakville, ON, Canada). The labelled [^{13}C-1]glycine (98%), [^{13}C-2]glycine (99%), [^{15}N]glycine (98%), [^{13}C-1]pyruvic acid sodium salt (99%), [^{13}C-2]pyruvic acid sodium salt (99%), [^{13}C-3]pyruvic acid sodium salt (99%), [^{13}C-1]alanine (99%), [^{13}C-2]alanine (99%), [^{15}N]alanine

(98%), were purchased from Cambridge Isotope Laboratories (Andover, MI). Fenugreek seeds were purchased from a local market.

8.3.2. Pyrolysis-Gas Chromatography-Mass Spectrometry (Py-GC/MS)

The Py-GC/MS analysis was performed according to procedure described by Guerra and Yaylayan (2010) with some modifications. A Varian CP-3800 GC equipped with a sample pre-concentration trap (SPT) filled with Tenax GR was coupled to a Varian Saturn 2000 Mass Spectrometry detector (Varian, Walnut Creek, CA, USA). The pyrolysis unit included a valved interface (CDS 1500), which was installed onto the GC injection port and connected to a CDS Pyroprobe 2000 unit (CDS Analytical, Oxford, PA, USA). The samples were analyzed on a DB-5MS column (5% diphenyl, 95% dimethyl-polysiloxane) with column dimensions of 50m length x 0.2mm internal diameter x 33μm film thickness (J&W Scientific, ON, Canada) using helium as the carrier gas. Two milligrams of sample mixtures containing 3:1 ratio of amino acid to the keto acid; 3:1:0.5 ratio of glycine to glyoxilic acid to pyruvic acid and 1:1 or 3:1 ratio of paraformaldehyde to pyruvic acid or three milligrams of ground fenugreek seed, were packed inside a quartz tube (0.3mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed at 200^{o}C for 20 seconds under helium atmosphere. For reactions performed under moisture, water (5μL) was added to silica gel (1mg) that was physically separated from the reaction mixture by a layer of glass wool and located at the exit end of the quartz tube. The volatiles after pyrolysis were concentrated on the sample pre-concentration trap (SPT), trapped at 50^{o}C and subsequently directed towards the GC column for separation. The GC column flow rate was regulated by an Electronic Flow Controller (EFC) and set at a delayed (30 s) pressure pulse of 70 psi for the first 4 minutes and maintained with a constant flow of 1.5mL/minute for the rest of the run. The GC oven temperature was set at –5^{o}C for first 5 minutes using CO_2 as the cryogenic cooling source and then increased to 50^{o}C at a rate of 50^{o}C/minute. Then, the oven temperature was again increased to 270^{o}C at a rate of 8^{o}C/minute and kept at 270^{o}C for 5 minutes. The MS transfer-line temperature was set at 250^{o}C, manifold temperature was set at 50^{o}C and the ion trap temperature was set at 175^{o}C. The ionization voltage of 70eV was used and EMV was set at 1500V. The reported percent label incorporation values (corrected for natural abundance and for percent enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

8.3.3. Tentative identification of 3-amino-4,5-dimethylfuran-2(5H)-one (1) and intermediate 2

The sotolone precursor was identified by comparison of its retention time and the mass spectrum to that generated from fenugreek seed and from commercial sotolone in the presence of excess ammonium chloride when pyrolyzed at 200^{o}C for 20 seconds and through NIST library matches in addition to isotope labelling data (see Table 24 andTable 25). The data reported in Table 24 toTable 26 are based on at least two replicate analyses with a percent standard deviation of < 15%. The proposed structure of intermediate **2** was based on the expected label incorporation pattern and the mass spectral fragmentations shown in Table 26.

Table 24. Percent incorporation of labelled atoms from glycine[a] and pyruvic acid[b] in m/z 127 of 3-amino-4,5-dimethyl-2(5H)-furanone (**1**).

Labelled reactant	M	M+1	M+2
[^{13}C-1]glycine[a]	0	0	0
[^{13}C-2]glycine[a]	0	100	0
[^{13}C-2]glycine[c]	85	15	0
[^{15}N]glycine[a]	0	100	0
[^{13}C-1]sodium pyruvate[b]	0	100	0
[^{13}C-2]sodium pyruvate[b]	0	0	100
[^{13}C-3]sodium pyruvate[b]	0	0	100

[a] Glycine /pyruvic acid model system (3:1). [b] Glycine.HCl/sodium pyruvate model system. [c] Glycine /pyruvic acid model system in the presence of unlabelled glyoxylic acid or paraformaldehyde

8.4 Results and Discussion

In an effort to investigate the role of 2-keto-acids such as glyoxylic and pyruvic acids in the Maillard reaction, glycine and alanine were chosen as model amino acids and their reaction mixtures were analyzed using Py-GC/MS. The results of these investigations have indicated that

such mixtures can generate 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) in addition to pyrazines and various other heterocyclic compounds that we reported previously (Guerra et al., 2010). Different model systems generated compound **1** in different intensities, however the most efficient system was that of glycine/pyruvic acid model (see Figure 30). The structure of compound **1** was confirmed through NIST library searches and by comparison of its retention time and the mass spectrum to that generated from pyrolysis of fenugreek seeds and from the reaction of commercial sotolone with ammonium chloride. Both fenugreek seeds and sotolone/NH_4Cl mixture generated peaks with identical retention times and mass spectra to that of the model systems and the NIST library with a very high match factor. As mentioned above, this compound was earlier reported (Rapior et al., 2000) in the cultures of *Lactarius helvus* a mushroom from Europe producing sotolone and in fenugreek seeds (*Trigonella foenum-graecum)* by Peraza-Luna et al., (2001). Due to the ease of conversion of 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) into sotolone, a potent aroma compound, its formation mechanism was further investigated to identify the role of its important precursors. Figure 30 indicates the ability of various model systems studied to generate compound **1** and the origin of label incorporation from different precursors (see also Table 24 andTable 25).

Table 25. Percent incorporation of labelled atoms from alanine[a] in m/z 127 of 3-amino-4,5-dimethyl-2(5H)-furanone (**1**).

Labelled reactant	M	M+1	M+2
[^{13}C-1]alanine[a]	0	0	0
[^{13}C-2]alanine [b]	0	0	100
[^{13}C-3]alanine [c]	0	0	100
[^{15}N]alanine[a]	0	100	0

[a] alanine /glyoxylic acid model system (3:1);[b] also implies the incorporation of C-3 atom of alanine; [c] based on the incorporation of C-2 of alanine

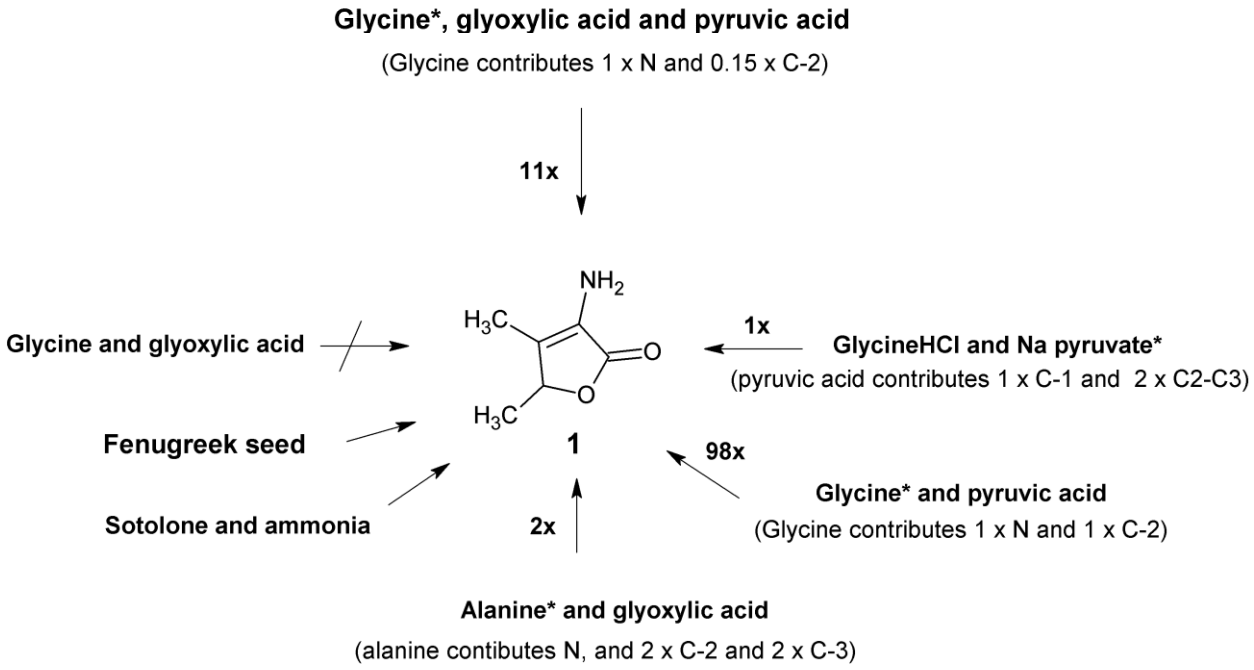


Figure 30. Different model systems capable of formation of 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**). Asterisk indicates labelled component in the reaction mixture. Efficiencies of formation (indicated over the arrows) are based on area/mole of amino acid reactant relative to the least efficient glycine.HCl/sodium pyruvate model.

