Unravelling the Maillard reaction network by multiresponse kinetic modelling

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Aos meus pais

e ao meu irmão.

CONTENTS

Abstract

Chapter 1 - General Introduction

1.1 Why study the Maillard reaction? 3
1.2 Why use multiresponse kinetic modelling to study the Maillard reaction? 4
1.3 Aim and outline of this thesis 5
1.4 References 7

Chapter 2 - A review of the Maillard reaction in food and implications to kinetic modelling

2.1 Introduction 11
2.2 Chemistry of the reaction 11
2.3 Influence of the Maillard reaction products on food properties 15
2.4 Role of kinetic modelling 17
2.5 Kinetic modelling in the Maillard reaction, an illustrative example 19
2.6 Conclusions 26
2.7 References 26

Chapter 3 - Identification and quantification of the main intermediates in the glucose/glycine Maillard reaction

3.1 Material and Methods 33
3.1.1 References 37

3.2 In situ trapping technique / carbohydrate degradation products 39
3.2.1 Introduction 40
3.2.2 Material and methods 41
3.2.3 Results and discussion 42
3.2.4 References 46

3.3 Melanoidins Extinction coefficient in the glucose/glycine Maillard reaction 47
3.3.1 Introduction 48
3.3.2 Material and methods 49
3.3.3 Results and discussion 51
3.3.4 Conclusions 59
3.3.5 References 59

Chapter 4 - Reaction network build-up: Unravelling the intermediate stage

4.1 Kinetic modelling of Amadori *N*-(1-deoxy-D-fructos-1-yl)-glycine degradation pathways. Part I - Reaction mechanism 63

4.1.1 Introduction 64
4.1.2 Results and discussion 66
4.1.3 Conclusions 78
4.1.4 References 78

4.2 Kinetic modelling of Amadori *N*-(1-deoxy-D-fructos-1-yl)-glycine degradation pathways. Part II - Kinetic analysis 81

4.2.1 Introduction 82
4.2.2 Results and discussion 83
4.2.3 Conclusions 101
4.2.4 References 102

Chapter 5 - A multiresponse kinetic model for the glucose/glycine Maillard reaction pathways

5.1 Introduction 105
5.2 Results and discussion 106
5.3 Conclusions 123
5.4 References 123

Chapter 6 - Kinetic model validation for the glucose/glycine Maillard reaction pathways: influence of pH and reactants initial concentration

6.1 Introduction 127
6.2 Results and discussion 130
6.3 Conclusions 147
6.4 References 148

Chapter 7 - General Discussion

7.1 Introduction 151
7.2 An overview of the main achievements 151
7.3 Has the objective been met? 154
7.4 Where to go from here 155
7.5 Use of the developed model in food quality prediction 156

Summary 159
Samenvatting 162
About the author 166
Publications 166
Acknowledgements 168

ABSTRACT

The Maillard reaction is an important reaction in food industry. It is responsible for the formation of colour and aroma, as well as toxic compounds as the recent discovered acrylamide. The knowledge of kinetic parameters, such as rate constants and activation energy, is necessary to predict its extent and, consequently, to optimise it.

Each of the chapters presented in this thesis can be seen as a necessary step to succeed in applying multiresponse kinetic modelling in a complex reaction, such as the Maillard reaction. Based on the established network for the intermediate stage a complete kinetic model for the whole glucose/glycine Maillard reaction pathways was developed. The estimated rate constants showed no dependence on the reactants initial concentration or ratio. However, concerning pH, the reaction mechanism was highly influenced with respect to which route prevailed and to the type of products formed. For the first time consistent pH dependence was derived for the estimated rate constants. For a pH drop $\leq$ 1 unit, the pH dependence was well captured by a power law relation, in line with a theoretical analysis. All in all, the model seemed to perform well and was consistent with the established reaction mechanism.

Multiresponse kinetic modelling has proved to be a powerful tool in unravelling complex chemical reactions such as the Maillard reaction. More than just deriving relevant kinetic parameters, multiresponse modelling gave also insight into the reaction mechanism. A main conclusion was that multireponse kinetic modelling goes beyond the Maillard reaction: it should also be applicable to other complex reactions in foods.

Chapter 1

General Introduction

1. General Introduction

This chapter introduces the reader to the subject of this thesis: the Maillard reaction and how multiresponse kinetic modelling can be a powerful tool to unravel such a complex reaction network. The aim of the performed research is defined and the structure and the contents of the present thesis are described based on the required steps to achieve the aim.

1.1. Why study the Maillard reaction?

For as long as food has been cooked, the Maillard reaction has played an important role in improving the appearance and taste of foods. It has been a central and major challenge in food industry, because the Maillard reaction is related to aroma, taste and colour, in particularly in traditional processes such as the roasting of coffee and cocoa beans, the baking of bread and cakes, the toasting of cereals and the cooking of meat (*1, 2*). Moreover, during the Maillard reaction a wide range of reaction products is formed with significant importance for the nutritional value of foods. This can be reduced by decrease of digestibility and possibly formation of toxic and mutagenic compounds, but can also be improved by the formation of antioxidative products (*3*). Recently, the Maillard reaction has also been associated with the formation of potentially carcinogenic compounds, such as the acrylamide (*4, 5*).

The Maillard reaction, also known as non-enzymatic browning, was first reported by Louis-Camille Maillard in a brief paper to the French Academy, in 1912 (*6*). The paper described what appeared to be a very simple phenomenon: upon gently heating sugars and amino acids in water, a yellow-brown colour developed. However, only in 1953, Hodge presented the first original comprehensive reaction scheme where it was shown that the chemistry underlying the Maillard reaction is indeed very complex (*7*). It encompasses not one reaction pathway but a whole network of various reactions. Ever since, the original scheme has been developed and elaborated by food technologists, so the understanding of the reaction is advancing steadily. Nevertheless the Maillard reaction is notoriously difficult to control.

The rate of the Maillard reaction and the nature of the products formed are mainly determined by the reaction conditions (*8*). These include the chemical composition (nature of the reactants and type of buffer), the pH and water activity, the presence of oxygen and metals, the temperature-time combination during heating and the presence of reaction inhibitors (like sulphur dioxide). However, it is the combined influence of time, temperature and pH, on the velocity of the reaction, which is most relevant for food systems. These factors interact in determining the development of the Maillard reaction during food processing, thereby having a high impact on the quality of processed foods.

The Maillard reaction strongly affects food quality. Quality is a broad concept, but from a food technologist point of view it means the control of chemical, physical and microbiological changes during processing and storage. In order to be able to control those changes in foods, the reactions of interest need to be studied in a quantitative way. Kinetic parameters describing such changes are thus needed. Not only their value but also the confidence in models in which such parameters play a role, is important.

1.2. Why use multiresponse kinetic modelling to study the Maillard reaction?

The Maillard reaction is a cascade of consecutive and parallel reaction steps, the complexity of which is well illustrated by the thousands of known reaction products and intermediates. Knowledge on kinetic parameters, such as rate constants and activation energy, is necessary to predict its extent and, consequently, to optimise it. The ultimate goal of applying kinetics is to have a tool to quantify reactions. With such a tool, it becomes possible to control and predict quality attributes in foods. Multiresponse kinetic modelling implies that one studies more than one reactant or one product. Rather, as many reactants, intermediates and products are studied at the same time.

Several works have tried to study the kinetics of the Maillard reaction just by fitting simple kinetic models (zero, first or second order reaction models) to one individual reaction pathway at the time (*9*). It is important to realize that applying simple kinetics in a complex reaction as the Maillard reaction is just a fitting procedure, since it gives no insight in the reaction mechanism. In contrast, applying multiresponse modelling techniques helps in building mechanistic models, which reaction route prevails and what kind of products are formed.

To develop a kinetic model for a chemical reaction as complex as the Maillard reaction, it is important to define, accurately, the system under study as well as to understand its mechanism. According to Van Boekel (*10*) the following steps should be taken into account: (i) identification and quantification of the reactants and main products formed; (ii) identification of reaction pathways based on reaction conditions; (iii) differentiate between primary and secondary reaction routes; (iv) propose a kinetic model based on the established reaction network; (v) test the hypothesized mechanism.

The added value of applying multiresponse modelling techniques is that the reaction pathways are considered in more detail and extra information about the reaction mechanism is obtained, since the reactants degradation is analysed simultaneously with the intermediates and end products formation. Moreover, kinetic modelling is an iterative process: propose a model, confront it with experimental data, criticize the model, adjust the model and confront the adapted model with experiments again, until an acceptable model results. What is acceptable is of course debatable, but it can be defined by discriminating between possible models. Also, by changing important reaction conditions, such as temperature and pH, the model can be strained and the reaction routes that prevail as well as the reaction products that are formed determined. All in all, multiresponse kinetic analysis can be a powerful tool to unravel complex chemical reaction mechanisms. Kinetic models can be tested more rigorously and, once the goodness of fit is deemed acceptable, estimation of the parameters can be done much more precisely.

1.3. Aim and outline of this thesis

Recent developments have led to detailed characterization of some of the many Maillard reaction products and to a sufficient understanding of their mechanism of formation. Nevertheless, much remains to be elucidated. Relevant kinetic data useful in optimising and controlling the reaction mechanism are in particular lacking.

For simplicity, the chemistry of the Maillard reaction was confined in this thesis to the reaction of glucose with a simple amino acid, glycine. Specifically to this thesis, different studies have been performed in order to achieve the ultimate goal: unravelling the glucose/glycine Maillard reaction network by multiresponse kinetic modelling under conditions relevant for food processing. The aims of the present thesis are therefore:

- Identify and quantify the main intermediates and end products in the glucose and glycine Maillard reaction;
- Establish the reaction pathways and propose a mechanistic model from a quantitative point of view;
- Reveal relevant kinetic data and determine the temperature and pH dependence of the estimated parameters.

Each of the following chapters can be seen as one of the necessary steps to succeed in applying multiresponse kinetic modelling in a complex reaction, such as the Maillard reaction. **Chapter 2** " **A Review of the Maillard reaction and its implications to kinetic modelling**" provides a more complete overview of the Maillard reaction, as well as its chemical and nutritional aspects together with the influence of Maillard reaction products on food properties. Moreover, an illustrative example is given on how kinetic modelling can be used to organize this plethora of information in a coherent manner.

To develop a kinetic model for a chemical reaction as complex as the Maillard reaction, it is important to define accurately the system under study, that is to identify and quantify the main intermediates involved. **Chapter 3** presents the **"Identification and quantification of the reactants and main products formed in the glucose/glycine Maillard reaction**". In the first section, **Section 3.1**, the *Materials and Methods* used are described. Special attention was paid to the identification and quantification of the amino acid, glycine; the sugars, glucose, fructose and mannose; the *N*-(1-deoxy-D-fructos-1-yl)glycine (Amadori rearrangement product); the organic acids, formic and acetic acid, and the α-dicarbonyl compounds, 1- and 3-deoxyglucosone together with methylglyoxal. Moreover, two specific techniques employed in this research step are presented, namely an *In situ trapping technique applied to the*

identification of carbohydrate degradation products (**Section 3.2**) and a radiolabelling technique used in determining the *Melanoidins extinction coefficient in the glucose/glycine Maillard reaction* (**Section 3.3**).

At the early stage of the Maillard reaction, the Amadori compound, a main intermediate with a central importance to the whole Maillard reaction, is formed. The chemistry of Amadori rearrangement provides a key to understanding the intermediate phase of the Maillard reaction network. In **Chapter 4 "Reaction network build-up: unravelling the intermediate stage"** the establishment of the main thermal degradation pathways of glucose/glycine Amadori compound (*N*-(1-deoxy-D-fructos-1-yl)glycine) is described. For clarity it was divided into two parts. The first (**Section 4.1**) focused on the 3 initial steps of multiresponse kinetic modelling: *Kinetic Modelling of Amadori N-(1-deoxy-D-fructos-1-yl)-glycine degradation pathways. Part I – Reaction mechanism*, whereas the second (**Section 4.2**) dealt with the remaining steps with a focus on the kinetics of the hypothesized mechanism: *Kinetic Modelling of Amadori N-(1-deoxy-D-fructos-1-yl)-glycine degradation pathways. Part II - Kinetic Analysis*. This chapter can be seen as a stepping stone for a complete kinetic analysis of the whole Maillard reaction, as will be described in the remaining chapters.

In **Chapter 5 "A multiresponse kinetic model for the glucose/glycine Maillard reaction pathways**" was established, using as basis the model proposed in Chapter 4. Special attention was given to the isomerisation and degradation reactions of the sugar as well as to the kinetic significance of the Amadori compound's reversibility in the early stages of the Maillard reaction. The established model was updated and strained by varying one of the most important reaction conditions, the temperature.

Once the model was established the next important step was to test the hypothesized mechanism by changing the reaction conditions. In **Chapter 6 "Kinetic model validation for the glucose/glycine Maillard reaction pathways"** was performed by confronting the model with five different initial reaction pHs, when it was allowed to fall and compared to when it was kept constant; also the initial concentration of the reactants was altered and the molar ratios 1:1, 1:2 and 2:1 compared. By changing important reaction conditions it was investigated how well the model performed and how accurate it was according to the established reaction mechanism.

Finally, in **Chapter 7** the main achievements of this thesis are summarized. It is discussed to what extent the objectives have been met and which questions still remain. An overview is given on how multiresponse kinetic modelling has become a powerful tool to unravel complex chemical reactions, such as the Maillard reaction.

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Chapter 2

A review of the Maillard reaction in food and implications to kinetic modelling

2. A review of Maillard reaction in food and implications to kinetic modelling

This chapter reviews some of the research designed to lead to an increased understanding of the chemistry of the Maillard reaction, based on recent developments, and its influence on food properties like colour, flavour and nutritional value. A critical analysis is given on how quality attributes associated with Maillard reaction can be predicted and controlled by kinetic modelling. Multiresponse modelling (taking more than one reactant and product into consideration in the modelling process) is a powerful tool to model complicated consecutive and parallel reactions, like the Maillard reaction. Such a multiresponse approach provides a major guidance in understanding the reaction mechanism. An illustrative example is given.

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2.1. Introduction

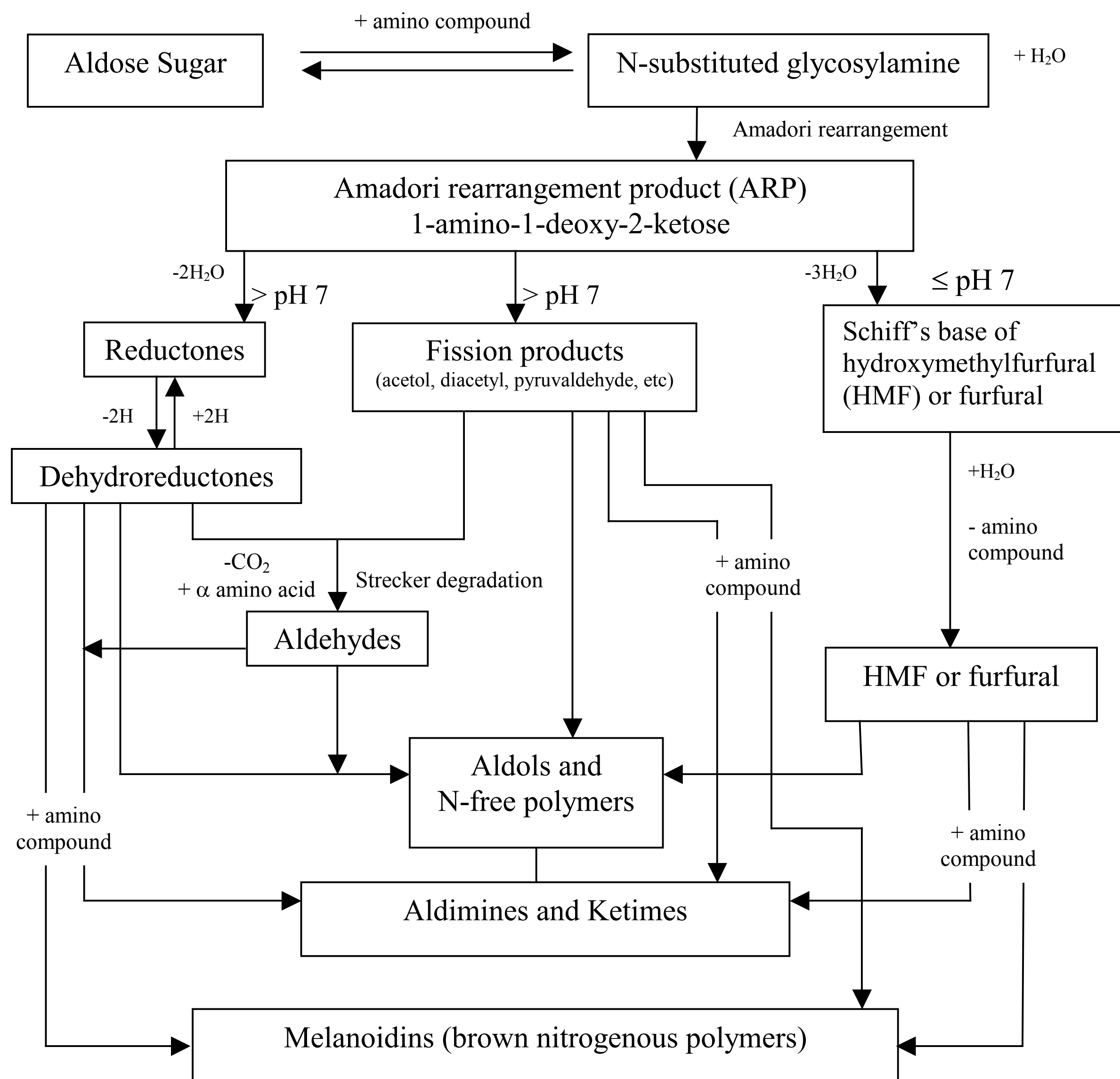
For as long as food has been cooked, the Maillard reaction has played an important role in improving the appearance and taste of foods. It has been a central and major challenge in food industry, since the Maillard reaction is related to aroma, taste and colour, in particularly in traditional processes such as the roasting of coffee and cocoa beans, the baking of bread and cakes, the toasting of cereals and the cooking of meat. Moreover, during the Maillard reaction a wide range of reaction products is formed with significant importance for the nutritional value of foods. This can be reduced by decrease of digestibility and possibly formation of toxic and mutagenic compounds, but can also be improved by the formation of antioxidative products.

The chemistry underlying the Maillard reaction is very complex. It encompasses not one reaction pathway but a whole network of various reactions. The original comprehensive reaction scheme of Hodge (1953) has been developed and elaborated by food technologists ever since, so the understanding of the reaction is advancing steadily. Nevertheless the Maillard reaction is notoriously difficult to control. Various factors involved in food processing influence it and they can be considered as food processing variables. The kinetic approach tends to present a complementing view of this mechanism, because it considers the rate-determining steps of the reaction. It is powerful because rate-determining steps provide control points.

This paper discusses a research approach designed to increase the understanding of (a) the chemistry of the reaction and its influence on food properties like colour, flavour and nutritional value, and (b) how quality attributes associated with the Maillard reaction, can be predicted and controlled by kinetic modelling.

2.2. Chemistry of the reaction

The Maillard reaction has been named after the French chemist Louis Maillard (1912) who first described it but it was only in 1953 that the first coherent scheme was put forward by Hodge (Scheme 2.1). In essence, it states that in an early stage a reducing sugar, like glucose, condenses with a compound possessing a free amino group (of an amino acid or in proteins mainly the ε-amino group of lysine, but also the α-amino groups of terminal amino acids) to give a condensation product N-substituted glycosilamine, which rearranges to form the Amadori rearrangement product (ARP). The subsequent degradation of the Amadori product is dependent on the pH of the system.

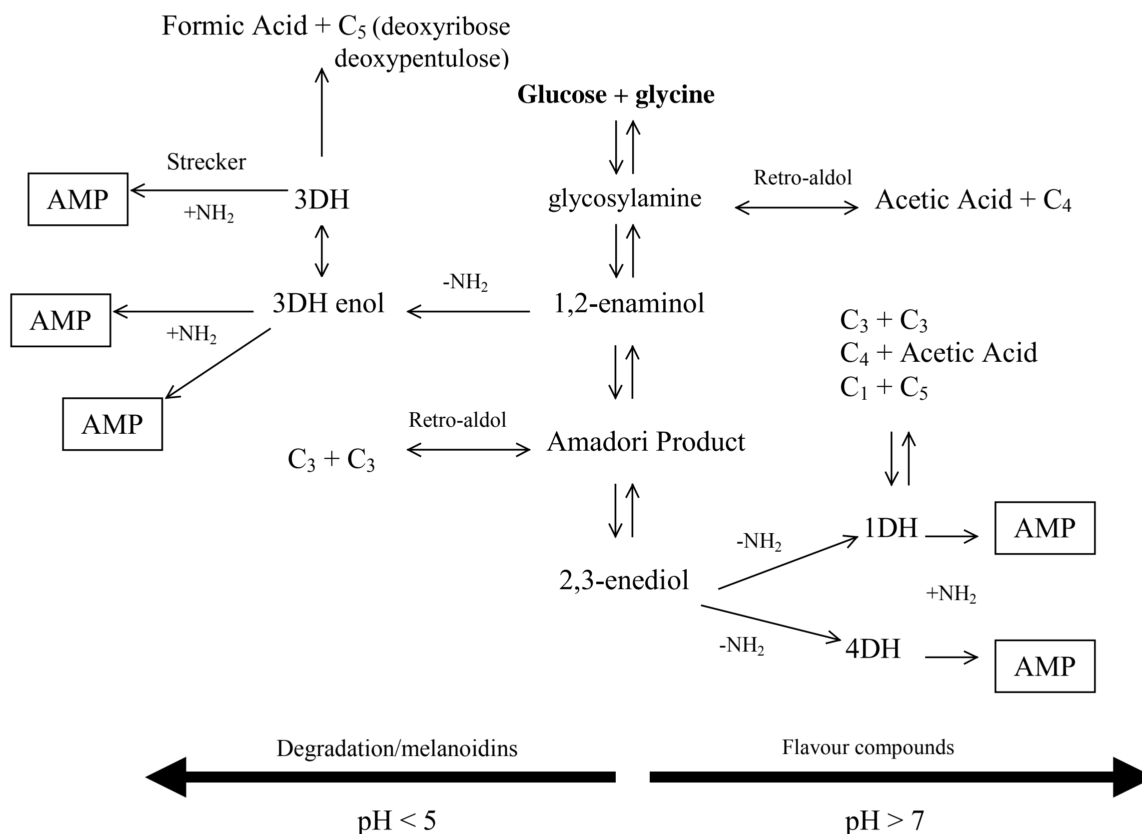


Scheme 2.1. Maillard reaction scheme adapted from Hodge (1953).

At pH 7 or below, it undergoes mainly 1,2-enolisation with the formation of furfural (when pentoses are involved) or hydroxymethylfurfural (HMF) (when hexoses are involved). At pH > 7 the degradation of the Amadori compound is thought to involve mainly 2,3 enolisation, where reductones, such as 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one (HMF^{one}), and a variety of fission products, including acetol, pyruvaldehyde and diacetyl are formed. All these compounds are highly reactive and take part in further reactions. Carbonyl groups can condense with free amino groups, which results in the incorporation of nitrogen into the reaction products. Dicarbonyl compounds will react with amino acids with the formation of aldehydes and α-aminoketones. This reaction is known as the Strecker degradation. Subsequently, in an advanced stage, a range of reactions takes place, including cyclisations, dehydrations, retroaldolisations, rearrangements, isomerisations and further condensations, which ultimately, in a final stage, lead to the formation of brown nitrogenous polymers and co-polymers, known as melanoidins.

The complexity and the variety of the Maillard reaction products has, throughout the years, raised the interest of scientists in different fields of research (Ericksson, 1981, Waller and Feather, 1983, Fujimaki, Namiki and Kato, 1986, Finot, Aeschbacher, Hurrel and Liardon, 1990, Labuza, Reineccius, Monnier, O'Brien and Baynes, 1994, Ikan, 1996, O'Brien, Nursten, Crabbe and Ames, 1998). New important pathways, not accounted for by Hodge, have been established. McWeeny, Knowels and Hearne (1974) reported that the most important intermediates in colour formation are 3-deoxyosuloses and 3,4-dideoxyosulos-3-enes, which in the case of glucose is 3-deoxyhexosulose (DH) and 3,4-dideoxyhexosuloses-3-ene (DDH). Later, Ghiron, Quack, Mahinney and Feather (1988) stated that 3-deoxy-2-hexosuloses, 1-deoxy-2,3-hexodiuloses and other α-dicarbonyl intermediates can undergo nucleophilic addition reactions with amino acids with subsequent decarboxylation to produce the "so-called" Strecker aldehyde (*RHC=O*). In 1990, Huber and Ledl isolated and characterised 1-deoxy- and 3-deoxyglucosones from heated Amadori products. More recently, in agreement with the previous reports, Tressl, Nittka and Kersten (1995) using ^{13}C-labeled sugars, have given a new perspective to the reaction mechanism (Scheme 2.2). It involves different reaction pathways, in which the key intermediates are the 1-, 3- and 4-deoxyhexosuloses. Moreover, a major influence of the pH is expressed. Along with enolization reactions, the Amadori product and its dicarbonyl derivatives can undergo concurrently retro-aldol reactions producing more reactive C_2, C_3, C_4 and C_5 sugar fragments, such as hydroxyacetone derivatives, glyceraldehyde and diketones. Retroaldol reactions become more important at higher pH values. Also, Yaylayan and Huyghues-Despointes (1996) stated that under basic conditions ARP could generate acetic acid and pyruvaldehyde and other lower sugars in addition to free amino acid. As a result, high pH is suggested to be the main pathway to flavour formation. In addition to retroaldol reactions, three redox mechanisms have been identified, in which α-

hydroxy carbonyls, α-dicarbonyls and formic acid are involved. Berg and Van Boekel (1994) reported formic acid as a main degradation reaction product for the Maillard reaction of lactose. Also, Van Boekel and Brands (1998) reported formic acid and acetic acid as two main degradation products for the Maillard reaction of glucose and fructose. In line with Tressl's perspective, Yaylayan (1997) proposed a classification for the Maillard reaction named the "chemical pools". This approach is based on the observation that during the Maillard reaction the amino acids and the sugars also undergo independent degradation, in addition to the conventional degradation where Amadori product is formed. Berg (1993) concluded that isomerization and degradation reactions of the sugar are, from a quantitative point of view, more important than the Maillard reaction, for conditions as in heated milk. Finally, the central importance of the Amadori product, formerly supposed to be the main intermediate of the reaction, has been questioned in both food (Molero-Vilchez and Wedzicha, 1997) and medical fields (Fu, Wells-Knecht, Blackledge, Lyons, Thorpe and Baynes, 1994). In spite of all the work that has been done, the mechanism of the Maillard reaction is still a controversial issue.



Scheme 2.2. Scheme glucose/glycine Maillard reaction adapted from Tressl (1995). AMP (Advanced Maillard Products); 1-DH (1-deoxy-2,3-diketose); 3-DH (3-deoxyaldoketose); 4-DH (4-deoxy-2,3-diketose).

2.3. Influence of Maillard reaction products on food properties

The origin of volatile compounds responsible for flavour is still relatively difficult to determine, due to their multiple origin. The interest shown by food industry stems from a desire to produce and control the characteristic aromas and colours obtained on cooking, baking and roasting. Once the analytical technique of combined gas-chromatography-mass spectrometry was developed for the separation and identification of relatively volatile substances, the search for compounds with specific odours was greatly intensified. The results have been summarised in a series of review articles (Buckholz, 1988, Ho, 1996, Taylor and Mottram, 1996, Chuyen, 1998). Like in brown colour formation, it is clear that both quantity and quality depend on the precursors, thermal processing parameters, pH, and quantitative ratio of amino nitrogen to reducing sugar. For example, Lane and Nursten (1983) reported a thorough study on odours produced in Maillard reaction systems. They identified 12 amino acids, five to seven of which they thought to produce bread, crusty biscuits, cake or toast aroma at each of the 4 temperatures studied, using single amino acid/glucose combinations at different temperatures. Also, Fors (1983) published a literature review of the sensory properties of volatile Maillard products and related compounds. It includes qualitative aroma and flavour descriptions and sensory threshold values for various compounds, classified according to the chemical structure. More recently Teranishi, Wick and Hornstein (1999) have reviewed the thermal generation of Maillard aroma.

From the fact that “we also eat with our eyes”, the significance of Maillard browning in processed foods, in consumer acceptance is obvious. The degree of browning (usually measured via absorbance at 420 nm) is often used analytically to assess the extent to which the Maillard reaction has taken place in foods. Nevertheless, it has been stated that fluorescent compounds are formed prior to brown compounds (Baisier and Labuza, 1992). In the final stage of the reaction, coloured intermediates and other reactive precursors (enaminol products, low-molecular-weight sugar analogues, unsaturated carbonyl products) condense and polymerise to form brown polymers, under acceleration by an amine catalyst. Some of their known properties are brown, high molecular weight, furan ring-containing and nitrogen-containing polymers; they may contain carbonyl, carboxyl, amine, amide, pyrrole, indole, azomethine, ester, anhydride, ether, methyl and/or hydroxyl groups (Ledl and Schleider, 1990, Ames, Apriyantono and Arnoldi, 1993, Tressl, Wondrack, Garbe, Krüger and Rewicki, 1998). Studies on melanoidins formation have been summarized in different review article (Feather, 1985, Namiki, 1988, Friedman, 1996, Rizzi, 1997). The isolation and identification of coloured Maillard products has so far been achieved only with model systems, mostly for low molecular weight (<500 Da) products. Hashiba (1982) concluded that browning was directly proportional to the reducing power of the sugar and to the amounts of glycine consumed, by comparing

different sugars with one single amino acid. However, more recently, Rizzi (1997) stated that many coloured products appear to be (retro)aldolization/dehydration products of sugars which may or may not be attached to proteins or other sources of amino nitrogen. Also Hofmann (1999) using dosage/activity relationship combined with chemical/instrumental techniques and visual/sensory measurments, identified carbohydrate degradation products as browning precursors (deoxyosones, glyoxal, methylglyoxal, hydroxy-2-propanone, 3-hydroxy-2-butanon and glycoaldehyde), which is in agreement with Tressl's *et al* (1995) scheme and demonstrated that their activity in producing browning substances changes during thermal treatment. So far, only partial structures of melanoidins have been elucidated. The origin and actual chemical species responsible for it remain largely undefined. Further investigations are needed.

One of the most obvious negative consequences of the Maillard reaction in food is the loss of nutritive value of proteins involved, with a loss of quality and a possible decrease of food safety. In various studies (Ericksson, 1981, Namiki, 1988, Friedman, 1996) this loss was attributed to decrease of digestibility, destruction and/or biological inactivation of amino acids, including essential amino acids like lysine and tryptophan, inhibition of proteolytic and glycolitic enzymes, and interaction with metal ions. Also, protein molecules can be crosslinked by Maillard reaction products (Chuyen, Utsunomiya and Kato, 1991, Pellegrino, Van Boekel, Gruppen, Resmini, Pagani, 1999). Moreover, the loss of nutritive value has also been associated with the formation of mutagenic compounds. Nagao, Takahashi, Yamanaka and Sugimura (1979) identified mutagenic compounds in instant and caffeine-free coffee. They consisted of dicarbonyl compounds, methylglyoxal, diacetyl and glyoxal, from which the methylglyoxal presented highest mutagenic activity; however, no quantitative correlation with carcinogenic properties was found. Also in both fried and grilled meat and fish, mutagenic compounds were identified, mainly stemming from heterocyclic amines (Arvidsson, Van Boekel, Skog and Jagerstad, 1998). However, flavones and flavonoids have been reported as inhibitors of the heterocyclic amine-type mutagens (Lee, Jiaan and Tsai, 1992) being defined as desmutagens. In the Maillard reaction, desmutagenic effects have also been reported (Yen, Tsai and LII, 1992, Lee, Chuyen, Hayase and Kato, 1994). The reaction mechanism seems to have a major influence in the mutagenicity of the reaction products. For instance, ketose sugars showed a higher mutagenic activity than the corresponding aldose sugars (Brands, Alink, Van Boekel and Jongen, 2000). With respect to food safety, the involvement of the Maillard reaction in the formation and elimination of mutagens is a matter that still needs to be elucidated. Up to date no reports correlated these compounds with human cancer.

Finally, the Maillard reaction has been shown to produce antioxidative components as well. One of the first observations was reported by Griffith and Johnson (1957), who demonstrated that the addition of 5% glucose to sugar cookies produced a marked browning in the cookies and resulted in a greater stability to oxidative rancidity. Since then, reaction

products from various amino compounds and sugars were studied with regard to antioxidative properties, able to protect food against lipid oxidation (Chuyen, Ijichi, Umetsu and Moteki, 1998).

Accordingly, many food quality aspects of the Maillard reaction, some desirable, others undesirable, should be taken into consideration in a food processing operation. The task of the food technologist is then to optimise by finding the best balance between the favourable and unfavourable effects of the reaction in a given process. It could be a question of minimising the nutritional losses while obtaining an optimal flavour production when roasting cereals; of maximising the antioxidant production while minimising flavour and colour production in milk drying, etc. Understanding the Maillard reaction is therefore an added value not only for the traditional processes of roasting, baking and cooking but also for the development of new technologies, like microwave and high pressure technology (Ames, 1998, Shahidi, Ho and Chuyen, 1998).

2.4. Role of Kinetic Modelling

Quality is a very elusive concept, which depends on many factors. The production management view is to maintain quality during production. For each stage of the production process, specific quality criteria are used to monitor and control that production stage (Sloof, Tijskens and Wilkinson, 1996). In a similar way, in a food technologist concept, quality is the result of the ability to control chemical, physical and microbiological changes during processing and storage. For this, kinetic modelling is gaining increasing interest in different fields of research (Nicolai and Baerdemaeker, 1998). It has been applied to food microbiology, with the development of mathematical models that describe how microrganisms behave in foods, referred to as predictive food microbiology (Buchanan, 1993). It can also be applied to chemical changes in food, for example colour as a function of time and temperature. If the rate and temperature dependence of a reaction is known, its occurrence can, in principle, be predicted and therefore controlled. Moreover, it can also help in understanding the chemistry and mechanism of the reaction.

2.4.1 – Basic principles

Van Boekel and Walstra (1995) have given a detailed explanation on the use of kinetics in food applications. Based on the general rate law, the disappearance of a compound (in a closed system with only one compound reacting) is:

$$-\frac{d[A]}{dt} = k[A]^n \tag{2.1}$$

in which the decrease in concentration of component A over time t is related to the concentration of that component, where k is the reaction rate constant and n (usually $0 \leq n \leq 2$) the reaction order. By integration of the differential equation to a chosen order, with respect to time, a zero-order reaction would be:

$$[A] = [A]_0 - kt \tag{2.2}$$

a first-order reaction would be:

$$[A] = [A]_0 \exp(-kt) \tag{2.3}$$

and a second-order reaction would be:

$$\frac{1}{[A]} = \frac{1}{[A]_0} + kt \tag{2.4}$$

In literature most works consider a first order, though zero and second order are also common. This approach, however, is only valid for simple reactions. The order of a reaction is a parameter that gives a mathematical description of time- or concentration-dependence, it does not necessarily give information on the reaction mechanism. This approach is quite suitable for modelling shelf-life or for instance enzyme inactivation during the process, but less useful for understanding chemical changes and mechanisms. In a complex reaction like Maillard reaction, one should be aware that an observed rate constant reflects a combination of elementary rate constants. If we attempt to unravel and explain a particular reaction in more detail, we should propose a reaction mechanism. This is another approach to kinetic modelling. In the case that we are able to analyse and model more than one component simultaneously, this approach is called Multiresponse Modelling (Van Boekel, 1996). The following steps should be taken into account:

- Identify the most important reactants and products and calculate the mass balance
- Identify species which are co-products of the same reaction pathway
- Differentiate between primary and secondary reaction routes
- Identify the influence of critical process parameters (pH, temperature, etc.)
- Determine the effect of reactant concentrations

- Propose a model mechanism for the reaction network based on elementary reaction-steps
- Test the hypothesised mechanism

2.5. Kinetic modelling in Maillard reaction, an illustrative example

As mentioned in Section 2.2, in the last years many analytical techniques have been developed in order to identify important intermediates in the Maillard reaction. The formation and degradation pathways are of major importance for kinetic modelling.

2.5.1 – Reaction routes

In our research group, we attempt to follow the various reaction steps as closely as possible, to take into account as many responses as possible at once, by analysing the reactants degradation, sugar isomers, the Amadori product and its degradation products and then apply the multiresponse modelling technique, as opposed to one response. From the Maillard reaction of a glucose/glycine model system, heated at pH 7, we identified as primary reactions:

1) isomerization of glucose via 1,2-enolization. Isomerisation occurs via the Lobry de Bruyn-Alberda van Ekenstein transformation (De Bruin, 1986, Van Boekel, 1998). Depending on the temperature, this step becomes more evident (Figure 2.1).
2) degradation of sugars into organic acids, namely formic acid, and C_5 fragments, designed as intermediate Maillard products, not easily experimentally accessible. Possible C_5 fragments are 2-deoxyribose (Berg, 1993) and 3-deoxypentulose (Hollnagel, 2000). Formic acid is supposedly formed via the 1,2-enediol by C_1-C_2 cleavage (De Bruin, 1986).
3) the Maillard reaction between sugars and amino groups leading to the so-called Amadori product, followed by its degradation into breakdown products, from which currently only acetic acid was identified. However, 1-deoxy and 3-deoxyglucosones are likely key intermediates of this pathway (Tressl et al., 1995, Hofmann, 1999).
4) the complete regeneration of glycine from the Amadori product. From studies with Amadori product (fructosylamine) heated at pH 7, on its own, at different temperatures, when the degradation of Amadori product starts, the regeneration of glycine is very prominent (Figure 2.2).
5) the interaction of breakdown products with amino groups into advanced Maillard products, such as colour compounds, known as melanoidines. The colour (measured as

absorption at 420 nm) is related to the melanoidins concentration, by the molar extinction coefficient *E* and can be expressed in terms of the number of glucose molecules incorporated, as described by Wedzicha and co-workers (Davies, Wedzicha and Gillard, 1997, Leong, 1999). In this way, colour can be linked quantitatively to sugar losses.

As secondary reactions were identified:

1) formation of hydroxymethylfurfural (not significant here because of the neutral pH range)
2) isomerisation of glucose via 2,3-enolization (acetic acid is believed to be a degradation product of 2,3-enediol by C_2-C_3 cleavage (De Bruin, 1986).
3) The Maillard reaction of fructose formed via isomerisation (not significant because of the low amount formed compared to glucose).

The big advantage of this approach is that the initial, intermediate and final stages become linked in this way, as well as the common intermediates stemming from sugar reactions, and this allows the interpretation of the quantitative importance of the different reaction paths.

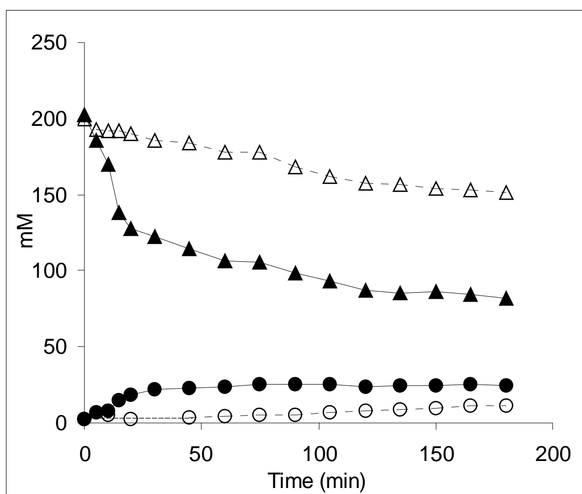


Figure 2.1. Isomerisation of glucose into fructose in the glucose/glycine model system (0.2 mol/L in phosphate buffer 0.1M, pH 6.8) when heated at 90°C (broken line) and 120°C (continuous line); glucose (Δ, 90°C; ▲, 120°C); fructose (○, 90°C; ●, 120°C).

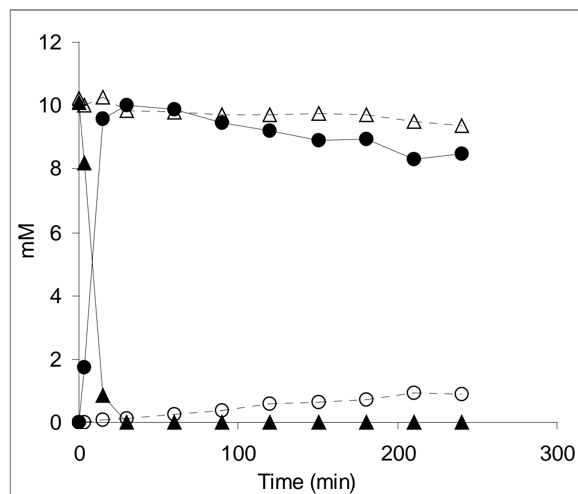


Figure 2.2. Glycine regeneration from Amadori decomposition when heated at 90°C (broken line) and 120°C (continuos line); Amadori (Δ, 90°C; ▲, 120°C); glycine (○, 90°C; ●, 120°C).

2.5.2 – Influence of critical process parameters

Various product and processing variables influence the Maillard reaction (Ames, 1990, Labuza and Baisier, 1992). Temperature and pH are believed to play a crucial role. By manipulating these variables, the balance of the various chemical pathways making up the Maillard reaction changes.

2.5.2.1 – Influence of temperature

Temperature and duration of heating were studied by Maillard (1912) himself, who reported that the rate of the reaction increases with temperature. Many workers have confirmed this observation (Labuza, *et al* 1994, O'Brien *et al*, 1998). An increase in temperature leads to an increase of the reactivity between the sugar and the amino group. The temperature dependence of a reaction rate constant k is often described by the well-known Arrhenius equation:

$$k = \mathrm{A} * \exp(-\frac{\mathrm{E_a}}{RT}) \tag{2.5}$$

where k is the rate constant; A the so-called frequency factor; E_a the activation energy; R the gas constant (8.3 J $mol^{-1}K^{-1}$) and T is the absolute temperature (K). However, it can also be described by the transition-state theory developed by Eyring:

$$k = \frac{k_B T}{h} \exp\left(-\frac{\Delta G^{\neq}}{RT}\right) = \frac{k_B T}{h} \exp\left(-\frac{\Delta H^{\neq}}{RT}\right) \exp\left(\frac{\Delta S^{\neq}}{R}\right) \tag{2.6}$$

where k_B is Boltzmann's constant (1.4×10^{-23} JK^{-1}); h is Planck's constant (6.6×10^{-34} Js); R is the gas constant (8.3 J $mol^{-1}K^{-1}$); T is the absolute temperature (K); $\Delta H^{\neq}$ is the activation enthalpy (J mol^{-1}); $\Delta S^{\neq}$ is the activation entropy (J $mol^{-1}K^{-1}$); $\Delta G^{\neq}$ is the activation Gibbs energy (J mol^{-1}). The relationship between rate constant and temperature is frequently taken by the Arrhenius equation. Still, it is an oversimplification (Van Boekel and Walstra, 1995). In the fundamental Eyring relation the reaction rates are determined by changes in activation entropy and enthalpy. Based on experimental data these parameters can be determined and the influence of temperature on the reactants reactivity modelled. It is therefore preferable to use the Eyring equation when studying more fundamental reaction kinetics, since not only an interpretation of the reaction rate data in terms of thermodynamic properties is considered, but also an insight on the reaction mechanism is given.

2.5.2.2 – Influence of pH

Just as for temperature, the reactivity of the sugar and amino group is also highly influenced by the pH. The open chain form of the sugar and the unprotonated form of the amino group, considered to be the reactive forms, are favoured at higher pH. Studying the two reactants separately, the following equilibrium can be written for the amino group:

$$R\text{-}NH_2 + H^+ \square R\text{-}NH_3^+ \quad (2.7)$$

The lower the pH, the more protonated amino group is present in the equilibrium and therefore, less reactive with the sugar. This equilibrium is dependent on the pH and the pKa of the amino group. The pKa of an amino group is defined as the pH value where 50% of the amino group is protonated. The pKa for the amine group in glycine is 9.6. Figure 2.3 shows the effect of pH on the amount of glycine available in the unprotonated form. The percentage of unprotonated amino group at room temperature and pH 7 is less then 1% and it decreases with lower pH values. Its dependence on the temperature is not yet known, however its pH dependence can be estimated by the plotted trend. In many articles the amino group effective concentration is considered to be equal to the total concentration. This assumption could be valid for high pH values, but not for neutral/acid conditions. A thorough study is still needed.

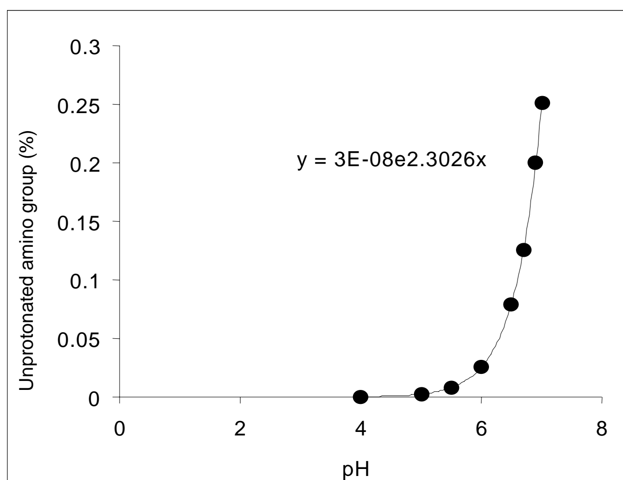


Figure 2.3. Effect of pH on the effective concentration of the amino group of glycine.

As for the other reactant, a similar approach can be used. The reducing sugar (ring form) in solution is in equilibrium with its open chain form, ionised form, enediol anion and isomers (De Bruin, 1986, Angyal, 1992). When we look to the isomerisation step, the equilibrium is established through the enediol anion, from the ionisation of the sugar. The pH dependence for isomerisation can be illustrated using the general model of Figure 2.4.

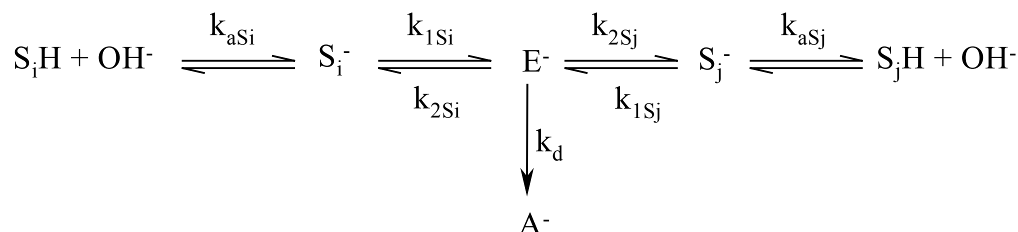


Figure 2.4. General model for alkaline isomerisation and degradation of monosaccharides (De Bruin, 1986). S_iH and S_jH: monosaccharides, S_i^- and S_j^-: monosaccharides anions, E^-: enediol anion, A^-: acidic degradation products.