According to Figure 30, unlike glycine/glyoxylic model system, glycine/pyruvic acid and alanine/glyoxylic acid systems were capable of formation of compound **1**. In addition, glyoxylic acid/alanine model system seems to alter the mechanism of formation of **1** as was indicted by the incorporation of four carbon atoms from the amino acid component as opposed to five carbon atoms from the keto-acid component in glycine pyruvic acid systems (see Figure 30, Table 24 and Table 25).These observations may indicate presence of different pathways for the generation of **1**. To explore in detail the different pathways of formation of **1** and as indicated in Figure 30 variously labelled glycine, alanine and pyruvic acid model systems were used. For model systems containing labelled pyruvic acid, glycine hydrochloride was used to neutralize the commercially available labelled sodium pyruvate. The efficiency of the pyrolytic generation of compound **1** was drastically reduced when the corresponding salts of glycine and pyruvic acid

were used as indicated in Figure 30. Furthermore, complete label incorporation patterns from different model systems are reported in Table 24 andTable 25.

8.4.1. Proposed mechanism of formation of 3-amino-4,5-dimethyl-2(5H)-furanone (1)

Based on the analysis of label incorporation patterns in different model systems (Table 24 andTable 25), we propose a two step formation pathway for the title compound (Figure 31). The first step involves the formation of 4,5-dimethylfuran-2,3-dione (**2**) and the second step involves its conversion into **1** through interaction with any amino acid. This proposition is based on the observation that model systems lacking amino acids generated only compound **2** such as paraformaldehyde and pyruvic acid (Table 26) and model systems containing amino acids generated only compound **1**. On the other hand, paraformaldehyde/glycine/pyruvic acid model systems when reacted in the presence of moisture generated only sotolone (see Table 27) confirming the ability of compound **1** to be hydrolyzed easily into sotolone. The data in Table 27 confirms the hypothesis that sotolone can be generated from 3-amino-4,5-dimethyl-2(5H)-furanone (**1**) in the presence of moisture.

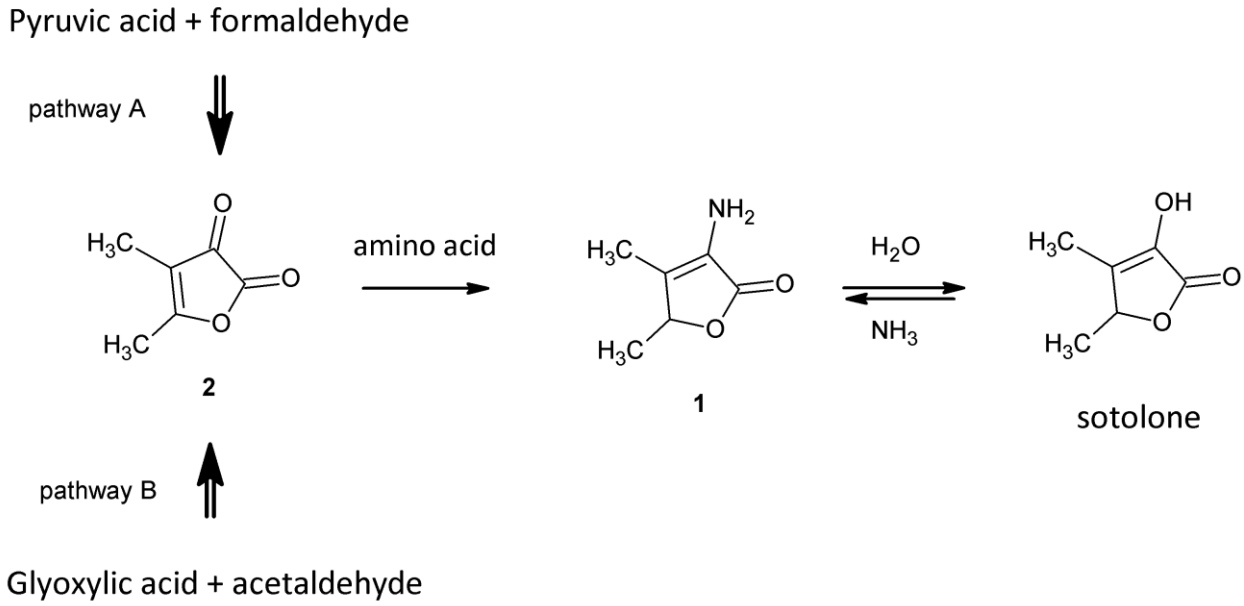


Figure 31. General pathways of formation of 3-amino-4,5-dimethyl-2(5H)-furanone (**1**).

Table 26. Percent incorporation of labelled atoms from pyruvic acid[a] in m/z 126 of 4,5-dimethylfuran-2,3-dione (**2**)[b].

Labelled reactant	M	M+1	M+2
[^{13}C-1]sodium pyruvate	0	100	0
[^{13}C-2]sodium pyruvate	0	0	100
[^{13}C-3]sodium pyruvate	0	0	100

[a] Paraformaldehyde/sodium pyruvate model system
[b] Retention time 15.55 min; m/z (% intensity) 127 (14.2), 126 (100), 98 (15.2), 97 (22.5), 84 (27.7), 83 (59.9), 69 (40.3), 55 (60.8), 41 (27.6), 39 (46.6)

8.4.1.1. Formation of 4,5-dimethylfuran-2,3-dione (2)

Based on the label incorporation patterns of different precursors (Table 24 andTable 25) the proposed intermediate 4,5-dimethylfuran-2,3-dione (**2**) can be formed by two different pathways shown in Figure 32. Pathway A requires only pyruvic acid and a formaldehyde source. In different model systems paraformaldehyde, glyoxylic acid or glycine was used as a source of formaldehyde, since glycine in the presence of pyruvic acid is known to generate formaldehyde (Guerra et al., 2010), similarly the decarboxylation of glyoxylic acid can yield formaldehyde. In the absence of a nitrogen source, model systems containing paraformaldehyde and pyruvic acid for example generated only compound **2** whereas in the presence of excess glycine only compound **1** was detected indicating conversion of **2** to **1** by the action of amino acid (see Figure 33). Pathway A requires aldol addition of two pyruvic acids molecules followed by decarboxylation and lactonization to form a furan-dione intermediate (**3)** that can undergo aldol addition with formaldehyde to form intermediate **4** followed by dehydration and isomerization to generate 4,5-dimethylfuran-2,3-dione (**2**). When selectively labelled pyruvic acids were reacted with paraformaldehyde as the source of formaldehyde, the compound (**2**) incorporated 1x C-1 and 2 x C2-C3 atoms (see Table 26) consistent with the proposed pathway A in Figure 32. However, in the presence of [^{13}C-2]glycine as the source of formaldehyde, pyruvic acid generated compound **1** with the same incorporation pattern of carbon atoms of pyruvic acid in addition to 100% incorporation of C-2 atom of glycine. When unlabelled glyoxylic acid was also added as a source of formaldehyde, the extent of incorporation of the [^{13}C-2]glycine label was

reduced to 15% indicating the higher efficiency of glyoxylic acid to generate formaldehyde relative to glycine (Table 24). On the other hand, unlike model system containing glyoxylic acid and glycine, the glyoxylic acid and alanine system also generated compound **1,** indicating the importance of alanine not only as a source of nitrogen but also as a precursor of the carbon atoms of the backbone of structure **1.** In the pathway B (Figure 32) the self-aldol addition product of acetaldehyde undergoes a second aldol reaction with glyoxylic acid and the subsequent lactonization generates intermediate **5** which can isomerize into **4** and similar to pathway A dehydrates to generate compound **2**.

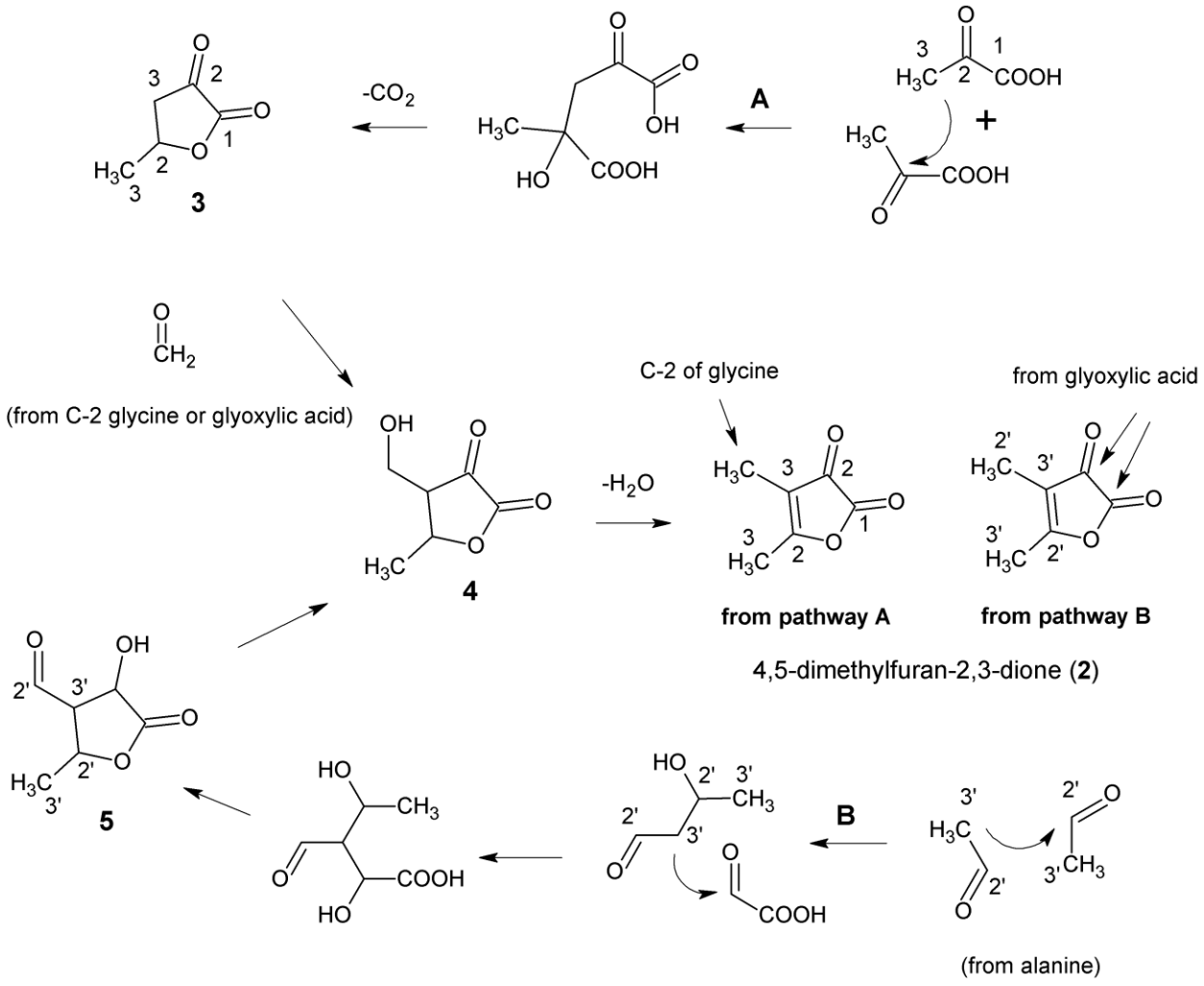


Figure 32. Proposed mechanism of formation of 4,5-dimethylfuran-2,3-dione through two pathways. Pathway A requires pyruvic acid and any formaldehyde source, pathway B requires glyoxylic acid and any acetaldehyde source.

Table 27. Comparison[a] of sotolone and 3-amino-4,5-dimethyl-2(5H)-furanone (**1**) formation in the presence and absence of moisture.

Model system	Sotolone	3-amino-4,5-dimethyl-2(5H)-furanone (1)
Paraformaldehyde /pyruvic acid/glycine (dry)	0	14.3×10^{10}
Paraformaldehyde/pyruvic acid/glycine (wet)	19.3×10^{10}	0

[a] in area/mole of glycine (values are based on the average of duplicate analysis with % RSD < 15)

8.4.1.2. Conversion of 4,5-dimethylfuran-2,3-dione (2) into 3-amino-4,5-dimethyl-2(5H)-furanone (1)

Both pathways A and B described above can generate compound **2** that can undergo an interesting variant of the Strecker reaction in the presence of any amino acid where the α, β-unsaturated carbonyl moiety of **2** is converted into the enamine moiety of **1** releasing in the process the Strecker aldehyde (Figure 33).

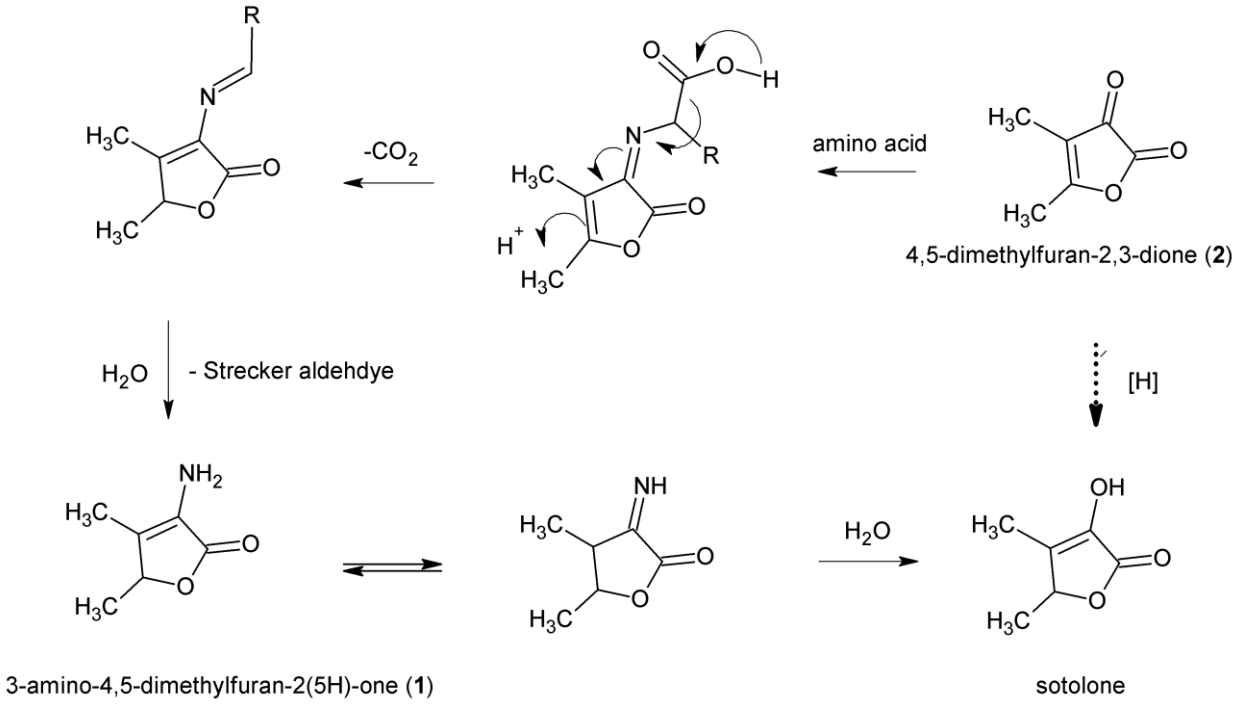


Figure 33. Proposed mechanism of conversion of 4,5-dimethylfuran-2,3-dione (**2**) into 3-amino-4,5-dimethyl-2(5H)-furanone (**1**) through Strecker-like reaction. R = amino acid side chain.

This transformation proceeds with retention of labelled carbon atom positions of intermediate **2** in the structure **1**. Structure **1** was confirmed not only through the NIST library search and mass spectral fragmentation patterns but also through the retention time of its naturally occurring counterpart desorbed from fenugreek seeds through pyrolysis and through its direct generation from the reaction of commercial sotolone with ammonia. Figure 32 summarizes the origin of the expected label incorporation in compound **2** from pathways A and B. Isotope label incorporation data supporting the proposed pathway A are shown in Table 24 and for pathway B in Table 25. Data in Table 24 indicate that in the presence of glycine and glyoxylic acid the main source of formaldehyde comes from glyoxylic acid decarboxylation (85%) and that there is 100% incorporation of one nitrogen atom from glycine. In addition, the data in Table 24 also confirm that the remaining five carbon atoms originate from pyruvic acid and as predicted from the proposed pathway, only one C-1 atom and two C2-C3 fragments were incorporated into the structure **1,** as shown in Figure 32. Regarding pathway B, data in Table 25 indicates incorporation of a single nitrogen atom of alanine, no incorporation of C-1 atom but double incorporation of C-2 and C-3 atoms from alanine consistent with the proposed pathway B shown in Figure 32. Out of six carbon atoms of structure **1** four were supplied by alanine indicating the remaining two atoms originated from glyoxylic acid as proposed by pathway B shown in Figure 32. This pathway although minor, may also shed some light on the mechanism of formation of sotolone as an off-flavor in citrus based soft drinks containing ascorbic acid during storage (Konig, Gutsche, Hartl, Hubscher, Schreier & Schwab, 1999). In this study ethanol along with ascorbic acid were identified as precursors of sotolone through incorporation of either one or two moles of ethanol into the sotolone backbone. Ascorbic acid is known to generate glyoxylic acid when incubated under storage conditions (Shin & Feather, 1990) and oxidation of ethanol into acetaldehyde during storage of alcoholic beverages has been documented (Pons, Lavigne, Landais, Darriet & Dubourdieu, 2010) indicating the possibility that pathway B shown in Figure 32 can provide a rational for the incorporation of two moles of ethanol into the sotolone backbone in citrus based soft drinks containing ascorbic acid.

Condensation of different keto-acids, dicarbonyl compounds and amino acids have been proposed in the literature as precursors of sotolone, however, identification of a nitrogen containing precursor such as 3-amino-4,5-dimethyl-2(5H)-furanone (**1**) that can be thermally generated from pyruvic acid and glycine was not reported so far. This knowledge may be used to

allow controlled generation of the intense aroma of sotolone in selected food products as demonstrated by the ability of pyruvic acid/glycine model system to generate sotolone when reacted in the presence of moisture.