As the ionisation of the sugar in alkaline medium is faster with respect to subsequent enediol anion formation it can be deduced (De Bruin, 1986) that:

$$\frac{-\mathrm{d}[S_t]}{\mathrm{d}t} = \mathrm{A} \times \frac{[\mathrm{OH}^-]}{\frac{k_w}{k_{as}} + [\mathrm{OH}^-]}[S_t] \qquad (2.8)$$

where the constant A comprises the elementary rate constants relating enolisation and isomerisation equilibrium, k_w is the water constant and k_{as} the sugar alkaline degradation constant. It follows from De Bruin (1986) that the concentration of the enediol is proportional to the sugar concentration, and therefore the enediol anion can be eliminated. The then pseudo first order rate constant includes the OH^- concentration and is dependent on the enolisation rate constant. A quantitative relation is thus given in equation (2.8), expressing the dependence of sugar disappearance on pH, as far as isomerisation is concerned. For the reaction with the amino group, the open chain form (S'), which is in equilibrium with the ring form, is required:

$$S_t \;\square\; S' + H^+ \qquad \text{or} \qquad S_t + OH^- \;\square\; S' \qquad (2.9)$$

The proportion between the sugar concentration and the open ring remains unknown. Taking the same line of thinking as for the derivation of equation (2.8), different assumptions should be taken into account. If the equilibrium in equation (2.7) and (2.9) is very fast (high pH), the effective concentration is directly proportional to the total concentration. However, this is still a controversial issue. According to Namiki (1988) the maximum condensation rate, involving aldoses and amines, occurs when the product of the concentrations [>C=O][RNH2] is maximum. Is believed that the condensation reaction is initiated by an attack of a nucleophilic amino nitrogen, with an unshared electron pair, on the carbonyl carbon. Protonation of the

carbonyl group should enhance its reactivity to the nucleophilic reagent. The rate of condensation would therefore, reach a maximum at weakly acidic pH.

This dependence of reactivity of both principal reactants on pH explains qualitatively the dependence of the Maillard reaction on pH. A thorough study is still needed and we are currently working on it.

2.5.3 – Numerical and statistical procedure

After proposing a reaction mechanism, where the influence of critical process parameters (like pH and temperature) is identified, a model can be built by setting up differential equations for every step in the reaction network. To clarify this, Figure 2.5 shows the kinetic model employed by us, adapted from Martins, Van Boekel and Jongen (2000).

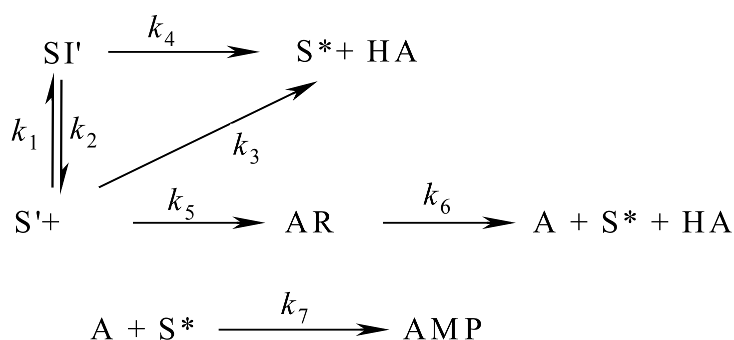


Figure 2.5. Kinetic model used to describe the parallel interaction of sugar isomerisation / degradation and Maillard reaction. S': open chain form of the sugar, SI': open chain form of sugar isomers, S*: reactive sugar fragments, HA organic acids formed (acetic + formic acid), ARP: Amadori product, AMP: advanced Maillard products (melanoidins among others) adapted from (Martins *et al*, 2000).

The coupled ordinary differential equations (ODE`s) describe the degradation/formation of the intermediates over time. For the glucose degradation, for example, we can write:

$$\frac{dS'}{dt} = -k_1[S'] + k_2[SI'] - k_3[S'] - k_5[S'][A] \tag{2.10}$$

This can be done for each component and the ODE`s can then be solved by numerical integration. Several algorithms are available and they should be able to handle stiff differential equations (Stewart, Caracotsios and Sørensen, 1992).

Once the model (i.e. the integrated rate equations) is proposed, it should be fitted to the

experimental data. In literature, the fit criterion is not always clearly indicated. The usual procedure seems to be the method of least squares minimization. In the above example, there are several responses at the same time (the concentrations of components S`, SI`, S*, A, HA, ARP, AMP at each time interval studied). As mentioned by Van Boekel (1996) there are several, rather strict, requirements for application of least squares, in particular in the case of multiresponse modelling. It turns out that for cases of multiresponse modelling the fit criterion to be used depends on the experimental error structure of the data. The variance-covariance matrix of the experimental errors is of importance. The least-squares minimisation can be attempted but the variance of each response must be known, which is not always possible. In this case, the best-fit criterion is the minimisation of the determinant of the matrix of cross-products of the various responses, so-called dispersion matrix. As a result, maximum likelihood estimate of the parameters would be obtained, even if the variance-covariance matrix is unknown. Moreover, if a fit seems adequate, this means that there is consistence between the data and the model. On the other hand if it appears to be bad, the model is obviously wrong and should be adjusted. This approach of numerical integration followed by fitting to the data is flexible and powerful because changing relevant differential equations can easily test different models. The quality of experimental data is therefore very important, in particular for the validity of the studied parameters.

Often, various mechanistic models can be generated. Alternative models may be formed from a candidate model by adding or deleting parameters. Criteria to identify a preferred model and assess its adequacy are desired. Questions like "which model is most probable according to the data?" or, "do any of the models represent the data adequately?" can arise. Stewart, Shon and Box (1998) addressed this problem for models of a multiresponse analysis. If more models seem to fit adequately we can search for model discrimination by Bayesian analysis (probability share). Judgement of the performance of the model: scrutiny of the residuals is recommended along with one of the multivariate tests of goodness of fit. This test is installed in a software package named Athena Visual Workbench (www.athenavisual.com). This goodness-of-fit test gives a sampling probability by which the adequacy of a model can be judged and then the probability function used as provided in the software package to see if the chosen model is good enough. A small value of this probability (0.01 or less) cast doubts on the hypotheses, whereas a value nearer to 1.0 supports it. The software package also offers several statistical indicators that allow judgement on the identifiability of parameters. For instance, a large confidence interval and high correlation coefficients may indicate that parameters are redundant (or that the data do not contain enough information to allow estimation).

2.6. Conclusions

The Maillard reaction is a cascade of consecutive and parallel reaction steps, whose complexity has been illustrated. It is of utmost importance for the food technologist to be able to control the extent of the Maillard reaction. For that reason, kinetic data are needed. The kinetic approach tends to present a much simpler view of the mechanism, because it is based only on the rate-determining steps of the reaction. It is powerful because rate-determining steps provide control points. The important requirement is to be able to identify these steps correctly and, when such knowledge is based on formation or loss of characterised intermediates, the multiresponse kinetics approach becomes more fundamental than the traditional global zero-, first or second-order approach. It is both helpful for deriving relevant kinetic parameters as well as for obtaining insight into reaction mechanisms. If a model is not consistent with the data, a new model can be proposed and easily tested by computer simulation. The only requirement is software that is capable of numerical integration of differential equations and the application of the appropriate statistical methods for multiresponse modelling. Such software is now available (www.athenavisual.com) and we are currently applying this to the Maillard reaction of glucose and glycine.

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Chapter 3

Identification and quantification of the main intermediates in the glucose/glycine Maillard reaction

3. Identification and quantification of the main intermediates in the glucose/glycine Maillard reaction

3.1. Material and Methods

In this Section are described the *Materials and Methods* used in the reaction mixtures preparation, in the analytical techniques developed for identification and quantification of the main intermediates as well as in the organic synthesis of the compounds not available commercially. Special attention was paid to the amino acid, glycine; the sugars, glucose, fructose and mannose; the *N*-(1-deoxy-D-fructos-1-yl)glycine (Amadori rearrangement product); the organic acids, formic and acetic acid, and the α-dicarbonyl compounds, 1- and 3-deoxyglucosone together with methylglyoxal.

In the following Sections two specific techniques employed in this research step are presented:

3.2. In situ trapping technique applied to the identification of carbohydrate degradation products

3.3. Melanoidins extinction coefficient in the glucose/glycine Maillard reaction

3.1. Material and Methods

Chemicals: The following compounds were obtained commercially: Glycine, D-glucose, D-fructose, D-mannose, formic acid, ammonium acetate (Merck, Darmstadt, Germany); acetic acid, methylglyoxal, 2-methylquinoxaline, 5-(hydroxymethyl)-furan-2-carboxaldehyde (Sigma-Aldrich, Germany); 1,2-diaminobenzene (78410, Fluka, Switzerland).

Preparation of the reaction mixtures (Chapter 4): *N*-(1-deoxy-D-fructos-1-yl)-glycine (0.237 g, 10 mmol) was dissolved in phosphate buffer (100 mL, 0.1 M K_2HPO_4 / KH_2PO_4, pH 6.8 and pH 5.5) and heated at 100°C for a maximum of 4 hours and at 120°C for a maximum of 3 hours, in an oil bath, in screw-capped glass tubes (Schott, 16 x 160 mm). At predetermined heating times, samples were taken and immediately cooled in ice water, prior to analyses. Each reaction mixture was prepared, heated and analysed in at least duplicate.

Preparation of reaction mixtures (Chapter 5): Equimolar solutions of glucose and glycine (0.2mol/L) were prepared in phosphate buffer (10 mL, 0.1 mol/L, pH 6.8), filtered (0.2 μm, Schleicher & Schuell) and heated at 80, 90, 100, 110 and 120°C in an oil bath, in screw-capped glass tubes (Schott, 16 x 160 mm). At predetermined heating times, samples were taken and immediately cooled in ice water, prior to analyses. Each reaction mixture was prepared, heated and analysed in at least duplicate.

Preparation of reaction mixtures (Chapter 6): Equimolar solutions of glucose and glycine (0.2mol/L) were prepared in phosphate buffer (10 mL, 0.1 mol/L), pH 4.8, 5.5, 6.0, 6.8 and 7.5, filtered (0.2 μm, Schleicher & Schuell) and heated at 100°C in an oil bath, in screw-capped glass tubes (Schott, 16 x 160 mm). At predetermined heating times, samples were taken and immediately cooled in ice water, prior to analyses. Each reaction mixture was prepared, heated and analysed in at least duplicate. Moreover, to strain the proposed kinetic model the reaction conditions were changed at pH 6.8. a) the reactants concentration varied to 100:200 mmol/L and 200:100 mmol/L and b) the pH was kept constant by addition of NaOH.

pH kept constant (Chapter 6). An aqueous solution of glycine (69 mL, 217.4 mmol/L) was introduced in a closed reaction vessel of approximately 100 mL, in an oil bath kept at 100°C. When the temperature was 100°C inside the vessel the set point of the pH stat controller (PHM290 Radiometer Copenhagen) was set to the pH at that moment because of the effect of temperature, this pH was lower than that at room temperature. The concentrated glucose solution (6 mL, 2.5 mol/L) was added and the final concentration inside the reaction vessel was an equimolar, 0.2 mol/L, glucose and glycine solution with an initial pH at room temperature

of 6.8. The pH stat controller monitored the pH and when it dropped below its set point an auto burette (ABU901 Radiometer Copenhagen) added a solution of NaOH (1 mol/L) to the reaction mix. The temperature was monitored by a Consort R305. At predetermined times the samples were taken through a septum with a Hamilton SampleLock (10 mL) into a glass tube and immediately cooled in ice.

Synthesis of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG): *Adapted from Kroh et al [1].* Glycine (5.25 g, 0.07 mol), D-glucose (50.3 g, 0.28 mol) and $Na_2S_2O_5$ - sodium disulphite (7.6 g, 0.04 mol) were added to a 1:1 MeOH-water solution (80 mL). The mixture was then stirred for 20 min at room temperature and subsequently refluxed for 8 hr. In the end a 1:1 EtOH-water solution (250 mL) was added. The final mixture was applied onto an ion-exchange resin column (Dowex 50W*8, 3 x 30 cm, H^+). The column chromatography of the residue was performed with 1:1 EtOH-water solution (750 mL), water (250 mL) and elution with ammonia solution (2.5 L, 0.1 mol/L). Fractions of 100 ml were collected. Biochrom 20 Amino Acid Analyzer, Pharmacia, was used to check the presence of DFG. The fractions containing only DFG were pooled and concentrated until dryness at 40°C in a rotavapor. The resulting syrup was dissolved in MeOH (using ultrasonic bath) and kept in the fridge overnight. A white precipitate was collected by filtration through a glass-sintered funnel (G4). The final yield was 19% based on the amount of glycine used. Anal. Calcd for $C_8H_{15}NO_7$: C, 40.50; H, 6.37; N, 5.91. Found: C, 40.13; H, 6.4; N, 5.89. mp: 160-163°C. On the Biochrom 20 Amino Acid Analyser the synthesised compound showed only one peak. In addition, *N*-(1-deoxy-D-fructos-1-yl)-glycine[3] was also kindly offered by Drs. Blank and Davidek, from Nestlé Research Center Lausanne, P.O.Box 44, Vers-Chez-les-Blanc, CH-1000 Lausanne 26, Switzerland.

Synthesis of 2-methyl-3-(1,2,3-trihydroxypropyl)quinoxaline: *Adapted from Hofmann et al [2].* A mixture of glucose (196 mmol), alanine (183 mmol) and 1,2 diaminobenzene (183 mmol) in phosphate buffer (800 mL, 0.5M, pH 6.8) was refluxed for 12h. A very black mixture was obtained, with a crude type precipitate at the bottom. After cooling to room temperature, the reaction mixture was extracted with methylene chloride (5 x 200 mL). The organic layers were then combined and dried over Na_2SO_4. After concentration till dryness, the residual was dissolved in ethyl acetate (EtOAc) and the separation was performed by column chromatography (35 x 400 mm) on silica gel (200 g, silica gel 60, Merck) conditioned with EtOAc. Elution was performed first with EtOAc (1.5L) followed by a 99:1 EtOAc-MeOH (1L). Fractions of 30 mL were collected and analysed by thin-layer chromatography (TLC) on silica gel (20 x 20 cm, 0.5 mm, Merck) using EtOAc-MeOH (99:1) as the mobile phase. The target compound was found at R_f = 0.3-0.4. It was visualized by UV and spraying with a $KMnO_4$ solution. Pure fractions were combined and concentrated till dryness and then

recrystalised from MeOH. In the end we obtained, fine, white yellowish crystals (0.02 % in yield). ^{1}H NMR (200 MHz; CD_3OD) δ 2.86 (s, 3H, $-CH_3$), 3.81-3.92 (m, 2H, $-CH_2OH$), 4.08 (m, 1H, -CH(OH)-), 5.10 (d, 1H; -CCH(OH)-), 7.77 (m, 2H, =CH-), 7.98 (m, 1H, =CH-), 8.096 (m, 1H, =CH-) which is in agreement with the reference used. Also the HPLC detection method revealed only one peak.

Quantification of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) and glycine: The reaction mixtures were diluted with water (1:10) and the pH adjusted with loading buffer (LB, Biochrom, England) to 2.2 in the proportion 100 µL of diluted sample to 900 µL of LB. The capsules were then loaded with 20 µL of sample, 20 µL of internal standard (glutamic acid; 25 µmol/mL) and 20 µL of LB. The analysis was performed on a Biochrom 20 Amino Acid Analyser, Pharmacia, England. The separation was done using the following program: lithium citrate buffer pH 2.8 for 16 min and column temperature 80°C; lithium citrate buffer pH 3.0 for 21 min and column temperature 75°C; lithium citrate buffer pH 3.15 for 12 min and column temperature 75°C; lithium citrate buffer pH 3.55 for 21 min and column temperature 75°C; lithium hydroxide solution for 6 min and column temperature 85°C; lithium citrate buffer pH 2.8 for 8 min and column temperature 85°C; lithium citrate buffer pH 2.8 for 20 min and column temperature 75°C; lithium citrate buffer pH 2.8 for 5 min and column temperature 80°C. All buffers were provided by Biochrom, England. After postcolumn derivatization with ninhydrine, the reaction products were determined photometrically at 570 nm: *N*-(1-deoxy-D-fructos-1-yl)-glycine (t_r 9.5 min); glycine (t_r 38.8 min). Quantification was done using external standards.

Quantification of 1-deoxy-2,3-hexodiulose (1-DG), 3-deoxy-2-hexosulose (3-DG) and methylglyoxal (MG): *Adapted from Hofmann [3].* At each reaction time, 1 mL of sample was withdrawn and put into 1 mL of water and 2 mL of a methanolic solution of 1,2-diaminobenzene (1 mol/L). The mixtures were maintained overnight at room temperature (25°C). With previous experimental tests it was established that this treatment ensured a complete derivatization of the target compounds into their quinoxaline form (*See section 3.2*). The mixtures were analysed by RP-HPLC using a solvent gradient starting with a mixture (10/90; v/v) of acetonitrile and ammonium acetate buffer (pH 3.5; 20 mmol/L) and increasing the acetonitrile content to 30 % within 50 min. The flow rate was 0.4 mL/min. By monitoring the effluent at a wavelength of 320 nm a similar chromatogram to the used reference was obtained. The peaks were identified by direct comparison of their retention times with that of authentic samples and by spiking the samples with standards. By comparison with 2-methyl-3-(1,2,3-trihydroxypropyl)quinoxaline the 2-(2,3,4-trihydroxybutyl)-quinoxaline was identified. The quantification was done using 2-methyl-3-(1,2,3-trihydroxypropyl)quinoxaline and

methylquinoxaline as external standards. Both quinoxalines have the same extinction coefficient. The same extinction coefficient was found for 2-methyl-3-(1,2,3-trihydroxypropyl)quinoxaline and 2-(1,2,3,4-tetrahydroxybutyl) quinoxaline *[4]*. Increasing the column temperature to 40°C optimised the peak separation. 1-deoxy-2,3-hexodiulose, 3-deoxy-2-hexosulose and methylglyoxal were detected as their corresponding quinoxaline derivatives at the retention times (t_r) given in parenthesis: 2-methyl-3-(1,2,3-trihydroxypropyl)quinoxaline (t_r 11.3 min); 2-(2,3,4-trihydroxybutyl) quinoxaline (t_r 12.4 min); 2-methylquinoxaline (t_r 38.8 min).

Quantification of sugars (D-glucose and D-fructose) and organic acids (formic and acetic acid): Diluted samples (1:10) were analysed by HPLC, using an ion-exchange column (ION-300, Interaction Chromatography Inc., San Jose, CA). Eluent 2.5 mmol/L H_2SO_4 in milipore water, flow 0.4 mL/min, column temperature 85°C. Sugars were detected by monitoring the refractive index: glucose (t_r 15.1 min), mannose (t_r 16.4 min); and organic acids by their absorbance at 210 nm: formic acid (t_r 21.6 min) and acetic acid (t_r 23.7 min). All compounds were quantified by external standards.

Quantification and separation of D-glucose, D-mannose and D-fructose: After heating, diluted samples (1:100) were analysed by a Dionex system (Sunnyvale, CA) using a CarboPack PA100 column. The separation was done using the following gradient: starting mixture (16/0/84, v/v) of NaOH (0.1N), sodium acetate (NaOAc) (1M in NaOH 0.1N) and water for 20 min. After NaOAc increased to (0/100/0) and was kept isocratic for 10 min, after which the gradient changed to (100/0/0) in 1 min and was kept isocratic for 5 min. The gradient was then brought to the initial starting mixture and kept for 25 min, before the next injection. The flow rate was 1 mL/min. Detection was performed by an electrochemical detector (model ED-40): D-glucose (t_r 12.95 min), D-mannose (t_r 13.9 min) and D-fructose (t_r 16.59 min). All compounds were quantified by external standards.

Determination of heterocyclic compounds: *Adapted from Knerr et al [5].* 5-(Hydroxymethyl)furan-2-carboxaldehyde (HMF), 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone (HHMF) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) were determined by RP-HPLC (Lichrosorb RP-18, Merck). The eluent was 7.5:100 MeOH-water solution, flow 0.6 mL/min, UV detection at 280 nm. Only HMF was quantified by external calibration line with 5-(hydroxymethyl)furan-2-carboxaldehyde (Sigma-Aldrich, Germany).

Melanoidins quantification: The browning intensity of the heated reaction mixtures was determined by measuring the absorbance at 470 nm with a spectrophotometer (Pharmacia Biotech, Upsala, Sweden). When necessary the samples were diluted with demi water. The absorbance was then recalculated to the concentration of the melanoidins by using the equation of Lambert-Beer. The extinction coefficient formed in glucose-glycine was measured to be 0.64 ± 0.03 $l.mmol^{-1}.cm^{-1}$ at 470 nm. (*See section 3.3*) The concentration of melanoidins is thus expressed as moles of sugar incorporated in the brown polymers.

Kinetic Modelling: Based on the established reaction network a kinetic model was proposed and translated into a mathematical model by setting-up differential equations for each reaction step. The software package Athena Visual Workbench *[6]* was used for numerical integration as well as for parameter estimation. The model parameters, the rate constants, were estimated by non-linear regression using the determinant criterion *[7],* that is to minimize the determinant of the matrix of cross-products of the various responses, so called dispersion matrix. To discriminate between models, a multivariate test of goodness of fit was used together with two model discrimination tests. The goodness of fit test is installed in the used software package. It gives a sampling probability by which the adequacy of the model can be judged and was based on replicate experiments. The model discrimination tests used were the posterior probability (PPB) *[8]*, which requires replicates or an estimation of experimental uncertainty, and the Akaike Criterion (AIC) *[9]*.

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3.2. In situ trapping technique applied to the identification of carbohydrate degradation products

The formation of deoxyosones and short-chain dicarbonyls via carbohydrate degradation are important intermediates in the overall mechanism of the Maillard reaction. From the identified carbohydrate fragments C_3 compounds were the predominant ones. The work presented in this section resulted from a short-term scientific mission (STSM) at the German research center for food chemistry, Garching, Germany, under the supervision of Dr. Thomas Hofmann.

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3.2.1. Introduction

The Maillard reaction has, throughout the years, been a matter of study, in many different fields. New important intermediates and pathways, not accounted for by Hodge (*1*), have been recognised, partly because of the development of better analytical techniques. McWeeny *et al* (*2*) reported that the most important intermediates in colour formation are 3-deoxyosuloses and 3,4-dideoxyosulos-3-enes, which in the case of glucose is 3-deoxyhexosulose (DH) and 3,4-dideoxyhexosuloses-3-ene (DDH). Later, Hayashi and Namiki (*3*) reported that the schiff base of aldoses undergoes a retroaldol cleavage to form a C_2 fragment (pyrazinium radicals). More recently, Rizzi (*4*), stated that many coloured products appear to be (retro)aldolization/dehydration products of sugars which may or may not be attached to proteins or other sources of amino nitrogen. Also Hofmann (*5*) identified carbohydrate degradation products, α-dicarbonyl and hydroxycarbonyl compounds, as colourless browning precursors.

As a result the formation of deoxyosones and short-chain dicarbonyls via carbohydrate degradation, appeared to be important intermediates in the Maillard reaction. Therefore, in previous quantitative studies these intermediates have also been determined. Carbohydrate/amino acid mixtures were heated in the presence of surplus amounts of 1,2-diaminobenzene, which transforms the formed α-dicarbonyl intermediates *in situ* into their stable quinoxaline derivatives (*6*, *7*, *8*). However, this technique gives no insight in the changes of the derivatised compounds during heating, because they are trapped; moreover, the derivatised agent may interfere with the reaction itself.

The aim of the present study was to learn an *in situ* trapping technique, as developed by Hofmann (*5*) and apply it to the identification of carbohydrate degradation products. It is well known from literature that deoxyosones are not stable, due to dicarbonyl reactivity, and can either be degraded to cyclic compounds with the carbon chain of the sugar intact, or be fragmented into short-chain carbonyl compounds upon C-C cleavage. We focused the following quantitative studies on the formation of C_2, C_3 and C_4 compounds. Two systems, one only with glucose and another with glucose and glycine, were compared. The formation of the α-dicarbonyls glyoxal, 2-oxopropanal, butane-2,3-dione and the hydroxycarbonyls glycolaldehyde, hydroxy-2-propanone and 2-hydroxy-3-butanone, was quantitatively followed during the heating of the aqueous solutions. The added value of this technique is that each system is heated without a trapping reagent. After cooling, the dicarbonyls formed are derivatised prior to quantification. This technique avoids the accumulation of derivatives during heating, giving therefore a better overview of the carbonyl intermediates formed.

In the scope of kinetic modelling (*9*), the identification and quantification of Maillard reaction intermediates allows presenting a complementing view of its mechanism, because it

considers the main intermediates as rate-determining steps of the reaction. Rate-determining steps provide control points in predicting quality attributes, like colour and flavour, in the Maillard reaction.

3.2.2. Materials and Methods

Sample preparation. Solutions of 40 mmol of glucose in 100 ml phosphate buffer (0.5mol/l, pH7) were refluxed on their own and in the presence of 40 mmol of glycine for the reaction times of 5-10-20-40-60-120-180-240 minutes. At each reaction time, up to 120 min, 1 ml of sample was withdraw and put into 5 ml of water, thereafter into 15 ml of water.

Separation and quantification of the hydroxyketones (glycolaldehyde, hydroxy-2-propanone and 2-hydroxy-3-butanone). After cooling, was added to each sample 60 µl of the internal standard, $[^{13}C_4]$-3-hydroxy-2-butanone, dissolved in methanol, and 1 ml of o-ethylhydroxylamine hydrochloric (1mol/l) solution. The pH was adjusted to 7.5 with aqueous sodium hydroxide (0.1 mol/l) and the samples were kept overnight at room temperature. The pH was then adjusted to 6 with hydrochloric acid (0.1 mol/l) and the mixtures were extracted with three volumes of 15 ml diethyl ether. After removing the water with sodium sulphate and filter, the organic layer was concentrated to about 1 ml. Then it was dissolved in the proportion 1:5 with ether and applied to a GC for a first identification (column SA5485; programme: 40°C (2 min); 40°/min 50°C (2min); 6°/min 240°C (5min)). The quantification was performed by MS using a GC coupled to an MS which scanned the pseudomolecular ions $[M+1]^+$ of the syn- and anti-o-ethyloxime obtained in the chemical ionization (CI) mode. The following results were obtained: GC/MS-CI of syn/anti-o-ethyl glycolaldehyde oxime (Rt 5.05 min), 104 (100, $[M+1]^+$); GC/MS-CI of syn/anti-o-ethyl hydroxy-2-propanone oxime (Rt 6.45/6.9 min), 118 (100, $[M+1]^+$); GC/MS-CI of syn/anti-o-ethyl 2-hydroxy-3-butanone oxime (Rt 8.68/9.2 min), 132 (100, $[M+1]^+$); GC/MS-CI of syn/anti-o-ethyl $[^{13}C_4]$-3-hydroxy-2-butanone oxime (Rt 15.79/15.94 min), 136 (100, $[M+1]^+$).

Separation and quantification of the diketones (glyoxal, methylglyoxal and butan-2,3-dione). After cooling, to each sample was added 0.5 ml of the internal standard, $[^{13}C_4]$-butane-2,3-dione, dissolved in methanol, and 1 ml of methanolic solution of 1,2-diaminobenzene (1mol/l) solution. The pH was adjusted to 6.5 with aqueous hydrochloric acid (0.1 mol/l) and the samples were kept overnight at room temperature. The pH was then adjusted to 5 with hydrochloric acid (0.1 mol/l) and the mixtures were extracted with three volumes of 15 ml

diethyl ether. After removing the water with sodium sulphate and filter, the organic layer was concentrated to about 1 ml. The liquid phase was then applied to the top of a column (4X30 cm) filled with 6 g of silica gel 60 and previously conditioned with *n* – pentane. The sample was flushed with *n* - pentane / diethylether (30/70, v/v). The first 20 / 25 ml were considered waste and the 10 ml thereafter collected and concentrated to 1 ml. The detection was performed by MS using a GC coupled to a MS which scanned the molecular ions of quinoxaline derivatives obtained in the EI mode. The following results were obtained: GC/MS-EI of quinoxaline (Rt 8.12 min): 130; GC/MS-EI of 2-methylquinoxaline (Rt 9.35 min): 144; GC/MS-EI of 2,3-dimethylquinoxaline (Rt 10.595): 158; GC/MS-EI of [$^{13}C_4$]-2,3-dimethylquinoxaline (Rt 10.595): 162.

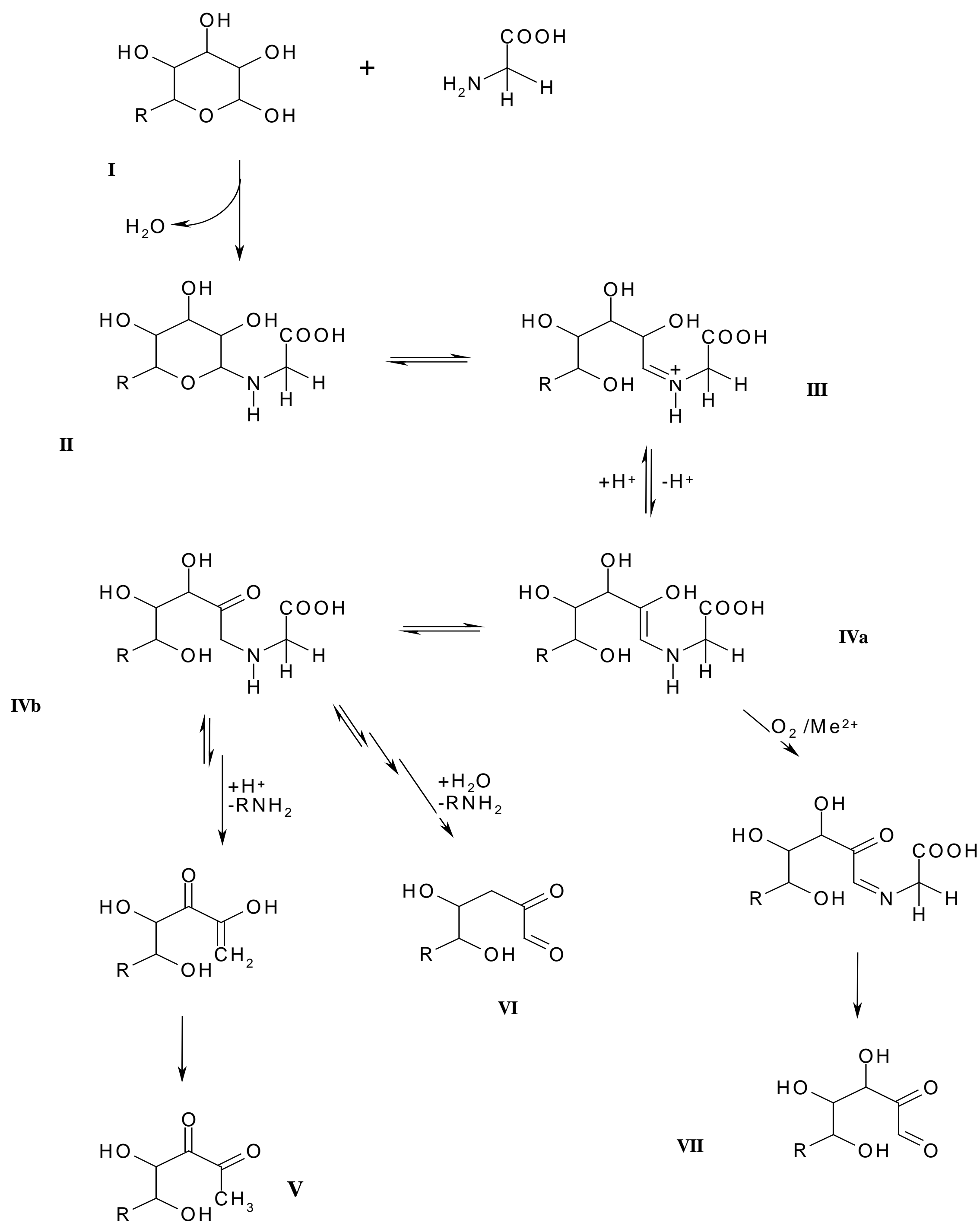
3.2.3. Results and Discussion

In order to gain insight into the role of carbohydrate degradation in the Maillard reaction and its importance as rate-determining steps in kinetic modelling, the separation and quantification methodology used has to be quite specific. The applied *in situ* trapping technique has been developed by Hofmann (*5*) and is based on the fact that 2-osuloses as well as 1- and 3-deoxyosones exist in aqueous solution as hemiacetal and hemiketal forms. As illustrated in Scheme 3.2.1, glycosylamines (II) are the first reaction products formed from the reaction of carbohydrates (I) with amino acids, being then transformed into deoxyglycosyl amino acid derivatives (IVa and IVb) via the so-called Amadori rearrangement (III).

These intermediates are not stable and are easily further degraded by loss of the amino acid to form 1- and 3-deoxyosones (V and VI). Besides these deoxyosones, 2-osuloses (VII) can also be formed from an enaminol intermediate of the Amadori rearrangement upon transitionmetal-catalized oxidation by oxygen in the air. Moreover, due to their reactive functional group, they can be easily degraded by cyclization to cyclic compounds with the carbon chain of the sugar intact, or be fragmented into short-chain carbonyl compounds upon C-C cleavage, leading to C_2, C_3 and C_4 carbonyl compounds.

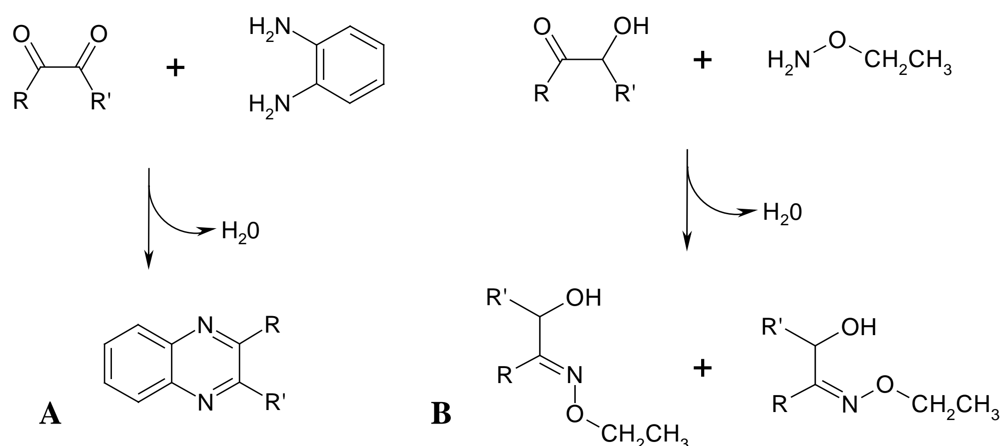
Because is known from literature that the C_2, C_3 and C_4 carbohydrate fragments formed are very reactive and hydrophilic, the dicarbonyls are derivatised with 1,2 – diaminobenzene as stable quinoxalines, and the hydroxycarbonyls are converted with ethoxamine into their stable syn/anti-*O*-ethyloximes. The derivatisation mechanism is illustrated in Scheme 3.2.2.

The added value of the used methodology is that the derivatising agent is added after withdrawing the sample. The mixture is heated without a trapping reagent.



Scheme 3.2.1. Formation of 2-osuloses, 1-deoxy-2,3-diuloses and 3-deoxy-2-osuloses from carbohydrates and amino acid, glycine.

After cooling, the dicarbonyls and hydroxycarbonyls formed are derivatised prior to quantification, avoiding the accumulation of derivatives during heating. Moreover, in order to guarantee exact quantitative data, the analysis were done using a stable isotope [$^{13}C_4$]-butane-2,3-dione and [$^{13}C_4$]-3-hydroxy-2-butanone, respectively, as internal standards for the dicarbonyl and the hydroxycarbonyl compounds. Quantification was then performed by MS by scanning the quinoxaline derivatives and the syn/anti-*O*-ethyloximes of the C_2, C_3 and C_4 carbohydrate fragments. We focused the quantitative studies on the formation of the dicarbonyls glyoxal, 2-oxopropanal, butane-2,3-dione and the hydroxycarbonyls glycolaldehyde, hydroxy-2-propanone and 2-hydroxy-3-butanone.



Scheme 3.2.2. Derivatisation mechanism of **A** – dicarbonyl carbohydrate fragments with 1,2 – diaminobenzene; **B** – hydroxycarbonyl carbohydrate fragments with *O*-ethoxamine

We were primarily interested in the actual amounts of these carbonyl compounds formed from the carbohydrate on its own. However, heating glucose alone in 0.5M phosphate buffer, during 4 hours, led to no significant amount of the analytes and therefore no quantification was possible. One of the possible reasons is the fact that without the amino group in the mixture, the degradation reaction proceeds quite slowly, since the amino group is believed to act as a catalyst in Maillard reaction. As a result, an equimolar solution of amino acid was added to the sugar solution. Heating glucose with glycine led to a drastic increase in the amount of analytes formed.

As illustrated in Figure 3.2.1 the concentration of glyoxal formed is considerably lower compared with 2-oxopropanal and butane-2,3-dione. However, its profile is rather similar to the butane-2,3-dione. Moreover, 2-oxopropanal, the C_3 compound, was the most predominant.

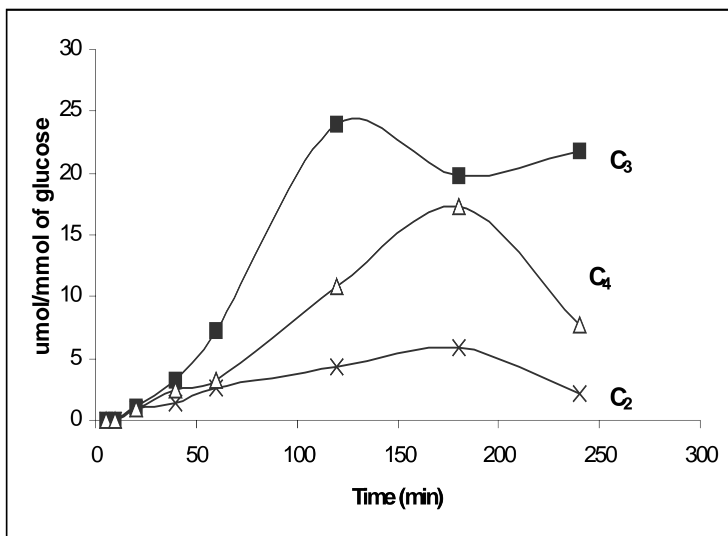


Figure 3.2.1. Dicarbonyls formation: glyoxal (×), 2-oxopropanal (■) and butane-2,3-dione (Δ) (C_2, C_3 and C_4 respectively).

At 120 min, its concentration reaches a maximum (24 μmol/mmol glucose) and is twice as much as butane-2,3-dione. Accordingly, with hydroxycarbonyls the C_3 compound had the highest concentration (Figure 3.2.2).

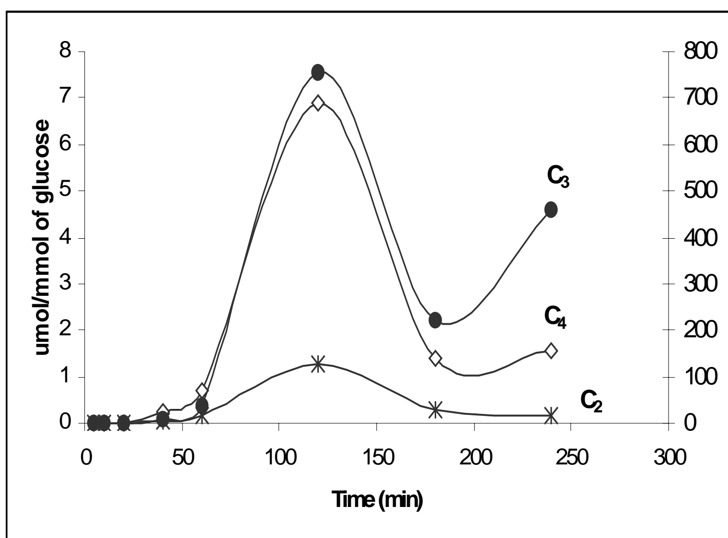


Figure 3.2.2. Hydroxycarbonyls formation: glycolaldehyde (∗), hydroxy-2-propanone (●) and 2-hydroxy-3-butanone (◇) (C_2, C_3 and C_4 respectively) (C_3 right scale).

Nevertheless, all three carbohydrate degradation compounds reached a maximum at the same time, an inducing period of nearly one hour can be observed. When compared the carbonyl with the hydroxycarbonyl the significance of the C_3 fragments is evident. Over the complete heating period, it can be noticed that hydroxy-2-propanone is the main intermediate formed, reaching a maximum of 0.7 mmol / mmol of glucose, after 2 hours of heating.

The application of appropriate quantification techniques offers the possibility of following the time course of the formation of reaction intermediates formed from carbohydrates and amino acids. These quantitative data give insight into the formation of

reactive carbohydrate degradation products, in the glucose / glycine system. However time did not allow drawing definitive conclusions. Nevertheless, is clear that C_3 compounds are predominant and therefore can be considered as possible rate-determining step in kinetic modelling. Further research will be done on this matter in the due course.

3.2.4. References

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3.3. Melanoidins extinction coefficient in the glucose/glycine Maillard reaction

Melanoidins (brown, nitrogenous polymers and co-polymers) are the final products of the Maillard reaction. The glucose/glycine melanoidins extinction coefficient was determined using ^{14}C-labelled glucose at three different reaction conditions. The absorbance was measured at different wavelengths (420, 450, 470 and 490 nm) and the extinction coefficient determined for each. The value of the extinction coefficient can be used to recalculate browning, measured as absorbance units, into melanoidins concentration in terms of sugar molecules incorporated. The amount of ^{14}C-labelled sugar molecules was estimated in melanoidins separated via dialysis with a cut-off value of 3500 Dalton. These melanoidins only represented ≈ 12% of the total colour formed. The extinction coefficient of the melanoidins remained constant during the observation period. At 470 nm, values of 0.65 (± 0.02) $l.mmol^{-1}.cm^{-1}$; 0.66 (± 0.02) $l.mmol^{-1}.cm^{-1}$ and 0.62 (± 0.05) $l.mmol^{-1}.cm^{-1}$, were obtained at 120°C pH 6.8, 100°C pH 6.8 and 100°C pH 5.5, respectively. The difference is not significant. The extinction coefficient appeared not to vary within the pH and temperature range studied. From the elemental analysis, the nondialysable melanoidins elementary composition seemed to be influenced by the reaction conditions, which was supposed to be related to the presence of side-chains on the melanoidin backbone. A trend was observed in the melanoidins C/N ratio: it decreased with increasing reaction pH as well as it changed to a lower level, of about 8, as the extent of browning increased.

The work presented in this section resulted from a short-term scientific mission (STSM) at the Procter Department at Leeds University, UK, under the supervision of Prof. B. L. Wedzicha.

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3.3.1. Introduction

In the Maillard reaction, melanoidins (brown nitrogenous polymers and co-polymers) are known as the main end product of the reaction. These brown polymers have significant effect on the quality of food, since colour is an important food attribute and a key factor in consumer acceptance. Up till now, browning is usually measured spectrophotometrically and expressed in absorbance units, which gives qualitative information in terms of colour formation but can not be related in quantitative terms to molecular concentration.

Studies on colour formation have been summarized in different review articles (*1*, *2*, *3*). Hashiba (*4*) concluded that browning was directly proportional to the reducing power of the sugar and to the amounts of glycine consumed, by comparing different sugars with one single amino acid. Rizzi (*5*) on the other hand stated that many coloured products appear to be (retro)aldolization /dehydration products of sugars which may or may not be attached to proteins or other sources of amino nitrogen. Up till now three main proposals for the structure of melanoidins have been put forward. Hofmann (*6*) detected low-molecular-weight (LMW) coloured substances, which were able to cross-link proteins via ϵ-amino groups of lysine or arginine to produce high-molecular-weight (HMW) coloured melanoidins. However one should keep in mind that with proteins there is always high-molecular-weight melanoidins, since proteins are by themselves HMW compounds. On the other hand, Tressl *et al*. (*7*) postulated a polymer consisting of repeating units of furans and/or pyrroles, formed during the advanced stages of the Maillard reaction and linked by polycondensation reactions. However, in a recent study (*8*) intact carbohydrate structures have been identified from acid hydrolysis of melanoidins indicating that sugars are not inevitably degraded to heterocycles such as furans and pyrroles. In a third structural proposal, the melanoidin skeleton is mainly built up from sugars degradation products, formed in the early stages of the Maillard reaction, polymerized through aldol-type condensation and linked by amino compounds, such as amino acids (*9*, *10*, *11*).

The mechanism of the formation of brown colour is not fully understood and the structure of melanoidins is largely unknown, which makes it difficult to quantify these compounds. However, this quantification is necessary when trying to predict or optimize browning in processed foods from a known molecular composition.

According to the Lambert-Beer equation ($A = \varepsilon * c * l$), there is a direct linear relation between absorbance (A) and concentration (c), through the extinction coefficient (ε), if the factor l, the length of the cuvette, is constant. Previous studies, not only in a sugar/amino acid system (*12*, *13*) but also in a sugar/protein system (*14*), have shown that it is possible to relate absorbance caused by nondialysable melanoidins to the number of sugar molecules incorporated in those melanoidins, by heating radioactive glucose (U-^{14}C glucose) with an

amino acid and/or protein. Experiments in a glucose-glycine system at 55°C and 90°C, pH 5.5 (*12*) suggested that the chromophores in melanoidins with Mr > 12000 Da formed in the early stages of heating are similar to those at later stages. However, under these conditions the amount of material with Mr > 12000 Da is believed to be very small (*15*). In a similar study (55°C, pH 5.5) but with the melanoidins cut off at 3500 Da it was also reported that the extinction coefficient remained constant throughout the heating time in glucose/amino acid systems (*13*). Values of ε at 470 nm were estimated to range from 0.34 $l.mmol^{-1}.cm^{-1}$ for alanine to 0.94 $l.mmol^{-1}.cm^{-1}$ for glycine. On the other hand in sugar/casein systems (120°C, pH 6.8), where ε also remained constant throughout the heating period, independently of the sugar, glucose or fructose, a constant value of 0.3 $l.mmol^{-1}.cm^{-1}$ was obtained (recalculated to ε at 470 nm) (*14*).

It may be speculated that melanoidins are formed as a result of random polymerization of carbohydrate degradation products or adducts of those with amino compounds. The regularity of the polymers with respect to nitrogen-containing and nitrogen-free subunits is still not clear. However, the starting materials, as well as reaction conditions have a strong influence on the elemental composition of melanoidins (*10*, *12*). The reaction conditions can determine the type of products that are formed during the Maillard reaction and as a consequence the followed pathways in the melanoidins formation (*16*).

The aim of the present study was to elucidate if and how the reaction conditions, pH and temperature, can influence the glucose/glycine melanoidins extinction coefficient. Melanoidins were, rather arbitrarily, defined as high-molecular-weight by a lower limit of 3500 Da, which was the nominal cut-off value in the dialysis system used. The average extinction coefficient of melanoidins was determined for three systems (A: pH 6.8, temperature 120°C; B: pH 6.8, temperature 100°C; C: pH 5.5, temperature 100°C) following the method of Leong (*13*). A benefit of this approach is that the molar extinction coefficient can be expressed in terms of the concentration of glucose molecules converted into melanoidins, even though the molecular weights of melanoidins are expected to span a very wide range of values. The C/N ratio of the high molecular weight products was also determined for the studied systems.

3.3.2. Materials and Methods

Materials. All chemicals were of analytical grade and were supplied by Sigma chemicals (United Kingdom). D-[U-^{14}C]-glucose (Specific Activity 111 MBq/mmol and 11.4 GBq/mmol) was obtained from Amersham Life Science Ltd (United Kingdom).

Samples Preparation. Reaction mixtures (100 ml) of glucose and glycine containing an equimolar final concentration of 0.2 M were prepared in phosphate buffer 0.1M, pH 6.8 or 5.5. Before making up to the final volume, 1 MBq of D-[U- ^{14}C]-glucose was spiked into the solution (the concentration of added labelled sugar was negligible). The reaction mixture was then distributed over glass, screw-capped, Schott tubes (16 x 160 mm), each containing a minimum of 10 ml. Samples were heated in at least duplicate at 120°C and 100°C (pH 6.8) and at 100°C (pH 5.5). The heating was carried out in an oil bath and the proper safety measures taken. At timed intervals, the samples were withdrawn, immediately cooled in ice and then dialyzed.