CHAPTER 9. GENERAL CONCLUSIONS, CONTRIBUTIONS TO KNOWLEDGE AND SUGGESTIONS FOR FUTURE RESEARCH

9.1 General Conclusions

The existence of alternative pathways of formation of pyrazines, other than traditional Strecker reaction, were identified based on the study of model systems composed of α-dicarbonyls, such as 2,3-butendione, 3,4-hexanedione and 2-keto-acids and amino acids. One such pathway proceeds through the double addition of amino acids to the α-dicarbonyl compound with a double Schiff base formation step, another pathway is initiated by the formation of α-amino carbonyls, similar to the standard Strecker reaction, but followed by a sequential Schiff base formation by Strecker aldehyde at the free amino group of the α-amino carbonyl intermediate and the addition of amino acid as a Schiff base at the carbonyl group. Both pathways contribute differently to the incorporation of the amino acid carbon atoms into the pyrazine ring structure. Alternatively, dimerization of azomethine ylides generated from the reaction of α-keto-acids with amino acids was also shown to lead to the formation of pyrazine moiety. This pathway also generates pyrazine ring structure with the incorporation of carbon atoms from the amino acid. In addition to its ability to initiate Strecker-type reactions, 2,3-butendione was also shown to undergo cyclocondensation reaction to generate 1,2-benzoquinone intermediate under Maillard reaction conditions. This intermediate was shown to behave in a similar fashion to the sugar derived α-dicarbonyl compounds undergoing Strecker-type reactions or double additions with amino acids generating 1,2-diaminobenzene moieties. The occurrence of the above reaction was extended to the interaction between the oxidized ring B of (+)-catechin and the amino acid glycine and their ability to form amino acid adducts was confirmed through high resolution TOF/MS analysis. Formation of such non-volatile (+)-catechin/amino acid adducts provides insight into how amino acids can have the potential of modifying the antioxidant properties of (+)-catechin and how catechin-type structures interfere with the Maillard reaction in food through scavenging of amino acids. Furthermore, preliminary investigations of the non-volatile portion of a model system consisting of two different amino acids glycine and cysteine and glucose, confirmed the occurrence of a variant of the double addition reaction mentioned above. The structure of an adduct identified in this system was consistent with occurrence of two consecutive Amadori rearrangement steps each with a different amino acid. The initial glycine Amadori compound was shown to undergo a second rearrangement with cysteine followed by a

dehydration step. Finally, it was also demonstrated that α-keto-lactones can react with amino acids similar to 2-keto acids, *o*-quinones, or any sugar-derived 1,2-dicarbonyl to incorporate a nitrogen atom into its structure. Such a transformation of 4,5-dimethylfuran-2,3-dione an α-keto-lactones with amino acids was shown to lead to the formation of sotolone an important aroma active compound generated enzymatically in various plants.

9.2 Contributions to knowledge

The work presented in this thesis provided evidence for the first time of the following:

1. The identification of two reaction mechanisms for the formation of pyrazine that result in the incorporation of amino acid carbon atoms in the ring structure of pyrazines.
2. A proposal of a comprehensive reaction mechanism of double addition of amino acids either to (i) a α-dicarbonyl moiety to form pyrazines, or (ii) to a reducing sugar through sequential double Amadori rearrangements.
3. Pyrolytic degradation of 1,2-dicarbonyls into shorter chain aldehydes and dicarbonyls such as glyoxal, acetaldehyde and pyruvaldehyde from 2,3-butendione.
4. Demonstration that *o*-quinones can undergo Strecker type reactions and this principle was extended to oxidized ring-B of flavanols.
5. Detection of aromatic *o*-diamine, 4,5-dimethyl-1,2-phenylendiamine in a Maillard model system.
6. A systematic study of the volatile and non-volatile adducts formed from the reaction of two different amino acids and a sugar was presented, as well as the confirmation of the mechanism of formation of thiazoles previously proposed.
7. Confirmation of formation of azomethine ylides during the Maillard reaction through the identification of their dimerization products.
8. Identification and thermal formation of an important enzymatically generated aroma active compound, sotolone during Maillard reaction.

9.3 Future Research

From the results presented here, the following future research could be proposed:

- Evaluation of the potential of α-keto acids in generating Maillard reaction products using different amino acids apart from glycine.
- Assessment of the role of different amino acids in modulating the antioxidant activity of (+)-catechin under thermal processing conditions.
- Further investigation of the role of sequential Amadori rearrangements in the generation of aroma/flavor active compounds using different combinations of sugars and amino acids.

REFERENCES

Adams, A., Polizzi, V., van Boekel, M., & De Kimpe, N. (2008). Formation of pyrazines and a novel pyrrole in Maillard model systems of 1,3-dihydroxyacetone and 2-oxopropanal. *Journal of Agricultural and Food Chemistry*, *56*(6), 2147-2153.

Ames, J. M. (1998). Applications of the Maillard reaction in the food industry. *Food Chemistry*, *62*(4), 431-439.

Amrani-Hemaimi, M., Cerny, C., & Fay, L. B. (1995). Mechanisms of Formation of alkylpyrazines in the Maillard reaction. *Journal of Agricultural and Food Chemistry*, *43*(11), 2818-2822.

Arts, I. C. W., van de Putte, B., & Hollman, P. C. H. (2000). Catechin contents of foods commonly consumed in The Netherlands. 2. Tea, wine, fruit juices, and chocolate milk. *Journal of Agricultural and Food Chemistry*, *48*(5), 1752-1757.

Aurelio, L., Box, J. S., Brownlee, R. T. C., Hughes, A. B., & Sleebs, M. M. (2003). An efficient synthesis of n-methyl amino acids by way of intermediate 5-oxazolidinones. *The Journal of Organic Chemistry*, *68*(7), 2652-2667.

Bailey, R. G., Ames, J. M., & Monti, S. M. (1996). An analysis of the non-volatile reaction products of aqueous Maillard model systems at pH 5, using reversed-phase HPLC with diode-array detection. *Journal of the Science of Food and Agriculture*, *72*(1), 97-103.

Barbieri, G., Bolzoni, L., Careri, M., Mangia, A., Parolari, G., Spagnoli, S., & Virgili, R. (1994). Study of the volatile fraction of Parmesan cheese. *Journal of Agricultural and Food Chemistry*, *42*(5), 1170-1176.

Bartowsky, E. J., & Henschke, P. A. (2004). The 'buttery' attribute of wine—diacetyl—desirability, spoilage and beyond. *International Journal of Food Microbiology*, *96*(3), 235-252.

Bauer, K., Garbe, D., & Surburg, H. (2001). Single fragrance and flavor materials. *Common Fragrance and Flavor Materials* (pp. 7-165): Wiley-VCH Verlag GmbH.

Bin, Q., Peterson, D. G., & Elias, R. J. (2012). Influence of phenolic compounds on the mechanisms of pyrazinium radical generation in the Maillard reaction. *Journal of Agricultural and Food Chemistry*, *60*(21), 5482-5490.

Bittner, S. (2006). When quinones meet amino acids: chemical, physical and biological consequences. *Amino Acids*, *30*(3), 205-224.

Blank, I., Lin, J., Fumeaux, R., Welti, D. H., & Fay, L. B. (1996). Formation of 3-hydroxy-4,5-dimethyl-2(5h)-furanone (sotolone) from 4-hydroxy-l-isoleucine and 3-amino-4,5-dimethyl-3,4-dihydro-2(5h)-furanone. *Journal of Agricultural and Food Chemistry*, *44*(7), 1851-1856.

Blank, I., Robert, F., Goldmann, T., Pollien, P., Varga, N., Devaud, S., Saucy, F., Huynh-Ba, T., & Stadler, R. (2005). Mechanisms of acrylamide formation. In: M. Friedman, & D. Mottram, *Chemistry and Safety of Acrylamide in Food*, vol. 561 (pp. 171-189): Springer US.

Blank, I., & Schieberle, P. (1993). Analysis of the seasoning-like flavour substances of a commercial lovage extract (*Levisticum officinale* Koch.). *Flavour and Fragrance Journal*, *8*(4), 191-195.

Blank, I., Sen, A., & Grosch, W. (1992). Potent odorants of the roasted powder and brew of Arabica coffee. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, *195*(3), 239-245.

Brudzynski, K., & Miotto, D. (2011). Honey melanoidins: Analysis of the compositions of the high molecular weight melanoidins exhibiting radical-scavenging activity. *Food Chemistry*, *127*(3), 1023-1030.

Buttery, R. G., Ling, L. C., & Stern, D. J. (1997). Studies on popcorn aroma and flavor volatiles. *Journal of Agricultural and Food Chemistry*, *45*(3), 837-843.

Cämmerer, B., Wedzicha, B. L., & Kroh, L. W. (1999). Nonenzymatic browning reactions of Retro-Aldol degradation products of carbohydrates. *European Food Research and Technology*, *209*(3-4), 261-265.

Carruthers, W., & Coldham, I. (2004). 1,3-Dipolar cycloaddition reactions. *Modern Methods of Organic Synthesis* (pp. 228-230): Cambridge University Press.

Cerny, C., & Davidek, T. (2003). Formation of aroma compounds from ribose and cysteine during the Maillard reaction. *Journal of Agricultural and Food Chemistry*, *51*(9), 2714-2721.

Cerny, C., & Guntz-Dubini, R. (2013). Formation of cysteine-s-conjugates in the Maillard reaction of cysteine and xylose. *Food Chemistry*, *141*(2), 1078-1086.

Challinor, J. M. (1996). A rapid simple pyrolysis derivatisation gas chromatography-mass spectrometry method for profiling of fatty acids in trace quantities of lipids. *Journal of Analytical and Applied Pyrolysis*, *37*(2), 185-197.