Dialysis. Approximately 2 ml of the reaction mixture were injected into dialysis cassettes (Mr > 3500) (Slide-A-Lyzer Dialysis Cassette, 3.5K MWCo, Pierce, USA) and dialyzed against distilled water. The optimum dialysis time was established by carrying out a test, where the retentate of the same sample was counted after different dialysis days. Each day corresponds to two water changes, 2 L each. The ^{14}C-activity reached equilibrium after seven days (14 water replacements) (Figure 3.3.1). Only then the contents were removed and the volume adjusted to 10 ml with distilled water.

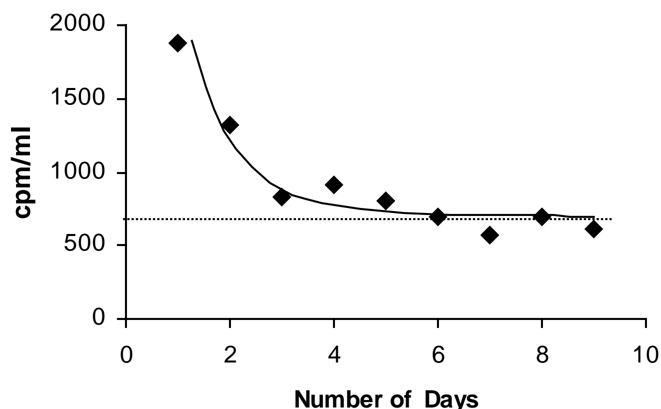

Figure 3.3.1. Change in ^{14}C-activity in the retentate as a function of dialysis time (2 water changes per day) at room temperature. Counts per minute (cpm).

Scintillation Counting. An aliquot (1ml) of the diluted dialysed fraction was pipetted into 10 ml of scintillation liquid (Emulsifier Scintillator Plus, Packard) in a plastic vial (Liquid Scintillation Vials, Wheaton Scientific), shaken thoroughly and counted immediately in a Liquid Scintillator Analyzer, 1600TR, Packard for 10 minutes. The count due to ^{14}C was corrected for quenching. Quenching is a phenomenon where the observed pulse height is lower

than the actual, and there is a shift of the energy spectrum of the isotope to lower energies. Chemical quenching is the result of impurities present in the solution that interfere with the energy transfer process, while colored solutions act as an optical filter causing color quenching. Quench correction was done by the internal standard method (*17*). After counting the sample was spiked with an accurately known amount of isotope in an unquenched form and recounted. The specific activity of ^{14}C-glucose in the reaction mixture was calculated from the counts obtained from 1ml of a 100-fold diluted unheated reaction mixture and was expressed as number of disintegration per minute (dpm) per mol of glucose. Once the quench-corrected number of counts for a certain sample was known the concentration of U-^{14}C-sugar incorporated into the high molecular weight fraction could be calculated by dividing the number of counts per minute by the specific activity of the sugar.

Spectrophotometric analysis. Browning was measured spectrophotometrically as the absorbance at 420, 450, 470 and 490 nm. Optical pathlength of the cuvette 1 cm. Spectrophotometer Cary 50 Bio, Varian.

Microanalysis. The reaction mixtures (without addition of radio labeled sugar) were dialyzed in the same way as described above and the retentates freeze-dried. Microanalysis was carried out using a CE Instruments Element Analyzer, Type EA 1110 CHN.

3.3.3. Results and Discussion

Extinction Coefficient

Browning measured, as absorbance at 470 nm was determined for each studied system before and after dialysis (Figure 3.3.2). Considerable browning was observed before dialysis with the smallest induction period at 120°C (pH 6.8). At the same pH but lower temperature (100°C), a longer heating time was required to achieve the same absorbance and at 100°C and pH 5.5, browning showed the longest induction period. An absorbance of 3 units was reached after 0.5h, 1.8h and 8h for systems A (120°C, pH 6.8), B (100°C, pH 6.8) and C (100°, pH 5.5), respectively.

After dialysis the absorbance results show that independently of the reaction conditions, more than 80% of browning, measured spectrophotometrically at 470 nm, passed into the dialysate, namely 88%, 88% and 82% in system A, B and C, respectively. The majority of colour was thus not retained in the HMW fraction (Mr > 3500) but below it.

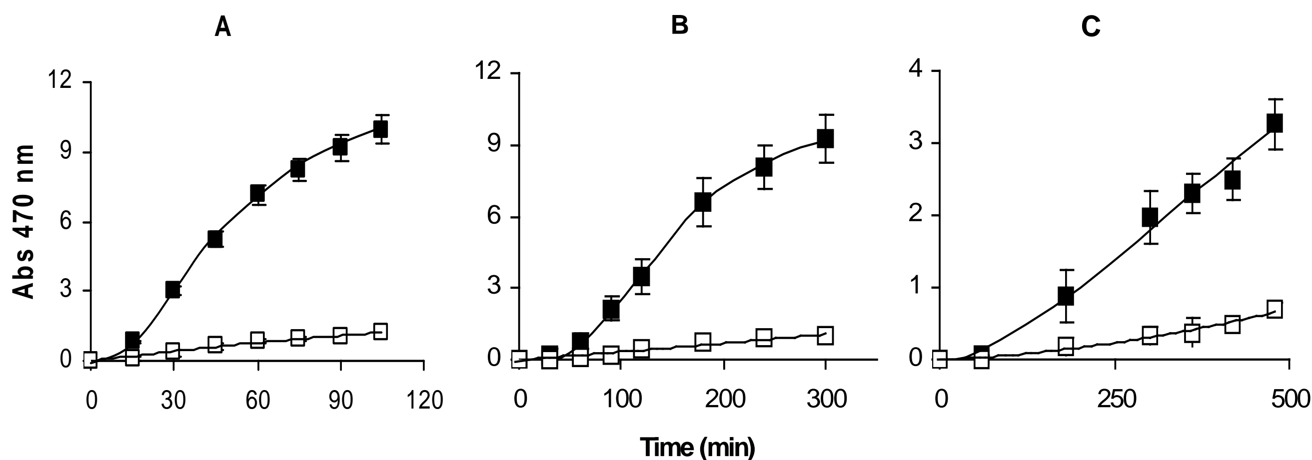

Figure 3.3.2. Browning (measured as Absorbance (Abs) at 470 nm) before (closed markers) and after (open markers) dialysis. A – Temperature 120°C pH 6.8; B - Temperature 100°C pH 6.8; C - Temperature 100°C pH 5.5. The error bars represent the standard deviation for each observation (n = 3).

This result is in line with literature describing browning in sugar-amino acid systems. Leong (*13*) observed that the HMW fraction (> 3500 Da) contributed only up to 10% of the absorbance of the glucose/glycine reaction mixture heated in acetate buffer at 55°C and pH 5.5. Also Hofmann (*15*) in both glucose/glycine and glucose/alanine systems heated in phosphate buffer for 4h at 95°C, pH 7, reported that only trace amounts of compounds with molecular weights greater than 3000 Da were formed. These results are in contrast with sugar/protein reactions. A much higher percentage of colour was detected in the HMW fraction (≥ 70%), which is as expected since the melanoidins are attached to the protein that is HMW by itself (*14*, *15*). Colour in the glucose/amino acid reaction mixtures is almost exclusively due to the LMW fraction. The fact that independently of the studied reaction conditions, the same % of high molecular weight fraction was obtained, suggests that the formation of nondialysable (HMW) melanoidins does not appear to be sensitive to temperature and pH.

As mentioned before, the extinction coefficient was calculated based on the Lambert-Beer equation ($A = \varepsilon * c * l$). Since the factor l is constant, there is a direct linear relation between absorbance (A) and concentration (c), through the extinction coefficient (ε). The concentration of nondialysable melanoidins was expressed as the concentration of U-^{14}C glucose incorporated. The extinction coefficient was then the slope of the plot of the absorbance of the retentate after dialysis versus the concentration of radiolabeled glucose incorporated into those melanoidins. In Figure 3.3.3 the results show that over the observation period the extinction coefficient of the nondialysable melanoidins remained constant, in the three systems studied.

The extinction coefficient was calculated taking the average of the repetitions carried out under the same conditions.

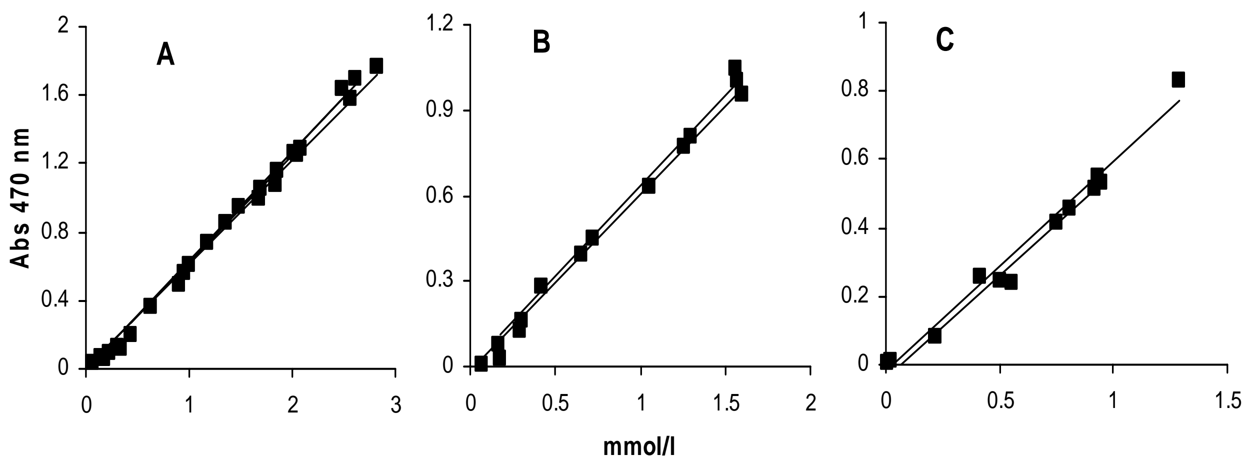

Figure 3.3.3. Browning (measured as absorbance (Abs) at 470 nm) as function of the melanoidin concentration (measured as incorporated sugar); A – Temperature 120°C pH 6.8; B – Temperature 100°C pH 6.8; C – Temperature 100°C pH 5.5. Each line corresponds to a repetition of the experiment. The slope of the lines determines the extinction coefficient value.

In Table 3.3.1 the values of ε are presented for different wavelengths. The chosen wavelengths result from the fact that in literature reports concerning brown colour, usually one of these 4 wavelengths are used, since there is no maximum in the visible spectrum (400 – 500 nm approximately) for brown colour (*17*). The differences in the extinction coefficient according to the used wavelength stress the importance of having standard procedures. The finding that the regression line not always passed through the origin was ascribed to the background measured in the system. Radioactive glucose, not incorporated into the melanoidins was possibly still retained inside of the retentate to some extent. A possible reason is the fouling of the membrane by the melanoidins, slowing down the diffusion, or a complex formation of the melanoidins with the sugar, which would also delay the diffusion.

The evidence is strong that the chromophores produced in the nondialysable melanoidins in the early stages of the reaction are similar to those at later stages, independently of the reaction conditions. In the present study the molar extinction coefficient averaged over the three systems, for glucose/glycine HMW melanoidins was calculated to be 0.64 ± 0.03 $l.mmol^{-1}.cm^{-1}$ at 470 nm. Leong (*13*) showed that at 470 nm the extinction coefficient for HMW and LMW glucose/glycine melanoidins was the same.

Table 3.3.1. Extinction Coefficient (ε) of glucose/glycine nondialysed melanoidins measured for different wavelengths under different reaction conditions.

	Wavelength (nm)	420	450	470	490
120°C pH 6.8	ε ($l.mmol^{-1}.cm^{-1}$)	**1.00 ± 0.03**	**0.77 ± 0.02**	**0.65 ± 0.02**	**0.53 ± 0.01**
	Intcp.[a] Lower 95%	-0.23	-0.18	-0.15	-0.12
	Intcp.[a] Upper 95%	-0.02	-0.002	-0.01	-0.002
100°C pH 6.8	ε ($l.mmol^{-1}.cm^{-1}$)	**1.01 ± 0.02**	**0.79 ± 0.02**	**0.66 ± 0.02**	**0.53 ± 0.01**
	Intcp.[a] Lower 95%	-0.12	-0.09	-0.09	-0.057
	Intcp.[a] Upper 95%	-0.02	-0.01	-0.004	0.01
100°C pH 5.5	ε ($l.mmol^{-1}.cm^{-1}$)	**0.97 ± 0.07**	**0.71 ± 0.05**	**0.62 ± 0.05**	**0.50 ± 0.04**
	Intcp.[a] Lower 95%	-0.20	-0.16	-0.14	-0.11
	Intcp.[a] Upper 95%	0.07	0.05	0.05	0.05

[a] Intercept with the origin (95% confidence interval).

However, the extinction coefficient that they reported was considerably higher for glucose/glycine systems, namely 0.94 $l.mmol^{-1}.cm^{-1}$ at 470 nm and it varied according to the type of amino acid. Using the kinetic modelling approach in the glucose/glycine system at pH 5.5 and 55°C, Leong and Wedzicha (*18*) estimated ε as a parameter to be 1.0 $l.mmol.^{-1}cm.^{-1}$ from absorbance measurements without radiolabelling. On the other hand, Wedzicha and Kaputo (*12*) by applying the same radiolabelled technique showed that ε was not affected by composition molar ratios of glucose and glycine, as well as by the heating temperature. Values of 0.41 $l.mmol^{-1}.cm^{-1}$ at 90°C and 0.37 $l.mmol^{-1}.cm^{-1}$ at 55°C were obtained, at 450 nm. Also, in a recent study Brands *et al.* (*14*) reported that the melanoidins extinction coefficient remained constant in a sugar/casein system, during the observation period (90 min at 120°C, pH 6.8) independently of the sugar. They found values of 0.48 $l.mmol^{-1}.cm^{-1}$ and 0.53 $l.mmol^{-1}.cm^{-1}$ for the glucose/casein and fructose/casein systems, respectively, at 420 nm. In the present study, under the same conditions the obtained extinction coefficient was 1.00 ± 0.03 $l.mmol^{-1}.cm^{-1}$ at 420 nm, about 2 times higher than the ε of sugar/casein melanoidins. This means that in sugar/amino acid reaction mixtures, less glucose molecules (or glucose fragments) have to be incorporated into the melanoidins than in the sugar/casein systems, to increase the absorbance by one unit.

If the degree of polymerization of melanoidins increases with the reaction time, the fact that the extinction coefficient does not seem to change with time implies that it does not depend on the degree of polymerization. Looking at each system individually, we observe that the ratio of absorbance due to LMW and HMW is constant in each system (Figure 3.3.4). Also, the yield of nondialysable melanoidins was proportional to the total colour formation.

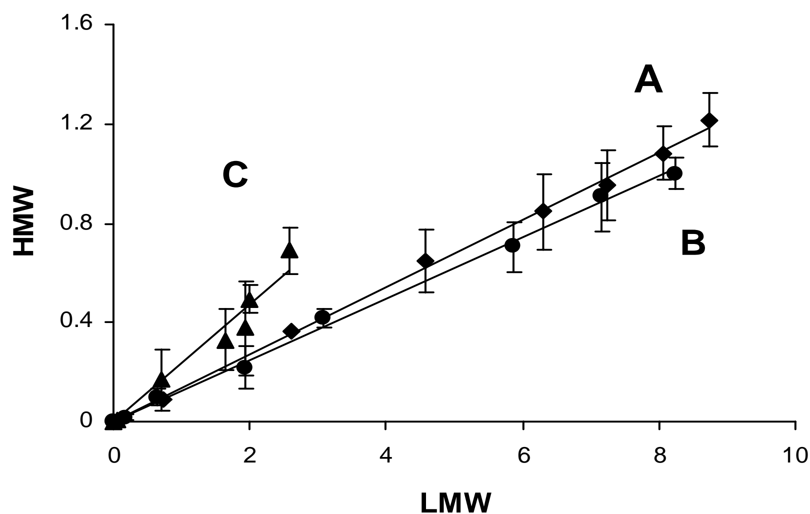

Figure 3.3.4. The absorbance at 470 nm of high molecular weight (HMW) fraction plotted against the low molecular weight (LMW) fraction. A – Temperature 120°C pH 6.8 (HMW = 0.12*LMW; R^2 = 0.99); B – Temperature 100°C pH 6.8 (HMW = 0.14*LMW; R^2 = 0.99); C – Temperature 100°C pH 5.5 (HMW = 0.24*LMW; R^2 = 0.96).

Leong (*13*) in a similar study, at 55°C and pH 5.5, reported that the ratio of HMW to LMW showed an initial lag phase, till 0.1 absorbance units of the HMW fraction, increasing afterwards proportionally with time. These results suggest that nondialysable melanoidins formation results from the built-up of LMW components into HMW structures. The chromophore formation can be explained, either by combination of isolated, low molecular weight, coloured chromophores only or together with low molecular weight non-coloured compounds. This combination, though, does not change the chromophore extensively (Figure 3.3.5). Moreover, the result that HMW colourants with molecular weight up to several thousand daltons could not be observed (*15*) suggests that the built up of LMW into HMW structures only reaches the oligomer size, approximately 13 molecules of glucose and glycine. Once the melanoidin chromophore is formed its concentration increases proportionally with time.

Moreover, other studies reported that changing the reaction conditions influence the elementary composition of the melanoidins (*10*, *12*). The observed differences were attributed to the possible presence of side chains that do not affect the chromophore extensively. In agreement with this a different slope was observed for the LMW and HMW ratio, in our three studied systems where the extinction coefficient remained constant throughout the heating period. We will come back to the melanoidins elementary composition in the following section.

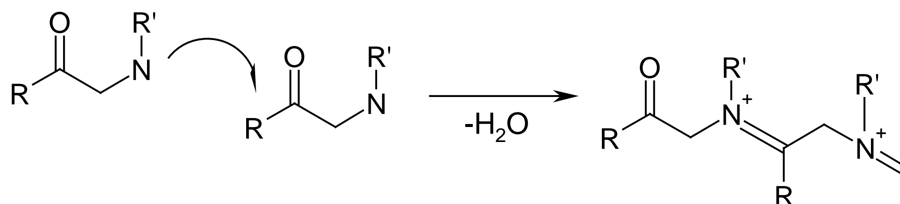

Figure 3.3.5. Formation of high molecular weight components through the combination of coloured low molecular weight subunits (adapted from Leong (1999)). R and R' may carry a chromophore which does not change extensively when combined with the high molecular weigh molecule.

Table 3.3.2. Microanalysis results reported in literature under different reaction conditions

Reference	Reaction Conditions	C/N
Cämmerer & Kroh (10)	H_20; 60°C; 160h; **pH5**; [gly] = [glu] = 0.1M	**7**
	H_20; 100°C; 10h; **pH5**; [gly] = [glu] = 0.1M	**9**
Wedzicha & Kaputo (12)	Acetate Buffer 0.2 M; 90°C; 22h; **pH 5.5**	
	[gly] = 1.0 M; [glu] = 1.0 M	**8**
Leong (13)	Acetate Buffer 0.2 M; 55°C; 90h; **pH 5.5**	
	[gly] = 0.5 M; [glu] = 1.0 M	**8**
Feather & Nelson (19)	100°C; 8h; **pH 3.5**	
	[gly] = 1.0 M; [glu] = 0.2 M	**10**
Bobbio et al (20)	Citrate Buffer 0.05 M	
	[gly] = 0.66 M; [glu] = 1.25 M	
	70°C; 415h; **pH 3.0**	**12**
	70°C; 415h; **pH 6.0**	**11**
	80°C; 80h; **pH 3.0**	**13**
	80°C; 80h; **pH 6.0**	**10**
Hayashi & Namiki (21)	[ala] = [glu] = 2 M	
	100°C; 190 min; **pH 2.3**	**12**
	100°C; 53 min; **pH 6.5**	**9**
	100°C; 25 min; **pH 9.2**	**7.5**
Olsson et al. (22)	H_20; 100°C; 120h; **pH 5**	
	[gly] = 0.5 M; [glu] = 0.75 M	**12**

Microanalysis

From microanalysis results reported in literature (Table 3.3.2) it seems that the elementary composition of the melanoidins is influenced by the reaction conditions. For the evaluation of the microanalysis data of the melanoidins in the present study, a stoichiometric reaction model was fitted to the results, as used before (*12*). The overall reaction for the formation of melanoidin is a combination of a molecules of sugar consisting of l, m and n atoms of C, H and O respectively and b molecules of amino acid consisting of p, q, r and s atoms of C, H, N and O, to give a melanoidin formula, where y is the number of water molecules: $C_{la+pb} H_{ma+qb-2y} N_{rb} O_{na+sb-y}$. In the glucose/glycine system $l = 6$, $m = 12$, $n = 6$, $p = 2$, $q = 5$, $r = 1$ and $s = 2$. Assuming $b = 1$, the unknowns a and y can be found by solving the following equations: $C = 6a+2$ and $H = 12a+5-2y$.

The number of carbon dioxide molecules was not calculated. The microanalysis data of nondialysable melanoidins derived at 120ºC and pH 6.8 are shown in Table 3.3.3. The results show that throughout the heating time the number of incorporated mol of sugar (or its corresponding degradation product) per amino acid remains constant, around 1.2. This is consistent with the fact that almost 80% of glycine was recovered after the reaction heating period. The same result was found for glucose/glycine heated at 100°C, pH 7 for 10h (*10*). Nevertheless, different reports have shown that the reaction conditions have influence on the number of sugar molecules incorporated into the polymer in glucose/glycine systems. In a H_2O/Methanol solution (*11*) at 65ºC, heated for 7h a value of 0.91 was reported, while in a solvent free system at 170ºC for 20 minutes a value of 2.19 was estimated (*10*). On the other hand, the number of molecules of water eliminated per mol of sugar or corresponding degradation product incorporated seemed to be constant independently of the reaction conditions. In the present study the estimated number was 3. Also Cämmerer and Kroh (*10*) independently of the temperature, pH, solvent free or water content came to the same conclusion, as well as Wedzicha and Kaputo (*12*). Also, Feather and Nelson (*19*) reported the same value for polymers derived from D-glucose and D-fructose with glycine. According to the proposed structure by Yaylayan and Kaminsky (*11*) Amadori products can polymerize through nucleophilic addition reactions of amino groups to the carbonyl moieties of a second molecule, followed by dehydration to form the zwitterionic polymer. Also, Cämmerer and Kroh (*10*) suggested that the melanoidin skeleton was mainly built up of sugar degradation products, formed in the early stages of the Maillard reaction. The hypothetical structure proposed was based on the reactions of dicarbonyl compounds (dehydrated, sugar-derived intermediates) that can react among themselves (aldol reaction or nucleophilic addition) as well as have substitution reactions with amino compounds. In these two last studies the position and the type of characteristic IR absorptions found for melanoidins was determined and it was not

affected by the different reaction conditions, which means that the main structure of the melanoidin chromophore was not influenced by the reaction conditions. The observed differences in the elementary analysis were attributed to the possible presence of side-chains that do not affect the chromophore. These results are well in line with the fact of the extinction coefficient remaining constant throughout the heating period, independently of the pH and temperature, as well as of the molar composition, the type of amino acid and type of sugar as observed in previous studies (*12*, *13*, *14*).

Table 3.3.3. Microanalysis results from system A.[a]

	120°C pH 6.8			
Time (min)	*C/N*	*a/b*	*y/a*	*Abs 470 nm*
15	**11**	1.2	1.8	3.6
30	**11**	1.2	2.6	6.3
45	**11**	1.2	3.1	8.1
60	**11**	1.2	3.2	9.2

[a] Calculated number of molecules of sugar (a) per molecule of amino acid (b) and calculated number of molecules of water (y) per molecule of sugar (a).

Table 3.3.4. Microanalysis results from systems B and C.

	100°C pH 6.8		*100°C pH 5.5*	
Time (min)	*C/N*	*Abs 470 nm*	*C/N*	*Abs 470 nm*
30	**15**	0.5		
60	**14**	2.0	**19**	0.1
120	**11**	4.6		
180	**11**	6.3	**16**	0.6
300	**11**	10.0	**12**	1.3
420			**12**	2.2
480			**12**	2.6

When we compare the C/N ratio values of system A (120°C, pH 6.8) (Table 3.3.3) with the ones of system B (100°C, pH 6.8) and C (100°C, pH 5.5) (Table 3.3.4) we observed that at higher pH and temperature, in a way the most favorable reaction conditions for formation of melanoidins, the C/N ratio remained constant throughout the heating period. If we decreased the temperature and/or the pH, we observed in the initial period of the reaction a higher value

for C/N followed by a decrease till a constant value was reached. It seems that the ratio is indeed lower at higher pH and therefore seems to depend on the reaction conditions. These results are consistent with the fact that at lower pH around 90% of glycine was recovered, in contrast with the 80% at higher pH, independently of the temperature. However, we should be careful with interpretation because elemental analysis could be sensitive to experimental errors. Consequently, the C/N values reported in literature are not consistent, either with pH or temperature (Table 3.3.2). However, there is a trend that the C/N ratio decreases with increasing pH (*20*, *21*) as well as it changes to a lower level, of about 8, as the extent of browning increases.

3.3.4. Conclusions

The results of the extinction coefficient are well in line with earlier investigations. There is strong evidence that chromophores formed in the nondialysable melanoidins in the early stages of the reaction are similar to those at the later stages and their formation does not appear to be sensitive to the reaction conditions. Alternative pathways in the formation of the same oligomer can be a possible explanation, as suggested by Yaylayan and Kaminsky (*11*). The microanalysis results showed that the melanoidins for which the extinction coefficient was determined are in line with those reported in literature. With this technique it was possible to determine the extinction coefficient of melanoidins formed in glucose/glycine systems at different reaction conditions. Through these results we are now able to translate the spectrophotometric data into concentrations of reacted sugars and to take browning into account in a kinetic model. This will allow a better prediction for browning in model systems as well as in foods.

3.3.5. References

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Chapter 4

Reaction network build-up:

Unravelling the intermediate stage

4. Reaction network build-up:

Unravelling the intermediate stage

4.1. Kinetic Modelling of Amadori *N*-(1-deoxy-D-fructos-1-yl)-glycine degradation pathways. Part I – Reaction mechanism.

4.2. Kinetic Modelling of Amadori *N*-(1-deoxy-D-fructos-1-yl)-glycine degradation pathways. Part II - Kinetic Analysis.

4.1. Kinetic Modelling of Amadori *N*-(1-deoxy-D-fructos-1-yl)-glycine degradation pathways. Part I – Reaction mechanism.

The fate of the Amadori compound *N*-(1-deoxy-D-fructos-1-yl)glycine (DFG) was studied in aqueous model systems as a function of pH and temperature. Special attention was paid to the formation of the free amino acid, glycine; parent sugars, glucose and mannose; organic acids, formic and acetic acid and α-dicarbonyls, 1- and 3-deoxyosone together with methylglyoxal. For the studied conditions decreasing the initial reaction pH with 1.3 units or increasing the temperature with 20°C has the same effect on the DFG degradation as well as on glycine formation. An increase in pH seems to favour the formation of 1-deoxyosone. The lower amount found comparatively to 3-deoxyosone, in all studied systems, seems to be related with the higher reactivity of 1-deoxyosone. Independently of the taken pathway, enolization or retro-aldolization, *N*-(1-deoxy-D-fructos-1-yl)-glycine degradation is accompanied by amino acid release. Together with glycine, acetic acid was the main end product formed. The rate of parent sugars formation increased with pH, but the type of sugar formed also changed with pH. Mannose was preferably formed at pH 5.5 whereas at pH 6.8 the opposite was observed, that is, glucose was formed in higher amounts than mannose. Also, independently of the temperature, at higher pH fructose was also detected. pH, more than temperature, had an influence on the reaction products formed. The initial steps for a complete multiresponse kinetic analysis have been discussed. Based on the established reaction network a kinetic model will be proposed and evaluated by multiresponse kinetic modelling in the subsequent section.

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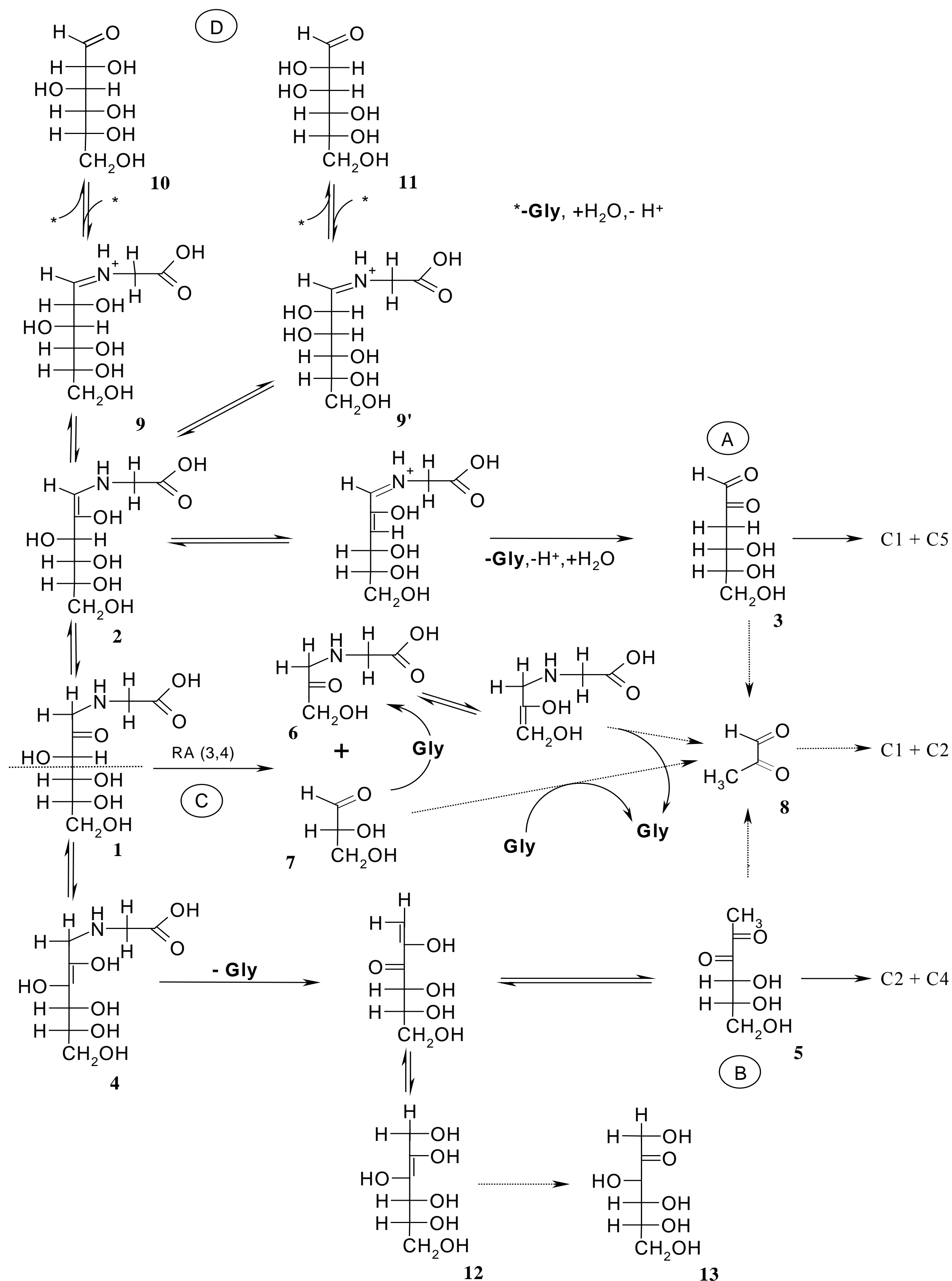
4.1.1. Introduction

Since Hodge (*1*) presented the first coherent Maillard reaction scheme in 1953, the Amadori compound *N*-substituted 1-amino-1-deoxy-ketose is believed to be the key intermediate in the early stages of the reaction. The accepted mechanism for the formation of Amadori compounds involves the initial reaction of a reducing sugar with an amino group to give the corresponding glycosylamine, that rearranges to the corresponding ketoseamine, also known as the Amadori rearrangement product (ARP). The set of reactions that occurs thereafter is of great importance in the processing of foods for the production of aroma, taste and colour. Furthermore, evidence strongly suggests that this intricate reaction cascade is involved in the pathology of diabetes and ageing.(*2*)

Due to the complexity of products that are formed from the degradation of the ARP, recent studies used the Amadori compound as the initial reactant (*3-6*). These studies showed that the 1,2- and 2,3-enolizations of Amadori compounds under acid/base catalysis conditions initiate β-elimination reactions, which eventually lead to the formation of reactive intermediates (Scheme 4.1.1). The degradation of *N*-(1-deoxy-D-fructos-1-yl)-glycine (**1**) by 1,2-enolization (pathway A) and 2,3-enolization (pathway B) leads to the formation of 3-deoxy-2-hexosulose (**3**) and 1-deoxy-2,3-hexodiulose (**5**), respectively. The formation of these intermediates is accompanied by amino acid release. In parallel, other α-dicarbonyls can also be formed from the ARP enolization. A recent study (*6*) presented a detailed scheme of Amadori compound degradation pathways through enolization, such as the formation of glucosones by transition-metal catalyzed oxidation of 1,2-enaminol (**2**) and 1-amino-1,4-dideoxy-2,3-diulose by elimination of the C4-OH group of the 2,3-enaminol (**4**).

Another possible mechanism of degradation of Amadori compounds involves a retro-aldol reaction, as shown in pathway C. Previous studies (*4,7*) have indicated that **1** generates 1-glycine-1-deoxy-D-glyceraldehyde (**6**) and glyceraldehyde (**7**) through a retro-aldol cleavage at C3-C4. **7** can react with free glycine and produce more of compound **6** that subsequently undergoes a β-elimination to form methylglyoxal (**8**) and release glycine. Moreover, **8** can also be produced from **7** through the catalytic action of the amino acid.

In spite of all the work done in Maillard reaction, the reversibility of ARP is still a controversial issue. Theoretically, as a series of equilibrium reactions, the Amadori rearrangement is expected to be a reversible process, that is to undergo a non-enzymatic reversal into enolamines and subsequently into free sugars and amino acid, as shown in pathway D. However, other pathways as side reactions from ARP enolization or aldol-type condensations between smaller sugar fragments generated from the decomposition of ARPs can also give rise to the original aldose.



Scheme 4.1.1. Amadori compound *N*-(1-deoxy-D-fructos-1-yl)glycine degradation pathways: enolization and retro-aldolization.

Under physiological conditions (pH 7.4 and 37°C), glucose and mannose were identified as major products formed from protein-bound Amadori compound fructoselysine, along with tetroses, pentoses and 3-deoxyglucosone (*8*). Up until recently the formation of parent sugars had only been reported for physiological conditions. However, quantitative data concerning the formation of parent sugars, glucose (**10**) and mannose (**11**), from *N*-(1-deoxy-D-fructos-1-yl)glycine at the early stage of the reaction have now been reported for conditions relevant to food processing (*6*). Besides **10** and **11**, formic and acetic acid were also detected, together with the free amino acid. Formic and acetic acid have also been identified as two major degradation products by heating the isolated Amadori compound from a glucose/casein system (*9*). The proposed mechanism suggests the C1-C2 cleavage of **3** for the formic acid formation and C2-C3 cleavage of **5** or a cleavage reaction of trioses intermediates, like **8**, for the formation of acetic acid. However, no attempt was made to identify and quantify any α-dicarbonyl compound to support such mechanism.

The aim of the present study was to establish the main thermal degradation pathways of *N*-(1-deoxy-D-fructos-1-yl)glycine by applying multiresponse kinetic modelling. The multiresponse kinetics analysis considers reaction pathways in more detail. It provides extra information about the reaction mechanism since the reactants degradation is analyzed simultaneously with the intermediates formation. First a reaction mechanism is proposed and then the multiresponse kinetic analysis is applied (*10*). The following steps should be taken into account: (i) identification and quantification of the reactants and main products formed; (ii) identification of reaction pathways based on reaction conditions; (iii) differentiate between primary and secondary reaction routes; (iv) propose a kinetic model based on the established reaction network; (v) test the hypothesized mechanism; (vi) estimate the rate constants. The present section, Part I, deals with the first 3 steps. Special attention was paid to the formation of the free amino acid, glycine; parent sugars, glucose and mannose; organic acids, formic and acetic acid and α-dicarbonyls, 1- and 3-deoxyosone together with methylglyoxal. The subsequent section, Part II, deals with the remaining steps with a focus on the kinetics of the hypothesized mechanism.

4.1.2. Results and Discussion

The effect of the combination of pH and temperature on the decomposition of **1** was studied at 100°C and 120°C, with initial pH values of 5.5 and 6.8. By analysing the reactant degradation and the formation of the main products, under different reaction conditions, the reaction routes could be established. The following steps were taken:

(i) Identification and quantification of the reactant 1 (DFG) and main products formed

Degradation of DFG. The rate of degradation of **1** increased with temperature and pH (Figure 4.1.1). After heating for 30 minutes at 120°C and pH 6.8, **1** was completely degraded whereas after 180 minutes at 100°C and pH 5.5, only approximately 57% of the initial concentration had reacted. Moreover, when comparing the system at 100°C and pH 6.8 with the system at 120°C and pH 5.5, a similar degradation rate was observed. These results indicate that decreasing the initial reaction pH with 1.3 units or increasing the temperature with 20°C has the same effect on the DFG degradation. However, this observation is only valid in the pH range between 5 and 7 (*6*).

Main products formed. Special attention was paid to the formation of the free amino acid, glycine; α-dicarbonyls, 1- and 3-deoxyosone together with methylglyoxal, parent sugars, glucose and mannose; and organic acids, formic and acetic acid.

Free amino acid formation. Similar to DFG degradation, glycine formation increased with pH and temperature (Figure 4.1.2). Also a decrease in the initial reaction pH of 1.3 units had a similar effect in glycine formation as increasing the temperature with 20°C. Moreover, when comparing the yield of glycine (amount of glycine formed / amount of DFG that reacted), we observed that at the early stage of the reaction the yield of glycine increased with time (Table 4.1.1). However, as the reaction proceeded a decrease was observed. Moreover, in all the studied systems the decrease in the yield of glycine was followed by an increase again. Also, the yield of glycine increased with increasing pH, independently of the temperature.

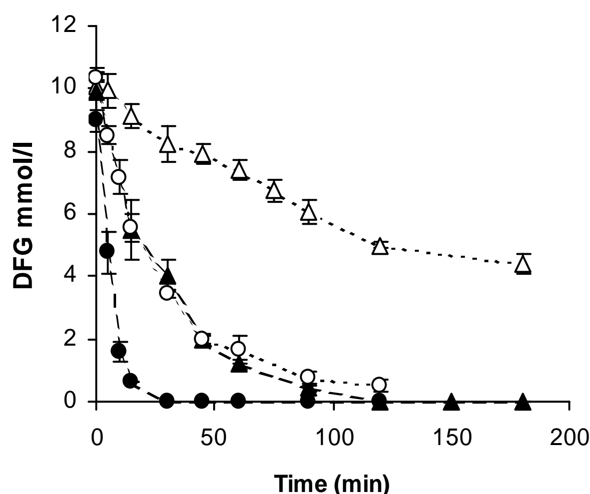


Figure 4.1.1. DFG thermal degradation at 100°C (Δ, pH 5.5; ▲, pH 6.8) and at 120°C (○, pH 5.5; ●, pH 6.8).

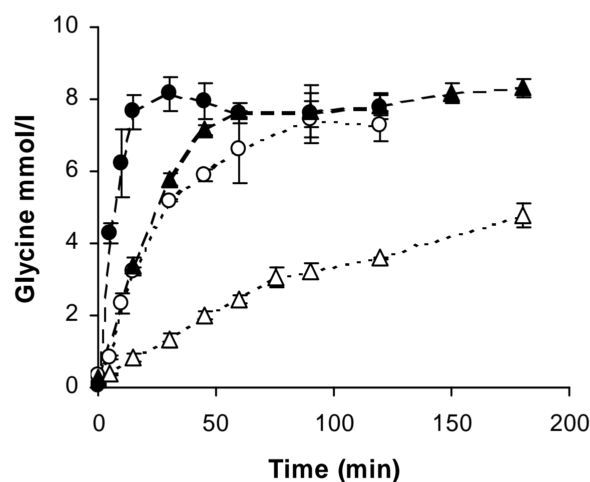


Figure 4.1.2. Glycine formation during thermal degradation of DFG at 100°C (Δ, pH 5.5; ▲, pH 6.8) and 120°C (○, pH 5.5; ●, pH 6.8).

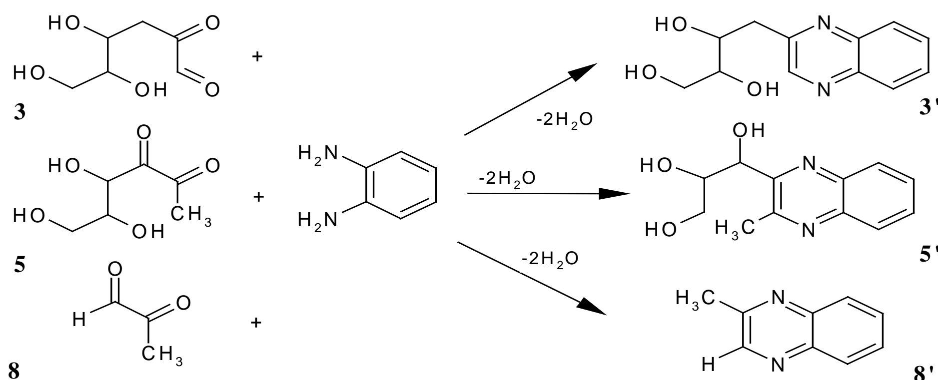
Table 4.1.1. Glycine yield from *N*-(1-deoxy-D-fructos-1-yl)-glycine heated in phosphate buffer (0.1 M) at different reaction conditions.

Heating Time (min)	Glycine Yield (%)[b]			
	A (T100°C pH 5.5)	B (T100°C pH 6.8)	C (T120°C pH 5.5)	D (T120°C pH 6.8)
5	43.40	n.a.	25.87	n.a.
10	n.a.	n.a.	62.05	83.92
15	57.89	71.02	59.56	94.72
30	53.61	93.85	69.79	95.82
45	78.23	87.44	66.20	92.36
60	79.69	84.91	71.95	84.41
75	81.46	n.a.	n.a.	n.a.
90	72.39	78.42	74.20	84.05
120	65.03	76.59	70.18	86.39
150	n.a.	80.14	n.a.	n.a.
180	78.31	81.45	n.a.	n.a.

[b] The yield of glycine was calculated based on the amounts of DFG reacted.
n.a.: not analyzed.

In a recently published study (*6*) the thermal degradation of **1** was compared at different pH, either in water or in phosphate buffer solution. It was observed that the yield of glycine in water increased with increasing pH, whereas in phosphate buffer solution the opposite was observed. Also NaOH was added to keep the pH constant throughout the reaction period. The authors suggested that phosphate slowed down the degradation of glycine at lower pH, mainly pH 5 and 6. However it has been reported (*11*) that phosphate ions act as a catalyst leading to enhanced degradation of Amadori compounds with an optimum in the pH range between 5 and 7, which contradicts their suggestion. A possible reason for the observed decrease of the glycine yield with increasing pH in phosphate buffer solution can be the increasing addition of NaOH with increasing pH. It may enhance the formation of alkyl(1*H*)pyrazinones, which can retain more than one glycine molecule (*7*). We will come back to glycine degradation pathways in the following step.

α-dicarbonyls formation. *a)* The deoxyosones, **3** (3-DG) and **5** (1-DG) were identified and quantified in their quinoxaline form (Scheme 4.1.2). As shown in Figure 4.1.3, at both studied pH values and temperatures, both deoxyosones were formed. Compared to the amount of DFG reacted, a quite high concentration of 1- and 3-DG was already present after 5 minutes at pH 5.5 and temperature 100°C (34%). Independently of the temperature, at lower pH 3-DG was present in larger amounts relatively to 1-DG. At pH 6.8 that difference decreased. An increase in pH seems to favour the 1-DG formation.



Scheme 4.1.2. α-Dicarbonyl derivatization with 1,2-diaminobenzene: 3-deoxy-2-hexosulose (**3**); 1-deoxy-2,3-hexodiulose (**5**); methylglyoxal (**8**) and the respective quinoxaline derivatives: 2-(2,3,4-trihydroxybutyl) quinoxaline (**3'**); 2-methyl-3-(1,2,3-trihydroxypropyl)quinoxaline (**5'**); 2-methylquinoxaline (**8'**).

These findings are in line with what has been reported (*12*). Under more alkaline conditions, relative to the p*K*a of the ARP (DFG p*K*a is 8.2), Amadori compound is believed to undergo 2,3-enolization, as well, with 1-DG formation. Also, independently of the pH and temperature, as the reaction proceeded the amount of 1-DG decreased much faster than the one of 3-DG. *b)* Another identified and quantified α-dicarbonyl was **8** (MG) as its quinoxaline derivative. Contrary to the temperature increase, the increase of pH had almost no influence on the MG formation (Figure 4.1.4). A very low increase in the peak area of methylquinoxaline, when the pH increased from 5 to 7, was also observed in a glucose/β-alanine reaction mixture heated in phosphate buffer for 12h (*13*). However, the influence of temperature is quite significant. An increase from 100°C to 120°C more than doubled the formation of MG. No decrease in its amount was observed with time.

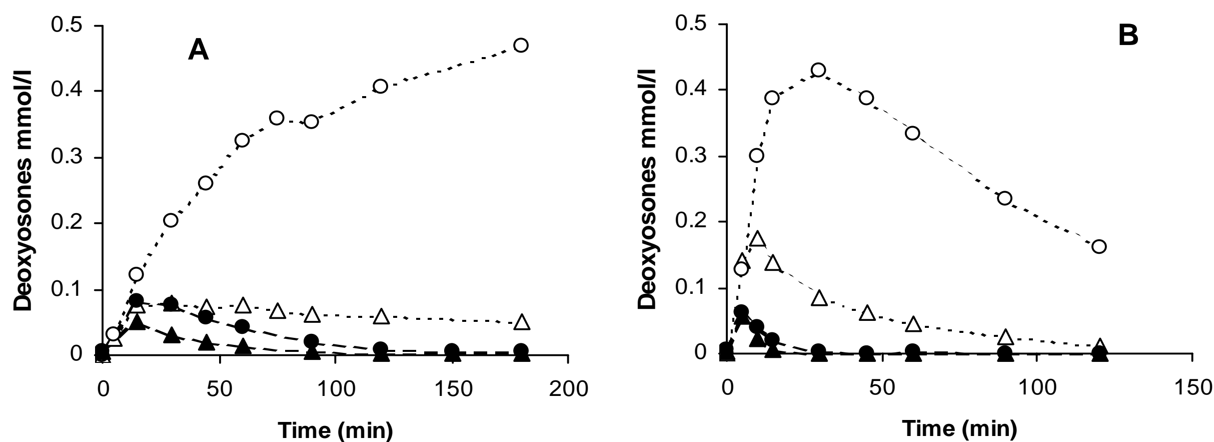


Figure 4.1.3. Deoxyosones formation during thermal degradation of DFG at 100°C (**A**) and 120°C (**B**): 1-DG (Δ, pH 5.5; ▲, pH 6.8) and 3-DG (○, pH 5.5; ●, pH 6.8).