Cho, I., Lee, S., Jun, H.-R., Roh, H.-J., & Kim, Y.-S. (2010). Comparison of volatile Maillard reaction products from tagatose and other reducing sugars with amino acids. *Food Science and Biotechnology*, *19*(2), 431-438.

Cho, I., & Peterson, D. (2010). Chemistry of bread aroma: A review. *Food Science and Biotechnology*, *19*(3), 575-582.

Chu, F. L., Sleno, L., & Yaylayan, V. A. (2011). Isotope labeling studies on the formation of multiple addition products of alanine in the pyrolysis residue of glucose/alanine mixtures by high-resolution ESI-TOF-MS. *Journal of Agricultural and Food Chemistry*, *59*(21), 11629-11634.

Chu, F. L., & Yaylayan, V. A. (2008a). Model studies on the oxygen-induced formation of benzaldehyde from phenylacetaldehyde using pyrolysis GC-MS and FTIR. *Journal of Agricultural and Food Chemistry*, *56*(22), 10697-10704.

Chu, F. L., & Yaylayan, V. A. (2008b). Post-Schiff base chemistry of the Maillard reaction. *Annals of the New York Academy of Sciences*, *1126*(1), 30-37.

Chu, F. L., & Yaylayan, V. A. (2009a). FTIR monitoring of oxazolidin-5-one formation and decomposition in a glycolaldehyde-phenylalanine model system by isotope labeling techniques. *Carbohydrate Research*, *344*(2), 229-236.

Chu, F. L., & Yaylayan, V. A. (2009b). Isotope labeling studies on the origin of 3,4-hexanedione and 1,2-butanedione in an alanine/glucose model system. *Journal of Agricultural and Food Chemistry*, *57*(20), 9740-9746.

Colahan-Sederstrom, P. M., & Peterson, D. G. (2004). Inhibition of key aroma compound generated during ultrahigh-temperature processing of bovine milk via epicatechin addition. *Journal of Agricultural and Food Chemistry*, *53*(2), 398-402.

Coleman, E. C., Ho, C.-T., & Chang, S. S. (1981). Isolation and identification of volatile compounds from baked potatoes. *Journal of Agricultural and Food Chemistry*, *29*(1), 42-48.

Cren-Olivé, C., Déprez, S., Lebrun, S., Coddeville, B., & Rolando, C. (2000). Characterization of methylation site of monomethylflavan-3-ols by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, *14*(23), 2312-2319.

Czerny, M., Mayer, F., & Grosch, W. (1999). Sensory study on the character impact odorants of roasted Arabica coffee. *Journal of Agricultural and Food Chemistry*, *47*(2), 695-699.

Davídek, T., Devaud, S., Robert, F., & Blank, I. (2006). Sugar fragmentation in the maillard reaction cascade: Isotope labeling studies on the formation of acetic acid by a hydrolytic β-dicarbonyl cleavage mechanism. *Journal of Agricultural and Food Chemistry*, *54*(18), 6667-6676.

Es-Safi, N.-E., Cheynier, V., & Moutounet, M. (2000). Study of the reactions between (+)-catechin and furfural derivatives in the presence or absence of anthocyanins and their implication in food color change. *Journal of Agricultural and Food Chemistry*, *48*(12), 5946-5954.

Fay, L. B., & Brevard, H. (2005). Contribution of mass spectrometry to the study of the Maillard reaction in food. *Mass Spectrometry Reviews*, *24*(4), 487-507.

Fayle, S. E., Gerrard, J. A., & Belton, P. S. (2002). What is the Maillard reaction?. *The Maillard Reaction* (pp. 1-8): The Royal Society of Chemistry.

Finot, P. A., Aeschbacher, H. U., Hurrell, R. F., & Liardon, R., eds (1990). *The Maillard Reaction in Food Processing, Human Nutrition, and Physiology*. Basel; Boston: Birkhäuser Verlag.

Frank, O., & Hofmann, T. (2002). Reinvestigation of the chemical structure of bitter-tasting quinizolate and homoquinizolate and studies on their Maillard-type formation pathways using suitable 13C-labeling experiments. *Journal of Agricultural and Food Chemistry*, *50*(21), 6027-6036.

Freeman, F., & Govindarajoo, G. (1995). Structures and dimerization reactions of acyclic azomethine ylides. *Reviews on Heteroatom Chemistry*, *13*, 123-147.

Gerrard, J. A., Brown, P. K., & Fayle, S. E. (2002). Maillard crosslinking of food proteins I: The reaction of glutaraldehyde, formaldehyde and glyceraldehyde with ribonuclease. *Food Chemistry*, *79*(3), 343-349.

Gerrard, J. A., Brown, P. K., & Fayle, S. E. (2003a). Maillard crosslinking of food proteins ii: The reactions of glutaraldehyde, formaldehyde and glyceraldehyde with wheat proteins in vitro and in situ. *Food Chemistry*, *80*(1), 35-43.

Gerrard, J. A., Brown, P. K., & Fayle, S. E. (2003b). Maillard crosslinking of food proteins III: The effects of glutaraldehyde, formaldehyde and glyceraldehyde upon bread and croissants. *Food Chemistry*, *80*(1), 45-50.

Gerrard, J. A., & Cottam, J. R. (2012). Protein cross-linking in food – Structure, applications, implications for health and food safety. *Food Biochemistry and Food Processing* (pp. 207-222): Wiley-Blackwell.

Gerrard, J. A., Meade, S. J., Miller, A. G., Brown, P. K., Yasir, S. B. M., Sutton, K. H., & Newberry, M. P. (2005). Protein cross-linking in food. *Annals of the New York Academy of Sciences*, *1043*(1), 97-103.

Girardon, P., Sauvaire, Y., Baccou, J., & Bessiere, J. (1986). Identification de la 3-hydroxy-4, 5-dimethyl-2 (5h)-furanone dans l'arome des graines de fenugrec (*Trigonella foenum graecum* L.). *Lebensmittel-Wissenschaft+ Technologie*, *19*(1), 44-46.

Gobert, J., & Glomb, M. A. (2009). Degradation of glucose: Reinvestigation of reactive α-dicarbonyl compounds. *Journal of Agricultural and Food Chemistry*, *57*(18), 8591-8597.

Granvogl, M., Beksan, E., & Schieberle, P. (2012). New insights into the formation of aroma-active Strecker aldehydes from 3-oxazolines as transient intermediates. *Journal of Agricultural and Food Chemistry*.

Guerra, P. V., & Yaylayan, V. A. (2010). Dimerization of azomethine ylides: an alternate route to pyrazine formation in the Maillard reaction. *Journal of Agricultural and Food Chemistry*, *58*(23), 12523-12529.

Guerra, P. V., & Yaylayan, V. A. (2012). Double Schiff base adducts of 2,3-butanedione with glycine: formation of pyrazine rings with the participation of amino acid carbon atoms. *Journal of Agricultural and Food Chemistry*, *60*(45), 11440-11445.

Guerra, P. V., & Yaylayan, V. A. (2013). Cyclocondensation of 2,3-butanedione in the presence of amino acids and formation of 4,5-dimethyl-1,2-phenylendiamine. *Food Chemistry*, *141*(4), 4391-4396.

Haffenden, L. J. W., & Yaylayan, V. A. (2005). Mechanism of formation of redox-active hydroxylated benzenes and pyrazine in 13C-labeled glycine/d-glucose model systems. *Journal of Agricultural and Food Chemistry*, *53*(25), 9742-9746.

Hanson, J. R. (2011). *The Organic Chemistry of Isotopic Labelling*. Royal Society of Chemistry.

Hartman, G. J., & Ho, C. T. (1984). Volatile products of the reaction of sulfur-containing amino acids with 2,3-butanedione. *Food Science & Technology*, *17*(3), 171-174.

Henle, T. (2003). Ages in foods: do they play a role in uremia? *Kidney International*, *63*, S145-S147.

Henle, T. (2005). Protein-bound advanced glycation endproducts (AGEs) as bioactive amino acid derivatives in foods. *Amino Acids*, *29*(4), 313-322.

Hidalgo, F. J., Alcón, E., & Zamora, R. (2013). Cysteine- and serine-thermal degradation products promote the formation of Strecker aldehydes in amino acid reaction mixtures. *Food Research International*, *54*(2), 1394-1399.

Hidalgo, F. J., Delgado, R. M., Navarro, J. L., & Zamora, R. (2010). Asparagine decarboxylation by lipid oxidation products in model systems. *Journal of Agricultural and Food Chemistry*, *58*(19), 10512-10517.

Ho, C.-T., & Coleman, E. C. (1980). Chemistry of baked potato flour: Futur identification of heterocyclic compounds in the volatile flavor of baked potato. *Journal of Food Science*, *45*(4), 1094-1095.

Ho, C. T., & Hartman, G. J. (1982). Formation of oxazolines and oxazoles in Strecker degradation of dl-alanine and l-cysteine with 2,3-butanedione. *Journal of Agricultural and Food Chemistry*, *30*(4), 793-794.

Hodge, J. E. (1953). Dehydrated foods, chemistry of browning reactions in model systems. *Journal of Agricultural and Food Chemistry*, *1*(15), 928-943.

Hofmann, T. (1999). Quantitative studies on the role of browning precursors in the Maillard reaction of pentoses and hexoses with l-alanine. *European Food Research Technology*, *209*, 113-121.

Hofmann, T. (2005). Taste-active Maillard reaction products: The "tasty" world of nonvolatile Maillard reaction products. *Annals of the New York Academy of Sciences*, *1043*(1), 20-29.

Hofmann, T., Münch, P., & Schieberle, P. (2000). Quantitative model studies on the formation of aroma-active aldehydes and acids by Strecker-type reactions. *Journal of Agricultural and Food Chemistry*, *48*(2), 434-440.

Hofmann, T., & Schieberle, P. (2000). Formation of aroma-active Strecker-aldehydes by a direct oxidative degradation of Amadori compounds. *Journal of Agricultural and Food Chemistry*, *48*(9), 4301-4305.

Hofmann, T. a. S., P. (1996). Studies on intermediates generating the flavour compounds 2-methyl-3-furanthiol, 2-acetyl-2-thiazoline and sotolon by Maillard-type reactions. *Flavour Science: Recent Developments* (pp. 182-187). London: Royal Society of Chemistry.

Hollnagel, A., & Kroh, L. W. (1998). Formation of α-dicarbonyl fragments from mono- and disaccharides under caramelization and Maillard reaction conditions. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A*, *207*(1), 50-54.

Horai, H., Arita, M., Kanaya, S., Nihei, Y., Ikeda, T., Suwa, K., Ojima, Y., Tanaka, K., Tanaka, S., Aoshima, K., Oda, Y., Kakazu, Y., Kusano, M., Tohge, T., Matsuda, F., Sawada, Y., Hirai, M. Y., Nakanishi, H., Ikeda, K., Akimoto, N., Maoka, T., Takahashi, H., Ara, T., Sakurai, N., Suzuki, H., Shibata, D., Neumann, S., Iida, T., Tanaka, K., Funatsu, K., Matsuura, F., Soga, T., Taguchi, R., Saito, K., & Nishioka, T. (2010). MassBank: A public repository for sharing mass spectral data for life sciences. *Journal of Mass Spectrometry*, *45*(7), 703-714.

Huyghues-Despointes, A., Yaylayan, V. A., & Keyhani, A. (1994). Pyrolysis/GC/MS analysis of 1-[(2'-carboxyl)pyrrolidinyl]-1-deoxy-d-fructose (proline Amadori compound). *Journal of Agricultural and Food Chemistry*, *42*(11), 2519-2524.

Johnstone, R. A. W. (1972). *Mass Spectrometry for Organic Chemists*. University Press.

Joucla, M., & Mortier, J. (1988). Parent and n-substituted azomethine ylides from alpha-amino-acids and formaldehyde. an easy access to 2,5-unsubstituted pyrrolidines. evidence for oxazolidin-5-ones as direct precursor of these reactive intermediates. *Bulletin De La Societe Chimique De France*(3), 579-583.

Kawamura, S. I. (1983). Seventy years of the Maillard reaction. *The Maillard Reaction in Foods and Nutrition*, vol. 215 (pp. 3-18): American Chemical Society.

Keeney, M., & Day, E. A. (1957). Probable role of the strecker degradation of amino acids in development of cheese flavor. *Journal of Dairy Science*, *40*(7), 874-876.

Keyhani, A., & Yaylayan, V. (1999). Origin of 2,3-pentanedione and 2,3-butanedione in d-glucose/l-alanine Maillard model systems. *Journal of Agricultural and Food Chemistry*, *47*(8), 3280-3284.

Keyhani, A., & Yaylayan, V. A. (1996). Elucidation of the mechanism of pyrazinone formation in glycine model systems using labeled sugars and amino acids. *Journal of Agricultural and Food Chemistry*, *44*(9), 2511-2516.

Keyhani, A., & Yaylayan, V. A. (1997). Glycine specific novel Maillard reaction products: 5-hydroxy-1,3-dimethyl-2(1h)-quinoxalinone and related compounds. *Journal of Agricultural and Food Chemistry*, *45*(3), 697-702.

Kobayashi, A. (1989). Sotolon: Identification, formation and effect on flavor. *Flavor Chemistry*, vol. 388 (pp. 49-59): American Chemical Society.

Koehler, P. E., Mason, M. E., & Newell, J. A. (1969). Formation of pyrazine compounds in sugar-amino acid model systems. *Journal of Agricultural and Food Chemistry*, *17*(2), 393-396.

Konig, T., Gutsche, B., Hartl, M., Hubscher, R., Schreier, P., & Schwab, W. (1999). 3-Hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon) causing an off-flavor: Elucidation of its formation pathways during storage of citrus soft drinks. *Journal of Agricultural and Food Chemistry*, *47*(8), 3288-3291.

Lang, R., Mueller, C., & Hofmann, T. (2006). Development of a stable isotope dilution analysis with liquid chromatography−tandem mass spectrometry detection for the quantitative analysis of

di- and trihydroxybenzenes in foods and model systems. *Journal of Agricultural and Food Chemistry*, *54*(16), 5755-5762.

Langler, J. E., Libbey, L. M., & Day, E. A. (1967). Identification and evaluation of selected compounds in Swiss cheese flavor. *Journal of Agricultural and Food Chemistry*, *15*(3), 386-391.

Lederer, M. O., & Klaiber, R. G. (1999). Cross-linking of proteins by Maillard processes: characterization and detection of lysine–arginine cross-links derived from glyoxal and methylglyoxal. *Bioorganic & Medicinal Chemistry*, *7*(11), 2499-2507.

Ledl, F., & Schleicher, E. (1990). New aspects of the Maillard reaction in foods and in the human body. *Angewandte Chemie International Edition in English*, *29*(6), 565-594.

Lee, M.-H., Ho, C.-T., & Chang, S. S. (1981). Thiazoles, oxazoles, and oxazolines identified in the volatile flavor of roasted peanuts. *Journal of Agricultural and Food Chemistry*, *29*(3), 684-686.

Ling, A. R. (1908). Malting. *Journal of the Institute of Brewing*, *14*, 494-521.

Ma, Y. L., Li, Q. M., Van den Heuvel, H., & Claeys, M. (1997). Characterization of flavone and flavonol aglycones by collision-induced dissociation tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, *11*(12), 1357-1364.

Maddila, S., & Jonnalagadda, S. B. (2012). Efficient one-pot synthesis of benzoxazole derivatives catalyzed by nickel supported silica. *Journal of the Chilean Chemical Society*, *57*, 1099-1100.

Maga, J. A., & Katz, I. (1982). Pyrazines in foods: An update. *C R C Critical Reviews in Food Science and Nutrition*, *16*(1), 1-48.

Maga, J. A., & Sizer, C. E. (1973). Pyrazines in foods. Review. *Journal of Agricultural and Food Chemistry*, *21*(1), 22-30.

Maillard, M. N., Soum, M. H., Boivin, P., & Berset, C. (1996). Antioxidant activity of barley and malt: relationship with phenolic content. *LWT - Food Science and Technology*, *29*(3), 238-244.

Martineau, B., Acree, T., & Henick-Kling, T. (1994). A simple and accurate gc/ms method for the quantitative analysis of diacetyl in beer and wine. *Biotechnology Techniques*, *8*(1), 7-12.

Masuda, M., Okawa, E.-i.-c., Nishimura, K.-i.-c., & Yunome, H. (1984). Identification of 4, 5-dimethyl-3-hydroxy-2(5*H*)-furanone (sotolon) and ethyl 9-hydroxynonanoate in botrytised wine and evaluation of the roles of compounds characteristic of it. *Agricultural and Biological Chemistry*, *48*(11), 2707-2710.

McIver, R. C., & Reineccius, G. A. (1986). Synthesis of 2-methoxy-3-alkylpyrazines by *Pseudomonas perolens*. *Biogeneration of Aromas*, vol. 317 (pp. 266-274): American Chemical Society.

Meade, S. J., Miller, A. G., & Gerrard, J. A. (2003). The role of dicarbonyl compounds in non-enzymatic crosslinking: a structure–activity study. *Bioorganic & Medicinal Chemistry*, *11*(6), 853-862.

Meyer, S., & Metzger, J. (2003). Use of electrospray ionization mass spectrometry for the investigation of radical cation chain reactions in solution: Detection of transient radical cations. *Analytical and Bioanalytical Chemistry*, *377*(7-8), 1108-1114.

Meynier, A., & Mottram, D. S. (1995). The effect of pH on the formation of volatile compounds in meat-related model systems. *Food Chemistry*, *52*(4), 361-366.

Miyazato, H., Nakamura, M., Hashimoto, S., & Hayashi, S. (2013). Identification of the odour-active cyclic diketone cis-2,6-dimethyl-1,4-cyclohexanedione in roasted Arabica coffee brew. *Food Chemistry*, *138*(4), 2346-2355.

More, S. S., Vartak, A. P., & Vince, R. (2012). The butter flavorant, diacetyl, exacerbates β-amyloid cytotoxicity. *Chemical Research in Toxicology*, *25*(10), 2083-2091.

Mottram, D. (2007). The Maillard reaction: source of flavour in thermally processed foods. In: R. Berger, *Flavours and Fragrances* (pp. 269-283): Springer Berlin Heidelberg.

Mottram, D. S. (1998). Flavour formation in meat and meat products: A review. *Food Chemistry*, *62*(4), 415-424.

Mottram, D. S., Low, M. Y., & Elmore, J. S. (2006). The Maillard reaction and its role in the formation of acrylamide and other protentially hazardous compunds in foods. In: J. A. K. Skog, *Acrylamide and Other Hazardous Compounds in Heat-Treated Foods* (pp. 3-22). Cambridge: Woodhead Publishing.