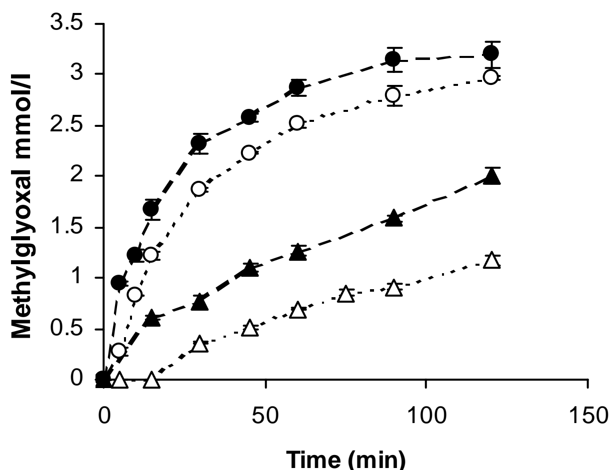


Figure 4.1.4. Methylglyoxal formation during thermal degradation of DFG at 100°C (Δ, pH 5.5; ▲, pH 6.8) and 120°C (○, pH 5.5; ●, pH 6.8).

Parent sugars formation. **10** and **11** have recently been reported as the DFG parent sugars (*6*). No other sugar was mentioned in that study, even though fructose (**13**) can also be formed by isomerisation of glucose and mannose (*14*). Based on those results (*6*), initial sugar analyses were carried out using an Ion exchange HPLC column, knowing that with this method mannose and fructose have the same retention time, but providing a good separation of these sugars with glucose. It was assumed that the fructose/mannose peak was mainly mannose. However, to check the assumption that no fructose was formed, samples from all the systems studied taken at 120 min were also analysed using the Dionex method, with which it was possible to separate fructose from mannose, besides glucose. Contrary to our expectations, fructose was also observed in addition to glucose and mannose, in samples heated at pH initial value 6.8. To gain insight whether fructose would be an isomerisation product from glucose and mannose, systems at pH 6.8 heated at 100°C and 120°C were analysed with the Dionex method. As shown in Figure 4.1.5 the formation of fructose showed no lag phase, indicating that it can be formed directly from DFG rather than from isomerisation of glucose and mannose. Moreover, independently of the temperature, at pH 5.5, mannose was formed in higher amounts than glucose (Figure 4.1.5-A and -C). Also, as the reaction proceeded at 120°C (Figure 4.1.5-C) mannose reached a maximum after 30 minutes, decreasing afterwards, whereas glucose did not decrease after 120 minutes of heating. At pH 6.8 the opposite was observed, that is, glucose not only was formed in higher amounts than mannose but also seemed to be more reactive, showed by the fast decrease at 120°C (Figure 4.1.5-B and -D). Fructose, even though formed in considerable amounts was always lower in concentration than the other two sugars. Also independently of the reaction conditions no decrease in the fructose amount was observed.

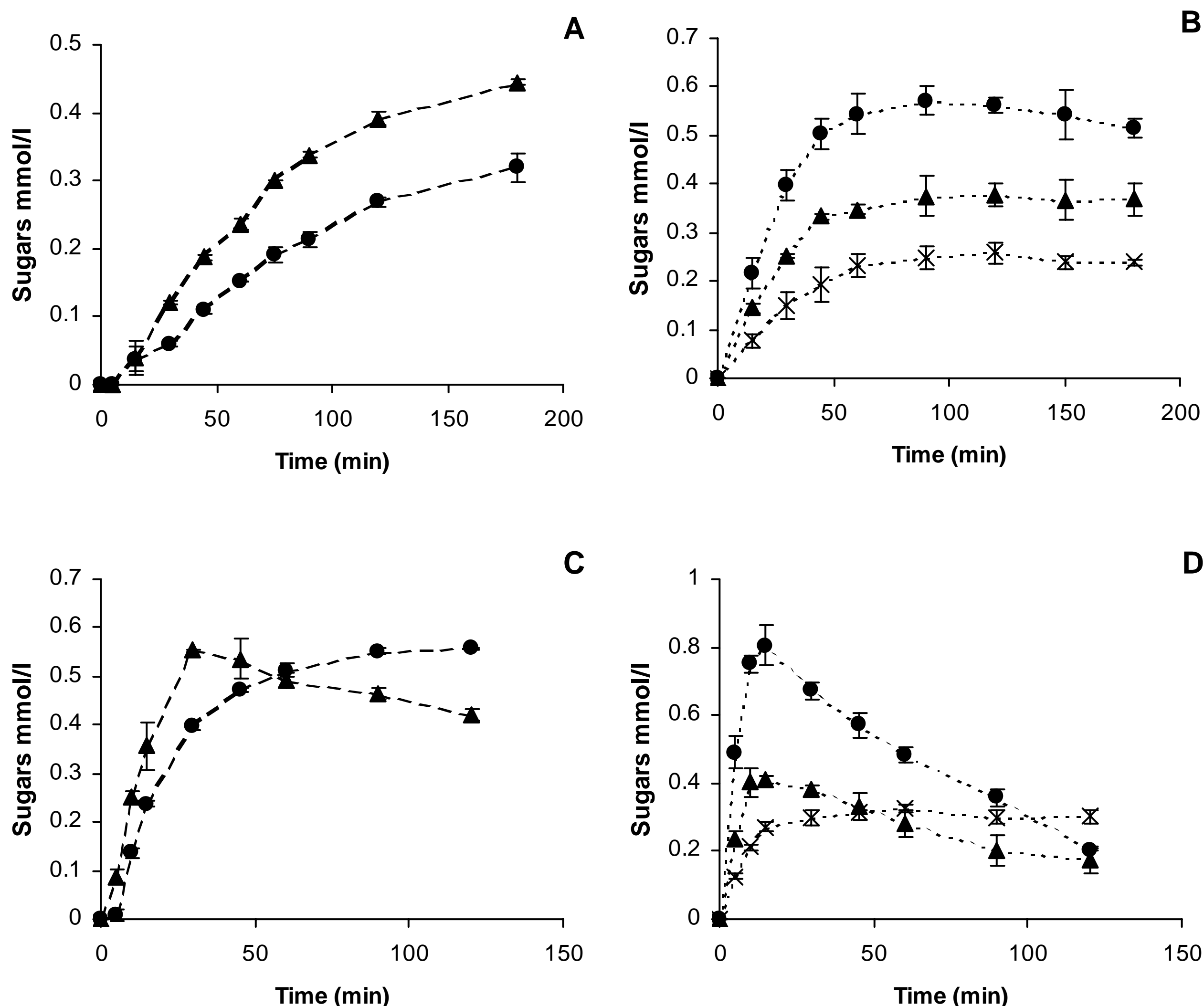


Figure 4.1.5 – Sugars formation during thermal degradation of DFG at pH 5.5 (---) and pH 6.8 (····). **A** and **B** heated at 100°C; **C** and **D** heated at 120°C. D-Glc (●); D-Man (▲); D-Fru (×).

Organic acids formation. The formation of formic acid and acetic acid was particularly favoured with increasing pH (Figure 4.1.6). At pH 5.5 the yield of formic and acetic acid was considerably lower relative to pH 6.8. Also an increase of temperature enhanced formation of both acids. The sum of the yields of both acids reached up to 0.8 mmol/mmol of DFG, which indicates that organic acids, in particular acetic acid, are important end products of the Maillard reaction. Independently of the reaction conditions, acetic acid was always formed in higher amounts than formic acid. The difference was more significant at pH 6.8.

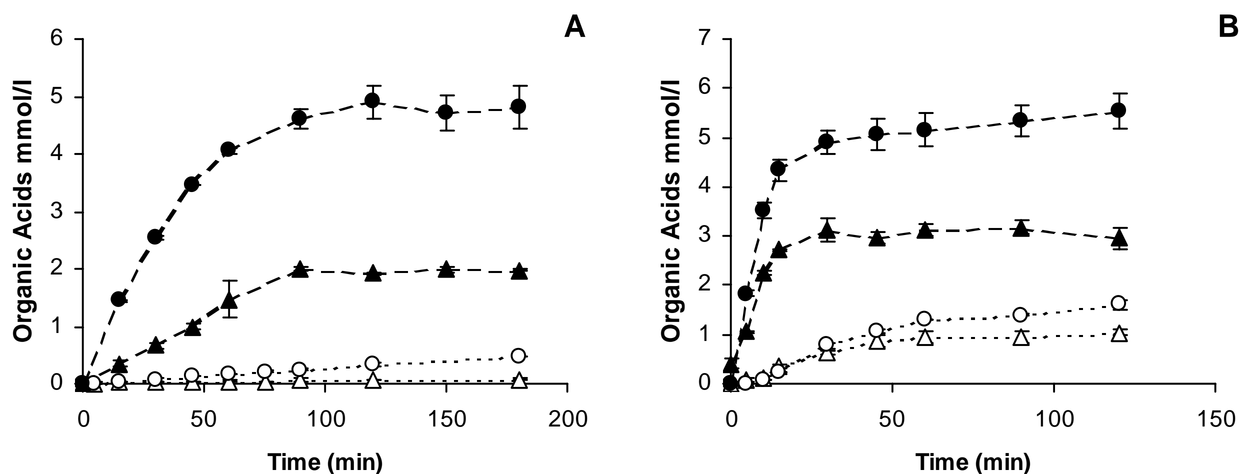


Figure 4.1.6. Organic acids formation during thermal degradation of DFG at 100°C (**A**) and 120°C (**B**): Acetic acid (○, pH 5.5; ●, pH6.8); Formic acid (Δ, pH 5.5; ▲, pH 6.8).

In the present study the reaction pH was not kept constant. As the reaction proceeded a pH drop was observed at both studied initial pHs. In Figure 4.1.7 the results are shown for initial pH 6.8. After 120 min at 120°C the pH drop was more significant than at 100°C after 180 min, 0.21 and 0.19 respectively. Due to the low buffer capacity at initial pH of 5.5 the pH drop for the same reaction conditions was 0.8 and 0.9, respectively, even though the sum of acids formed was lower. Davidek et al. (*6*) observed in their study that the total amounts of acetic and formic acid were generally lower than the amounts of NaOH added to keep the pH constant, suggesting that other acids might be formed. It would be interesting though to study the reaction mass balance to see how much is missing. One of the consequences of allowing the pH to fall is the inhibition of the reaction by the pH drop. However, the level-off observed on organic acids formation (Figure 4.1.6-B) matched the complete degradation of DFG, which implies direct involvement of DFG or its early degradation product in the formation of organic acids.

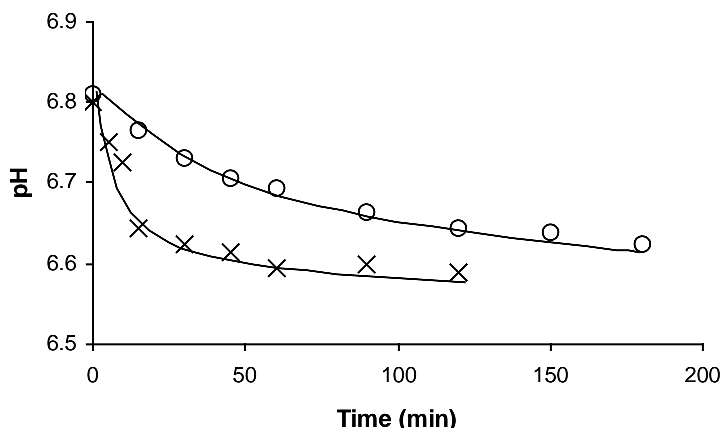


Figure 4.1.7. pH drop during thermal degradation of DFG at initial reaction pH 6.8. (○, 100°C; ×, 120°C).

Melanoidins formation. The concentration of melanoidins (nitrogenous brown polymers) was calculated from the absorbance data through the extinction coefficient. As observed in Figure 4.1.8, pH more than temperature had a strong influence in melanoidins formation from DFG. Moreover, the observed colour formation is much less than when one starts with glucose/glycine system (*15*), even though the amount of DFG formed then is approximately the same as we used as reactant in the present study.

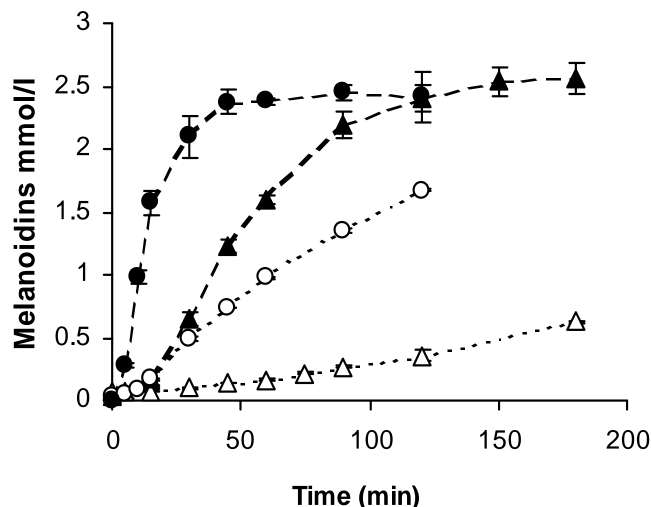


Figure 4.1.8. Melanoidins formation during thermal degradation of DFG at 100°C (Δ, pH 5.5; ▲, pH 6.8) and 120°C (○, pH 5.5; ●, pH 6.8).

(ii) Identification of the reaction pathways based on reaction conditions

Degradation of DFG. Two possible degradation pathways of DFG are the enolization pathway and the retro-aldolization pathway. As mentioned before, through enolization the aminoenol intermediates can lead to either **3** or **5**. Although only small amounts may be present at a given time, large amounts of ARP may degrade via one or both of these pathways. The reaction conditions, especially pH, are believed to determine the degradation pathway taken (*16*). At lower pH, 1,2-enolization is believed to be favoured and therefore a higher yield of 3-DG is expected, which is in agreement with the results obtained in the present study. On the other hand as the pH increases to neutral/alkaline values, 2,3-enolization is believed to be favoured and therefore 1-DG should be present in higher amounts. In literature the results are however contradictory. By heating 1-deoxy-1-propylamino-D-fructose in phosphate buffer for 10h under reflux the 1-DG / 3-DG ratio was 8:5 at pH 4.5 and 20:1 at pH 7 (*17*). For quantification of deoxyosones several researchers reacted Maillard mixtures in the presence of excess amounts of 1,2-diaminobenzene (*13,17)* This *in situ* trapping technique leads to an accumulation of the quinoxalines, but does not offer insights into the relative changes in the concentration of the deoxyosones or any other dicarbonyl compound (*18*) We, therefore, derivatized the

deoxyosones produced with 1,2-diaminobenzene after rapid cooling of the thermally treated DFG. From the results presented in the previous section, independently of the pH, 3-DG was always present in higher concentration than 1-DG. Hofmann et al (*19*) in a heated aqueous solution of glucose and L-alanine under reflux at pH 7 found 3-DG in 4 times higher amounts than 1-DG. In the present study at 100°C and pH 6.8 a ratio of 3.2 was found.

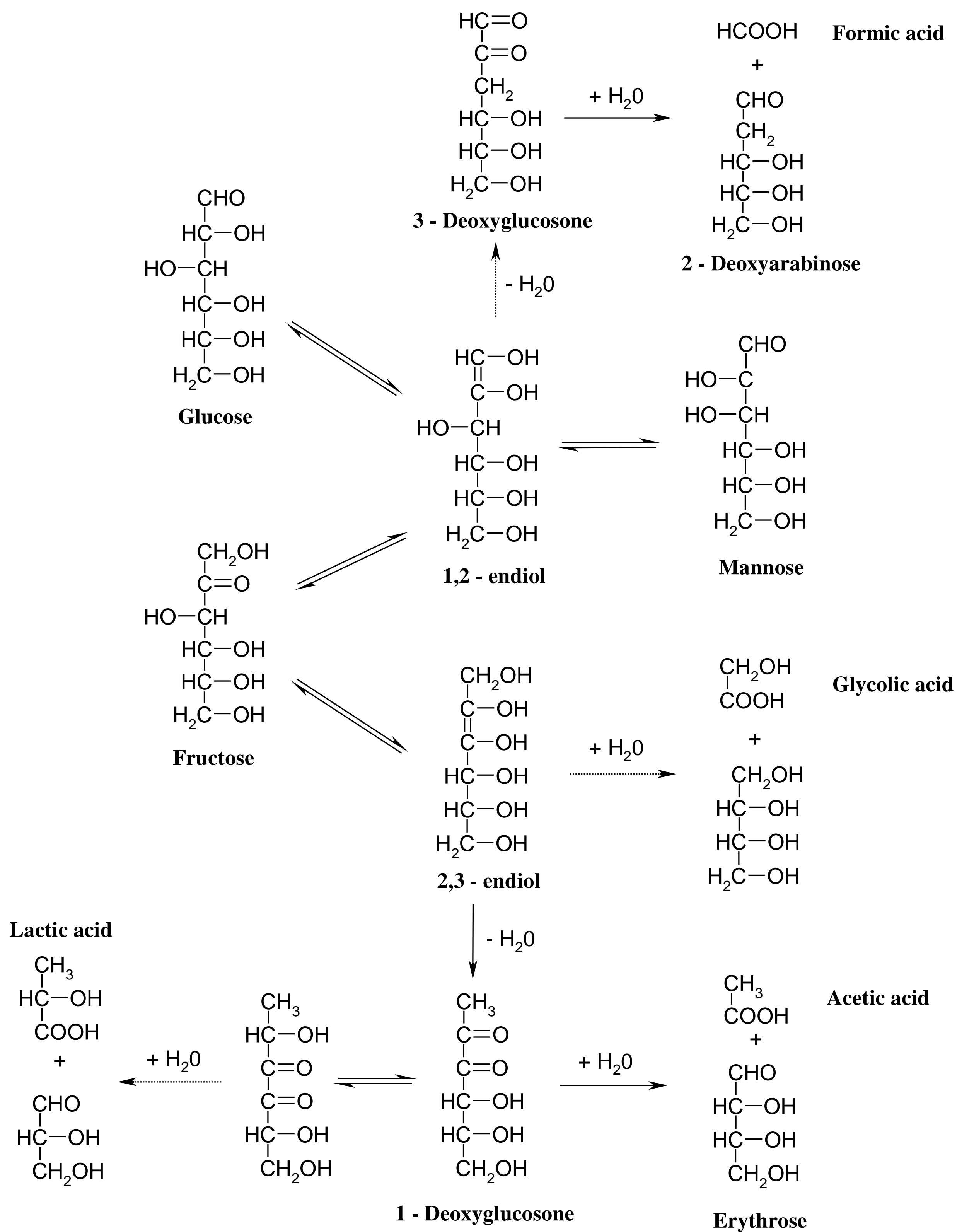
To gain insight whether the amount of 1-DG is lower because its formation is not favoured or because it is more reactive than 3-DG, compounds formed by cyclization/condensation of the deoxyosones were also analysed. They include 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2H)-furanone (HHMF), 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one (DDMP) and 5-(hydroxymethyl)-2-furfural (HMF). Both the furanone (HHMF) and the dihydropyranone (DDMP) derive from 1-DG and are regarded as the 2,3-enolization indicator (*20)*. In the present study both HHMF and DDMP were identified at both pHs, which indicates that 2,3-enolization occurs not only at pH 6.8 but also at pH 5.5. Unfortunately, their quantification was not possible since no reference material was available. However, judging by the response factor of HMF they were formed in the order of magnitude of mmoles. On the other hand, HMF was not formed at pH 6.8 and only μmole amounts at pH 5.5. The relatively low reactivity of 3-deoxyglucosone as well as its relatively poor methylated pyrazine precursor has already been observed in previous studies (*21, 22*) Also HMF formation has only been reported in more acidic conditions, such as pH 3.5 (*23*). Besides enolization, intact Amadori products can also undergo retro-aldol reactions or acid/base-catalyzed thermal degradations without deoxyosone formation, to produce a variety of other reactive intermediates that retain the amino acid, such as 1-amino-1,4-dideoxy-2,3-dicarbonyl compounds (*24*) These compounds can release the free amino acid later by β-elimination, which can justify the oscillation observed in the yield of glycine.

Formation of glycine. Under the studied conditions the formation of glycine from DFG can occur via both 1,2- and 2,3-enolization. In theory, 1 mol of DFG should yield 1 mol of glycine. The results obtained in the present study show that the yield of glycine increased in the beginning of the reaction and decreased as the reaction proceeded. However, the decrease in the yield of glycine was followed by an increase again. This indicates that glycine is first liberated and then reacts with other compounds present in the reaction mixture (e.g α-dicarbonyls and hydroxycarbonyls). These reactions may include: (i) the formed α-dicarbonyls can further react with glycine through Strecker degradation to produce the corresponding amines, carboxylic acids and Strecker aldehydes; (ii) formation of pyrazinones and pyrazines (*25*); (iii) chain elongation of reactive C2 and C3 α-dicarbonyls by one carbon unit originating from C2 atom of glycine (*26*); (iv) incorporation of glycine into melanoidins. However, because after a decrease an increase was observed, glycine is suggested to react with other

compounds present in the reaction mixture and subsequently released again. These reactions may include not only the conversion of carbohydrate fragments by catalytic action of glycine, but also glycine can be formed directly from DFG with the formation of **6** that subsequently undergoes β-elimination to produce MG and release free glycine (*4,7*).

Methylglyoxal formation. In a previous study (*22*) a mechanism for the MG formation was proposed through retro-aldolization of both 1- and 3-deoxyosones. This would justify the absence of pH influence observed in MG formation. Another possibility would be the direct cleavage of DFG into MG as presented in Scheme 4.1.1. This process of conversion of glyceraldehyde into MG by catalytic action of amino acid was also observed with glucose/glycine model systems (*27*). Literature results thus give us some indication that DFG degrades also by retro-aldolization into MG with release of glycine. Moreover, MG is also believed to degrade further into formic and acetic acid (*4*).

Parent sugars formation. The formation of glucose and mannose together with the free amino acid, at early stages of the reaction could be considered as sufficient evidence for the reversibility of the rearrangement, as shown in pathway D in Scheme 4.1.1. However, this pathway does not explain the formation of fructose at the early stage of the reaction. The fact that no lag phase was observed made us exclude the isomerisation possibility from glucose and mannose. According to the Lobry-de-Bruyn-Alberda-van-Eckenstein-rearrangement all three sugars, glucose, mannose and fructose are in equilibrium with the same intermediate, the 1,2-endiol. However, fructose is also in equilibrium with the 2,3-endiol (*14*). The formation of 1,2-endiol from the respective enaminol (**2**) in Scheme 4.1.1 is not so likely to happen, however, by release of the amino acid the enaminol **4** can originate its endiol, 2,3-endiol (**12**), which through enolization can lead to sugars formation (*12*), in particular fructose (**13**). This indicates that fructose can be formed from DFG by its 2,3-enolization step, whereas mannose and glucose can only be formed via the 1,2-enaminol, through the schiff base (**9** and **9'**). Moreover, it could also be argued that the sugars might arise by aldol-type condensations between smaller sugar fragments generated from the decomposition of DFG. This hypothesis has, however been excluded by labelling studies (*28*). To get a better insight in the sugars enolization pathways two additional experiments were performed. Glucose and fructose were heated alone in an aqueous phosphate buffer (0.1 mol/L) solution at 120°C and pH 6.8. It was observed (results not shown) that glucose isomerised preferably into fructose, whereas mannose was only formed in small amounts. On the other hand the amount of glucose and mannose formed from fructose was very low relatively to its decrease. Fructose degraded preferably into acids.



Scheme 4.1.3. Reaction scheme for organic acids formation from primary thermal degradation products of DFG (adapted from Ginz et al[29]).

It is suggested that not only 2,3-enolization is favoured under these conditions with fructose preferably formed from glucose, but also that the reversibility of 2,3-endiol into 1,2-endiol occurs in a quite low extent. These findings are in agreement with what we have observed before, namely that 2,3-enolization gains importance in the reaction mechanism as pH and temperature increased.

(iii) Differentiate between primary and secondary reaction routes

Based on the discussion above we suggest the following primary and secondary routes: *Primary routes:*

- DFG degradation through enolization occurs through two intermediates, designed as E_1 and E_2, which can be the Schiff's base, the cation form of the Schiff's base, the 1,2 enaminol or the 2,3-enaminol. These intermediates haven't been isolated yet from the Maillard reaction due to their reactivity, however, according to previous studies (*31, 32*) ARPs undergo 1,2- or 2,3-enolizations while the amino acid moiety is still attached. E_1 is favoured at lower pH whereas E_2 is favoured at higher pH.
- The intermediates E_1 and E_2 by release of the amino acid lead to the formation of 3-DG and 1-DG, respectively.
- Glucose (Glu), mannose (Man) and 3-DG are formed through the same intermediate (E_1) whereas fructose (Fru) is formed by E_2. Moreover, Man and Glu can isomerise into each other as well as degrade into C_n ($n \leq 6$) carbonyl compounds.
- DFG degradation through retro-aldol cleavage leads to MG formation, with amino acid release.
- DFG, 3-DG and 1-DG due to their reactive functional group can easily degrade to produce reactive C_n carbonyl compounds.
- Formic acid and acetic acid are formed from DFG degradation pathways through E_1 by 3-DG dicarbonyl cleavage and through E_2 by 1-DG dicarbonyl cleavage, respectively.
- Melanoidins formation results from the interaction of C_n carbonyl compounds with glycine.

Secondary routes:

- Products formed by cyclization/condensation of the deoxyosones that include HMF, HHMF and DDMP.
- Incorporation of glycine in pyrazinones and pyrazines, as well as in chain elongation of α-dicarbonyls. Also degradation of glycine through Strecker degradation to produce the

corresponding amines, carboxylic acids and Strecker aldedehydes, which can be included in the melanoidins formation.

- MG formation from deoxyosones retro-aldol reaction and its degradation into carboxylic acids.
- Isomerisation of glucose and mannose into fructose, as well as direct degradation of glucose into 3-DG.
- Fructose formation from E_1 as well as its degradation into reactive C_n carbonyl compounds.
- Carboxylic acids formation from sugars enolization or scission products from C_n carbonyl compounds.

4.1.3. Conclusions

The main degradation pathways for *N*-(1-deoxy-D-fructos-1-yl)-glycine have been established. pH, more than temperature, has an influence on the reaction products formed. An increase in pH seems to favour the formation of 1-deoxyosone. The lower amount found comparatively to 3-deoxyosone, in all studied systems, seems to be related with the higher reactivity of 1-deoxyosone. Independently of the pathway taken, enolization or retro-aldolization, *N*-(1-deoxy-D-fructos-1-yl)-glycine degradation is accompanied by amino acid release. Together with glycine, acetic acid was the main end product formed. At this stage we are able to propose a kinetic model based on the established reaction network. In part II the following steps for a complete multiresponse kinetic analysis will be taken.

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4.2. Kinetic Modelling of Amadori *N*-(1-deoxy-D-fructos-1-yl)-glycine degradation pathways. Part II - Kinetic Analysis.

A kinetic model for *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) thermal decomposition was proposed. Two temperatures (100°C and 120°C) and two pHs (5.5 and 6.8) were studied. The measured responses were DFG, 3-deoxyosone, 1-deoxyosone, methylglyoxal, acetic acid, formic acid, glucose, fructose, mannose and melanoidins. For each system the model parameters, the rate constants, were estimated by non-linear regression, via multiresponse modelling. The determinant criterion was used as the statistical fit criterion. Model discrimination was preformed by both chemical insight and statistical tests (Posterior Probability and Akaike criterion). Kinetic analysis showed that at lower pH DFG 1,2-enolization is favoured whereas with increasing pH 2,3-enolization becomes a more relevant degradation pathway. The lower amount observed of 1-DG is related with its high reactivity. It was shown that acetic acid, a main degradation product from DFG, was mainly formed through 1-DG degradation. Also from the estimated parameters 3-DG was found to be the main precursor in carbohydrate fragments formation, responsible for colour formation. Some indication was given that as the reaction proceeded other compounds besides DFG become reactants themselves with the formation among others of methylglyoxal. The multiresponse kinetic analysis was shown to be both helpful in deriving relevant kinetic parameters as well as in obtaining insight into the reaction mechanism.

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4.2.1. Introduction

The control of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) degradation pathways is a subject in chemical kinetics and can be treated in the same way as any chemical reaction, that is by measuring the concentrations of the reactants, intermediates and products with time. General kinetic data describing Maillard reaction pathways are lacking. Also, most studies apply simple kinetics, which does not provide any understanding of the reaction mechanism (*1,2*). The use of kinetic simulation techniques for predicting and controlling properties of interest for the chemical and food industry is becoming increasingly important. The kinetic approach is useful in the sense that it describes not only the way in which the rate of the slowest step of a reaction changes with reaction variables, e.g. temperature and pH, but also that those changes can be predicted in a quantitative way.

Concerning Amadori compound decomposition kinetics only few studies have been published (*3-6*), from which only the first had ARP as starting reactant and still simple kinetics was applied. The multiresponse kinetics analysis considers reaction pathways in more detail. It provides extra information about the reaction mechanism since the reactants degradation is analyzed simultaneously with the intermediates formation. The advantage is that the information in various responses can be used simultaneously so that more precise parameter estimates and more realistic models can be determined. The following steps should be taken into account (*7*): (i) identification and quantification of the reactants and main products formed; (ii) identification of reaction pathways based on reaction conditions; (iii) differentiate between primary and secondary reaction routes; (iv) propose a kinetic model based on the established reaction network; (v) test the hypothesized mechanism; (vi) estimate the rate constants. In part I (Section 4.1) the chemistry behind the degradation pathways of DFG was extensively discussed, dealing with the initial 3 steps. The present section (part II) is a follow up for the complete multiresponse kinetic analysis of the thermal degradation pathways of *N*-(1-deoxy-D-fructos-1-yl)-glycine. It deals with the last 3 steps: the kinetic analysis of the reaction network model proposed. One should keep in mind that kinetic modelling is an iterative process: propose a model, confront it with experimental data, criticize the model, adjust the model and confront the adapted model with experiments again, until an acceptable model results. What acceptable is, is of course debatable and we will discuss it by comparing two different possible models.

This chapter can be seen as a stepping stone for a complete kinetic analysis of the whole Maillard reaction, as will be described in the remaining chapters.

4.2.2. Results and Discussion

Most literature reports regarding the kinetics of Maillard reaction use simple kinetics to describe reactants degradation or products formation. Therefore a comparison with multiresponse kinetic analysis was made in the present study.

Simple Kinetics: Disregarding the actual mechanism or the number of steps involved, Yaylayan and Forage (*3*) assumed a pseudo-first-order reaction in determining the degradation kinetics of Amadori product of tryptophan and D-glucose. Taking this result into account, when applying simple kinetics to the thermal degradation of *N*-(1-deoxy-D-fructos-1-yl)-glycine we observed that DFG disappearance followed indeed a first-order reaction model; that is $C = C_0 \exp(-kt)$, where C is the concentration of the reactant ($mmol.l^{-1}$), C_0 is the initial reactant concentration, k the reaction rate constant and t time (minutes). However, no real distinction could be made between other kinetic orders, in particular for the reaction conditions at 100°C and pH 5.5 (Figure 4.2.1). First- and second-order plots are almost the same. In fact, when optimising the fit of the curve to the data by minimizing the sum of squares, the reaction order was estimated to be 1.6 with a 95% confidence interval of 0.83 to 2.4. These results show that the use of simple kinetics is very limited. It is important to realize that the simple kinetics approach is actually only a mathematical fit procedure disregarding the actual mechanism or the number of steps involved.

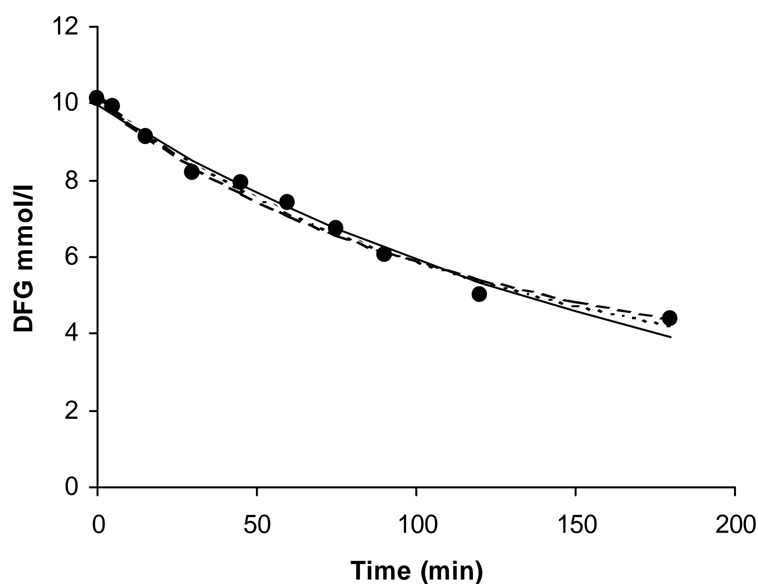
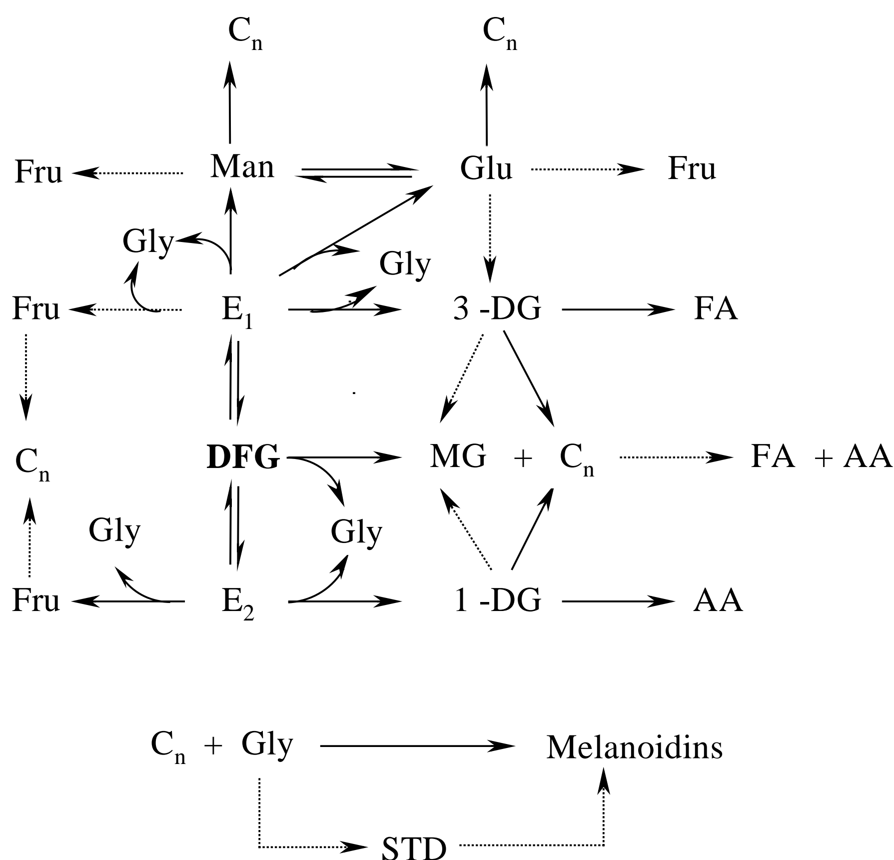


Figure 4.2.1. Simple kinetics analysis of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) thermal degradation at 100°C, pH 5.5. Comparison of a first-order (——), estimated order of 1.6 (- - -) and second-order (— —) plot.

Complex kinetics – Multiresponse kinetic analisys: A step further in the kinetic analysis is to consider reaction paths in more detail. The chemistry behind the degradation pathways of DFG has been extensively discussed in Section 4.1, and from this, a reaction network model was established which is summarized in Scheme 4.2.1. The main pathways (primary routes) for the DFG degradation are presented together with alternative pathways that at this stage are considered as secondary. However, confronted with the results some of the secondary routes might turn into primary routes and vice-versa. As stated above, one should be aware that kinetic modelling is an iterative process.



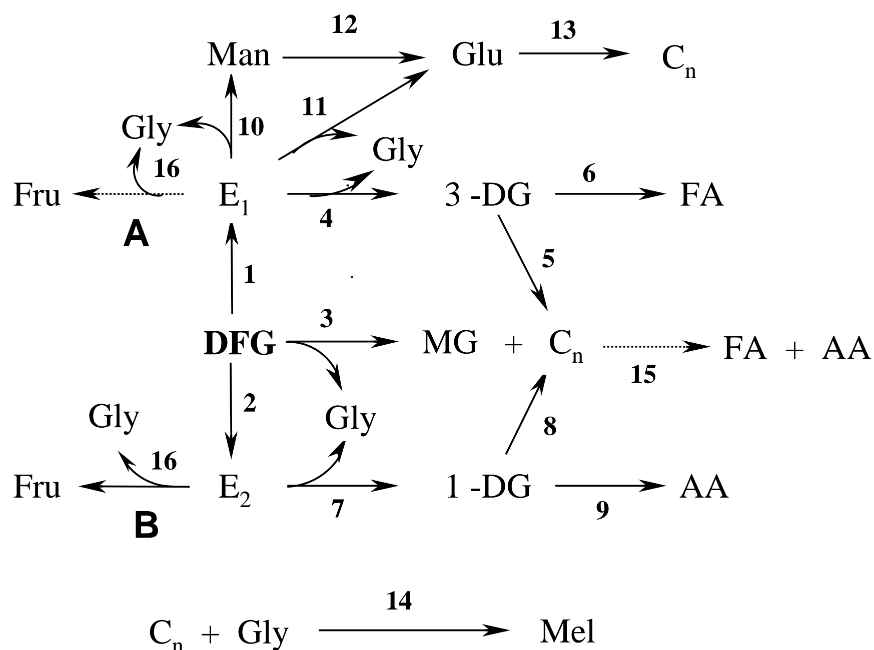
Scheme 4.2.1. Established reaction network for *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) thermal degradation. Primary routes (——); Secondary routes (- - -). E_1 and E_2 are unidentified key compounds involved in rate-determining steps that can be the Schiff's base, the cation form of the Schiff's base, the 1,2 enaminol or the 2,3-enaminol, respectively. Glycine (Gly); methylglyoxal (MG); Glc; Man; fructose (Fru); acetic acid (AA); formic acid (FA); 3-deoxyosone (3-DG); 1-deoxyosone (1-DG); unidentified carbohydrate fragments (C_n); Strecker degradation products (STD).

(iv) Propose a kinetic model based on the established reaction network

For modelling purposes the reaction network model presented was simplified. The degradation of DFG through enolization requires two intermediates E_1 (1,2-enolization) and E_2 (2,3-enolization), which was assumed to have the amino acid still incorporated (*8, 9*). Due to their reactivity these compounds haven't been isolated yet from the Maillard reaction. However, in terms of modelling it is an important step to take into account. Moreover, besides the enolization step, DFG also degrades through retro-aldolization reaction with the formation of methylglyoxal (MG) together with unidentified carbonyl compounds (C_n) and release of the amino acid (*10, 11*). It was assumed to be a fast step with no intermediate in between.

The intermediates E_1 and E_2 by release of glycine lead to the formation of 3-deoxyosone (3-DG) and 1-deoxyosone (1-DG), respectively. 3-DG and 1-DG, due to their reactive functional group, can easily degrade to produce reactive C_n ($n \leq 6$) carbonyl compounds as well as organic acids (*12*). As we mentioned in Section 4.1 (part I) the acids formation occurs preferably by direct cleavage of 3-DG and 1-DG with formic and acetic acid formation, respectively. Concerning the sugars it was assumed that glucose (Glu) and mannose (Man) were formed through the same intermediate (E_1) whereas fructose (Fru) was preferably formed by E_2, even though theoretically it can also be formed through E_1. The main reason for this is that fructose was not detected at the lower pH studied and, while E_1 is favoured at lower pH, E_2 is favoured at higher pH (*13, 14*). However, for modelling purposes both hypotheses were compared. Moreover, mannose and glucose can isomerise into each other as well as degrade into C_n ($n \leq 6$) carbonyl compounds. In the sugar isomerization step it was concluded that neither glucose nor mannose isomerised into fructose, since no lag phase was observed in its formation.

From a previous study (*15*) where the kinetics of monosaccharides isomerization was studied in alkaline conditions it was reported that mannose isomerised into glucose at a rate of 18×10^{-3} min^{-1} while the reverse was approximately half (7×10^{-3} min^{-1}), as well as the degradation of mannose into C_n ($n \leq 6$) compounds. Also MacLaurin and Green (*16*) came to the same conclusion. These literature results suggest that mannose degrades preferably into glucose rather than into carbonyl compounds. Concerning glucose the rate of its degradation into C_n ($n \leq 6$) compounds was found to be higher than its rate of isomerization into mannose (*15, 16*). As a result the degradation step of glucose into mannose was neglected. The degradation of glycine was assumed to occur only by reaction with carbonyl compounds leading to melanoidins (Mel) formation. The Strecker degradation (STD) to produce the corresponding amines, carboxylic acids and Strecker aldedehydes was considered to be a minor step. These assumptions lead us to the model presented in Scheme 4.2.2.



Scheme 4.2.2. First proposed kinetic model, **Model 1** (M_1), based on the established reaction network. E_1 and E_2 are unidentified key compounds involved in rate-determining steps that can be the Schiff's base, the cation form of the Schiff's base, the 1,2 enaminol or the 2,3-enaminol, respectively. Glycine (Gly); methylglyoxal (MG); Glc; Man; fructose (Fru); acetic acid (AA); formic acid (FA); 3-deoxyosone (3-DG); 1-deoxyosone (1-DG); unidentified carbohydrate fragments (C_n); melanoidins (Mel).

To fit the model to the experimental data, the reaction network presented in Scheme 4.2.2 needs to be translated into a mathematical model. This is done by setting-up differential equations for each reaction step, using the law of mass action:

$$\frac{\mathrm{d}[DFG]}{\mathrm{d}t} = -k_1[DFG] - k_2[DFG] - k_3[DFG] \tag{4.2.1}$$

$$\frac{\mathrm{d}[E1]}{\mathrm{d}t} = k_1[DFG] - k_4[E1] - k_{10}[E1] - k_{11}[E1] \tag{4.2.2}$$

$$\frac{\mathrm{d}[E2]}{\mathrm{d}t} = k_2[DFG] - k_7[E2] - k_{16}[E2] \tag{4.2.3}$$

$$\frac{\mathrm{d}[MG]}{\mathrm{d}t} = k_3[DFG] \tag{4.2.4}$$

$$\frac{\mathrm{d}[3DG]}{\mathrm{d}t} = k_4[E1] - k_5[3DG] - k_6[3DG] \tag{4.2.5}$$

$$\frac{\mathrm{d}[1DG]}{\mathrm{d}t} = k_7[E2] - k_8[1DG] - k_9[1DG] \tag{4.2.6}$$

$$\frac{\mathrm{d}[Man]}{\mathrm{d}t} = k_{10}[E1] - k_{12}[Man] \tag{4.2.7}$$

$$\frac{\mathrm{d}[Glu]}{\mathrm{d}t} = k_{11}[E1] + k_{12}[Man] - k_{13}[Glu] \tag{4.2.8}$$

$$\frac{\mathrm{d}[Fru]}{\mathrm{d}t} = k_{16}[E2] \tag{4.2.9}$$

$$\frac{\mathrm{d}[FA]}{\mathrm{d}t} = k_6[3DG] \tag{4.2.10}$$

$$\frac{\mathrm{d}[AA]}{\mathrm{d}t} = k_9[1DG] \tag{4.2.11}$$

$$\frac{\mathrm{d}[Gly]}{\mathrm{d}t} = k_3[DFG] + k_4[E1] + k_{10}[E1] + k_{11}[E1] + k_7[E2] + k_{16}[E2] - k_{14}[Gly][Cn] \tag{4.2.12}$$

$$\frac{\mathrm{d}[Cn]}{\mathrm{d}t} = k_3[DFG] + k_5[3DG] + k_8[1DG] + k_{13}[Glu] - k_{14}[Gly][Cn] \tag{4.2.13}$$

$$\frac{\mathrm{d}[Mel]}{\mathrm{d}t} = k_{14}[Gly][Cn] \tag{4.2.14}$$

These coupled differential equations are difficult to solve analytically but can be solved by numerical integration. Nowadays different software packages are able to this, like Nsolve or Mathematica (*5*). In the present study the used subroutine was DDAPLUS, which is available in the software package Athena Visual Workbench (*17*). The results of the fit for the experimental data at 120°C are presented in Figure 4.2.2.

For the reaction conditions at pH 5.5 the model fitted the data reasonably well (Figure 4.2.2-A). Note that the observations were done in at least duplicate. Glycine was slightly overestimated, while formic acid and melanoidins were underestimated at the beginning and overestimated at the end of heating period. Similar results were obtained at 100°C, pH 5.5. When the pH was increased to 6.8 (Figure 4.2.2-B) there was clearly a miss fit for the organic acids formation, namely for formic acid, as well as for glucose. Both compounds were underestimated. Also a lack of fit was observed for MG formation. It was overestimated at the beginning and underestimated as the reaction proceeded. The results forced us to reconsider the kinetic model. This shows the power of the iterative modelling approach. To begin with, an extra step was added to the model. Incorporating step 15 would induce both formic acid (C_1) and acetic acid (C_2) formation from MG (C_3). Not only the underestimation of formic acid could be solved as well as it would induce 3-DG, instead of glucose, to form more C_n ($n \leq 6$).

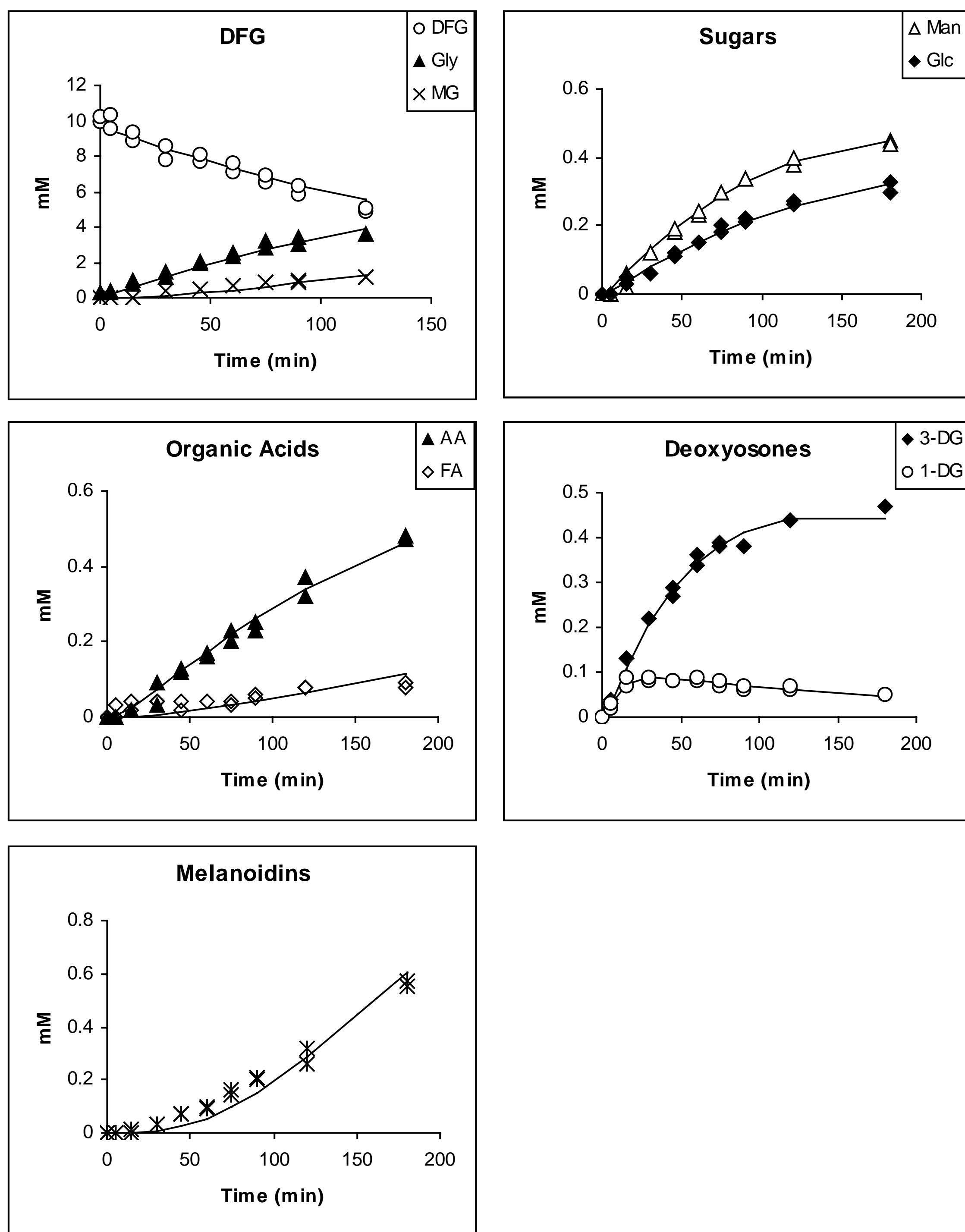


Figure 4.2.2–A. Model 1 fit (lines) to experimental data (dots) of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) thermal degradation at **120°C and pH 5.5**. Glycine (Gly); methylglyoxal (MG); Glc; Man; fructose (Fru); acetic acid (AA); formic acid (FA); 3-deoxyosone (3-DG); 1-deoxyosone (1-DG).

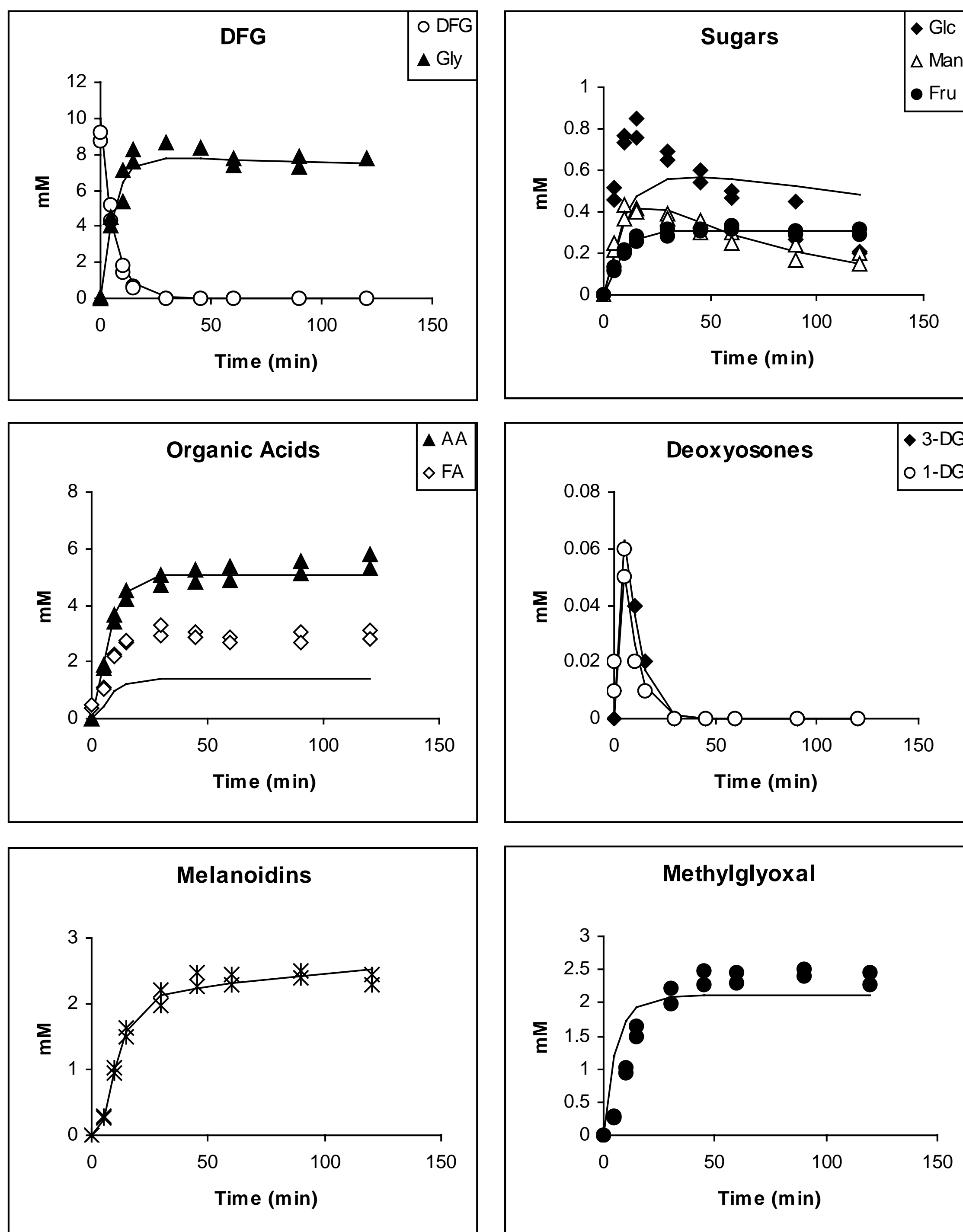
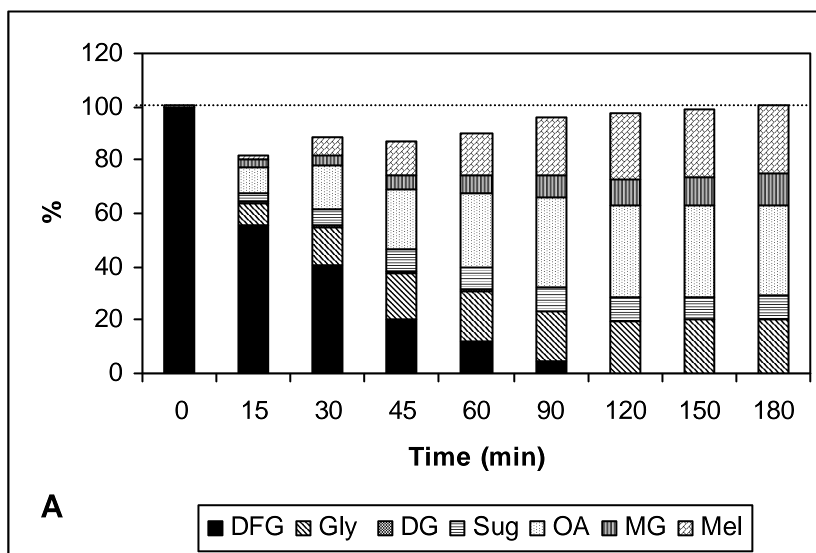


Figure 4.2.2-B. Model 1 fit (lines) to experimental data (dots) of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) thermal degradation at **120°C and pH 6.8**. Glycine (Gly); methylglyoxal (MG); Glc; Man; fructose (Fru); acetic acid (AA); formic acid (FA); 3-deoxyosone (3-DG); 1-deoxyosone (1-DG).

However, it appeared that the estimation of this parameter lead to an indeterminate result for all the studied systems, which means that this step is not important for the model. Also it suggests that the organic acids formation does not result from MG degradation.

From a chemical point of view it is clear that the model is not completely correct. Isbell and co-workers (*18*) have postulated the existence of cis- and trans-isomers of the 1,2-endiol to explain the transformation of various sugars. It was suggested that both enolization and ring opening might be involved in the rate-determining steps of the process. Moreover, the relative rates of enolization for glucose and mannose were found to be 1.0 h^{-1} and 0.5 h^{-1}, respectively. If that is the case then glucose has a higher ability to transform into 1,2-endiol and not so much to degrade into sugar fragments (C_n) involved in colour formation. Apparently, the transformation step of glucose into 3-deoxyosone that initially has been considered as a secondary route might be in fact a primary route. Also, the observation that at the beginning of the reaction MG was overestimated leads us to the assumption that a rate-determining intermediate might be formed previously to MG. The initial assumption that MG formation from DFG was a fast step might not be correct.

When calculating the reaction mass balance (evolution of each intermediate towards the reactant (DFG) initial concentration) we observe that at the initial stage of the reaction the products identified and quantified in the present study do not count for the total DFG degradation. However, as the degradation reaction proceeded, within experimental error, 100% was reached. In Figure 4.2.3 we can observe the results for pH 6.8. Besides melanoidins, and glycine, the other main end products obtained were the organic acids, in particular acetic acid, as mentioned in Section 4.1. A possible explanation for the observed gap is that, as the reaction proceeds besides DFG other compounds formed during its degradation become reactants themselves with the formation of the same end products.



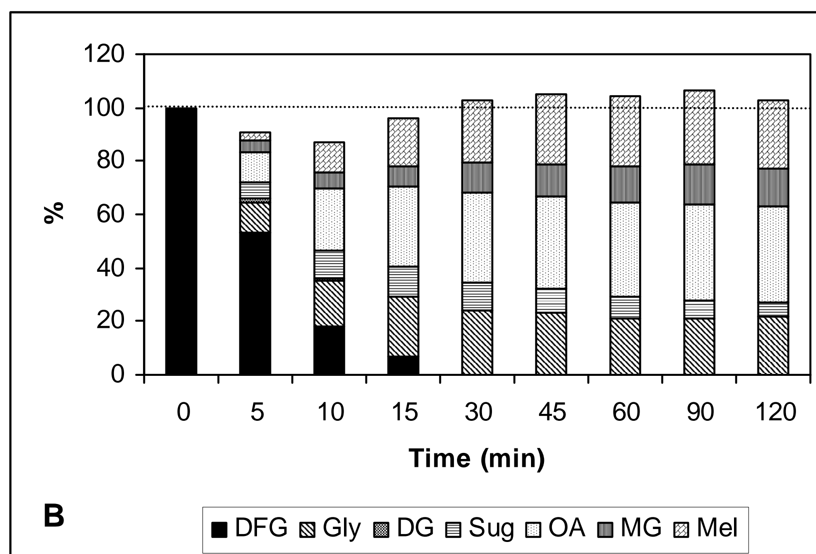
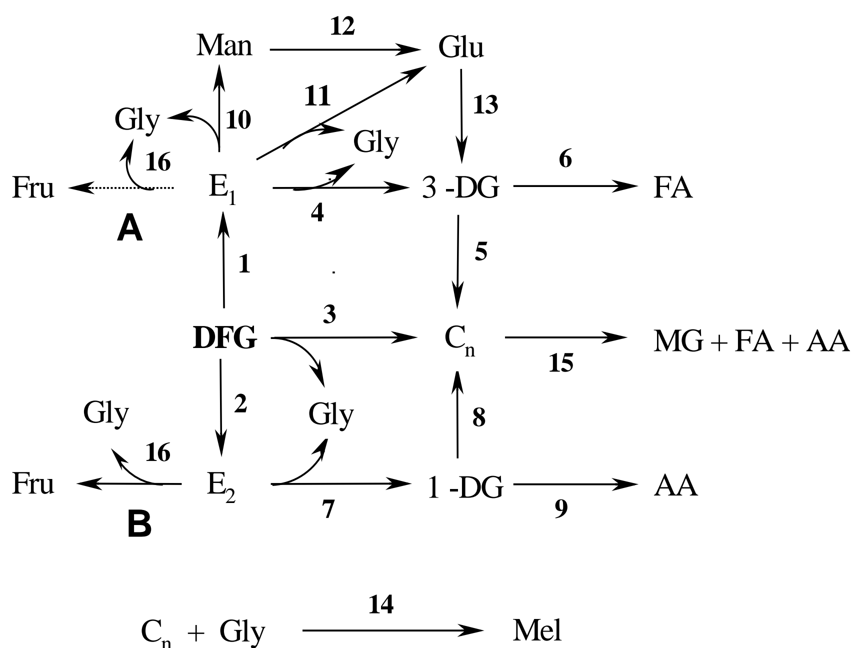


Figure 4.2.3. Mass Balance: evolution of each intermediate towards the reactant initial concentration in heated *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) at 100°C, pH 6.8 (A) and 120°C, pH 6.8 (B). Glycine (Gly); Deoxyosones (DG); Sugars (Sug); organic acids (OA); methylglyoxal (MG); melanoidins (Mel).

As a result a modified simplified model (*M2*) is proposed, as shown in Scheme 4.2.3. To fit *M2* to the experimental data, the same procedure was taken by coupling differential equation to each reaction step. The approach of numerical integration followed by fitting to the data is flexible because changing relevant differential equations and fitting them to experimental data can easily test different models. The results of the fit of *M2* for the experimental data taken at all the studied systems are presented in Figures 4.2.4 – 4.2.7.

Scheme 4.2.3. Second proposed kinetic model, **Model 2** (M_2), based on the established reaction network. E_1 and E_2 are unidentified key compounds involved in rate-determining steps that can be the Schiff's base, the cation form of the Schiff's base, the 1,2 enaminol or the 2,3-enaminol, respectively. Glycine (Gly); methylglyoxal (MG); Glc; Man; fructose (Fru); acetic acid (AA); formic acid (FA); 3-deoxyosone (3-DG); 1-deoxyosone (1-DG); unidentified carbohydrate fragments (C_n); melanoidins (Mel).



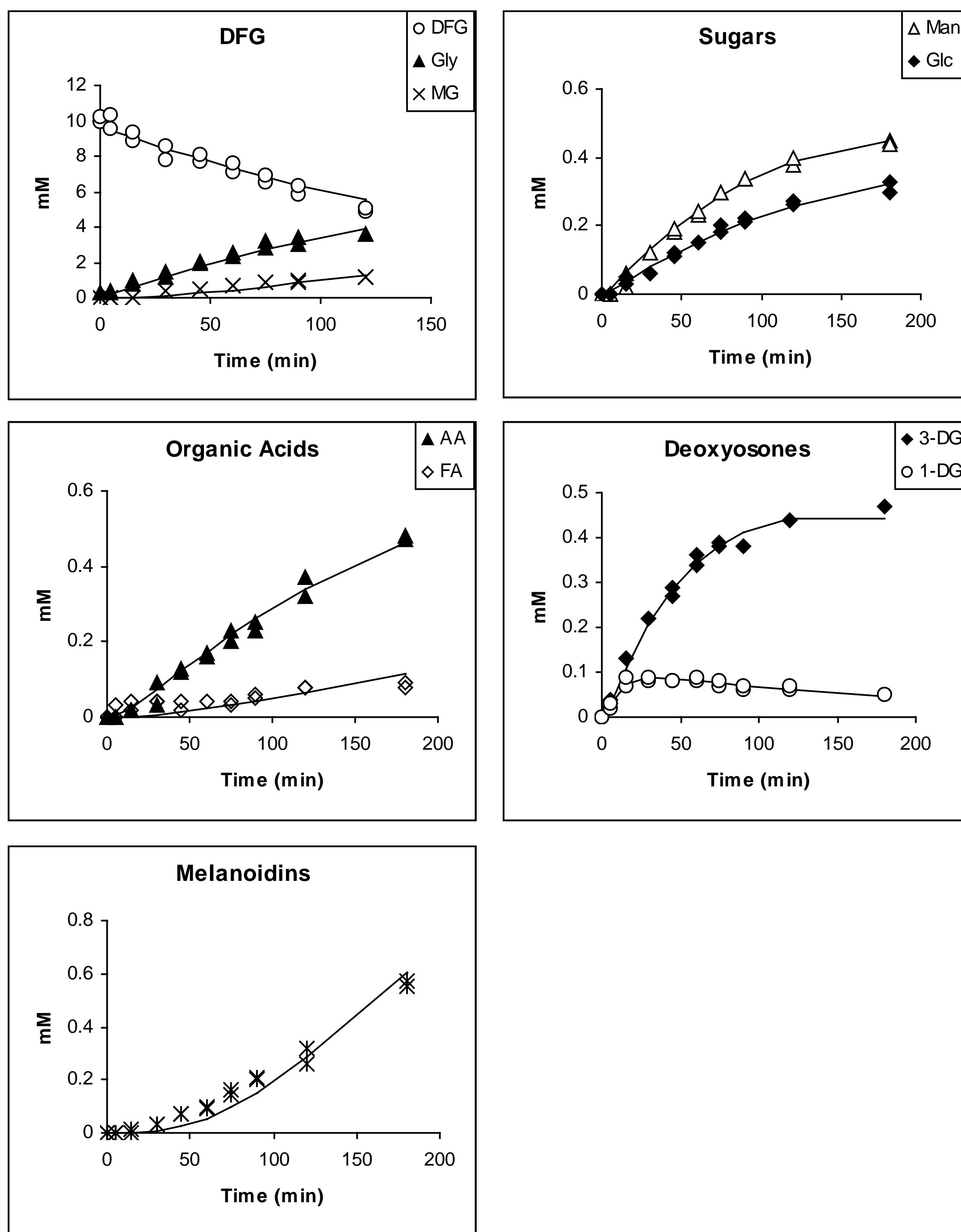


Figure 4.2.4. Model 2 fit (lines) to experimental data (dots) of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) thermal degradation at **100°C and pH 5.5**. Glycine (Gly); methylglyoxal (MG); Glc; Man; acetic acid (AA); formic acid (FA); 3-deoxyosone (3-DG); 1-deoxyosone (1-DG).

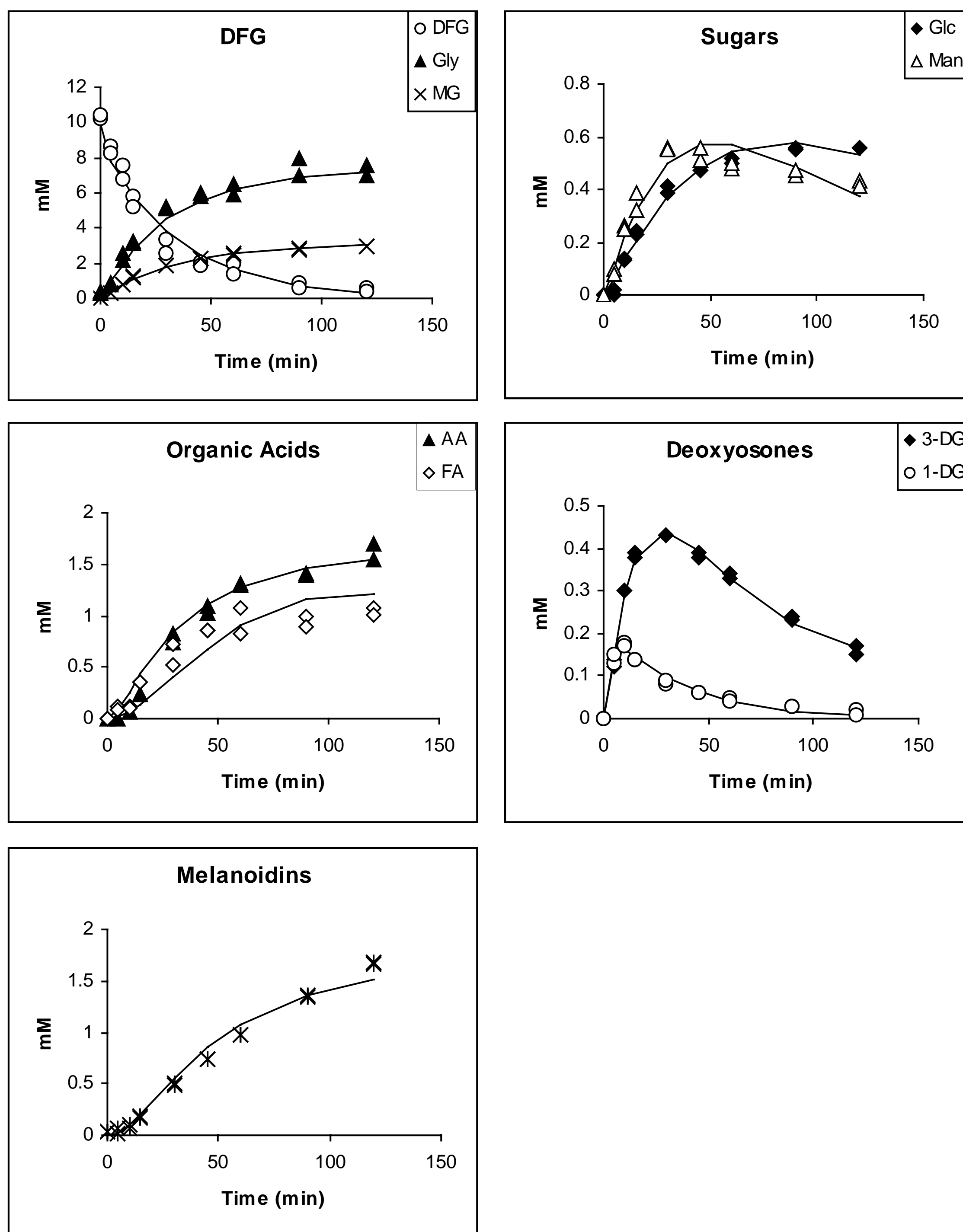


Figure 4.2.5. Model 2 fit (lines) to experimental data (dots) of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) thermal degradation at **120°C and pH 5.5**. Glycine (Gly); methylglyoxal (MG); Glc; Man; acetic acid (AA); formic acid (FA); 3-deoxyosone (3-DG); 1-deoxyosone (1-DG).

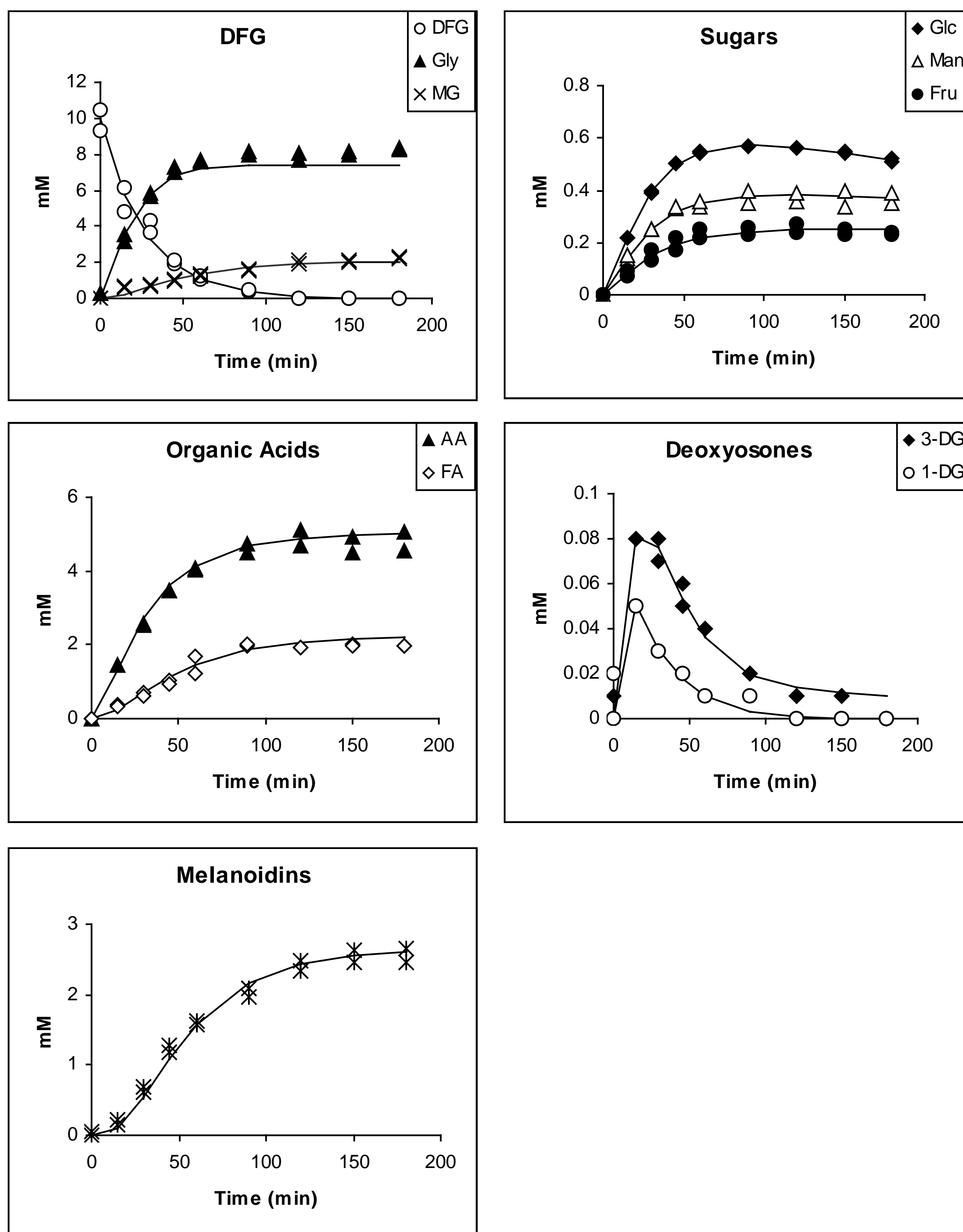


Figure 4.2.6. Model 2 fit (lines) to experimental data (dots) of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) thermal degradation at **100°C and pH 6.8**. Glycine (Gly); methylglyoxal (MG); Glc; Man; acetic acid (AA); formic acid (FA); 3-deoxyosone (3-DG); 1-deoxyosone (1-DG).

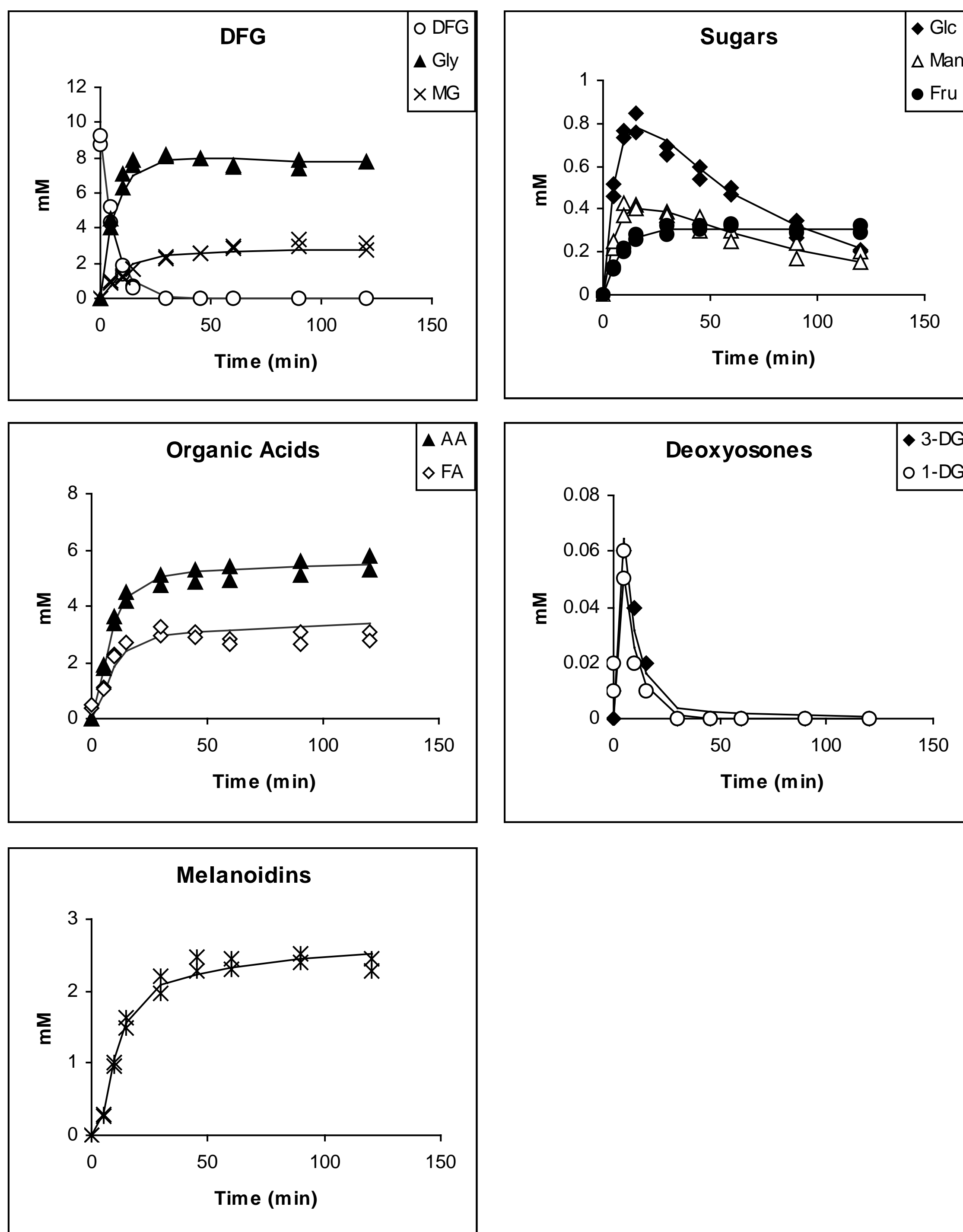


Figure 4.2.7. Model 2 fit (lines) to experimental data (dots) of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) thermal degradation at **120°C and pH 6.8**. Glycine (Gly); methylglyoxal (MG); Glc; Man; acetic acid (AA); formic acid (FA); 3-deoxyosone (3-DG); 1-deoxyosone (1-DG).

A major improvement in the organic acids fit was observed, as well as in the sugars and MG formation. Independently of the reaction conditions the model seems to fit the experimental data quite well. However, an important question is still not clear: is fructose preferably formed through E_1 or E_2? To answer this question both hypotheses (A and B, respectively) were tested for both proposed models, through model discrimination.

(v) Test the hypothesized mechanism (goodness of fit and model discrimination)

The question how well the proposed model describes the experimental data must be addressed from a statistical point of view. One could be led to choose a poor model because it was *not as bad* as the other models. To avoid such a mistake it is recommended to use a multivariate test of goodness-of-fit (or its counterpart lack-of-fit) (*19*). This test is installed in the same software package as before (*17*) and was based on replicate experiments. Apart from each model there is an error associated with the data, the experimental error. If this is too high any model is in principle able to fit the data. However if the experimental error is small the discrimination between the data and the model estimation gives a better understanding how good the model fits the data. As can be seen in the Figures 4.2.4 – 4.2.7, the scatter in replicates was not very high which allowed a goodness-of-fit test. The quality of experimental data is therefore very important. The fit of the mathematical model to the data was done simultaneously with parameter estimation, which will be addressed in section (vi).

As mentioned before, kinetic modelling is an iterative process: propose a model, confront it with experimental data, criticise the model, adjust the model and confront the adapted model with experiments again, until an acceptable model results. What is acceptable though, is of course debatable. Alternative models may be formed from a candidate model by adding or deleting parameters. Once models show an acceptable fit, there may still be many models left, so the next step is model discrimination. According to the Bayesian concept (*20*), on which Athena Visual Workbench is based, the plausibility of a model results from the combination of likelihood (given by the data) with prior probability (given by previous results or personal belief). This combination is called Posterior Probability (PPB) (*21*). The model with the highest posterior probability performs then the best. It is required to have replicates (or an estimate of experimental uncertainty) in order to execute model discrimination. It is a relative concept, not an absolute one, meant for comparison. It provides information about the most plausible model, not necessarily the true one. In model construction additions of parameters are favored when they yield higher posterior probabilities; deletions are favored when they do not make the PPB appreciably worse.

However, it is also known that the higher the number of parameters the better fit will be

(*1*). In order to have a second opinion the model discrimination was also performed by using the Akaike criterion (AIC) (*22*) which, can be expressed in the case of least-squares approximation as:

$$AIC = n\ln(\sigma^2) + 2(p+1) \tag{4.2.15}$$

and the maximum likelihood estimator for the variance is:

$$\sigma^2 = \frac{SS}{n} \tag{4.2.16}$$

in which p is the number of estimated parameters (+1 to include the variance estimate). The $2p$ term is the penalty in the Akaike criterion for the use of more parameters. When the number of data points n is relatively small compared to p (say $n/p < 40$) the corrected AIC should be used:

$$AIC_c = n\ln(\sigma^2) + 2(p+1)\left(\frac{n}{n-p}\right) \tag{4.2.17}$$

Because the AIC criterion is on a relative scale it is common practice to calculate AIC differences, taking the model with the lowest value (AIC_{min}) as the reference:

$$\Delta_{AIC} = AIC - AIC_{\min} \tag{4.2.18}$$

A rule of thumb is that models with $\Delta_{AIC} \leq$ 2-3 are worthwhile to consider, values of Δ_{AIC} between 4 and 7 indicate that models are less supported, and values higher than 10 indicate that models may be discarded.

In Table 4.2.1 and Table 4.2.2 the results of the model discrimination tests are shown for M_1 (Scheme 4.2.2) and M_2 (Scheme 4.2.3) using PPB and the AIC criterion. At lower pH (Table 4.2.1) according to the Akaike criterion the results are contradictory. At 100°C, M_2 is less supported whereas at 120°C M_1 can be discarded, which was also supported by the PPB values. It should be taken into account that under these conditions (pH 5.5), for the studied heating period, the DFG degradation rate is quite small as well as the amount of products formed, which gives the models more flexibility to fit the experimental data. When the pH was increased to 6.8 (Table 4.2.2), besides M_1 and M_2 model discrimination, hypotheses A and B for fructose formation via E_1 and E_2, respectively, were also tested. Note that this test is only relevant for the systems at pH 6.8, since at lower pH fructose was not detected.

Table 4.2.1. - Model discrimination tests for the systems studied at pH 5.5

System	Model*	Parameters	SS	n	AIC_c	Δ_{AICc}	PPB
A	M_1	14	5.14	100	-261.92	**0**	**34.11**
(100°C)	M_2	15	5.58	100	-250.96	10.96	32.28
B	M_1	13	17.01	90	-117.22	11.50	14.78
(120°C)	M_2	15	14.05	90	-128.72	**0**	**17.65**

* Model 1 (M_1) presented in Scheme 4.2.2; Model 2 (M_2) presented in Scheme 4.2.3.

Table 4.2.2. - Model discrimination tests for the systems studied at pH 6.8

System	Hypotheses	Model*	Parameters	SS	n	AIC_c	Δ_{AICc}	PPB
C	**A**	M_1	14	50.05	99	-32.60	173.32	16.20
(100°C)		M_2	14	8.69	99	-205.92	**0**	**19.80**
D	**A**	M_1	13	49.85	99	-35.69	127.86	15.78
(120°C)		M_2	15	12.96	99	-163.55	**0**	**21.25**
C	**B**	M_1	12	754.67	99	230.67	423.88	Indt.[a]
(100°C)		M_2	15	9.61	99	-193.21	**0**	**29.09**
D	**B**	M_1	13	49.08	99	-37.22	123.62	14.59
(120°C)		M_2	15	13.32	99	-160.85	**0**	**21.77**

* Model 1 (M_1) presented in Scheme 4.2.2; Model 2 (M_2) presented in Scheme 4.2.3.
[a] Indeterminate (low trust region).

According to the Akaike criterion, independently of the chosen hypothesis A or B, M_2 always performed better than M_1. The results for M_1 indicated that this model could be discarded ($\Delta_{AIC} \geq 10$). These findings are confirmed by the obtained PPB values that were always higher in M_2. As for both model discrimination tests, it was clear that M_2 performed better than M_1. Also the obtained fits at pH 6.8 come into agreement with this conclusion. When comparing hypotheses A and B for M_2 the PPB values as well as the AIC values of hypothesis B are higher than those of hypothesis A.

These results suggest that fructose was mainly formed through the intermediate E_2, supporting the assumption made in Section 4.1. Also that model M_2 is more likely than model M_1, giving some evidence that the transformation step of glucose into 3-deoxyosone that

initially has been considered as a secondary route might be in fact a primary route and that the initial assumption that MG formation from DFG was a fast step was indeed not correct.

(vi) Estimate the rate constants

As mentioned before, once the basic form of the functional part of the model is established, the next step is to fit the model to the experimental data to obtain estimates for the model parameters, i.e. the rate constants. In general, this is accomplished by solving an optimization problem in which the objective function (the function being minimized or maximized) relates the response variable and the functional part of the model, in a way that will produce parameter estimates that will be closest to the true, unknown parameter values. In previous studies (*4, 5*) with multi-step kinetic analysis of glucose-amino acid Maillard reaction the approach used was to minimize the overall residual sum of squares (RSS) from all the responses. However, the RSS criterion is based on the assumption that the data on each response have the same variance and there is no correlation between the variances of the individual measurements of the response, which is not very realistic. In the present study several responses were measured from which some were measured from the same sample (e.g. formic and acetic acid) and with different degrees of precision. For cases of multiresponse modelling the fit criterion to be used depends on the experimental error structure of the data. Box and Draper (*20*) provided a solution for this problem assuming normally distributed errors. The best-fit criterion is the minimization of the determinant of the matrix of cross-products of the various responses, the so-called dispersion matrix from the responses. If the determinant of the dispersion matrix is minimized, the most probable estimates of the parameter will be found. The resulting parameter estimates for the hypothesized B mechanism in Scheme 4.2.3 (M_2) using the determinant criterion are shown in Table 4.2.3. The assumptions made in Section 4.1, can now be confronted with the estimated rate constants.

DFG was assumed to degrade preferably through E_1 (1,2-enolization) at lower pH. In fact at pH 5.5 step 1 prevailed to steps 2 and 3, especially when the temperature was increased. At higher pH (6.8) on the other hand step 2 gained importance, which is evident at lower temperature, suggesting that 2,3-enolization becomes more relevant by increasing the pH. Moreover, from DFG enolization step it becomes clear that independently of the reaction conditions, deoxyosones formation prevail to sugars formation. The rate constant for step 4 is always higher than for step 10 and for step 11. Also, fructose formation is only a minor step from the 2,3-enolization pathway. The rate constant for step 7 is always higher than for step 16. Moreover, the results suggest that sugar formation is mainly pH dependent.

Table 4.2.3 – Rate constants (10^{-2}) ± 95% Highest Posterior Density (HPD) as found by kinetic modelling for hypothesis B of Model 2 (M_2).

Rate Constant (min^{-1})	A (100°C, pH 5.5)	B (120°C, pH 5.5)	C (100°C, pH 6.8)	D (120°C, pH 6.8)
k_1	0.19 ± 0.02	1.11 ± 0.07	0.57 ± 0.03	8.89 ± 0.83
k_2	0.10 ± 0.04	0.86 ± 0.40	1.56 ± 0.09	6.29 ± 0.66
k_3	0.18 ± 0.01	0.88 ± 0.28	1.55 ± 0.11	8.62 ± 0.36
k_4	20.14 ± 3.37	31.13 ± 9.97	7.94 ± 1.51	215.79 ± 50.08
k_5	1.38 ± 0.39	2.23 ± 1.17	9.07 ± 3.45	506.69 ± 62.87
k_6	0.19 ± 0.04	4.30 ± 0.69	2.74 ± 2.82	30.41 ± 2.17
k_7	60.17 ± 9.11	76.79 ± 10.65	21.25 ± 9.18	55.93 ± 2.14
k_8	5.90 ± 4.06	15.41 ± 1.71	0.00 ± 0.00	0.00 ± 0.00
k_9	3.93 ± 0.21	22.50 ± 1.81	190.85 ± 22.85	653.55 ± 94.08
k_{10}	11.31 ± 1.94	19.42 ± 6.84	7.07 ± 1.11	25.07 ± 5.28
k_{11}	6.42 ± 1.07	10.96 ± 4.25	11.31 ± 1.81	50.55 ± 10.63
k_{12}	0.39 ± 0.13	1.27 ± 0.24	0.08 ± 0.05	1.06 ± 0.17
k_{13}	0.73 ± 0.28	1.41 ± 0.26	0.22 ± 0.05	2.03 ± 0.23
k_{14}	0.12 ± 0.03	70.68 ± 3.93	0.34 ± 0.06	2.47 ± 0.99
k_{15}	1.45 ± 0.42	5.15 ± 0.99	1.59 ± 0.22	16.82 ± 5.91
k_{16}	——	——	1.34 ± 0.59	4.51 ± 1.72

At lower pH mannose formation was favored towards glucose and no other sugar was detected, whereas at higher pH not only fructose was formed but also glucose was formed in higher amounts than mannose. Besides enolization, DFG was also assumed to degrade through retro-aldolization, where the rate determining step leads to C_n, the fragment without the amino group. In a previous multi-step kinetic analysis study (*5*) 1-morpholino-1-deoxy-D-fructose degradation was also assumed to undergo retro-aldolization reaction but in that case the resulting product kept the amino group. No attempt was made however in identifying and quantifying any reaction product that might be formed from it. The same assumption was tested in the present study and it showed a lack-of-fit in particular in the MG as well as in the organic acids formation. These results support the hypothesis that from DFG retro-aldolization the rate-determining step leads to the fragment without the amino group.

In Scheme 4.2.3 both deoxyosones are presented as essential steps in organic acids formation. In fact, steps 6 and 9 are the most significant in formic and acetic acid formation, respectively. The same result was observed in a previous study with different sugars and casein. (*23*). In Section 4.1, the obtained results suggested that 1-DG was more reactive than 3-DG. Also, acetic acid was identified as one of the main end products of DFG decomposition. From the kinetic analysis these results are supported. Not only the rate constant for the degradation of 1-DG into acetic acid (step 9) prevailed to the degradation of 1-DG into

carbohydrate fragments (C_n) (step 8), in particular at pH 6.8, but also under these conditions the rate constant of step 9 is the highest of the system. However, in terms of colour formation the influence of 1-DG seems to be very low compared with 3-DG. As the pH increased 1-DG is no longer involved in carbohydrate fragments responsible for colour formation, but mainly in acetic acid formation. Under these conditions 3-DG becomes the main precursor in colour formation, through step 5. In a previous study (*6*) the significance of DFG in colour formation in Maillard reaction has been questioned. This result in combination with the fact that 3-DG is the main precursor of carbohydrate fragments involved in colour formation raises an important question about the importance of ARP reversibility in Maillard reaction. This question will be addressed in the following chapter.

4.2.3. Conclusions

The multiresponse kinetic analysis was shown to be both helpful in deriving relevant kinetic parameters as well as in obtaining insight into the reaction mechanism. It becomes more fundamental than simple kinetics. It is important to realize that multiresponse kinetics analysis, contrary to uniresponse kinetic analysis is based on the rate-determining steps of the reaction, regarding both the reaction mechanism and the number of steps involved. The multiresponse modelling approach as used in this study is a helpful tool to unravel complicated reaction routes. Acetic acid, identified as a main end product in DFG thermal degradation, is according to the kinetic analysis mainly formed through 1-DG degradation. Also 3-DG was determined as a main precursor in carbohydrate fragments responsible for colour formation. As the reaction proceeded other compounds besides DFG are suggested to become reactants themselves with the formation among others, of methylglyoxal. Kinetic modelling is an iterative process: propose a model, confront it with experimental data, criticise the model, adjust the model and confront the adapted model with experiments again, until an acceptable model results. If models are possible from a scientific point of view, then a statistical treatment may help to choose. We would like to stress that scientific insight should be the first and the foremost discrimination tool in discussing model discrimination. Model discrimination is not about finding out whether or not the model is right or wrong, but rather to find the best performing model, from a scientific and statistical point of view.

4.2.4. References

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Chapter 5

A multiresponse kinetic model for the glucose/glycine Maillard reaction pathways

5. A multiresponse kinetic model for the glucose/glycine Maillard reaction pathways

A complete kinetic model for the glucose/glycine Maillard reaction was proposed. Special attention was paid to both reactants and intermediates: fructose, *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG), 1-deoxy and 3-deoxyglucosone (3-DG), formic and acetic acid, methylglyoxal and 5-(hydroxymethyl)furan-2-carboxaldehyde (HMF). The proposed model was updated and strained by varying one of the most important reaction conditions, the temperature. The estimated rate constants showed Arrhenius-like temperature dependence. As far as sugar kinetics was concerned, glucose isomerisation was one of the main degradation pathways, approximately 17% of the degraded glucose and highly temperature dependent. The organic acids were found to be stable end products and 3-DG to be involved in colour formation by reaction with glycine. The significance of DFG reversibility was studied by model discrimination. The results suggested that the reaction path from DFG into its parents, glucose and glycine, is not important from a quantitative point of view. More than just a fitting procedure, multiresponse modelling was shown to be a powerful tool in unravelling complicated reaction routes as occur in the Maillard reaction.

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5.1. Introduction

The Maillard reaction is a type of non-enzymatic browning of fundamental interest to food scientists and food processors, since it affects the quality of a food product, in particular the sensory properties: colour, flavour and taste. It results from an initial reaction of a reducing sugar with an amino compound, followed by a cascade of consecutive and parallel reactions to form a variety of coloured and colourless products which range from flavour volatiles (low molecular weight) to melanoidins (high molecular weight, brown, nitrogenous chromophores). Brown pigment formation is desired during some types of food processing (baking, cocoa and coffee roasting, cooking of meat) while it is undesirable in other technologies (milk drying, thermal treatments for the stabilisation of milk, fruit juices and tomatoes). Besides the sensory characteristics of foods, the Maillard reaction can also have negative consequences on the nutritional value (amino acids and protein unavailability for the human metabolism) as well as on the formation of mutagenic compounds (*1*) or even potentially carcinogenic, as the recently discovered acrylamide (*2, 3*).

The rate of the Maillard reaction and the nature of the products formed is mainly determined by the reaction conditions. Various factors influence the Maillard reaction and they can be considered as food processing variables. They include the chemical composition (nature of the reactants and type of buffer), the pH and water activity, the presence of oxygen and metals, the presence of any reaction inhibitors like sulphur dioxide, and the temperature-time combination during heating, which is, together with pH, believed to be the most important factor for the velocity of the reaction. Knowledge on kinetic parameters, such as rate constant and activation energy, is necessary to predict the extent of a specific chemical reaction and, consequently, to optimise it.

Several authors (*4*, *5*, *6*) have tried to study the kinetics of the Maillard reaction just by fitting simple kinetic models to one individual reaction pathway at the time. Bell et al. (*7*) reported that the glycine loss followed a second order reaction model in a glucose/glycine mixture in a glassy state at 25°C. However, no real distinction could be made between the first- and the second order plot (*8*). Also, Carabasa-Giribet and Ibarz-Ribas (*9*) reported that both zero- and first-order reaction models gave acceptable fits for brown colour formation in glucose and amino acid systems heated at four different temperatures. It is important to realize that applying simple kinetics in a complex reaction as the Maillard reaction is just a fitting procedure, since it gives no insight on the reaction mechanism. In contrast, applying multiresponse modelling techniques helps in building mechanistic models (*8*, *10*). The basic idea is to take into account simultaneously as many responses as possible. The main advantages of this approach are that models can be tested more rigorously and, once the goodness of fit is deemed acceptable, estimation of the parameters can be done much more

precise.

To develop a kinetic model for a chemical reaction as complex as the Maillard reaction, it is important to define, accurately, the system under study as well as to understand its mechanism. In the Maillard reaction the Amadori rearrangement product is believed to be the main intermediate formed in the early stage of the reaction (*4*, *5*, *6*). In the previous Chapter the thermal degradation pathways of the isolated *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) Amadori compound were investigated. Special attention was given to the formation of deoxyosones and methylglyoxal through DFG enolization and retro-aldolization, respectively. Also both parent sugars, glucose and mannose, and free amino acid, glycine were detected. Based on the reaction mechanism, a kinetic model was proposed and by multiresponse modelling strained and validated. These results raised an important question concerning the Amadori product reversibility in the Maillard reaction.

The aim of the present study was to develop a complete kinetic model for the whole glucose/glycine Maillard reation pathways, using as a base the kinetic model proposed for the *N*-(1-deoxy-D-fructos-1-yl)-glycine degradation mechanism. Both reactants and intermediates: fructose, formic and acetic acid, 1-deoxy and 3-deoxyglucosone, DFG, methylglyoxal and HMF (5-(hydroxymethyl)furan-2-carboxaldehyde) were identified and quantified upon thermal degradation of glucose and glycine mixtures at five different temperatures. The used temperatures were 80, 90, 100, 110 and 120°C. The model proposed was updated and tested at the various reaction conditions, and the model parameters (rate constants and activation energies) estimated. Special attention was given to the isomerisation and degradation reactions of the sugar as well as to the kinetic significance of Amadori compound reversibility in the early stages of the Maillard reaction.