Müller, R., & Rappert, S. (2010). Pyrazines: Occurrence, formation and biodegradation. *Applied Microbiology and Biotechnology*, *85*(5), 1315-1320.

Mulders, E. J. (1973). Volatile components from the non-enzymic browning reaction of the cysteine/cystine-ribose system. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, *152*(4), 193-201.

Murai, K., Morishita, M., Nakatani, R., Fujioka, H., & Kita, Y. (2008). Oxidative decarboxylative synthesis of 2-h-imidazolines from glyoxylic acid and 1,2-diamines. *Chemical Communications*(37), 4498-4500.

Murray, K. E., & Whitfield, F. B. (1975). The occurrence of 3-alkyl-2-methoxypyrazines in raw vegetables. *Journal of the Science of Food and Agriculture*, *26*(7), 973-986.

Nagaraj, R. H., Shipanova, I. N., & Faust, F. M. (1996a). Protein cross-linking by the Maillard reaction. *Journal of Biological Chemistry*, *271*(32), 19338-19345.

Nagaraj, R. H., Shipanova, I. N., & Faust, F. M. (1996b). Protein cross-linking by the Maillard reaction: Isolation, characterization, and in vivo detection of a lysine-lysine cross-link derived from methylglyoxal. *Journal of Biological Chemistry*, *271*(32), 19338-19345.

Nikolantonaki, M., & Waterhouse, A. L. (2012). A method to quantify quinone reaction rates with wine relevant nucleophiles: A key to the understanding of oxidative loss of varietal thiols. *Journal of Agricultural and Food Chemistry*, *60*(34), 8484-8491.

Nikolov, P. Y., & Yaylayan, V. A. (2012). Role of the ribose-specific marker furfuryl-amine in the formation of aroma active 1-(furan-2-ylmethyl)-1h-pyrrole (or furfuryl-pyrrole) derivatives. *Journal of Agricultural and Food Chemistry*, *60*(40), 10155-10161.

Noda, Y., & Peterson, D. G. (2007). Structure−reactivity relationships of flavan-3-ols on product generation in aqueous glucose/glycine model systems. *Journal of Agricultural and Food Chemistry*, *55*(9), 3686-3691.

Nursten, H. E. (1981). Recent developments in studies of the Maillard reaction. *Food Chemistry*, *6*(3), 263-277.

Nursten, H. E. (2005). *The Maillard reaction. Chemistry, biochemistry and implications*. Royal Society of Chemistry.

Ota, M., Kohmura, M., & Kawaguchi, H. (2006). Characterization of a new Maillard type reaction product generated by heating 1-deoxymaltulosyl-glycine in the presence of cysteine. *Journal of Agricultural and Food Chemistry*, *54*(14), 5127-5131.

Padwa, A. (2002). Synthetic applications of 1,3-dipolar cycloaddition chemistry toward heterocycles and natural products. In: A. Padwa, & W. H. Pearson, *Chemistry of Heterocyclic Compounds*, vol. 59 (pp. 820-822): Wiley.

Paine III, J. B., Pithawalla, Y. B., & Naworal, J. D. (2008). Carbohydrate pyrolysis mechanisms from isotopic labeling: Part 2. The pyrolysis of d-glucose: general disconnective analysis and the formation of C1 and C2 carbonyl compounds by electrocyclic fragmentation mechanisms. *Journal of Analytical and Applied Pyrolysis*, *82*(1), 10-41.

Patiny, L., & Borel, A. (2013). ChemCalc: A building block for tomorrow's chemical infrastructure. *Journal of Chemical Information and Modeling*, *53*(5), 1223-1228.

Patton, S. (1954). The mechanism of sunlight flavor formation in milk with special reference to methionine and riboflavin1. *Journal of Dairy Science*, *37*(4), 446-452.

Peraza-Luna, F., Rodríguez-Mendiola, M., Arias-Castro, C., Bessiere, J.-M., & Calva-Calva, G. (2001). Sotolone production by hairy root cultures of *Trigonella foenum-graecum* in airlift with mesh bioreactors. *Journal of Agricultural and Food Chemistry*, *49*(12), 6012-6019.

Perez Locas, C., & Yaylayan, V. A. (2008). Further insight into thermally and pH-induced generation of acrylamide from glucose/asparagine model systems. *Journal of Agricultural and Food Chemistry*, *56*(15), 6069-6074.

Pietta, P.-G. (2000). Flavonoids as antioxidants. *Journal of Natural Products*, *63*(7), 1035-1042.

Piloty, M., & Baltes, W. (1979). Investigations on the reaction of amino-acids with alpha-dicarbonyl compounds .2. volatile products of the reaction of amino-acids with diacetyl (2,3-butanedione). *Zeitschrift für Lebensmittel-Untersuchung und-Forschung*, *168*(5), 374-380.

Pisarnitskiĭ, A. F., Bezzubov, A. A., & Egorov, I. A. (1987). Nonenzymatic formation of 4,5-dimethyl-3-hydroxy-2(5h)-furanone in foodstuffs. *Prikladnaia Biokhimiia i Mikrobiologiia*, *23*(5), 642-646.

Pons, A., Lavigne, V. R., Landais, Y., Darriet, P., & Dubourdieu, D. (2010). Identification of a sotolon pathway in dry white wines. *Journal of Agricultural and Food Chemistry*, *58*(12), 7273-7279.

Porter, Q. N., & Baldas, J. (1971). *Mass Spectrometry of Heterocyclic Compounds*. New York :: Wiley-Interscience.

Pozo-Bayón, M. A., Guichard, E., & Cayot, N. (2006). Flavor control in baked cereal products. *Food Reviews International*, *22*(4), 335-379.

Pripis-Nicolau, L., de Revel, G., Bertrand, A., & Maujean, A. (2000). Formation of flavor components by the reaction of amino acid and carbonyl compounds in mild conditions. *Journal of Agricultural and Food Chemistry*, *48*(9), 3761-3766.

Qian, M., & Reineccius, G. (2002). Identification of aroma compounds in Parmigiano-Reggiano cheese by gas chromatography/olfactometry. *Journal of Dairy Science*, *85*(6), 1362-1369.

Raghavan, S. K., Ho, C.-T., & Daun, H. (1986). Identification of soy protein in meat by pyrolysis—high-resolution gas chromatography. *Journal of Chromatography A*, *351*(0), 195-202.

Rapior, S., Fons, F., & Bessière, J.-M. (2000). The fenugreek odor of *Lactarius helvus*. *Mycologia*, *92*(2), 305-308.

Reddy, V. P., & Beyaz, A. (2006). Inhibitors of the Maillard reaction and AGE breakers as therapeutics for multiple diseases. *Drug Discovery Today*, *11*(13), 646-654.

Reese, G., & Baltes, W. (1992). Model reactions on roast aroma formation. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A*, *194*(5), 417-421.

Rijkens, F., & Boelens, H. (1975). The future of aroma research. In: *International Symposium on Aroma Research* (pp. 203-220).

Rizzi, G. P. (2006). Formation of strecker aldehydes from polyphenol-derived quinones and α-amino acids in a nonenzymic model system. *Journal of Agricultural and Food Chemistry*, *54*(5), 1893-1897.

Rizzi, G. P. (2008). The Strecker Degradation of amino acids: Newer avenues for flavor formation. *Food Reviews International*, *24*(4), 416-435.

Romano, P., & Suzzi, G. (1996). Origin and production of acetoin during wine yeast fermentation. *Applied and Environmental Microbiology*, *62*(2), 309-315.

Rössnes, J., Velíšek, F. P., & Davídek, J. (2001). Strecker degradation products of aspartic and glutamic acids and their amides. *Czech Journal of Food Science*, *19*, 41-45.

Sang, S., Tian, S., Wang, H., Stark, R. E., Rosen, R. T., Yang, C. S., & Ho, C.-T. (2003). Chemical studies of the antioxidant mechanism of tea catechins: Radical reaction products of epicatechin with peroxyl radicals. *Bioorganic & Medicinal Chemistry*, *11*(16), 3371-3378.

Sauler, M., & Gulati, M. (2012). Newly recognized occupational and environmental causes of chronic terminal airways and parenchymal lung disease. *Clinics in Chest Medicine*, *33*(4), 667-680.

Schieberle, P. (2005). The Carbon Module Labeling (CAMOLA) Technique: A useful tool for identifying transient intermediates in the formation of Maillard-type target molecules. *Annals of the New York Academy of Sciences*, *1043*(The Maillard Reaction: Chemistry at the Interface of Nutrition, Aging, and Disease), 236-248.

Schieberle, P., Köhler, P., & Granvogl, M. (2005). New aspects on the formation and analysis of acrylamide. In: M. Friedman, & D. Mottram, *Chemistry and Safety of Acrylamide in Food*, vol. 561 (pp. 205-222): Springer US.

Schnermann, P., & Schieberle, P. (1997). Evaluation of key odorants in milk chocolate and cocoa mass by aroma extract dilution analyses. *Journal of Agricultural and Food Chemistry*, *45*(3), 867-872.

Shen, S.-C., Tseng, K.-C., & Wu, J. S.-B. (2007). An analysis of Maillard reaction products in ethanolic glucose–glycine solution. *Food Chemistry*, *102*(1), 281-287.

Shibamoto, T. (1986). Odor threshold of some pyrazines. *Journal of Food Science*, 51(4), 1098-1099.

Shibamoto, T. (2014). Diacetyl: occurrence, analysis, and toxicity. *Journal of Agricultural and Food Chemistry*, 62(18), 4048-4053.