5.2. Results and Discussion

5.2.1 - Identification and quantification of reactants and main products formed

During heating of glucose and glycine the concentration of the reactants decreased, as expected. However, independently of the temperature, the loss of glucose was faster than the loss of glycine (Figure 5.1). The same observation was reported by Baisier and Labuza (*5*) in glucose/glycine liquid model systems containing varying initial ratio concentrations in phosphate buffer (0.1 mol/L, pH 7) at 37°C. The amino acid showed an initial decrease in concentration followed by regeneration and attainment of steady state after 40 days.

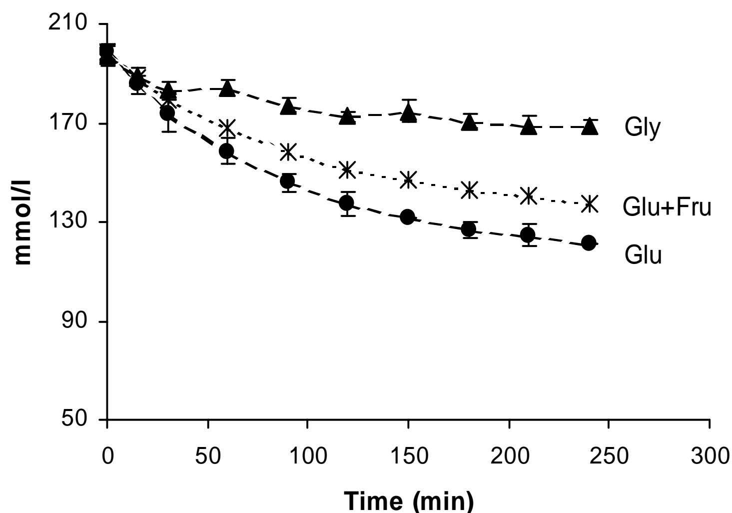


Figure 5.1. Loss of glycine (▲) and glucose (●), and the sum of glucose and its isomerisation product fructose (✳), in glucose/glycine (0.2M) solution heated at 100°C and pH 6.8.

For the first time a kinetic model was proposed taking into account the amino acid regeneration. Van Boekel and Martins (*11*) when studying the fate of glycine at varying temperatures in a glucose/glycine system also reported that the loss of glucose was considerably higher than that of glycine at all temperatures studied. The observed difference in reaction kinetics was accounted by glycine regeneration from the initial condensation products, like the Amadori rearrangement product. However, as the temperature increased the regeneration of the amino acid became less apparent. On the other hand Vernin et al. (*12*) and Debrauwer et al. (*13*) have considered the formation of diglucosylamine to account for the faster disappearance of glucose than amino acids. However, the rate constant for its formation was measured by quantifying only the amino acid and the Amadori compound, which may not be enough information to quantify diglucosylamine efficiently and independently (*8*).

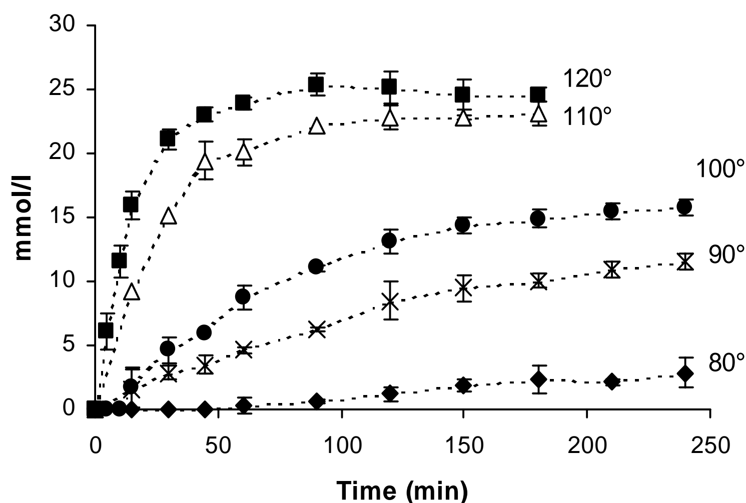


Figure 5.2. Formation of fructose during thermal degradation of glucose (0.2M) in an equimolar sugar/glycine aqueous solutions at pH 6.8 and 80°C (♦); 90°C (✳); 100°C (●); 110°C (Δ) and 120°C (■).

Sugar Isomerisation and degradation products. As far as sugar kinetics is concerned, it must be realized that the sugar is subject to other reactions than the Maillard reaction, namely isomerisation and degradation reactions. Brands and Van Boekel (*14*) reported that the main reaction product detected in a heated glucose/casein system at 120°C under neutral conditions was fructose. In the present study the formation of fructose was strongly influenced by the temperature, which became more significant for temperatures above 80°C (Figure 5.2). Moreover, the degradation products of lactose found in heated milk appeared to be mostly the same as those mentioned for the alkaline degradation of sugars (*15*). To gain insight into the sugar isomerisation and degradation products, glucose was heated alone at 100°C and pH 6.8. As is shown in Figure 5.3-A, under these conditions approximately 25% mol of glucose isomerised into fructose. Besides sugar isomers, organic acids, namely formic and acetic acid, were also detected when heating glucose alone. Formic acid was formed in higher amounts than acetic acid, 1.2 % mol and 0.65 % mol, respectively (Figure 5.3-B). In the glucose/glycine system, the sugar isomerisation accounted for 17% mol of glucose degradation at 100°C and pH 6.8. However, after correcting for isomerisation by adding up glucose and fructose concentrations, the loss of glucose in the Maillard reaction (Figure 5.1) did not account for all of the difference observed between glucose and glycine loss. It seems from a quantitative point of view that sugar isomerisation and degradation reactions should not be neglected when proposing a Maillard reaction kinetic model for temperatures above 80°C at pH 6.8.

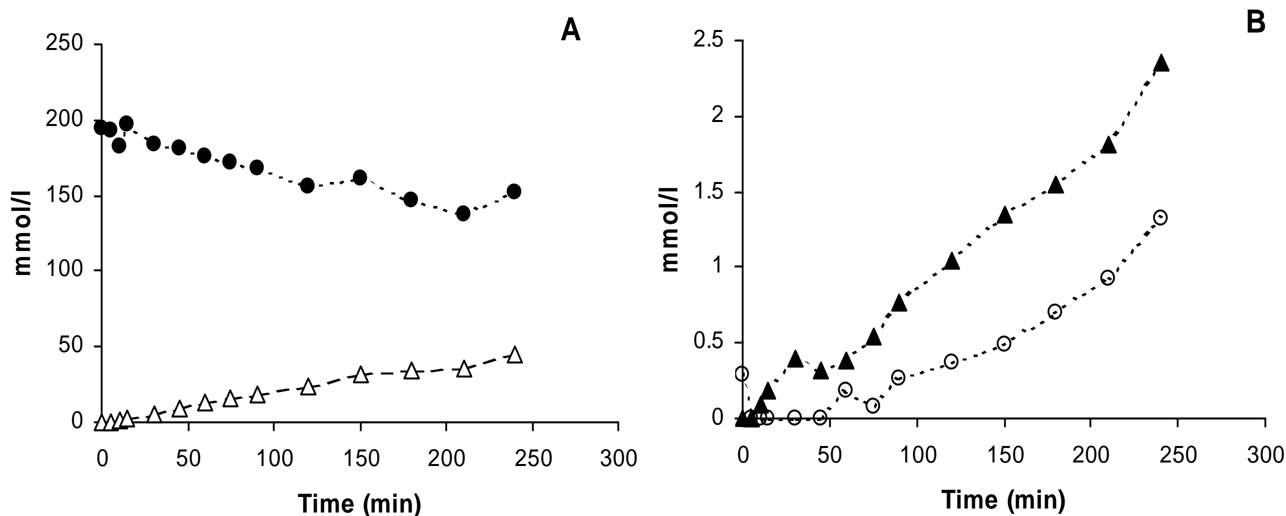


Figure 5.3. Isomerisation (**A**) and degradation products (**B**) of glucose (0.2M) heated alone (i.e. without glycine) at 100°C and pH 6.8. Glucose (●); fructose (Δ); formic acid (▲) and acetic acid (○).

Sugar/Amino-acid Maillard reaction products. Organic acids formation was investigated in the glucose/glycine systems for the different temperatures studied. Interestingly, independent of the temperature acetic acid was always formed in higher concentrations than formic acid (Figure 5.4). After heating the system for 4h at 100°C and pH 6.8, 25% of the degraded glucose was acetic acid whereas only 5% was formic acid. The same results were observed previously in Section 4.1. From these results it seems that acetic acid is preferably formed during the Maillard reaction. The importance of carboxylic acids is that they cause considerable reduction in the pH (Figure 5.4), slowing down the reaction. This phenomenon is particularly important at higher temperatures.

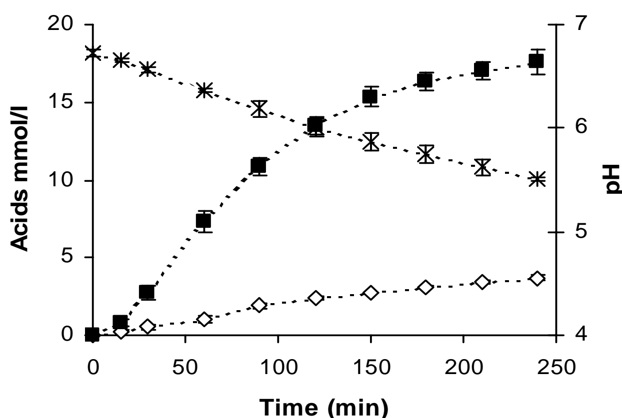


Figure 5.4. Organic acids formation with pH decrease in glucose/glycine (0.2 M) solution heated at 100°C and pH 6.8. Acetic acid (■), formic acid (◊) and pH drop (✳).

Another main intermediate identified and quantified in the glucose/glycine Maillard reaction was the Amadori compound *N*-(1-deoxy-D-fructos-1-yl)-glycine. Its behaviour is typical of an intermediate: at first a built up followed by a slow or fast decrease depending on the temperature (Figure 5.5).

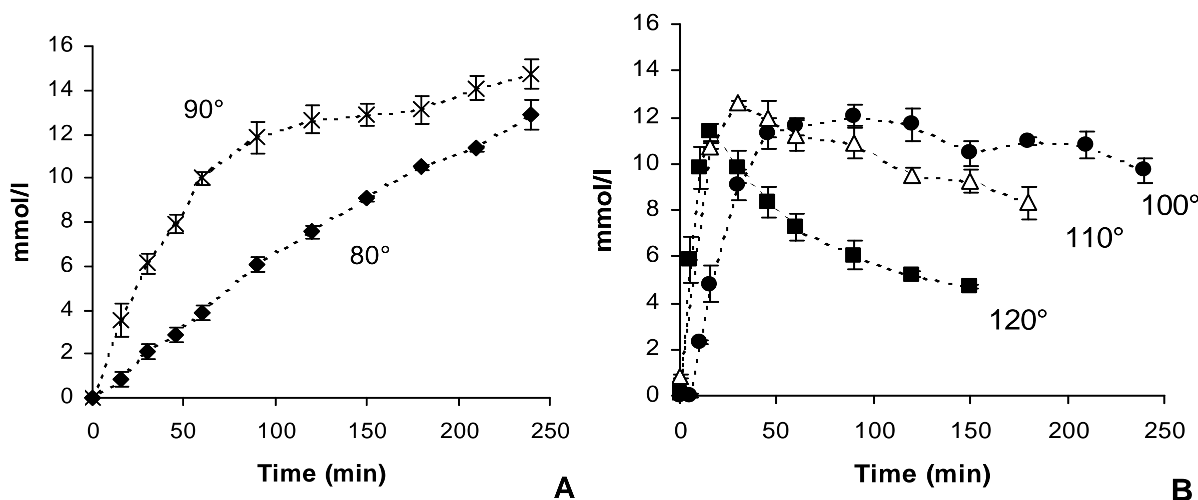


Figure 5.5. Amadori compound *N*-(1-deoxy-D-fructos-1-yl)-glycine formation upon thermal treatment of glucose and glycine (0.2 M) aqueous solutions at pH 6.8. **A** - 80°C (♦); 90°C (✳). **B** – 100°C (●); 110°C (Δ); 120°C (■).

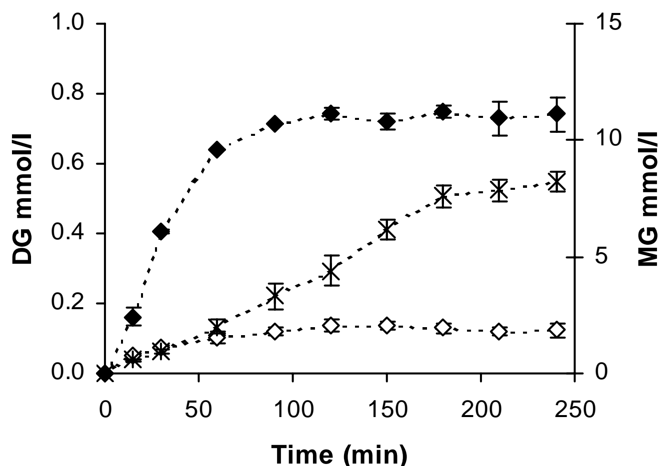


Figure 5.6. Formation of α-dicarbonyls upon thermal treatment of glucose and glycine aqueous solutions at 100°C and pH 6.8: 3-deoxyosone (♦); 1-deoxyosone (◊)and methylglyoxal (✳). All detected α-dicarbonyl compounds were in their quinoxaline form.

The degradation pathways of the DFG were studied in more detail in Chapter 4. In summary it resulted in regeneration of the amino acid and sugar fragments, including organic acids and α-dicarbonyl compounds. Comparatively, the thermal treatment of glucose/glycine solution led to an increase in the concentration of both organic acids and α-dicarbonyl compounds, deoxyglucosones (DG) and methylglyoxal (MG) (Figure 5.6). For all the temperatures studied at pH 6.8, 3-deoxyglucosone (3-DG) was always formed in higher amounts than 1-deoxyglucosone (1-DG). At 100°C, as shown in Figure 5.6, independently of the reaction time the concentration of 1-DG was 4 times lower than that of 3-DG. In parallel with the increase of deoxyglucosones, the concentration of MG also increased, reaching a yield of 3.8 % after a reaction time of 180 minutes.

Hollnagel and Kroh (*16*) detected α-dicarbonyl compounds, namely glyoxal, methylglyoxal and diacetyl, in glucose/glycine quasi-water free mixtures at 100°C and concluded that methylglyoxal was the predominating α-dicarbonyl detected, 5.07 % after 60 min. Deoxyosones were also determined quantitatively as breakdown products of glucose/L-alanine aqueous solution heated under reflux at pH 7 (*17*). In this study the concentration of 3-DG was 3 times higher than that of 1-DG, which is in the same range as observed in the present study.

Another identified compound was HMF, however in only very small amounts. After 30 minutes of heating, HMF reached a maximum of 20 μmol/L at 120°C and pH 6.8. HHMF and DDMP even though identified could not be quantified because no reference material was available, however judging by HMF response factor these compounds were also formed in very low amounts. Brands and Van Boekel (*14*) by studying both glucose and fructose together with casein at 120°C and pH 6.8 also detected only very small amounts of HMF, 40 μmol/L.
Finally the concentration of melanoidins (high molecular weight, brown, nitrogenous chromophores), also known as Maillard reaction end products responsible for colour formation, was calculated from the absorbance data through the extinction coefficient (*E*).

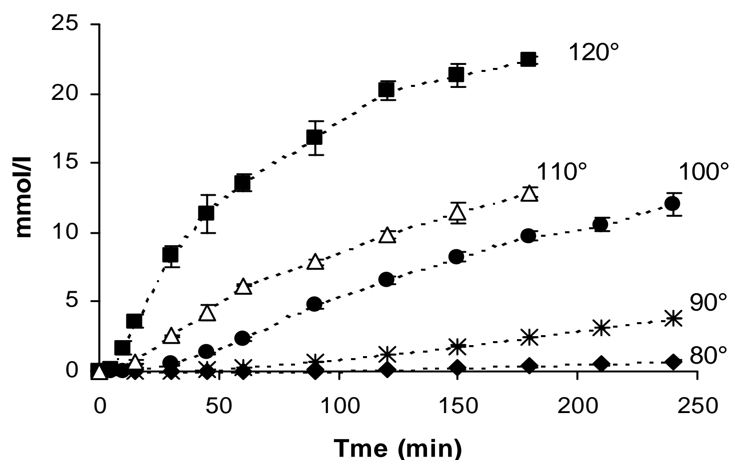


Figure 5.7. Melanoidins formation upon thermal treatment of glucose and glycine aqueous solutions at five different temperatures and pH 6.8 (80°C (♦); 90°C (✳); 100°C (●); 110°C (Δ);120°C (■)).

The molar extinction coefficient was expressed in terms of the concentration of glucose molecules converted into melanoidins. The value of E was derived experimentally by using radio labelled glucose (Section 3.3). As observed in Figure 5.7, an increase in temperature had a strong influence in melanoidins formation. Also, for the studied reaction conditions the melanoidins concentration was always higher in the glucose/glycine system than when the Amadori compound was heated alone.

Mass Balance. The results of the mass balance calculations (Figure 5.8) showed an almost negligible amount of missing compounds after 240 minutes of heating at 100°C and pH 6.8. The sum of all the intermediates identified and quantified reached 96% of the glucose initial concentration. In the Maillard reaction numerous products are formed, however the fact that we came to an almost 100% recovery indicates that the acids formed are stable end products of scission reactions leading to C_1-C_5 reaction products. Moreover, the complete loss of glycine was accounted for by the Amadori product and melanoidins formation.

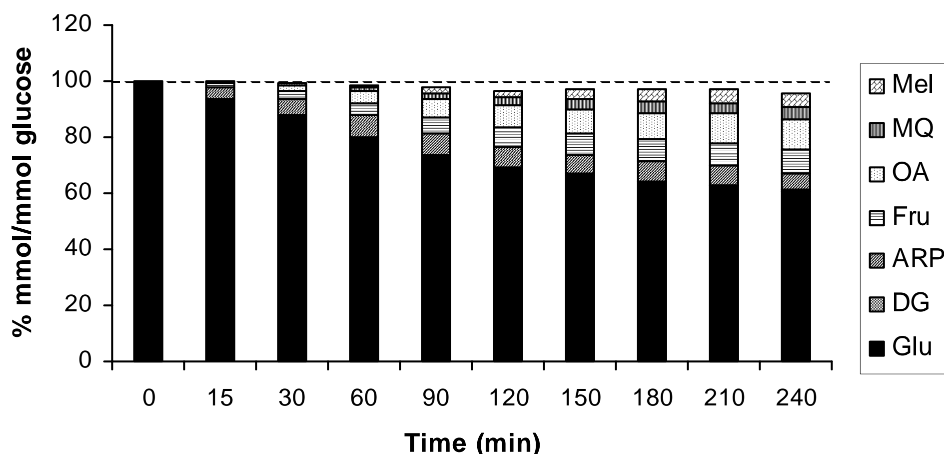


Figure 5.8. Mass Balance of reactants and reaction products in heated glucose-glycine system at 100°C and pH 6.8.

5.2.2 - Identification of the reaction pathways

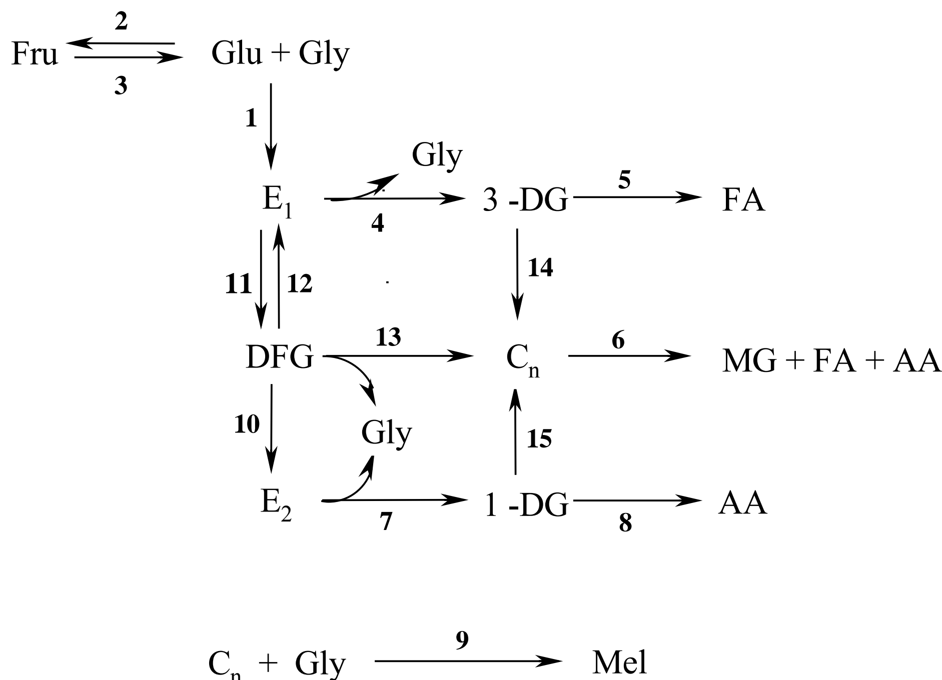
Sugar isomerisation, in which aldoses and ketoses isomerise into each other, is one of the main reaction pathways in the glucose/glycine systems. Glucose and fructose can isomerise into one another via the Lobry de Bruyn-Alberda van Ekenstein transformation (*18*), where the 1,2-enediol anion is the key intermediate. Through this reaction pathway mannose can also be formed, however it was not detected in the heated model systems. Besides isomerisation, label experiments have indicated that the sugar moiety can undergo C_2/C_4 and C_3/C_3 cleavage in addition to the formation of C_6 reactive intermediates: 1-deoxy and 3-deoxyglucosones (*19*). These α-dicarbonyl compounds are unstable and undergo either benzilic acid rearrangement yielding saccharinic acids or a cleavage reaction (at the C-C bond) resulting in both formic and acetic acid from 1- and 3-DG, respectively (*20*). The link with the Maillard reaction is that sugar degradation products are to a large extent similar to those formed through Amadori degradation pathways.

Methylglyoxal was the predominant α-dicarbonyl fragment detected in quasi-water free mixtures of glucose/glycine and glucose alone systems heated at 100°C (*16*). However its formation was enhanced greatly when glycine was present in the reaction mixture. The authors concluded that methylglyoxal was mainly formed from intermediates that arise during the Maillard reaction, such as the 1-deoxyglucosone or the Amadori rearrangement product. These results are well in line with our previous work presented in Section 4.2 where through multiresponse kinetic analysis an indication was given that methylglyoxal formation occurred mainly through Amadori retro-aldolization.

5.2.3 - Proposal of a kinetic model based on the established reaction network

The kinetic scheme proposed for the thermal degradation of *N*-(1-deoxy-D-fructos-1-yl)-glycine has been applied to the glucose/glycine Maillard reaction pathways as presented in Scheme 5.1 (*M1*).

In the first coherent scheme of the Maillard reaction (*21*) the Amadori compound had a main role in the intermediates formation. As a result previous kinetic studies were based on the Amadori compound as the initial main product formed (*22*). However, more recently, new pathways have been established (*23*, *24*) which question the central importance of the Amadori product. At the University of Leeds, a kinetic model was proposed for glucose/glycine systems at pH 5.5, in which 3-deoxyglucosone was the key intermediate in colour formation and it was formed from the 1,2-enediol, precursor of the Amadori compound (*25*). Also Debrauwer et al. (*13*) have postulated a kinetic scheme for glucose/amino acids where the glucosylamine was formed prior to the Amadori compound.



Scheme 5.1. Kinetic model (*M1*) proposed for the glucose/glycine Maillard reaction based on the established network for *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) thermal degradation (*11*). Glucose (Glu); glycine (Gly); fructose (Fru); E_1 and E_2 unidentified key compounds involved in rate-determining steps that can be the Schiff's base, the cation form of the Schiff's base, the 1,2 enaminol or the 2,3-enaminol, respectively; 3-deoxglucosone (3-DG); 1-deoxyglucosone (1-DG); methylglyoxal (MG); acetic acid (AA); formic acid (FA); unidentified carbohydrate fragments (C_n); melanoidins (Mel).

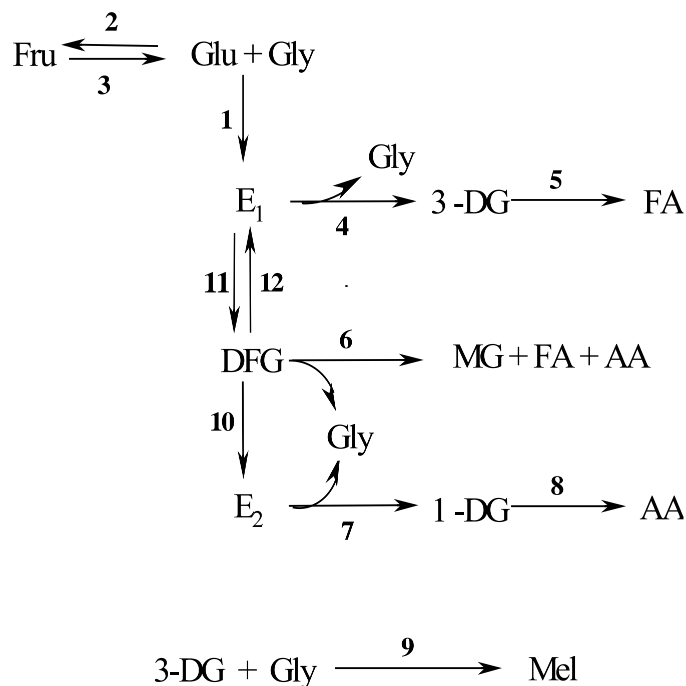
In *M1* the glucose/glycine Maillard reaction pathways result in an intermediate, designed as a E_1, which can be the Schiff's base, the cation form of the Schiff's base or the 1,2 enadiol with the amino acid still incorporated (*26*). This intermediate hasn't been isolated yet from the Maillard reaction due to its reactivity, however, for modelling purposes it has been included. Through multiresponse modelling the proposed model was strained and validated. Moreover, to get more insight, the significance of DFG reversibility was studied by model discrimination, as will be discussed in the following section.

5.2.4 - Test of the hypothesized mechanism (goodness of fit and model discrimination)

To fit the model to the experimental data, the reaction network presented in Scheme 5.1 was translated into a mathematical model by setting-up differential equations for each reaction step using the law of mass action. These coupled differential equations were solved by numerical

integration. The used subroutine was DDAPLUS, which is available in the software package Athena Visual Workbench (*27*). The model was first fitted to data obtained at 100°C and pH 6.8, but the subroutine failed to converge to an acceptable solution giving an error message. This could be related with the high number of parameters relatively to the number of responses. Moreover, the obtained plots for the experimental data and the predictions by the model showed a lack of fit for both methylglyoxal and melanoidins formation. In both cases the model predicted a lag-phase in their formation, which was not observed in the experimental data. Similar results were obtained at higher and lower temperatures. These results forced us to review the proposed kinetic model.

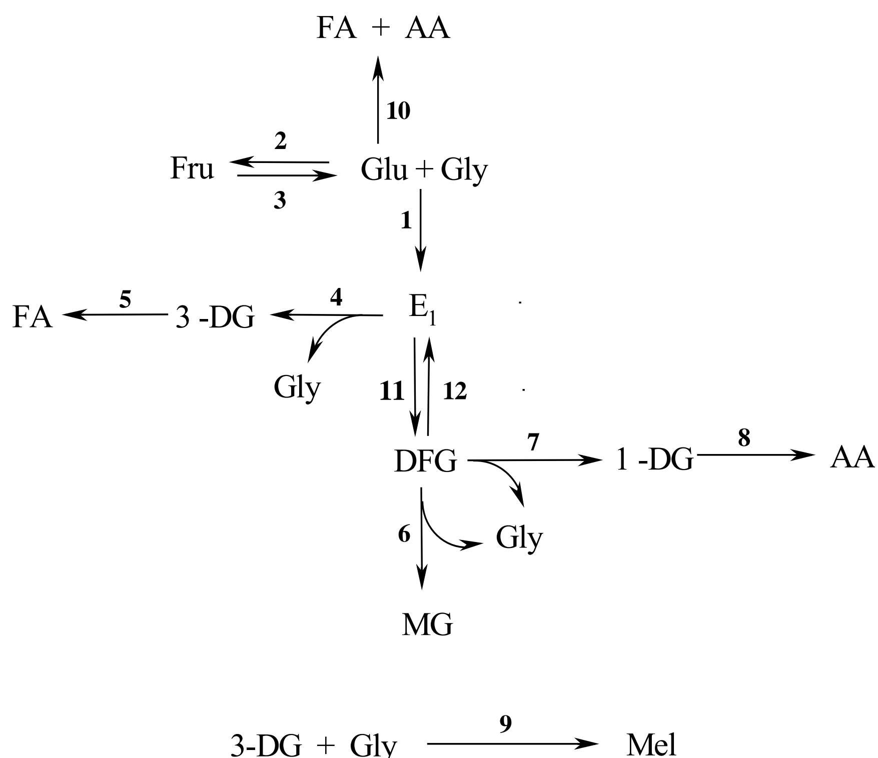
To begin with, a decrease in the number of parameters in *M1* was advised. Moreover, the mechanism of the formation of brown colour is not fully understood and the structure of melanoidins is largely unknown. However, recent studies (*28*, *29*) suggested that the melanoidin skeleton was mainly built up from sugars degradation products, formed in the early stages of the Maillard reaction, polymerized through aldol-type condensation and linked by amino compounds, such as amino acids. In the model proposed for *N*-(1-deoxy-D-fructos-1-yl)-glycine thermal degradation (Section 4.2) the results showed that 3-DG more than 1-DG was involved in the formation of carbohydrate fragments (C_n) responsible for colour formation. Moreover, the hypothetical melanoidin structure proposed by Cämmerer and Kroh (*28*) was based on the reactions of α-dicarbonyl compounds, in particular 3-deoxyglucosone. These findings together with the lag-phase predicted in melanoidins formation made us reconsider step 9. Instead 3-DG was assumed to be the main precursor of colour formation by reaction with glycine, as shown in Scheme 5.2 (*M2*).



Scheme 5.2. Modified kinetic model (*M2*) for the glucose/glycine Maillard reaction. Glucose (Glu); glycine (Gly); fructose (Fru); E_1 and E_2 unidentified key compounds involved in rate-determining steps that can be the Schiff's base, the cation form of the Schiff's base, the 1,2 enaminol or the 2,3-enaminol, respectively; *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG); 3-deoxglucosone (3-DG); 1-deoxyglucosone (1-DG); methylglyoxal (MG); acetic acid (AA); formic acid (FA); melanoidins (Mel).

A major improvement was obtained. Not only were all parameters estimated, indicating that indeed we had too many, but also in melanoidins formation a good fit was observed, as well as in the MG formation. However, there was clearly a miss fit for the organic acids and 1-DG formation. Both formic and acetic acid were underestimated at the beginning and overestimated at the end of the heating period. In fact, the assumption of direct cleavage of Amadori into formic and acetic acid (step 6) is not very likely to happen, not from a chemical point of view. This shows the power of the iterative modelling approach: propose a model, confront it with experimental data, criticize the model and confront it with experimental data again. More than just a fitting procedure, multiresponse modelling gives insight on the reaction mechanism and helps to differentiate between assumptions. From the sugar isomerisation and degradation products it was concluded that for temperatures above 80°C at pH 6.8, besides glucose isomerisation into fructose the sugar degradation step into organic acids should be included. Also, the observation that at the beginning of the reaction 1-DG was underestimated lead us to the assumption that 1-DG formation from DFG was a fast step, without E_2 as a rate-determining intermediate.

As a result a modified model *M3* was once again proposed (Scheme 5.3). To fit the model to the experimental data, the same procedure was taken by coupling differential equation to each reaction step. The approach of numerical integration followed by fitting to the data is flexible because by changing relevant differential equations and fitting them again to the experimental data, different models can easily be tested.



Scheme 5.3. Modified kinetic model (*M3*) for the glucose/glycine Maillard reaction. Glucose (Glu); glycine (Gly); fructose (Fru); E_1 unidentified key compound involved in rate-determining step that can be the Schiff's base, the cation form of the Schiff's base or the 1,2 enaminol; *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG); 3-deoxglucosone (3-DG); 1-deoxyglucosone (1-DG); methylglyoxal (MG); acetic acid (AA); formic acid (FA); melanoidins (Mel).

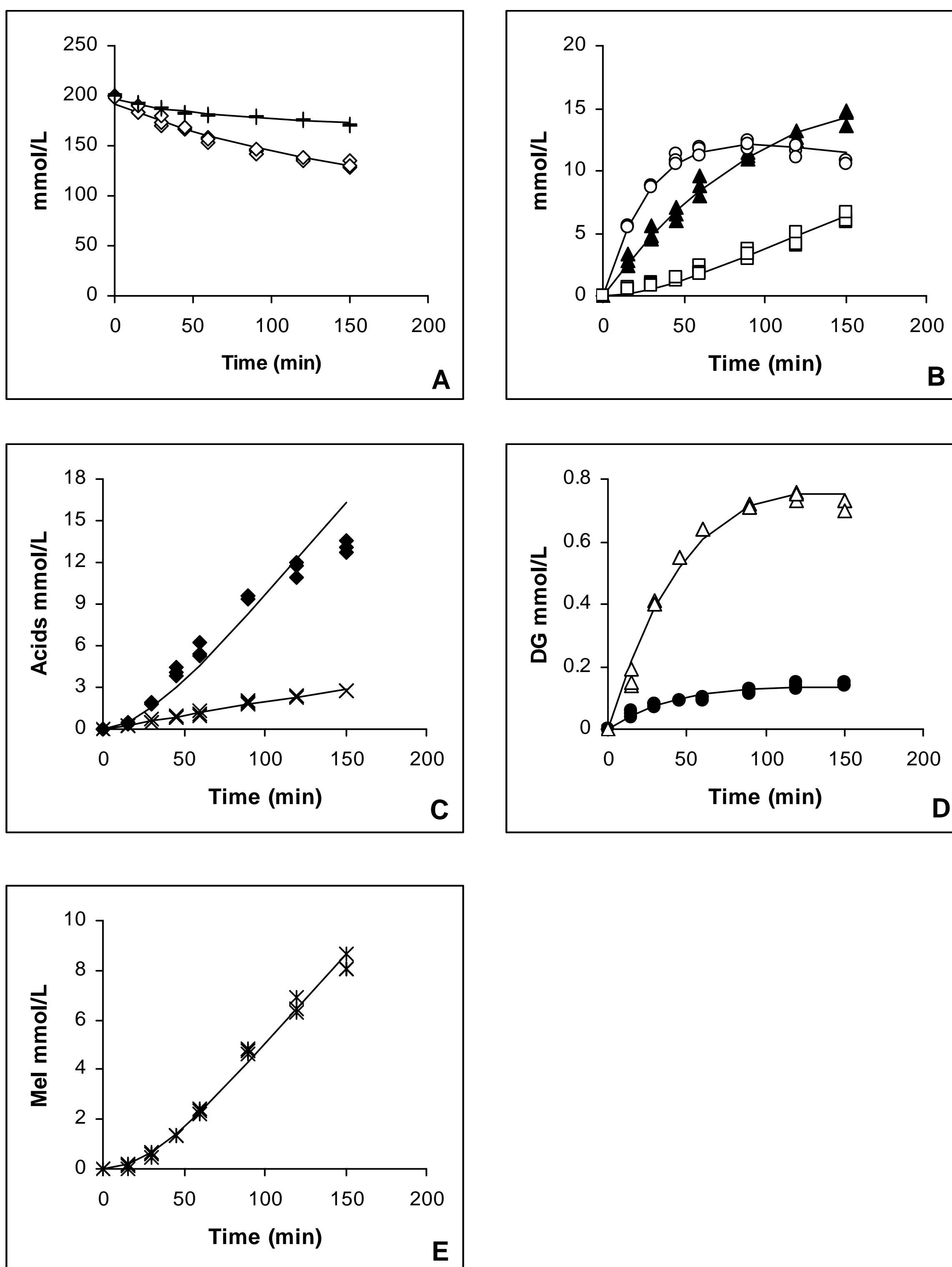


Figure 5.9. Scheme 5.3 (*M3*) model fit (lines) to experimental data (dots) of glucose/glycine aqueous system heated at 100°C and pH 6.8. **A**: Glucose (◊), Glycine (+); **B**: Fructose (▲), *N*-(1-deoxy-D-fructos-1-yl)-glycine (○), methylglyoxal ( ); **C**: Formic acid (×), Acetic acid (♦); **D**: 1-deoxyglucosone (●), 3-deoxyglucosone (Δ); **E**: Melanoidins (∗).

The results of the fit of *M3* to the experimental data taken at 100°C and pH 6.8 are presented in Figure 5.9. For most of the identified and quantified intermediates, the model described the experimental data quite well. From a statistical point of view a multivariate test of goodness-of-fit was used. This test is installed in the same software package as before and was based on replicates experiments. As can be seen in Figure 5.9 the scatter in replicates was not very high.

It should be noted that for the acetic acid formation as the reaction proceeded a levelling-off was observed, but this was not predicted by the model. This could be a result of two phenomena: first one could consider that the acetic acid would be involved in oxidation/reduction reactions decreasing its concentration (*30*); second the observed pH drop should be considered since this may inhibit the reaction rate. Both hypotheses are valid and a kinetic analysis should take this into account. However this issue will be addressed in more detail in the subsequent Chapter where the influence of pH was studied in the glucose/glycine systems. For the present study the effect of pH is concealed in the rate constants.

5.2.5 - Model discrimination

The significance of DFG reversibility was studied by model discrimination. Despite the significant effort in the past decades, many questions remain concerning the Maillard reaction pathways. Among others, the reversibility of the Amadori product. The formation of parent sugars from the corresponding Amadori compound has been reported under physiological conditions (*31*), pyrolysis conditions (*32*) and under conditions relevant to food processing (*33*). In most of those studies the hypothesis of reversible Amadori rearrangement was supported. However, the mechanism of reversion is still not full understood nor it is known if it is relevant in the Maillard reaction.

In a recent study Ge and Lee (*6*) proposed a kinetic model for the early stage of the Maillard reaction in which the Schiff base, formed prior to the Amadori compound, was reversible into the parent sugar and amino acid. However, in that study they used an equilibrium constant in their derivation and estimated this constant from the data. Still, the components measured were very likely not at their equilibrium concentration and therefore a wrong estimate of the equilibrium constant could have been obtained (*8*). Moreover under physiological conditions it has been suggested that the ketoamine adducts of aldoses with the ε-amino groups of RNase A undergo enolization and rearrangement to form the Schiff base (*31*). Keeping this in mind, from the model presented in Scheme 5.3, three possible reaction mechanisms arose for the early stage of the glucose/glycine Maillard reaction:

(glucose (Glu); glycine (Gly); *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG); E_1 unidentified key compound)

All 3 hypotheses have been confronted with the experimental data and hypothesis B showed the lowest goodness-of-fit, in particular in DFG formation. However, acceptable fits were obtained for the other intermediates. Therefore model discrimination was performed for all the 3 suggested mechanisms.

Model discrimination is a relative concept, not an absolute one. It provides information about the most plausible model, not necessarily the true one. In the present study the model discrimination tests used were the posterior probability (PPB) (*34*), which requires replicates or an estimation of experimental uncertainty, and the Akaike Criterion (AIC) (*35*). PPB is given by the software used and results from the combination of likelihood (given by the data) with prior probability (given by previous results or personal belief). It is a Bayesian concept. The model with the highest PPB performs the best. The Akaike criterion (AIC) can be expressed in the case of least-squares (*SS*) approximation as:

$$AIC = n\ln\left(\frac{SS}{n}\right)^2 + 2(p+1) \tag{5.1}$$

where n is the number of data points and p the number of estimated parameters. Because it is on a relative scale it is common practice (*21*) to calculate Δ_{AIC} by difference, taking the model with the lowest value as reference. The model with the lowest Δ_{AIC} performs the best.

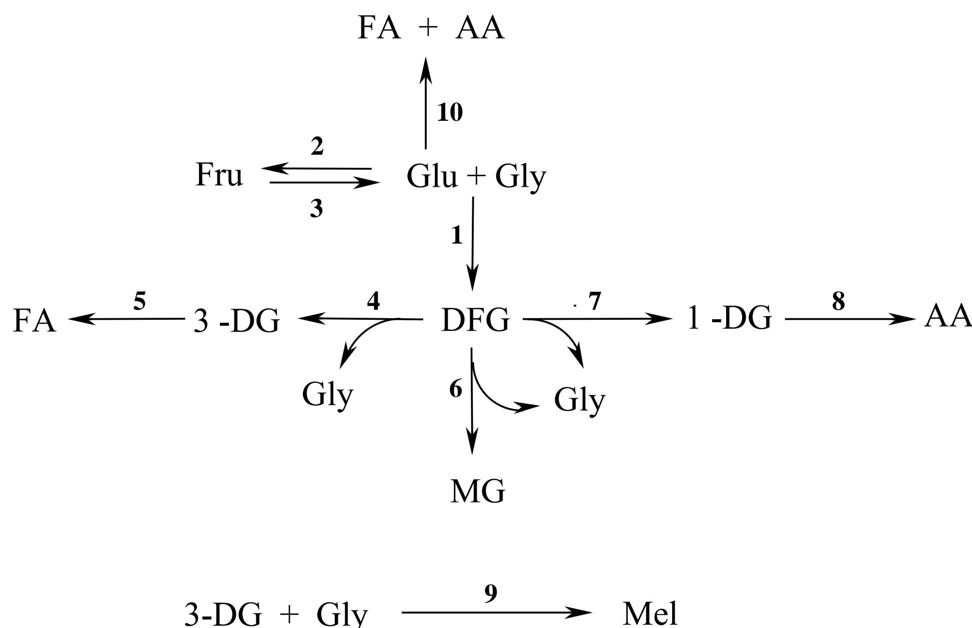
In Table 5.1 the results of the model discrimination tests are shown. Both tests support hypothesis A, where it is suggested that a key intermediate is formed prior to the DFG formation from the glucose/glycine thermal degradation and it is not reversible into the parent sugar and amino acid for temperatures above 80°C at pH 6.8. Moreover, it is the main precursor of α-dicarbonyl compounds responsible for colour formation and it is in equilibrium with the Amadori rearrangement product. These results suggest that the reaction path from DFG into its parents, glucose and glycine, is not important from a quantitative point of view, even though the step prior to DFG formation is reversible.

Table 5.1. Model discrimination tests for Hypothesis A, B and C.

Hypothesis	*p*	SS	*n*	AIC_c	Δ_{AICc}	PPB
A	13	$8.07 \times 10^{+2}$	720	110.13	**0**	**-19.05**
B	13	$1.20 \times 10^{+3}$	720	397.59	276.00	-24.11
C	13	$1.18 \times 10^{+3}$	720	386.13	287.46	-23.91

p (number of parameters); SS (Residual Sum of Squares); *n* (number of data points including the replicates); PPB (Posterior Probability); AIC (Akaike Criterion); Δ_{AIC} (AIC difference taking the smallest value as reference).

However, since E_1 is not measurable, the question arises if the reaction network in Scheme 5.3 can be further simplified for modelling purposes. Such simplification is presented in Scheme 5.4 (*M4*). The same fitting procedure to the experimental data was performed. This model fitted the data equally well as in Figure 5.9.



Scheme 5.4. Simplified kinetic model for the glucose/glycine Maillard reaction. Glucose (Glu); glycine (Gly); fructose (Fru);. *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG); 3-deoxglucosone (3-DG); 1-deoxyglucosone (1-DG); methylglyoxal (MG); acetic acid (AA); formic acid (FA); melanoidins (Mel).

5.2.6 - The Influence of Temperature

To strain the model proposed (*M4*) the reaction conditions should be altered, in particular the temperature. The Maillard reaction depends greatly on the temperature with respect to which reaction route prevails and what pattern of intermediates and end products are formed. Consistent temperature dependence is an additional indication that a model is acceptable. In order to be able to predict the reaction rates at various temperatures, the temperature dependence had to be determined. The relationship between the rate constant (k) and temperature (T) is frequently indicated by the Arrhenius equation:

$$k = k_0 \exp\left(\frac{-E_a}{RT}\right) \tag{5.2}$$

where k_0 is the so-called frequency factor, R the gas constant (8.314 $J.mol^{-1}.K^{-1}$) and E_a the activation energy, the kinetic energy of reactant molecules. E_a is usually determined experimentally from the plot of ln k versus $1/T$. The Arrhenius equation is a simplification. The estimated rate constants by both *M3* and *M4* at each temperature separately showed that the temperature dependence was Arrhenius like, in the sense that a straight line was obtained when ln k was plotted against $1/T$. However, when estimating the activation energies a high correlation is found between the parameters. The experimental range of studied temperatures is small compared to the absolute temperature range over which the Arrhenius equation would apply. As a result the equation should be reparameterised, as follows:

$$k = X \exp(-YE_a) \tag{5.3}$$

$$X = k_0 \exp\left(\frac{-E_a}{RT_{av}}\right) \tag{5.4}$$

$$Y = \frac{1}{R}\left(\frac{1}{T} - \frac{1}{T_{av}}\right) \tag{5.5}$$

$$T_{av} = \frac{\sum T}{n} \tag{5.6}$$

The model presented in Scheme 5.4 was fitted to all the data at the same time, taking into account the temperature dependence by including the reparameterised Arrhenius equation at the five heating temperatures simultaneously.

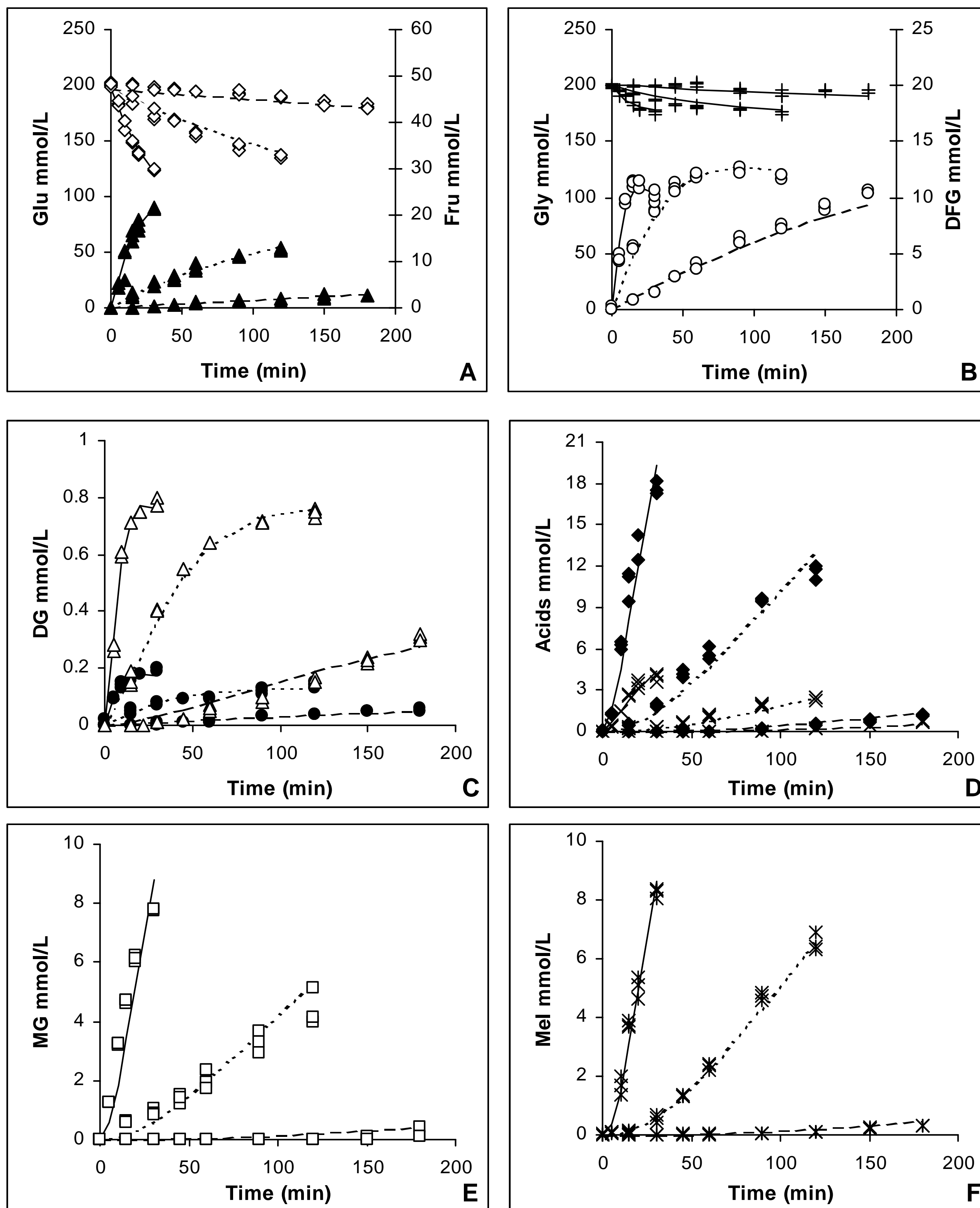


Figure 5.10. Scheme 5.4 (*M4*) model fit (lines) to experimental data (dots) of glucose/glycine aqueous system heated at 80°C (– – –), 100°C (- - -) and 120°C (——). **A**: Glucose (Glu) (◊), Fructose (Fru) (▲); **B**: Glycine (Gly) (+), *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) (○); **C**: 1-deoxyglucosone (1-DG) (•), 3-deoxyglucosone (3-DG) (Δ); **D**: Formic acid (×), Acetic acid (♦); **E**: methylglyoxal ( ); **F**: Melanoidins (Mel) (∗).

In Figure 5.10 the results of the fit for the systems heated at 80, 100 and 120°C are given as example. An increase in temperature leads to a higher loss of the reactants and an increase in the formation of the reaction products. The estimates of the activation energies and their 95%-confidence interval are shown in Table 5.2. The consistent temperature dependence together with how well the model fitted the data is an indication that the model is acceptable. The assumption that glycine was regenerated from the Amadori compound degradation steps was well predicted by the model (Figure 5.10-B), as well as that colour formation was mainly related with the reaction between 3-DG and glycine (Figure 5.10-E). From the results the glucose isomerisation step becomes more important at higher temperatures, in particular in the formation of the organic acids (E_a = 237 kJ/mol). Comparatively 3-DG and 1-DG show low temperature dependence in formic (E_a = 30 kJ/mol) and acetic acid (76 kJ/mol) formation, respectively. The low amount of formic acid detected relatively to acetic acid at pH 6.8 suggested that 3-DG degraded preferably into melanoidins formation (95 kJ/mol) as the temperature increased whereas 1-DG degraded mainly into acetic acid. Evidence is given that acetic acid is a main end product from the Maillard reaction (Figure 5.10-D). In fact, it could be considered as an indicator of the progress of the Maillard reaction at pH 6.8. Moreover, under these reaction conditions DFG degradation shows lower temperature dependence in 3-DG formation (97 kJ/mol) and higher in MG formation (125 kJ/mol). It was suggested that DFG degrades first through the enolisation step. This result is well in line with the model discrimination results where DFG is believed to be in equilibrium with an intermediate (Schiff's base, the cation form of the Schiff's base or the 1,2 enadiol) formed previously and responsible for the formation of 3-DG, the main colour precursor. This result is supported by the relatively low tendency for DFG to form brown colour under the same reaction conditions, as observed in Section 4.1 and by Molero-Vilchez & Wedzicha (*36*).

Table 5.2. Estimated parameters with temperature dependence included as found by kinetic modelling for M4.

Reaction Step	X^a	E_a (kJ / mol)[b]
1	1.61×10^{-5}	97 ± 3
2	1.64×10^{-3}	123 ± 5
3	9.15×10^{-3}	93 ± 3
4	1.11×10^{-2}	97 ± 2
5	3.45×10^{-2}	30 ± 9
6	7.08×10^{-3}	125 ± 5
7	1.57×10^{-2}	107 ± 7
8	$1.45 \times 10^{+0}$	76 ± 4
9	8.12×10^{-4}	95 ± 2
10	4.41×10^{-5}	237 ± 36

[a]Reparameterised Arrhenius equation as shown in equation 5.4.[b]Activation energy.

5.3. Conclusions

The iterative process: propose a model, confront it with the experimental data and criticise the model, arrived at a kinetic model that was largely consistent with all results obtained. More than just a fitting procedure, multiresponse modelling was shown to be both helpful in deriving relevant kinetic parameters (rate constants and activation energies) as well as in obtaining insight on the reaction mechanism. It is therefore a powerful tool for the unravelling of complicated reaction routes as occur in the Maillard reaction.

5.4. References

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Chapter 6

Kinetic model validation for the glucose/glycine Maillard reaction pathways: influence of pH and reactants initial concentration

6. Kinetic model validation for the glucose/glycine Maillard reaction pathways: influence of pH and reactants initial concentration

To strain the kinetic model for the glucose/glycine Maillard reaction, as proposed in the previous chapter, the reaction conditions were altered, namely the reactants initial concentration (1:2 and 2:1 molar ratios were compared to the 1:1 ratio) and the reaction initial pH (4.8, 5.5, 6.0, 6.8 and 7.5). By changing important reaction conditions it was investigated how well the model performs and how accurate it is according to the established reaction mechanism. The estimated rate constants showed no dependence on the glucose and glycine initial concentration. The reaction mechanism was highly influenced by the pH with respect to which reaction route prevailed and to the type of products formed. For a pH drop ≤ 1 unit, the model showed consistent pH dependence, which was well captured by a power law relation: $k = \beta \times pH^{pD}$, in line with a theoretical analysis for sugar reactions where the rate constant was shown to be inversely proportional to the concentration of H^+. This is an additional indication that the model performed well and that it was consistent with the established reaction mechanism. A sensitivity analysis of the model was performed to see which responses are most strongly influenced by which parameters. This helped in highlighting the important steps, as well as finding a possible redundant step for the formation of formic acid. Multiresponse kinetic modelling has proved to be a powerful tool to unravel complex chemical reactions such as the Maillard reaction.

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6.1. Introduction

The reaction between carbonyls and amines, known as the Maillard reaction, strongly affects the quality of foods. It plays a major role in food sensorial properties, flavor and color formation, as well as in its nutritional and health aspects (*1*-5). Quality is a broad concept. However, from a technological point of view it means the control of chemical, physical and microbiological changes during processing and storage. In order to be able to control those changes in foods, the reactions of interest need to be studied in a quantitative way. Kinetic parameters describing such changes are thus needed. Not only their value but also the confidence in models where those changes play a key role is important, and, consequently the validation of proposed kinetic models needs to be considered (*6*).

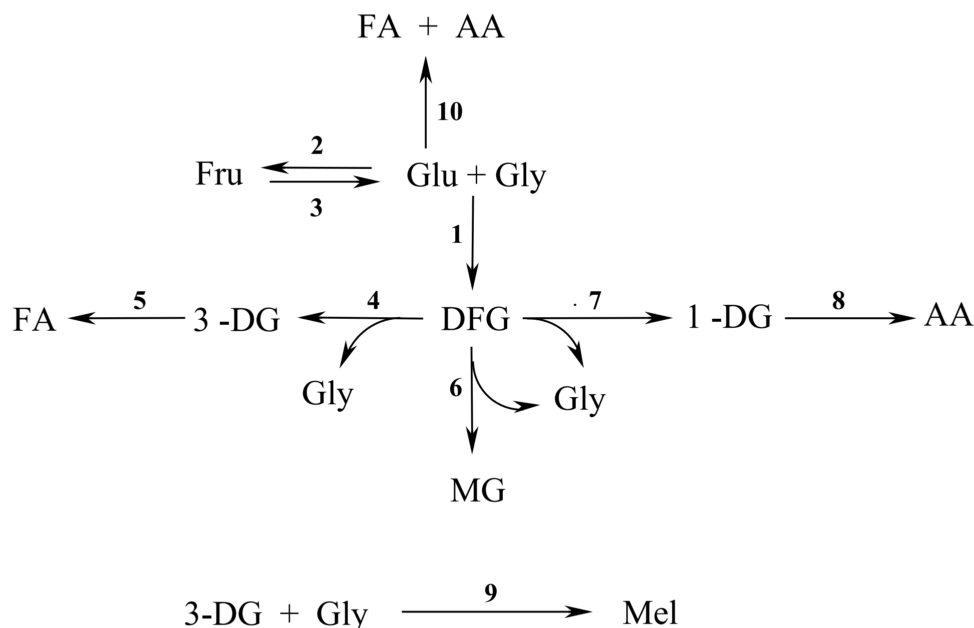
The development of the Maillard reaction can be affected by many factors including the temperature, chemical composition, type of buffer, water activity and pH. The type of sugar and amino group influence the rate of reaction as well as the products formed (*7, 8*). The concentration and ratio of reactants also have a significant impact on the reaction. O'Brian and Morrissey (*9*) stated that "an excess of sugar over the amino compound promotes the rate of Maillard browning" the explanation being that there are "mechanistic differences" in the destruction of the sugar compared to the amino acid. It should be noted that the rate of browning and the rate constant of the step in the reaction network that results in color formation, are two different things. In fact, increasing the initial reactant concentration should not influence the reaction rate constants, since

$$-\frac{\mathrm{dS}}{\mathrm{d}t} = -\frac{\mathrm{dA}}{\mathrm{d}t} = k_1[\mathrm{S}][\mathrm{A}] \tag{6.1}$$

the overall rate of loss of the sugar (S) and the amino acid (A) is equal to the rate constant times their concentration.

However, certain reaction parameters such as temperature and pH have a strong influence on the rate constants values. They influence each step of reaction mechanism differently and therefore determine which reaction route prevails and what pattern of intermediates and end products is formed. Concerning temperature, in Chapter 5 the estimated rate constants for the proposed simplified glucose/glycine kinetic model (Scheme 6.1) showed that the temperature dependence was Arrhenius like but obviously with varying parameter values. The glucose degradation step into organic acids showed the highest activation energy. With regard to the influence of pH on the Maillard reaction, it is generally accepted that the Maillard reaction proceeds faster when the pH is increased (*10*). It has been stated that the substrate loss increases with increasing pH, up to a pH of about 10, with little if any browning

occurring below pH 6 (*8*). Also, the degradation pathways of the Amadori compound, enolisation and retro-aldolization, are strongly dependent on the reaction pH (*11*, *12*).



Scheme 6.1. Simplified kinetic model for the glucose/glycine Maillard reaction pathways. Glucose (Glu); glycine (Gly); *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG); fructose (Fru); 3-deoxyglucosone (3-DG); 1-deoxyglucosone (1-DG); methylglyoxal (MG); formic acid (FA); acetic acid (AA); Melanoidins (Mel).

The pH dependence of the Maillard reaction can be related to the of amount unprotonated form of the amino group (the reactive form), which is favored at high pH, as described in the following equation:

$$R\text{-}NH_2 + H^+ \rightleftharpoons R\text{-}NH_3^+ \qquad (6.2)$$

The amino group reacts with the C═O group of the sugar through a nucleophilic attack. At basic conditions the nucleophile, i.e. the amino group will be more likely deprotonated, and therefore more nucleophilic. However, under acidic conditions the amino group becomes a weaker nucleophile, which requires that the C═O group be activated prior to the nucleophilic attack (Figure 6.1). This can be done using an acid catalyst. The carbonyl group becomes more electrophilic by protonation. The influence of the pH on the sugar can be written as follows:

$$SH \rightleftharpoons S^- + H^+ \qquad (6.3)$$

Where the total amount of the sugar (S_t) is:

$$S_t = SH + S^- \tag{6.4}$$

The sugar electrophilic form (S^-) is related with its open chain form and it is only a fraction of the total amount of sugar (S_t) present. The higher the pH, the more open chain form is present in the equilibrium and therefore more sugar is reactive with the amino acid. The lower the pH, the more protonated amino group is present in the equilibrium and therefore less reactive with the sugar. The initial step of the Maillard reaction is, therefore, highly influenced by the reaction initial pH. Also the amino-proton equilibrium is dependent on the pKa of the amino group as shown in Chapter 2.

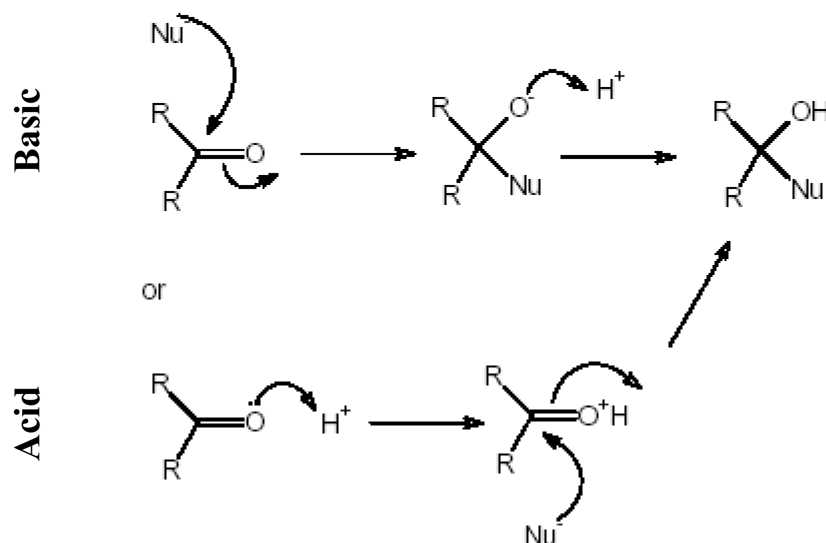


Figure 6.1. Nucleophilic addition under basic and acidic conditions.

In many articles the amino group and effective sugar concentration are considered to be equal to the total concentration. This assumption is more true for high pH values, but certainly not for neutral/acid conditions. As a consequence, pH is not often included in kinetic models. However, it is an important reaction parameter and its effect cannot be neglected.

The present study deals with the kinetic analysis of the reaction network proposed in the previous Chapter for the glucose/glycine degradation pathways (Scheme 6.1). Relevant kinetic parameters were estimated and the model was strained by changing the important reaction parameters, pH and reactants initial concentration. In spite of the complexity of the Maillard reaction, the previously developed multiresponse kinetic model allowed us to consider pH effects for individual steps in the reaction network.

6.2. Results and Discussion

Validation of the proposed kinetic model: influence of the reaction conditions

To strain the model proposed in Scheme 6.1 the reaction conditions should be altered. By changing important reaction parameters an idea is obtained how well the model performs and how accurate it is according to the established reaction mechanism.

6.2.1 - Influence of reactants initial concentration

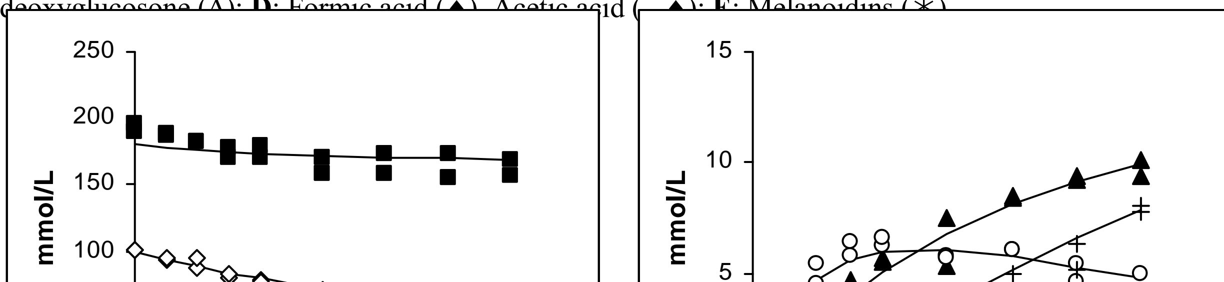
A series of reactions was investigated in which the reactants initial concentration was changed without altering the further reaction conditions. The equimolar system at 100°C and pH 6.8 was used as control and the concentration of glucose and glycine was changed to give molar ratios of 2:1 and 1:2, respectively. If the kinetic model is consistent, the reaction rate constants should be independent of the concentration of the reactants and therefore no significant difference should result from the estimated parameters. As observed in Table 6.1 the obtained results showed, as expected, some variation, but on the whole it could be concluded that the variation in the values remained in many cases within the 95% confidence intervals.

Table 6.1. Rate constants (*k*) estimation ± 95% HPD[a] interval at different initial concentration of the reactants.

k	**1:1**	**2:1**	**1:2**
1 ($l.mol^{-1}.min^{-1}$)	$1.1\cdot10^{-5} \pm 4\cdot10^{-6}$	$1.5\cdot10^{-5} \pm 1\cdot10^{-6}$	$1.4\cdot10^{-5} \pm 8\cdot10^{-7}$
2 (min^{-1})	$8.9\cdot10^{-4} \pm 4\cdot10^{-5}$	$1.2\cdot10^{-3} \pm 2\cdot10^{-4}$	$1.1\cdot10^{-3} \pm 2\cdot10^{-4}$
3 (min^{-1})	$5.9\cdot10^{-3} \pm 8\cdot10^{-4}$	$5.1\cdot10^{-3} \pm 3\cdot10^{-3}$	$3.5\cdot10^{-3} \pm 2\cdot10^{-3}$
4 (min^{-1})	$6.5\cdot10^{-3} \pm 3\cdot10^{-4}$	$8.0\cdot10^{-3} \pm 9\cdot10^{-4}$	$8.5\cdot10^{-3} \pm 9\cdot10^{-4}$
5 (min^{-1})	$4.1\cdot10^{-3} \pm 5\cdot10^{-4}$	$3.1\cdot10^{-3} \pm 1\cdot10^{-4}$	$2.9\cdot10^{-3} \pm 2\cdot10^{-4}$
6 (min^{-1})	$5.2\cdot10^{-3} \pm 2\cdot10^{-4}$	$5.3\cdot10^{-3} \pm 6\cdot10^{-4}$	$8.5\cdot10^{-3} \pm 6\cdot10^{-4}$
7 (min^{-1})	$1.3\cdot10^{-2} \pm 4\cdot10^{-4}$	$1.1\cdot10^{-2} \pm 1\cdot10^{-3}$	$1.3\cdot10^{-2} \pm 3\cdot10^{-3}$
8 (min^{-1})	$1.3\cdot10^{+0} \pm 2\cdot10^{-1}$	$9.3\cdot10^{-1} \pm 9\cdot10^{-2}$	$9.6\cdot10^{-1} \pm 9\cdot10^{-2}$
9 (min^{-1})	$9.1\cdot10^{-4} \pm 2\cdot10^{-5}$	$1.4\cdot10^{-3} \pm 3\cdot10^{-4}$	$1.0\cdot10^{-3} \pm 1\cdot10^{-4}$
10 (min^{-1})	$1.0\cdot10^{-4} \pm 5\cdot10^{-5}$	$1.0\cdot10^{-4} \pm 6\cdot10^{-5}$	$2.1\cdot10^{-4} \pm 5\cdot10^{-5}$

Samples heated at 100°C and reaction initial pH 6.8; [a]Highest Posterior Density.

Figure 6.2-A. Reactants molar ratio 2:1. Scheme 1 model fit (lines) to experimental data (dots) glucose/glycine aqueous system heated at 100°C and pH 6.8. **A**: Glucose (◊), Glycine (■); **B**: Fructose (▲), *N*-(1-deoxy-D-fructos-1-yl)-glycine (○), methylglyoxal (+); **C**: 1-deoxyglucosone (•), 3-deoxyglucosone (Δ); **D**: Formic acid (◆), Acetic acid (□); **E**: Melanoidins (✳).



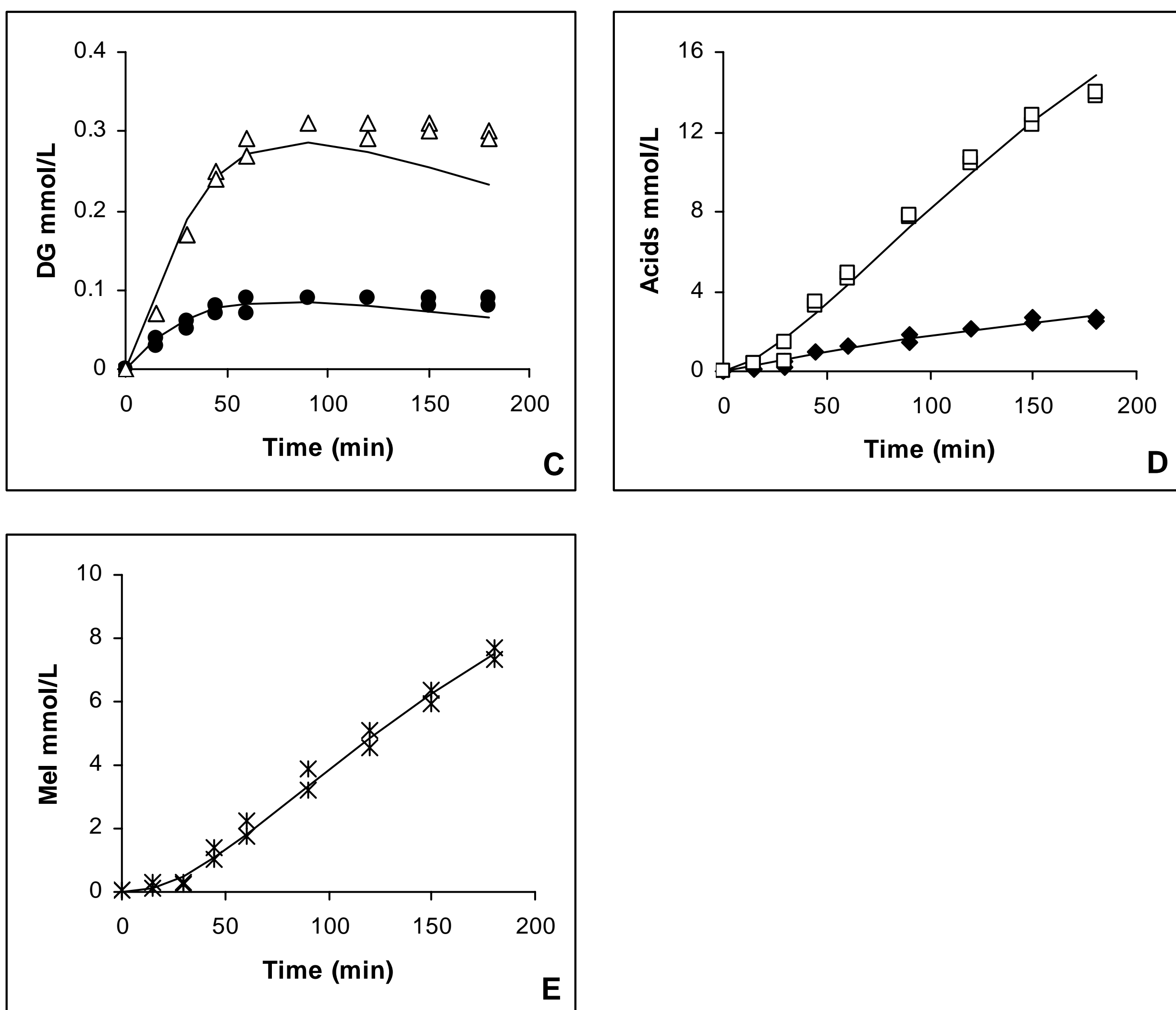


Figure 6.2-B. Reactants molar ratio 1:2. Scheme 1 model fit (lines) to experimental data (dots) of glucose/glycine aqueous system heated at 100°C and pH 6.8. **A**: Glucose (◊), Glycine (■); **B**: Fructose (▲), *N*-(1-deoxy-D-fructos-1-yl)-glycine (○), methylglyoxal (+); **C**: 1-deoxyglucosone (•), 3-deoxyglucosone (Δ); **D**: Formic acid (♦), Acetic acid (♦); **E**: Melanoidins (✳).

Also the resulting plots show that the model is consistent with changing the reactants initial concentration (Figure 6.2-A and -B). It is interesting to note that decreasing the concentration

of one of the reactants to half, the formation of DFG only reaches half of what was detected when the glucose:glycine ratio was 1:1 as observed in Chapter 5. Also, a small increase in the methylglyoxal formation occurred when the initial concentration of glycine was higher (Figure 6.2-B-B).

6.2.2 - Influence of the pH

To study the effect of pH on the glucose/glycine reaction kinetics the samples were heated at 100°C for up to 4 h at five different initial pHs (4.8, 5.5, 6.0, 6.8 and 7.5). As the initial pH increased the decrease of glucose concentration was faster (Figure 6.3). A maximum of 50 % degradation was observed at pH 7.5, whereas for the same heating time only 6% of glucose was degraded at pH 4.8. The sugar reactivity seems to be highly influenced by the reaction initial pH. In line with these findings is the fact that the higher the pH the higher the observed increase in the concentration of most of the detected intermediates (fructose, *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG), organic acids: formic and acetic acid, α-dicarbonyl compounds: 3-deoxyglucosone (3-DG), 1-deoxyglucosone (1-DG) and methylglyoxal (MG), and melanoidins). In contrast the formation of 5-hydroxymethylfurfural (HMF) increased with decreasing pH, however its amounts were one order of magnitude lower (i.e. in the micromolar range) than the other detected reaction products.

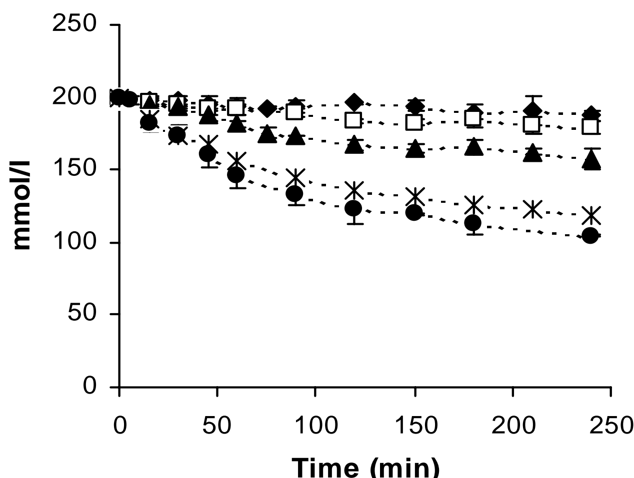
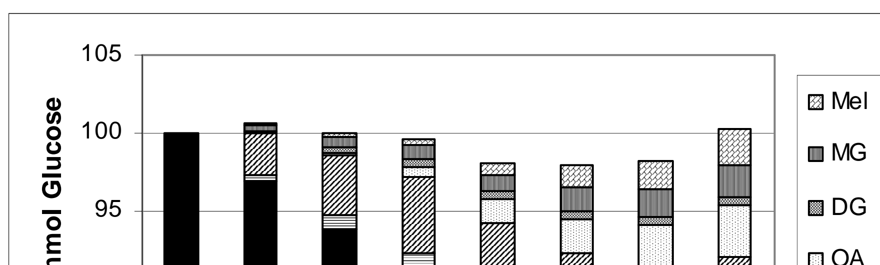


Figure 6.3. Glucose degradation during the Maillard reaction at 100°C and pH 4.8 (♦); pH 5.5 ( ); pH 6.0 (▲); pH 6.8 (∗) and pH 7.5 (•).

The results of the mass balance calculations showed that for pH ≤ 6.0 the main quantified compound was the Amadori compound (DFG).



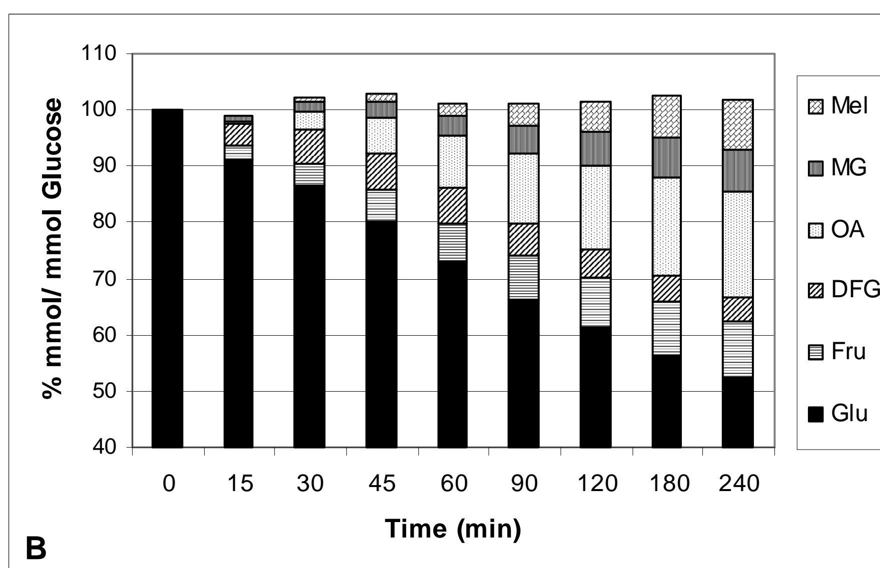


Figure 6.4. Mass Balance of reactants and reaction products in heated glucose-glycine system at 100°C and (**A**) **pH 6.0** and (**B**) **pH 7.5**. Glucose (Glu); Fructose (Fru); Amadori compound (DFG); sum of 1-deoxy and 3-deoxyglucosone (DG); sum of formic and acetic acid (OA); methylglyoxal (MG); melanoidins (Mel).

In Figure 6.4-A the results for pH 6.0 are shown. The gap observed between 45 min and 180 min suggested that other products, not identified in the present study, were also formed. However the fact that we came to 100% recovery eventually indicates that the acids formed are stable end products of scission reactions leading to C_1-C_5 reaction products. Moreover, under these reaction conditions (pH ≤ 6.0) the predominance of 3-DG over 1-DG was clear (Figure 6.5), which is also evident by the presence of a slightly higher amount of formic acid towards acetic acid in the beginning of the heating period at pH 5.5 (Figure 6.6).

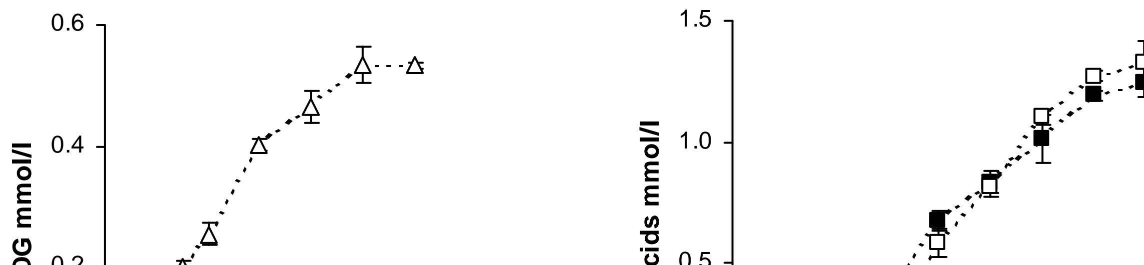


Figure 6.5. Deoxyglucosones (DG) formation at 100°C and pH 5.5. 3-deoxyglucosone (Δ); 1-deoxyglucosone (▲).

Figure 6.6. Organic acids formation at 100°C and pH 5.5. Formic acid (■); Acetic acid ( ).

However, when the pH was increased the opposite was observed. At pH 7.5 (Figure 6.4-B) from the nearly 50 % of degraded glucose, 20% represented both formic and acetic acid, from which only 6% resulted from formic acid. For reactions with initial pH > 6 acetic acid became the main end product formed. The importance of carboxylic acids formation was that they caused a considerable pH drop, slowing down the reaction. This phenomenon was particularly important at higher pH values (Figure 6.7). The same observation has been reported by Waller et al (*13*) when heating arginine and xylose systems with different initial pH values. Moreover, the sugar isomerisation step with the formation of fructose also became more important for higher pH values.

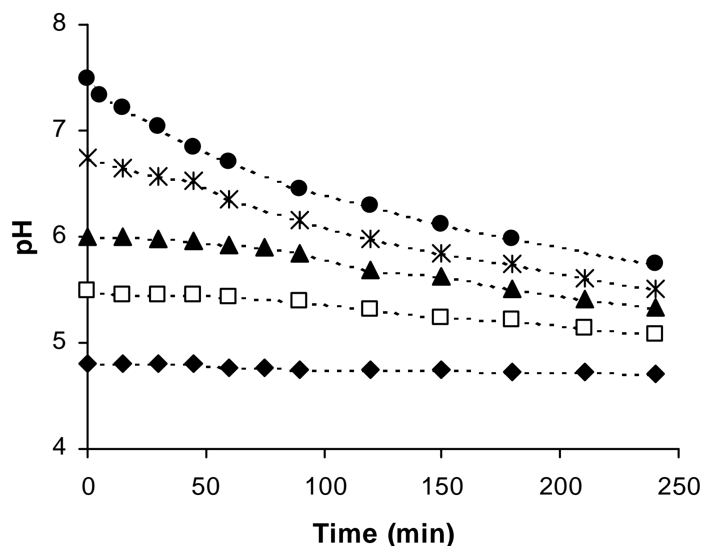


Figure 6.7. pH drop in glucose/glycine samples heated at 100°C and pH 4.8 (♦); pH 5.5 ( ); pH 6.0 (▲); pH 6.8 (✳) and pH 7.5 (•).

From the results it is clear that the reaction mechanism is highly influenced by the pH, with respect to which reaction route prevails and to the type of intermediates and end products that

are formed. If consistent pH dependence can be accounted for in a model, this is an additional indication that the model is acceptable. However, we do not know for every step where H^+ ions interfere. In the following section an attempt was made to determine the pH dependence of the overall glucose/glycine pathways (Scheme 6.1) based on a theoretical analysis.

6.2.3 - pH dependence

The dependence of reactivity of both principal reactants on pH explains qualitatively the dependence of the Maillard reaction on pH, as shown above. However, in order to be able to predict the reaction rates at various pHs, the pH dependence has to be determined in a quantitative way.

According to De Bruin (*14*) the reducing sugar (ring form) in solution is in equilibrium with its ionized form, endiol anion and isomers. From that study the pH dependence for the isomerisation of sugars in alkaline solution was deduced as follows:

$$[\mathrm{S}^-] = \frac{[\mathrm{OH}^-]}{\frac{k_w}{k_{as}} + [\mathrm{OH}^-]}[\mathrm{S_t}] \tag{6.5}$$

assuming that the ionization of the sugar $[S^-]$ in alkaline medium is fast with respect to subsequent endiol anion, the change in total sugar concentration $\frac{-\mathrm{dS_t}}{\mathrm{d}t} = \frac{-\mathrm{dS}^-}{\mathrm{d}t}$, and therefore

$$\frac{-\mathrm{d}[\mathrm{S_t}]}{\mathrm{d}t} = \mathrm{A} \times [\mathrm{S}^-] = \mathrm{A} \times \frac{[\mathrm{OH}^-]}{\frac{K_w}{K_{as}} + [\mathrm{OH}^-]}[\mathrm{S_t}] = k' \times [\mathrm{S_t}] \tag{6.6}$$

where the constant A comprises the elementary rate constants relating enolisation and isomerisation equilibrium, K_w is the water dissociation constant and K_{as} the sugar alkaline dissociation constant. The pseudo first order rate constant (k') includes the OH^- concentration and is dependent on the enolisation rate constant. Taking the same line of thinking, at acidic/neutral reaction conditions, we can define the sugar dissociation as:

$$K_{as} = \frac{[\mathrm{S}^-][\mathrm{H}^+]}{[\mathrm{SH}]} \tag{6.7}$$

where [SH] = $[S_t] - [S^-]$

$$[S^-] = \frac{K_{as}}{[H^+]}[SH] = \frac{K_{as}}{[H^+]}\left([S_t] - [S^-]\right) = \frac{K_{as}}{[H^+]}[S_t] \quad (6.8)$$

assuming that under acid/neutral conditions the ionization of the sugar $[S^-]$ <<< $[S_t]$. The combination of equation 6.6 with equation 6.8 results in:

$$\frac{-\,d[S_t]}{dt} = A \times \frac{K_{as}}{[H^+]}[S_t] = k' \times [S_t] \quad (6.9)$$

where the pseudo rate constant (*k*') includes the H^+ concentration and can be rewritten as:

$$k' = A \times \frac{K_{as}}{[H^+]} \quad (6.10)$$

From a theoretical point of view it thus follows that the value of the rate constant (*k*) for the sugar reaction is inversely proportional to $[H^+]$, that is, the higher the pH, the higher the value of *k*, since $[H^+] = 10^{-pH}$, and thus the log *k* should be proportional to the pH. Although we cannot prove it, it stands to a reason that similar pH dependence can be expected in the Maillard reaction. It was reported earlier that the Maillard reaction resembles alkaline sugar reactions to some extent (*15*). With respect to the proposed kinetic model, in principle it would be best to have a mechanistic base to explain the pH dependence, on which the model would be built. However, we do not know where and how the H^+ ions interfere in the Maillard reaction mechanism. Therefore, we took a pragmatic approach and we observed how rate constants were affected by the pH.

For each studied pH, the simplified model proposed for the glucose/glycine Maillard reaction (Scheme 6.1) was fitted. The method used is described in Chapter 3. Moreover, to avoid an interfering affect of reaction rate inhibition due to the pH drop occurring during the Maillard reaction (Figure 6.7), only data points were taken for which the pH drop was not higher than 1 unit. As shown in Table 6.2 the estimated rate constants, which now include the influence of the H^+ concentration and a pH drop ≤ 1 unit, increased with increasing the initial reaction pH.

Table 6.2. Rate constants (*k*) estimation by the simplified glucose/glycine kinetic model at different initial reaction pH.

k	pH 4.8	pH 5.5	pH 6.0	pH 6.8	pH 7.5
1 ($l.mol^{-1}.min^{-1}$)	7.9×10^{-7}	2.3×10^{-6}	4.9×10^{-6}	1.1×10^{-5}	1.8×10^{-5}
2 (min^{-1})	8.8×10^{-6}	5.5×10^{-5}	2.3×10^{-5}	8.9×10^{-4}	2.1×10^{-3}
3 (min^{-1})	0.00	0.00	2.8×10^{-3}	5.9×10^{-3}	1.4×10^{-2}
4 (min^{-1})	1.5×10^{-3}	3.1×10^{-3}	4.5×10^{-3}	6.5×10^{-3}	1.5×10^{-2}
5 (min^{-1})	0.00	6.3×10^{-3}	2.0×10^{-2}	4.1×10^{-3}	1.9×10^{-2}
6 (min^{-1})	5.0×10^{-4}	9.1×10^{-4}	2.7×10^{-3}	5.2×10^{-3}	1.1×10^{-2}
7 (min^{-1})	7.7×10^{-4}	4.0×10^{-4}	3.1×10^{-3}	1.3×10^{-2}	1.5×10^{-2}
8 (min^{-1})	9.9×10^{-2}	6.2×10^{-2}	1.0×10^{-1}	1.3×10^{0}	4.5×10^{-1}
9 (min^{-1})	5.0×10^{-5}	2.1×10^{-4}	2.4×10^{-4}	9.1×10^{-4}	5.3×10^{-4}
10 (min^{-1})	0.00	2.1×10^{-5}	6.3×10^{-5}	1.0×10^{-4}	3.1×10^{-4}

Samples heated at 100°C, pH allowed to drop; pH drop ≤ 1 unit.

By plotting the estimated k values versus the initial reaction pH, the pH dependence of k was determined to be a power function for most of the reaction steps. In Figure 6.8 the results for k_1 estimations are given as example.

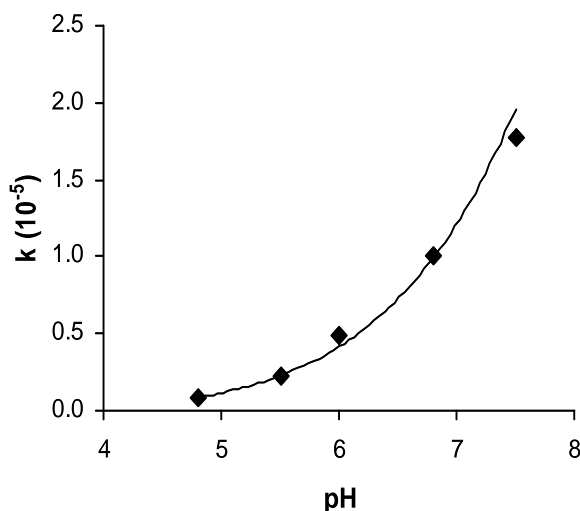


Figure 6.8. Power function trendline for k_1 pH dependence.

The pH dependence equation was determined as follows:

$$k = \beta \times \text{pH}^{\text{pD}} \tag{6.11}$$

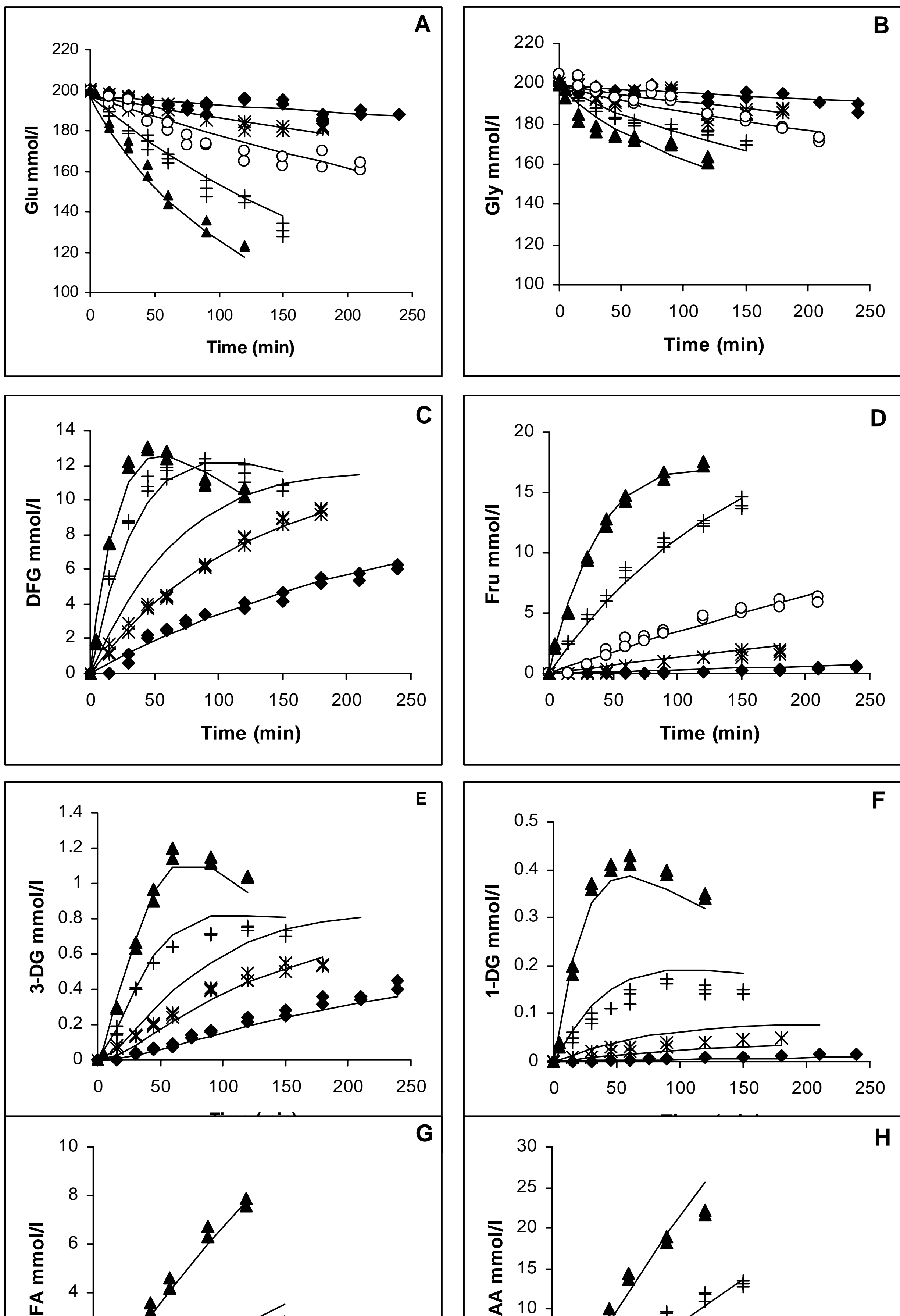
where β is the equation coefficient and pD is the pH dependence of *k*. The rate constant is inversely proportional to the concentration of H^+. The higher $[H^+]$ the lower is the value of *k*. This result is well in line with what has been deduced from a theoretical point of view for sugars reactions.

The used approach allowed us to find a relationship between *k* and pH, just like the Arrhenius equation describes the relation between *k* and *T* (temperature). Taking into account the determined pH dependence (Equation 6.11), the model presented in Scheme 6.1 was fitted to all the data simultaneously for all the reaction initial pH values studied. In Figure 6.9 the results of the fit are shown. As can be observed the deduced power law relation captured the pH dependence well. The estimates of the pH dependence (pD) and their 95%-confidence interval for each reaction step are shown in Table 6.3.

These results show that the sugar isomerisation and degradation steps 2, 3 and 10 are highly pH dependent, stronger than the initial Maillard reaction step 1. In fact, the sugar degradation pathway results preferably in formic acid as observed in the previous chapters. As a result a strong increase in formic acid formation was observed at pH 7.5 (Figure 6.9-G). Moreover, in the enolisation step of the Amadori compound (DFG), the 1,2-enaminol route with the formation of 3-deoxyglucosone (step 4) is less pH dependent than the 2,3-enaminol route (step 7). In line with what has been reported in earlier studies, 1-deoxyglucosone is favored at higher pHs (Figure 6.9-F). However, its degradation into acetic acid was determined not to be pH dependent (step 8). This suggests that 1-DG, which is a very reactive α-dicarbonyl compound in scission reactions leading to C_1-C_5 reaction products, leads to stable end products such as acetic acid. In Chapter 4, where DFG was heated alone at different reaction conditions, the lower amount of 1-DG detected relative to 3-DG was related with its higher reactivity. Moreover, 3-DG also shows low pH dependence in formic acid formation (step 5). Accordingly formic acid was detected in slightly higher amounts than acetic acid at pH 5.5 (Figure 6.6). However, the fact that formic acid was formed in such lower amounts, when the pH increased, suggests that at higher pH 3-DG degrades preferably into color formation by reaction with glycine (step 9) (Figure 6.9-J). Moreover, retroaldol reactions become more important at higher pH values, that is the formation of methylglyoxal from the Amadori compound (step 6). Also Huyghues-Despointes and Yaylayan (*12*) stated that under basic conditions ARP could generate methylglyoxal and other lower carbohydrate fragments, such as glyceraldehydes, in addition to free amino acid.

The model seems to perform reasonably well and is consistent with the chemistry behind the reaction scheme, discussed in the previous chapters. Moreover, the pH dependence of rate-

constants was successfully determined by a power law relation, which is in line with the theoretical analysis for sugar reactions.