Shibamoto, T., & Bernhard, R. A. (1977). Investigation of pyrazine formation pathways in sugar-ammonia model systems. *Journal of Agricultural and Food Chemistry*, *25*(3), 609-614.

Shin, D. B., & Feather, M. S. (1990). The degradation of l-ascorbic acid in neutral solutions containing oxygen. *Journal of Carbohydrate Chemistry*, *9*(4), 461-469.

Shu, C.-K. (1998). Pyrazine Formation from amino acids and reducing sugars, a pathway other than Strecker degradation. *Journal of Agricultural and Food Chemistry*, *46*(4), 1515-1517.

Shu, C.-K. (1999). Pyrazine formation from serine and threonine. *Journal of Agricultural and Food Chemistry*, *47*(10), 4332-4335.

Shu, C. K., Hagedorn, M. L., Mookherjee, B. D., & Ho, C. T. (1985). Volatile components of the thermal degradation of cystine in water. *Journal of Agricultural and Food Chemistry*, *33*(3), 438-442.

Silva Ferreira, A. C., Barbe, J.-C., & Bertrand, A. (2003). 3-Hydroxy-4,5-dimethyl-2(5H)-furanone: A key odorant of the typical aroma of oxidative aged port wine. *Journal of Agricultural and Food Chemistry*, *51*(15), 4356-4363.

Smith, M. B., & March, J. (2006). Mechanisms and methods of determining them. *March's Advanced Organic Chemistry* (pp. 296-327): John Wiley & Sons, Inc.

Sobeih, K. L., Baron, M., & Gonzalez-Rodriguez, J. (2008). Recent trends and developments in pyrolysis–gas chromatography. *Journal of Chromatography A*, *1186*(1–2), 51-66.

Stitt, A. W. (2005). The Maillard reaction in eye diseases. *Annals of the New York Academy of Sciences*, *1043*(1), 582-597.

Strecker, A. (1862). Notiz über eine eigenthümliche oxydation durch alloxan. *Justus Liebigs Annalen der Chemie*, *123*(3), 363-365.

Tai, C.-Y., & Ho, C.-T. (1997). Influence of cysteine oxidation on thermal formation of Maillard aromas. *Journal of Agricultural and Food Chemistry*, *45*(9), 3586-3589.

Takahashi, K., Tadenuma, M., & Sato, S. (1976). 3-hydroxy-4,5-dimethyl-2(5h)-furanone, a burnt flavoring compound from aged sake. *Agricultural and Biological Chemistry*, *40*(2), 320-330.

Terashima, M. (1982). A facile synthesis of 2-substituted benzoxazoles. *Synthesis*, *1982*(06), 484.

Tokitomo, Y., Kobayashi, A., Yamanishi, T., & Muraki, S. (1980). Studies on the "sugar flavor" of raw cane sugar. III. Key compound of the sugary flavor. *Proceedings of the Japan Academy*, *56*(7), 457-462.

Totlani, V. M., & Peterson, D. G. (2005). Reactivity of epicatechin in aqueous glycine and glucose Maillard reaction models: quenching of C2, C3, and C4 sugar fragments. *Journal of Agricultural and Food Chemistry*, *53*(10), 4130-4135.

Totlani, V. M., & Peterson, D. G. (2006). Influence of epicatechin reactions on the mechanisms of Maillard product formation in low moisture model systems. *Journal of Agricultural and Food Chemistry*, *55*(2), 414-420.

Tressl, R., Helak, B., Kersten, E., & Rewicki, D. (1993). Formation of proline- and hydroxyproline-specific Maillard products from [1-13C]glucose. *Journal of Agricultural and Food Chemistry*, *41*(4), 547-553.

Tressl, R., Helak, B., Koppler, H., & Rewicki, D. (1985). Formation of pyrrolidines and piperidines on heating l-proline with reducing sugars. *Journal of Agricultural and Food Chemistry*, *33*, 924-928.

Tsuge, O. (1987). Simple generation of nonstabilized azomethine ylides through decarboxylative condensation of α-amino acids with carbonyl compounds via 5-oxazolidinone intermediates. *Bulletin of the Chemical Society of Japan*, *60*(11), 4079.

Tsuge, O., & Kanemasa, S. (1989). Recent advances in azomethine ylide chemistry. In: R. K. F. Alan, *Advances in Heterocyclic Chemistry*, vol. Volume 45 (pp. 231-349): Academic Press.

Umano, K., Hagi, Y., Nakahara, K., Shyoji, A., & Shibamoto, T. (1995). Volatile chemicals formed in the headspace of a heated d-glucose/l-cysteine Maillard model system. *Journal of Agricultural and Food Chemistry*, *43*(8), 2212-2218.

Van Praag, M., Stein, H. S., & Tibbetts, M. S. (1968). Steam volatile aroma constituents of roasted cocoa beans. *Journal of agricultural and food chemistry*, *16*(6), 1005-1008.

Weenen, H., Kerler, J., & van der Ven, J. G. M. (1997). The Maillard reaction in flavour formation. *Flavours and Fragrances* (pp. 153-170). Cambridge: Cambridge, Woodhead Publishing Ltd.

Wnorowski, A., & Yaylayan, V. A. (2000). Influence of pyrolytic and aqueous-phase reactions on the mechanism of formation of Maillard products. *Journal of Agricultural and Food Chemistry*, *48*(8), 3549-3554.

Wu, C.-H., & Yen, G.-C. (2005). Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *Journal of Agricultural and Food Chemistry*, *53*(8), 3167-3173.

Xu, Q., Liu, J., Song, H., Zou, T., Liu, Y., & Zhang, S. (2013). Formation mechanism of volatile and non-volatile compounds in peptide–xylose Maillard reaction. *Food Research International*, *54*(1), 683-690.

Yaylayan, V., Perez Locas, C., Wnorowski, A., & O'Brien, J. (2005a). Mechanistic pathways of formation of acrylamide from different amino acids. In: M. Friedman, & D. Mottram, *Chemistry and Safety of Acrylamide in Food*, vol. 561 (pp. 191-203): Springer US.

Yaylayan, V. A. (1997). Classification of the Maillard reaction: A conceptual approach. *Trends in Food Science & Technology*, *8*(1), 13-18.

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

Yaylayan, V. A., Haffenden, L., Chu, F. L., & Wnorowski, A. (2005b). Oxidative pyrolysis and postpyrolytic derivatization techniques for the total analysis of Maillard model systems: investigation of control parameters of Maillard reaction pathways. *Annals of the New York Academy of Sciences*, *1043*(1), 41-54.

Yaylayan, V. A., & Haffenden, L. J. W. (2003a). Mechanism of imidazole and oxazole formation in [13C-2]-labelled glycine and alanine model systems. *Food Chemistry*, *81*(3), 403-409.

Yaylayan, V. A., & Huyghues-Despointes, A. (1994). Chemistry of Amadori rearrangement products: Analysis, synthesis, kinetics, reactions, and spectroscopic properties. *Critical Reviews in Food Science and Nutrition*, *34*(4), 321-369.

Yaylayan, V. A., & Keyhani, A. (1999). Origin of 2,3-pentanedione and 2,3-butanedione in d-glucose/l-alanine maillard model systems. *Journal of Agricultural and Food Chemistry*, *47*(8), 3280-3284.

Yaylayan, V. A., & Keyhani, A. (2000). Origin of carbohydrate degradation products in l-alanine/d-[13C]glucose model systems. *Journal of Agricultural and Food Chemistry*, *48*(6), 2415-2419.

Yaylayan, V. A., & Keyhani, A. (2001). Carbohydrate and amino acid degradation pathways in l-methionine/d-[13C] glucose model systems. *Journal of Agricultural and Food Chemistry*, *49*(2), 800-803.

Yaylayan, V. A., Wnorowski, A., & Perez Locas, C. (2003b). Why asparagine needs carbohydrates to generate acrylamide. *Journal of Agricultural and Food Chemistry*, *51*(6), 1753-1757.

Yu, A.-N., Tan, Z.-W., & Wang, F.-S. (2012). Mechanism of formation of sulphur aroma compounds from l-ascorbic acid and l-cysteine during the Maillard reaction. *Food Chemistry*, *132*(3), 1316-1323.

Yu, A.-N., & Zhang, A.-D. (2010). Aroma compounds generated from thermal reaction of l-ascorbic acid with l-cysteine. *Food Chemistry*, *121*(4), 1060-1065.

Zeppa, G., Conterno, L., & Gerbi, V. (2001). Determination of organic acids, sugars, diacetyl, and acetoin in cheese by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*, *49*(6), 2722-2726.

Zhang, Y., & Ho, C. T. (1991). Comparison of the volatile compounds formed from the thermal reaction of glucose with cysteine and glutathione. *Journal of Agricultural and Food Chemistry*, *39*(4), 760-763.

Zhang, Y., Ren, Y., & Zhang, Y. (2009). New research developments on acrylamide: Analytical chemistry, formation mechanism, and mitigation recipes. *Chemical Reviews*, *109*(9), 4375-4397.

Zhu, N., Huang, T.-C., Yu, Y., LaVoie, E. J., Yang, C. S., & Ho, C.-T. (2000). Identification of oxidation products of (−)-epigallocatechin gallate and (−)-epigallocatechin with H_2O_2. *Journal of Agricultural and Food Chemistry*, *48*(4), 979-981.