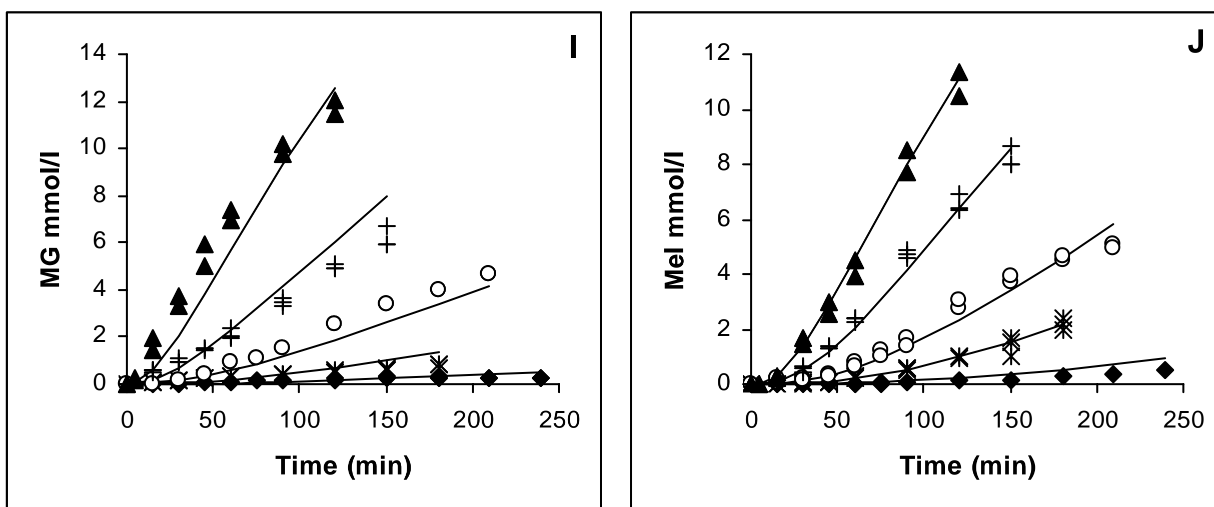


Figure 6.9. pH dependence included in Scheme 1. Model fit (lines) to experimental data (dots) of glucose/glycine aqueous system heated at 100°C and pH 4.8 (♦); pH 5.5 (✳); pH 6.0 (○); pH 6.8 (+) and pH 7.5 (▲). **A**: Glucose (Glu); **B**: Glycine (Gly); **C**: *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG); **D**: Fructose (Fru); **E**: 3-deoxyglycosone (1-DG); **F**: 1-deoxyglucosone (1-DG); **G**: Formic acid (FA); **H**: Acetic acid (AA); **I**: Methylglyoxal (MG); **J**: Melanoidins (Mel).

Table 6.3. pH dependence (pD) ± 95% HPD[a] interval as found by kinetic modelling.

k[b]	β[c]	pD ± HPD[a]
1	$4.6\cdot10^{-11} \pm 9\cdot10^{-12}$	6.4 ± 0.1
2	$3.2\cdot10^{-13} \pm 1\cdot10^{-13}$	11.3 ± 0.3
3	$5.1\cdot10^{-16}$ ± Indt.[d]	15.4 ± 0.04
4	5.3	3.8 ± 0.2
5	1.9	1.2 ± 1.1
6	2.4	6.4 ± 0.5
7	1.7	6.8 ± 0.4
8	5.2	0.00
9	2.6	3.9 ± 0.3
10	1.0	6.6 ± 0.04

[a]Highest Posterior Density; [b]Rate constant; [c]Power function coefficient; [d]Indeterminate.

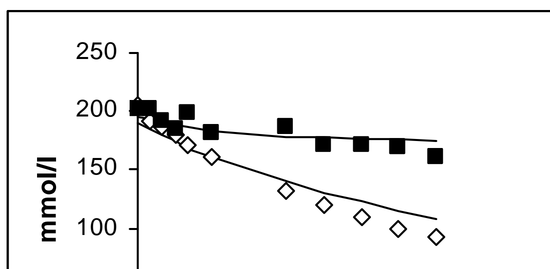
6.2.4 - pH kept constant

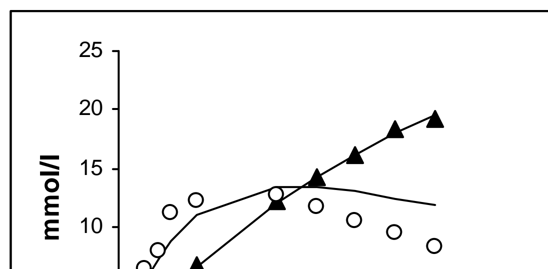
From the previous section it should be clear that the rate constants in the model include the observed pH drop (≤ 1 unit). In order to investigate the influence of that pH drop, an additional experiment was performed where the pH was kept constant during heating, using a pH Stat Controller. The used conditions were 100°C with an initial pH of 6.8 at room temperature. The results of the model fits to the experimental data are shown in Figure 6.10 and the estimated rate constants are presented in Table 6.4. In general the model performed reasonably well. When the pH was kept constant, most of the estimated rate constant did not change drastically when compared to the system where the pH was allowed to fall. This shows that, at least for pH drop ≤ 1 unit, the deduced pH dependence is quite accurate. The exception is for both formation and degradation of 3-DG into formic acid, steps 4 and 5 respectively, as well as for step 10, the degradation of glucose into organic acids and to a lesser extent step 3, the isomerisation of fructose into glucose. For these steps the pH drop is seen to have a considerable influence.

Table 6.4. Rate constants (*k*) estimation ± 95% HPD[a] intervals: pH allowed to fall vs. pH kept constant.

	pH drop ≤ 1	**pH constant**
1 ($l.mol^{-1}.min^{-1}$)	$1.1 \cdot 10^{-5} \pm 4 \cdot 10^{-6}$	$1.8 \cdot 10^{-5} \pm 1 \cdot 10^{-6}$
2 (min^{-1})	$8.9 \cdot 10^{-4} \pm 4 \cdot 10^{-5}$	$1.3 \cdot 10^{-3} \pm 1 \cdot 10^{-4}$
3 (min^{-1})	$5.9 \cdot 10^{-3} \pm 8 \cdot 10^{-4}$	$2.5 \cdot 10^{-3} \pm 1 \cdot 10^{-3}$
4 (min^{-1})	$6.5 \cdot 10^{-3} \pm 3 \cdot 10^{-4}$	$1.3 \cdot 10^{-2} \pm 9 \cdot 10^{-4}$
5 (min^{-1})	$4.1 \cdot 10^{-3} \pm 5 \cdot 10^{-4}$	$1.3 \cdot 10^{-1} \pm 1 \cdot 10^{-2}$
6 (min^{-1})	$5.2 \cdot 10^{-3} \pm 2 \cdot 10^{-4}$	n.a.
7 (min^{-1})	$1.3 \cdot 10^{-2} \pm 4 \cdot 10^{-4}$	$2.0 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$
8 (min^{-1})	$1.3 \cdot 10^{+0} \pm 2 \cdot 10^{-1}$	$8.7 \cdot 10^{-1} \pm 5 \cdot 10^{-2}$
9 (min^{-1})	$9.1 \cdot 10^{-4} \pm 2 \cdot 10^{-5}$	$7.8 \cdot 10^{-4} \pm 3 \cdot 10^{-5}$
10 (min^{-1})	$1.0 \cdot 10^{-4} \pm 5 \cdot 10^{-5}$	$5.7 \cdot 10^{-4} \pm 4 \cdot 10^{-5}$

Samples heated at 100°C, pH 6.8; [a]Highest Posterior Density. n.a. not analysed (formation of methylglyoxal).





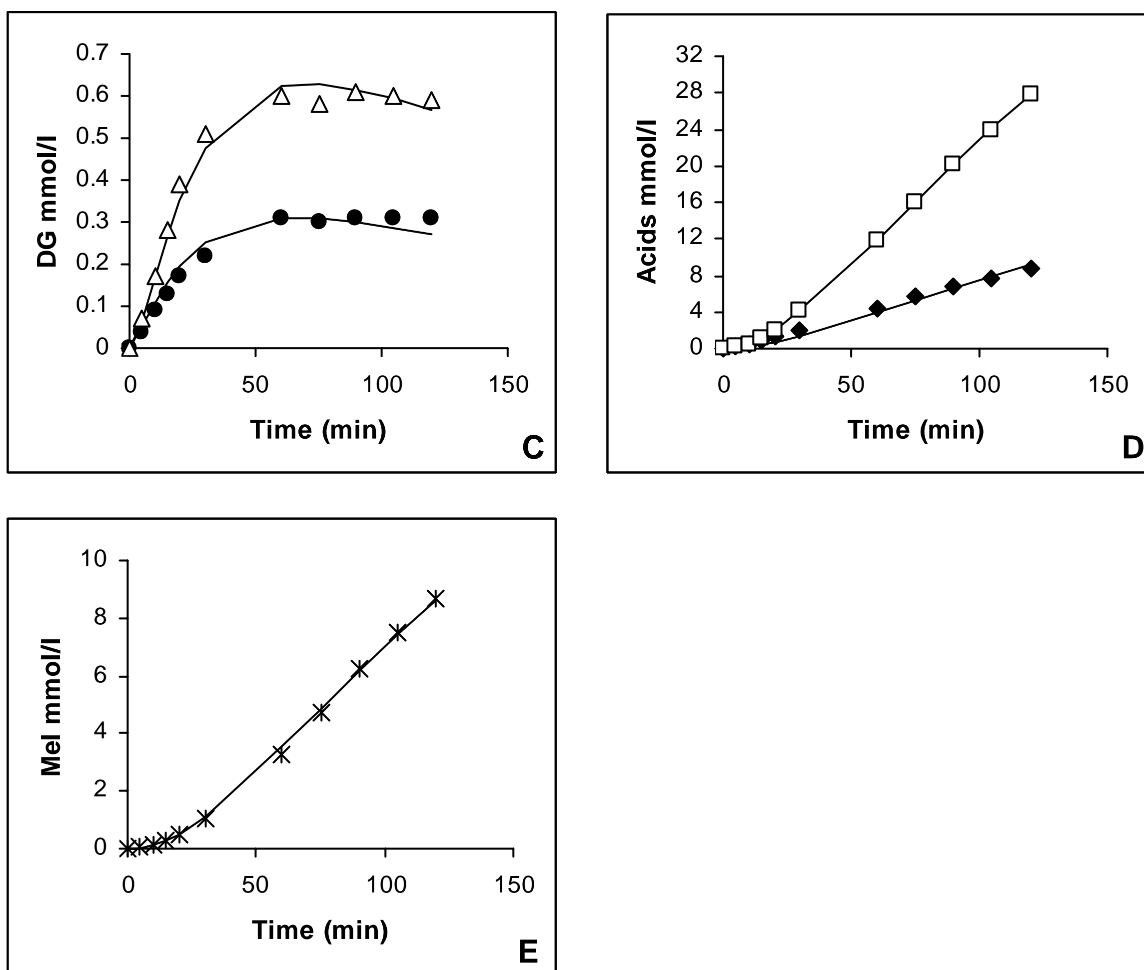
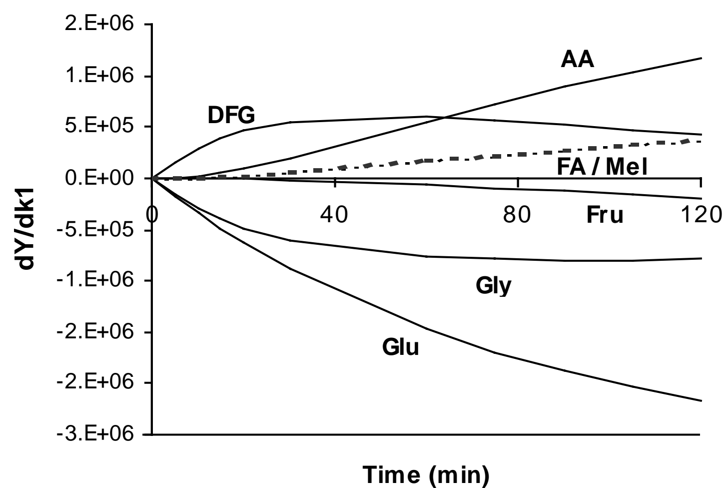
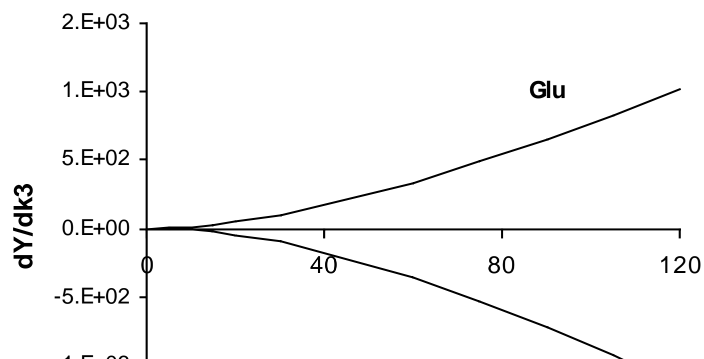


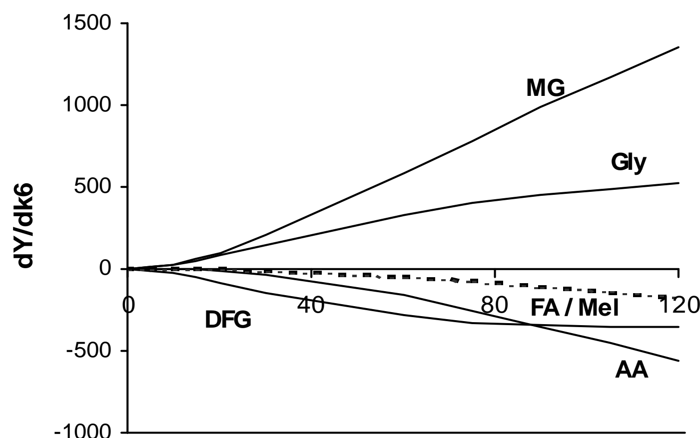
Figure 6.10. pH kept constant. Scheme 1 model fit (lines) to experimental data (dots) of glucose/glycine aqueous system heated at 100°C and pH 6.8. **A**: Glucose (◊), Glycine (■); **B**: Fructose (▲), *N*-(1-deoxy-D-fructos-1-yl)-glycine (○), methylglyoxal (+); **C**: 1-deoxyglucosone (•), 3-deoxyglucosone (Δ); **D**: Formic acid (♦), Acetic acid (♦); **E**: Melanoidins (✳).

6.2.5 - Sensitivity Analysis

Clearly, further experiments are needed to test this model for its validity. A useful aid in this respect is a sensitivity analysis, which shows the sensitivity of a response towards a parameter, expressed as the partial derivative of that response to a chosen parameter. Such an analysis gives an idea of which parameters are most influential and may help to determine unimportant reactions so that the model may be simplified. The same software package as used for the parameters estimation (Chapter 3) supplies this information. The results for k_1, k_3, k_6 and k_8 are shown in Figure 6.11. These steps were chosen, firstly to see the impact of k_1 on the overall mechanism, and secondly to determine the importance of certain steps which might cast some doubts, like the isomerisation of fructose into glucose, the retro-aldolisation of the Amadori compound, as well as the formation of acetic acid from 1-deoxyglucosone.







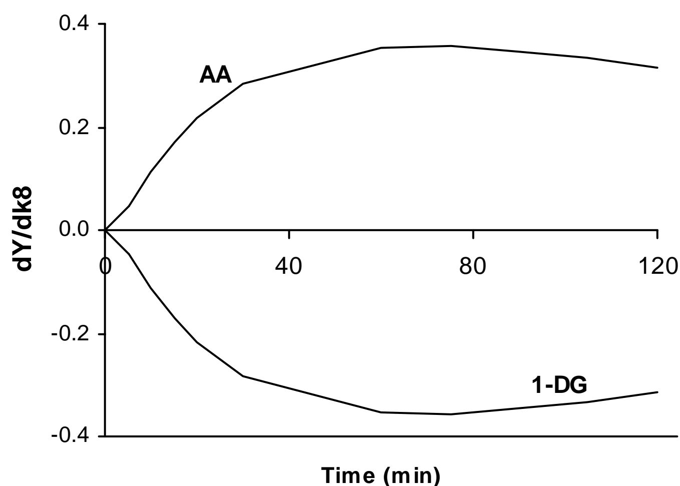


Figure 6.11. Sensitivity analysis of the responses depicted in Scheme 1 for rate constants k_1, k_3, k_6 and k_8. The sensitivity was zero for responses not shown. Glucose (Glu); glycine (Gly); fructose (Fru); *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG); acetic acid (AA); formic acid (FA); 1-deoxyglucosone (1-DG); 3-deoxyglucosone (3-DG) and Melanoidins (Mel).

As expected k_1 has a strong influence in the main products formed throughout the Maillard reaction. Moreover, together with k_4 and k_7 (DFG enolization step), the DFG retro-aldolisation step (k_6) appeared not to be a redundant parameter. Besides methylglyoxal formation it has a positive influence in glycine regeneration. Also, the isomerisation step of fructose into glucose (k_3) becomes more evident for longer heating periods, which may elucidate its dependence on the pH drop. In acetic acid formation, besides k_1, k_7 also shows a strong positive influence (results not shown), which explains the low sensitivity of acetic acid to k_8. This result is well in line with the observed low pH dependence of k_8. Moreover in Chapter 5, k_8 also showed low temperature dependence. It is suggested that 1-DG is indeed very reactive in the formation of scission products that lead to acetic acid formation, in agreement with the results of Chapter 4.

The limiting factor on the amount of acetic acid is the formation of 1-deoxyglucosone.

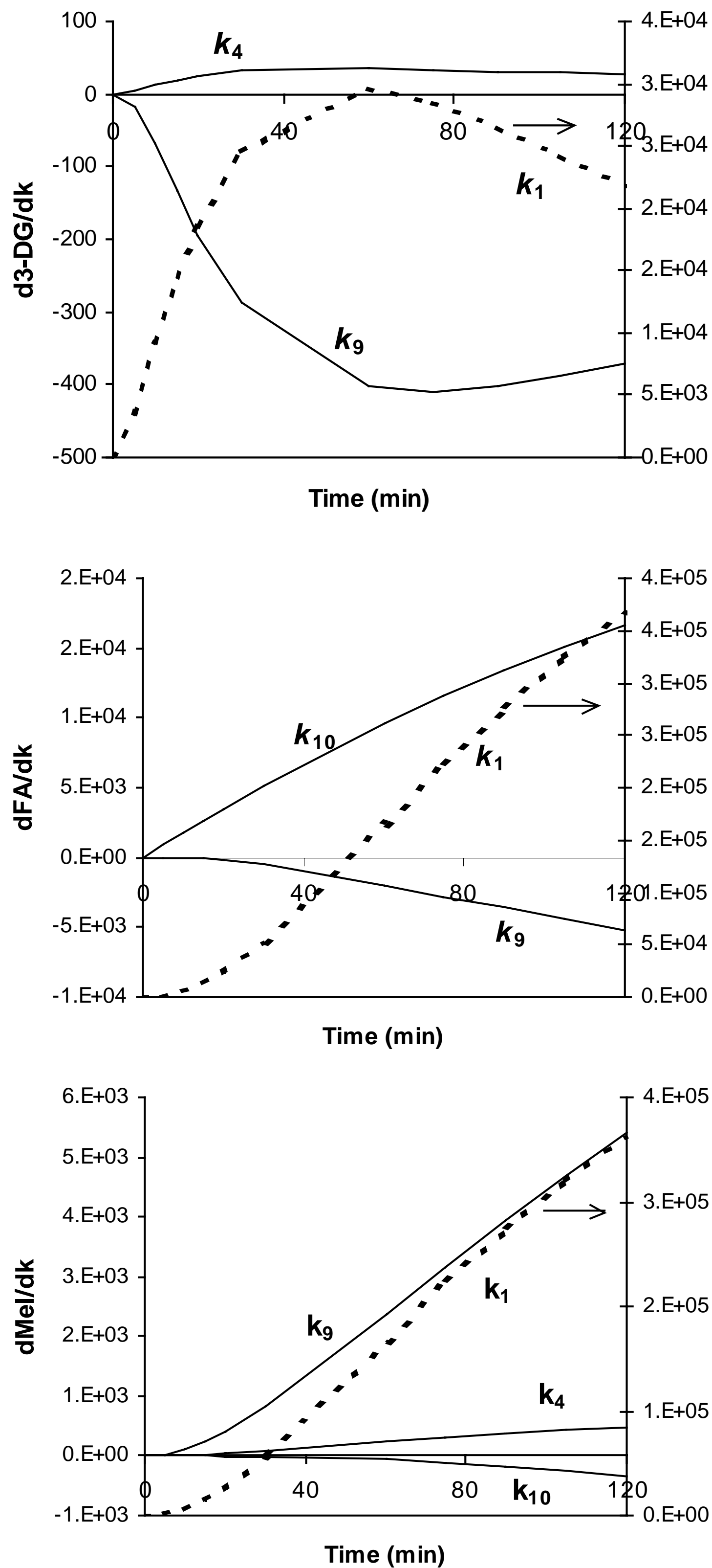


Figure 6.12. Sensitivity analysis of the responses 3-deoxyglucosone (3-DG), formic acid (FA) and melanoidins (Mel) for the rate constants depicted in Scheme 6.1. The sensitivity was zero for the rate constants not shown.

In Figure 6.12 the sensitivity analysis results are shown in

particular for 3-deoxyglucosone, formic acid and melanoidins formation. As mentioned before k_1 has a strong positive influence in all products formed through the Maillard reaction. Besides k_1, 3-DG formation and degradation is mainly influenced by k_4 and k_9, respectively. In line with this result is the negative influence of k_9 on formic acid formation, which supports what previously has been mentioned that 3-DG degrades preferably into melanoidins formation as the reaction proceeds. Also, the formation of formic acid is mainly affected by k_{10} and not by k_5, which suggests that k_5 might not be an important step in formic acid formation. Moreover, it was interesting to observe that more than the isomerisation step, the sugar degradation step into organic acids had a negative influence on the melanoidins formation. A more detailed study on the chemistry behind these pathways is still required.

6.3. Conclusions

The proposed model is able to deal with varying initial concentration of the reactants as well as varying the initial pH. For pH drops ≤ 1 unit, the pH dependence was well captured by a power law relation, which is in line with a theoretical analysis for sugar reactions. Moreover, the observed consistency for most of the estimated parameters when the pH was kept constant gives an additional indication that the deduced pH dependence is quite accurate. The sensitivity analysis suggested that the reaction governed by k_5 might be a redundant step. All in all, the model seems to perform well and is consistent with the established reaction mechanism. Multiresponse kinetic modelling has proved to be an extraordinary tool to unravel complex chemical reactions such as the Maillard reaction.

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Chapter 7

General Discussion

7. General Discussion

The main achievements of this thesis are summarized. It is discussed to what extent the objectives have been met and which questions still remain. An overview is given on how multiresponse kinetic modelling has become a powerful tool to unravel complex chemical reactions such as the Maillard reaction.

7.1. Introduction

The Maillard reaction is a cascade of consecutive and parallel reaction steps, the complexity of which has been illustrated. Cascades of reactions, in which several reactants and products take part, frequently occur in foods. This work shows that kinetic modelling of such reactions having parameters in common is much more powerful when using a multiresponse rather than a uniresponse approach (i.e. analyzing more than one reactant/product at the same time rather than only one reactant or product). With the multiresponse approach, kinetic models can be tested rigorously for their validity and the precision of the parameters estimated is much increased. Kinetic models describing the Maillard reaction in foods were proposed and multiresponse modelling was shown to give a better insight into and, indeed improve such kinetic models.

7.2. An overview of the main achievements

The Maillard reaction strongly affects food quality, which from a food technologist point of view means the control of chemical, physical and microbiological changes during processing and storage. The present thesis focused in particular on controlling chemical changes in the glucose/glycine Maillard reaction under conditions of food processing. The main quality attribute studied was colour.

- **Quantification of colour**

In the Maillard reaction, melanoidins (brown, nitrogenous polymers and co-polymers) are known as the main end product of the reaction. These brown polymers have a significant effect on the perception of quality of food, since colour is a key factor in consumer acceptance. The mechanism of formation of brown colour is not fully understood and the structure of the melanoidins is largely unknown. However, quantification is necessary when trying to predict or optimise browning from a known molecular composition in processed foods.

In **Chapter 3** a detailed explanation is given on the “Melanoidins extinction coefficient in the glucose/glycine Maillard reaction” which was determined using ^{14}C-labelled glucose. The benefit of this approach was that the molar extinction coefficient could be expressed in terms of the concentration of glucose molecules converted into melanoidins, even though the molecular weights of melanoidins are expected to span a very wide range of values and even

though their molecular structure as such is unknown. From the obtained results we concluded that the chromophores formed in the nondialysable melanoidins in the early stages of the reaction are similar to those at later stages and their formation does not appear to be sensitive to the tested reaction conditions, namely pH and temperature. Through these results we were able to translate the spectrophotometric data into concentrations of reacted sugars and to take browning quantatively into account in a kinetic model. This will allow a better, quantitative prediction for browning in model systems as well as in foods.

- **Quantification of important intermediates: Elucidation of the role of the Amadori Rearrangement Product (ARP)**

The chemistry underlying the Maillard reaction is very complex. It encompasses not one reaction pathway but a whole network of various reactions. In the first comprehensive reaction scheme of Hodge (Chapter 2) the ARP played a central role. It was the main intermediate formed in the early stage of the Maillard reaction from which all the flavour and colour compounds would be formed. However, because of the development of better analytical techniques, new important intermediates, not accounted for by Hodge, have been recognised and described by Tressl's scheme in Chapter 2. This can be seen as the modern revised scheme of Hodge. The main difference was that 3-deoxyglucosones were reported to be the most important intermediate in colour formation, formed through an intermediate prior to the ARP.
In the present thesis a major step was taken in identifying and quantifying 10 important intermediates in the glucose/glycine Maillard reaction: glucose, fructose, glycine, *N*-(1-deoxy-D-fructos-1-yl)-glycine, 1-deoxy-2,3-hexodiulose, 3-deoxy-2-hexosulose, methylglyoxal, formic acid, acetic acid, 5-(hydroxymethyl)furan-2-carboxaldehyde, and linking these intermediates quantitatively to each other by kinetic modelling.

In **Chapter 4** the main degradation pathways of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) are established. Significant results were obtained, in particular that ARP's undergo 1,2- or 2,3-enolizations while the amino moiety is still attached, and it leads to 3-deoxy-2-hexosulose and 1-deoxy-2,3-hexodiulose, respectively. Moreover, the Amadori product also undergoes retro-aldolisation with methylglyoxal formation and amino acid release. At this stage we were able to propose a kinetic model based on the established reaction network. The multiresponse modelling approach as used in this study appeared to be a helpful tool to unravel complicated reaction routes. Several models were tested and improved by confronting them with the data. Kinetic analysis showed that acetic acid, identified as a main end product in DFG thermal degradation, is mainly formed through 1-DG degradation. Also 3-DG was determined as a main precursor for carbohydrate fragments responsible for colour formation. As the

reaction proceeds other compounds besides DFG are suggested to become reactants themselves with the formation among others, of methylglyoxal.

- **Quantitative Kinetic Model**

A major achievement of this thesis is the establishment of a simplified kinetic model for the whole glucose/glycine Maillard reaction, as described in **Chapter 5**. The main conclusion is that DFG was most likely in equilibrium with an intermediate (Schiff's base, the cation form of the Schiff's base or the 1,2-enaminol) formed previously and responsible for the formation of 3-DG, the main colour precursor. This result is supported by the relatively low tendency for DFG to form brown colour under the same reaction conditions. However, due to its reactivity, this intermediate has not yet been quantified. Also, the postulated equilibrium allowed us to simplify the model where from a quantitative point of view the Amadori compound was the first intermediate to be formed in the glucose/glycine Maillard reaction. Quoting Einstein: *Models should be as simple as possible but no simpler than that*. The model has been simplified as much as possible but is still in line with the chemistry behind it.

The multiresponse kinetic analysis was shown to be both helpful in deriving relevant kinetic parameters as well as in obtaining insight into the reaction mechanism. It leads to more fundamental insight than simple kinetics. It is important to realize that multiresponse kinetics analysis, contrary to uniresponse kinetic analysis is based on the rate-determining steps of the reaction, regarding both the reaction mechanism and the number of steps involved. From the several models proposed, the most acceptable one, based on chemical and statistical criteria (goodness-of-fit and model discrimination techniques) was chosen. We would like to stress that scientific insight should be the first and the foremost discrimination tool in discussing model discrimination. Model discrimination is not about finding out whether or not the model is right or wrong, but rather about finding the best performing model, from a scientific and statistical point of view.

- **Straining of the Simplified Model**

An important step in modelling is to criticize a model to see how robust it is. If a model is only valid for the conditions on which it was built, its use is limited. If however, the model also holds for other conditions, it becomes much more useful. One can see this criticism, or straining of the model, as an attempt to falsify a hypothesis as propagated by Popper. If the model stands the test, it becomes more credible.

To strain the simplified kinetic model proposed for the glucose/glycine Maillard reaction pathways, important reaction conditions were altered: temperature, reactants initial concentration and pH. By changing important reaction conditions it was investigated how well the model performed and how accurate it was according to the established reaction mechanism. It was rewarding to see that the model was robust with respect to all the 3 variables studied. The estimated parameters showed Arrhenius-like temperature dependence. Moreover, for the first time the pH dependence has been established quantitatively in the Maillard reaction. For pH drops $\leq$ 1 unit, the pH dependence was well captured by a power law relation ($k = \beta\ pH^{pD}$), which is in line with a theoretical analysis for sugar reactions that resemble the Maillard reaction. Moreover, the observed consistency for most of the estimated parameters when the pH was kept constant gave an additional indication that the deduced pH dependence is quite accurate. The model seems to perform well and is consistent with the established reaction mechanism.

7.3. Has the objective been met?

Yes! A quantitative kinetic model is now available, able to predict main Maillard reaction intermediates as well as quality attributes such as colour. It has been well established for the glucose/glycine reaction and it will serve as basis for other reactants. Moreover, extensive use was made of the multiresponse modelling concept and it has proved to be very useful in: 1) building models; 2) giving mechanistic insight in combination with the chemistry behind it; 3) providing accurate estimates of the relevant kinetic parameters (rate constants, activation energies, pH dependence parameters).

A better insight into the Maillard reaction has been obtained and some of the controversial issues of the Maillard reaction have been answered, such as the Amadori compound reversibility within the Maillard reaction. Of course, several questions remain as yet unsolved. We have put emphasis on the main intermediates from a quantitative point of view, but did not pay much attention to other intermediates in low concentration. This is not to say that they are not important. The Strecker degradation, for instance plays a main role in the formation of flavour compounds which are very important. Even though they are present in low amounts some have low threshold, which determines the aroma and therefore the quality of food. An interesting compound is HMF, occurring in rather low concentration but frequently used as an indicator for the Maillard reaction. The question arises now if it is a good indicator.

The mechanism of formation of HMF has been reported and its main precursor is the 3-deoxyglucosone, which can either be formed through the Maillard reaction or from the degradation of the sugars. The very low amount of HMF detected in all studied systems in the

present thesis, could perhaps be explained by assuming that HMF is incorporated in the melanoidins, since the proposed model strongly suggests that 3-deoxyglucosones are the main precursor of melanoidins; but it could also be that HMF is formed in small amounts, i.e. a minor reaction path. Perhaps even more important is the fact that HMF is also formed from sugars in the absence of the Maillard reaction.

From the results presented in this thesis, we could put acetic acid forward as a main end product of the Maillard reaction (Chapter 4, 5 and 6). Its mechanism of formation was found to be highly dependent on the formation of its precursor, 1-deoxyglucosone. Contrary to 3-deoxyglucosone, 1-deoxyglucosone can only be formed through the degradation of the Amadori compound in the Maillard reaction. Moreover, the low concentration levels of 1-deoxyglucosone found in the studied systems were directly correlated with its reactivity in the formation of scission products such as acetic acid. The fact that we came to 100% recovery in the mass balance studies indicates that the acids formed are stable end products of scission reactions.

A suggestion is made here that for processed foods under conditions relevant for the Maillard reaction, the detection of acetic acid may be a better indicator of the extent of the Maillard reaction than compounds such as HMF.

7.4. Where to go from here?

Straining of the proposed model with respect to other reaction conditions: type of buffer and concentration; trace metals; effect of O_2/oxidation reactions; other reactants; synergy/antagonism between reactants.

Elucidate colour formation. In the proposed model colour formation was predicted in just one reaction step, where 3-deoxyglucosone was the main precursor. However, from a chemical point of view it is known that it is indeed a very complex step and other pathways may also be involved. More detailed information on this matter would allow to better test the robustness of the model in estimating colour formation.

Prediction of other food quality attributes for which the Maillard reaction is also responsible, as for instance flavour and mutagenicity. Kinetic modelling can be used to identify the main intermediates that are linked to flavour and mutagenicity (pyrazines, pyranones, heterocyclic amines, acrylamide etc.). These compounds can therefore be linked to each other and the mechanism of formation can be better understood.

7.5. Use of the developed model in food quality prediction

Food quality can be defined as the sum of the characteristics of a food that determine the satisfaction of the consumer and compliance to legal standards. Thus, food quality is a combination of numerous factors. The Maillard reaction happens to affect many of these, such as organoleptic properties (e.g. colour, flavour, texture), nutritional value (e.g. decrease of digestibility, destruction and/or biological inactivation of amino acids), safety (e.g formation of mutagenic and/or carcinogenic compounds).

Kinetic modelling proved to be a powerful tool to predict and control quality attributes associated with the Maillard reaction. To determine food quality, quality indicators are needed. The developed model can be a useful tool to get insights into the importance of the Maillard reaction intermediates as well as to optimise colour formation, based on quantitative predictions as function of concentration, temperature and pH (F(Conc, T, pH)). As a result, in food quality prediction the developed model is useful to:

- Predict main intermediates in the Maillard reaction as F(Conc, T, pH)
- Predict colour formation as F(Conc, T, pH)
- Give an insight in the Maillard reaction mechanism

Multiresponse kinetic modelling proved to be an important tool to predict chemical changes in foods and to gain insight into the reaction mechanism i.e. to determine important intermediates, possible quality indicators, and to understand how they are linked in the reaction network. Cascades of reactions, in which several reactants and products take part, frequently occur in foods. We conclude that multiresponse kinetic modelling goes beyond the Maillard reaction: it should also be applicable to other cascade reactions in foods.

SUMMARY

The present thesis entitled "Unravelling the Maillard reaction network by multiresponse kinetic modelling" was designed to increase the understanding of (a) the chemistry of the reaction and its influence on food properties and (b) how quality attributes associated with the Maillard reaction can be predicted and controlled by multiresponse modelling.

The Maillard reaction, also known as non-enzymatic browning, was first reported in 1912 by Louis-Camille Maillard. Ever since it has been a central and major challenge in food industry, since it plays an important role in food sensory properties. The Maillard reaction is related to aroma, taste and colour in processes as the roasting of the coffee and cocoa beans, the baking of bread and cakes, the toasting of cereals and the cooking of meat. Moreover, during the Maillard reaction a wide range of reaction products is formed with significant importance for the nutritional value of foods. This can be reduced by decrease of digestibility and possibly formation of toxic compounds, but can also be improved by the formation of antioxidative products.

The chemistry underlying the Maillard reaction is very complex. It encompasses not one reaction pathway but a whole network of various reactions. In essence, it states that in a early stage a reducing sugar, like glucose, condenses with a compound possessing a free amino group (of a protein or of an amino acid) to give a condensation product N-substituted glycosilamide, which rearranges to form the Amadori rearrangement product. Subsequently, a range of reactions takes place including cyclisations, dehydrations, retroaldolisations and further condensations, which ultimately in a final stage lead to the formation of brown nitrogenous polymers and co-polymers, known as melanoidins. The rate and the nature of the products formed are mainly determined by the reaction conditions. These include the chemical composition (nature of the reactants and type of buffer), the pH and water activity, the presence of oxygen and metals, the temperature-time combination during heating and the presence of reaction inhibitors, like sulphur dioxide.

The Maillard reaction is a cascade of consecutive and parallel reaction steps. The knowledge of kinetic parameters, such as rate constants and activation energy, is necessary to predict its extent and, consequently, to optimise it. When developing a kinetic model it is important to define accurately the system under study as well as to understand the reaction mechanism. The following steps should be taken into account:

(i) identification and quantification of the reactants and main products formed;
(ii) identification of reaction pathways based on reaction conditions;
(iii) differentiate between primary and secondary reaction routes;
(iv) propose a kinetic model based on the established reaction network;
(v) test the hypothesized mechanism;
(vi) estimate the rate constants.

Each of the chapters presented in this thesis can be seen as a necessary step to succeed in applying multiresponse kinetic modelling in a complex reaction, such as the Maillard reaction. In **Chapter 1** a general introduction was given followed by a more complete overview of the Maillard reaction in **Chapter 2**. At this stage the possible routes and the type of products that are formed according to the reaction conditions were summarized. This is an important step to establish which intermediates are of relevance when developing a kinetic model.

Chapter 3 presented the identification and quantification methods of the main products, from a quantitative point of view, formed in the glucose/glycine Maillard reaction. A specific technique was described for the carbohydrate degradation products, in particular dicarbonyls and hydroxycarbonyls (**Section 3.2**). From this study it was clear that C_3 compounds (methylglyoxal and acetol) were predominant and therefore should be considered as compounds involved in possible rate-determining steps in kinetic modelling. Moreover, in the Maillard reaction, melanoidins are believed to be the main end product of the reaction. However, the mechanism of their formation is not fully understood and the structure of the melanoidins is largely unknown, which makes it difficult to quantify these compounds. In **Section 3.3** a radiolabelling technique was described to determine melanoidins extinction coefficient (EC). The value of EC can be used to recalculate browning, measured as absorbance units, into melanoidins concentration. It was concluded that the EC did not vary within the pH and temperature range studied. Strong evidence was given that melanoidin chromophores formed in the early stages of the reaction are similar to those at the later stages and that their formation does not appear to be sensitive to the reactions conditions. From the elemental analysis, on the other hand, the melanoidins elementary composition seemed to be influenced by the reaction conditions. This seemed to be related to the presence of side-chains on the melanoidin backbone. Through these results we were able to take browning into account in kinetic modelling.

Due to the complexity of the Maillard reaction the intermediate stage was unravelled first. In **Chapter 4** the main thermal degradation pathways of *N*-(1-deoxy-d-fructos-1-yl)glycine (the Amadori compound of the glucose/glycine reaction) were established. Special attention was given to free amino acid, glycine, parent sugars, glucose and mannose, organic acids, formic and acetic acid and α-dicarbonyls, 1- and 3-deoxyosone together with methylglyoxal. It was concluded that independently of the pathway taken, enolization or retro-aldolization, *N*-(1-deoxy-d-fructos-1-yl)glycine degradation was accompanied by amino acid release. Together with glycine, acetic acid was the main end product formed. According with the kinetic analysis 1-deoxyosone was more reactive than 3-deoxyosone and its formation was favoured at higher pH values. Also, it was determined that 3-deoxyosone was a main precursor in carbohydrate fragments responsible for the colour formation. At this stage a kinetic model was suggested for the intermediate stage.

Based on the established network for the intermediate stage a complete kinetic model for the whole glucose/glycine reaction pathway was developed in **Chapter 5**. Relevant kinetic data were estimated and the temperature dependence of the rate constants determined. Also the significance of the Amadori compound reversibility was studied by model discrimination (Posterior probability and Akaike criterion). The results suggested that from a quantitative point of view the degradation path of *N*-(1-deoxy-d-fructos-1-yl)glycine into its parents, glucose and glycine, was not important. Moreover, as far as sugar kinetics was concerned, glucose isomerisation was one of the main degradation pathways.

In **Chapter 6** the reaction conditions of the glucose/glycine Maillard reaction were altered. By changing important reactions conditions it was investigated how well the model performed and how accurate it was according to the established reaction mechanism. The estimated rate constants showed no dependence on the reactants initial concentration or ratio. However, concerning pH, the reaction mechanism was highly influenced with respect to which route prevailed and to the type of products formed. For the first time consistent pH dependence was derived for the estimated rate constants. For a pH drop $\leq$ 1unit, the pH dependence was well captured by a power law relation, in line with a theoretical analysis. Moreover, a sensitivity analysis of the model was performed, which helped highlighting the important steps, as well as finding possible redundant steps. All in all the model seemed to perform well and was consistent with the established reaction mechanism.

Multiresponse kinetic modelling has proved to be a powerful tool in unravelling complex chemical reactions such as the Maillard reaction. More than just deriving relevant kinetic parameters, multiresponse modelling gave also insight into the reaction mechanism. In **Chapter 7** an overview is given of the main achievements and where to go from here. A main conclusion was that multireponse kinetic modelling goes beyond the Maillard reaction: it should also be applicable to other cascade reactions in foods.

SAMENVATTING

Dit proefschrift getiteld "Het ontrafelen van het Maillard reactienetwerk door middel van multirespons kinetisch modelleren" is bedoeld om inzicht te verschaffen in (a) de chemische reacties teweeg gebracht door de Maillard reactie en hun invloed op de eigenschappen van voedsel en (b) hoe met behulp van multirespons modelleren de met Maillard reactie samenhangende kwaliteitseigenschappen voorspeld en beheerst kunnen worden.

De Maillard reactie, ook bekend als het proces van niet-enzymatische bruinkleuring, is in 1912 voor het eerst ontdekt door Louis-Camille Maillard. Sindsdien vormt de reactie een van de grootste uitdagingen voor de voedseltechnologie omdat hij zeer belangrijk is voor alle waarneembare eigenschappen van voedsel. De reactie speelt heeft invloed op het aroma, de smaak en de kleur in processen als het roosteren van koffie- en cacaobonen, het bakken van brood en cake, het toasten van corn-flakes en het bereiden van vlees. Bovendien ontstaan tijdens de Maillard reactie een reeks reactieproducten die een belangrijke invloed hebben op de voedingswaarde van een voedingsmiddel. Zo kan het enerzijds minder makkelijk verteerbaar worden en kunnen er toxische componenten ontstaan terwijl anderzijds ook anti-oxidanten gevormd kunnen worden.

Het chemische proces van de Maillard reactie is zeer complex. Het bestaat niet uit één reactiepad maar uit een uitgebreid netwerk van verschillende reacties. In essentie gaat het in een vroeg stadium om een reducerende suiker, zoals glucose, die condenseert met een component waarbij een vrije aminogroep (van een eiwit of een aminozuur) hoort. Dat geeft het condensatieproduct N-gesubstitueerd glycosilamide, wat zich omgevormd wordt tot het Amadori product. Daarop volgen een aantal reacties waaronder cyclisaties, dehydrataties, retroaldolisaties en verdere condensaties die uiteindelijk leiden tot de vorming van bruine

stikstof-houdende polymeren en co-polymeren, de zogeheten melanoidinen. Het soort producten dat wordt gevormd en de snelheid waarmee dat gebeurt worden voornamelijk bepaald door condities waaronder de reactie plaatsvindt, zoals chemische samenstelling (type reactanten and het soort buffer), de pH en wateractiviteit, de aanwezigheid van zuurstof en metalen, de temperatuur-tijd combinatie gedurende het verhitten en de aanwezigheid van remmers, zoals sulfite.

De Maillard reactie is een complex gebeuren van opeenvolgende en parallelle reactie stappen. De kennis van kinetische parameters, zoals reactiesnelheidsconstanten en activerings energie is nodig om de effecten van de reactie te voorspellen en die vervolgens te optimaliseren. Bij het ontwikkelen van een kinetisch model is het belangrijk om het te bestuderen systeem nauwgezet te definiëren en het reactiemechanisme te begrijpen. Daarvoor moeten de volgende stappen genomen worden:

(i) identificatie en kwantificering van de ontstane reactanten en hoofdproducten;
(ii) identificatie van reactiepaden gebaseerd op reactiecondities;
(iii) differentiatie tussen primaire en secundaire reactiepaden;
(iv) opstellen van een kinetisch model gebaseerd op het vastgestelde reactienetwerk;
(v) testen van het veronderstelde mechanisme;
(vi) vaststellen van de reactiesnelheidsconstanten;

Elk hoofdstuk van dit proefschrift kan gezien worden als een noodzakelijke stap voor een geslaagde modelstudie met behulp van multirespons kinetiek, van een complexe reactie zoals die van Maillard.

Hoofdstuk 1 is een algemene inleiding gevolgd door **Hoofdstuk 2** met daarin een completer overzicht van de Maillard reactie. Hierin worden de mogelijke reactiepaden en producten bij verschillende reactiecondities samengevat. Dit is een belangrijke stap om vast te stellen welke tussenproducten relevant zijn bij het ontwikkelen van het kinetische model.

Hoofdstuk 3 beschrijft de identificatie en kwantificering methodes voor de belangrijkste producten, in termen van hoeveelheid, die gevormd worden bij de glucose/glycine Maillard reactie. Zo wordt in **Sectie 3.2** een dergelijke techniek beschreven voor koolhydraat afbraak degradatie producten, met name hydroxycarbonyl en dicarbonyl verbindingen. De conclusie was dat het toepassen van een geschikte bepalingsmethode de

mogelijkheid biedt om het ontstaan van tussenproducten van de reactie in de tijd te volgen. Gebleken is dat C_3 producten zoals methylglyoxal en acetol een belangrijke rol spelen.

Bovendien worden melanoidines verondersteld het belangrijkste eindproduct van de reactie te zijn. Toch is het mechanisme van hun ontstaan en hun structuur niet volledig bekend, waardoor het moeilijk is ze te kwantificeren. In **Sectie 3.3** wordt een radiolabelings techniek beschreven om de extinctiecoëfficiënt van de melanoidinen te bepalen. Deze coëfficiënt kan gebruikt worden om de bruine kleur, gemeten in absorptie eenheden bij een bepaalde golflengte, om te rekenen in melanoidinen gehalten. Het bleek dat de extinctiecoëfficiënt niet varieerde in het bestudeerde pH en temperatuur bereik. Er is een sterke aanwijzing dat de melanoidine chromophoren die in het begin van de reactie worden gevormd dezelfde zijn als die, in een later stadium ontstaan. Het blijkt dat dit niet gevoelig is voor de reactie condities. Uit elementaire analyse leek echter dat de elementaire samenstelling van de melanoidines wel beïnvloed werd door de reactie condities. Dit zou kunnen komen door vertakkingen van de meladoine hoofdketen. Deze resultaten stelden ons in staat om de bruinkleuring kwantitatief mee te nemen bij het kinetisch modelleren.

Vanwege de complexiteit van de Maillard reactie is eerst het tussenstadium uiteen gerafeld. In **Hoofdstuk 4** zijn de belangrijkste thermische degradatie paden van *N*-(1-deoxy-d-fructos-1-yl)glycine (de Amadori component van de glucose/glycine reactie) bepaald. Speciale aandacht kregen hierbij de vorming van het vrij aminozuur, glycine, de samenstellende suikers, glucose en mannose, organische zuren, mierezuur en azijnzuur en α-dicarbonyl, 1- and 3-deoxyosone verbindingen samen met methylglyoxal. Hieruit kon worden geconcludeerd dat onafhankelijk van het reactiepad, enolisatie of retro-aldolisatie, *N*-(1-deoxy-d-fructos-1-yl)glycine degradatie gepaard ging met het vrijkomen van aminozuur. Naast glycine was azijnzuur het belangrijkste gevormde eindproduct. Volgens de kinetische analyse was 1-deoxyosone reactiever dan 3-deoxyosone en ontstond het makkelijker bij hogere pH-waarden. Bovendien werd vastgesteld dat 3-deoxyosone de belangrijkste verbinding was waaruit koolhydraat fragmenten gevormd werden die op hun beurt de basis vormen voor de gekleurde melanoidinen. Gebaseerd op deze resultaten is een kinetisch model voorgesteld voor het tussenstadium van de reactie.

Op basis van het vastgestelde netwerk voor dit tussenstadium is in **Hoofdstuk 5** een compleet kinetisch model ontwikkeld voor de gehele glucose/glycine reactie. Relevante kinetische data en de temperatuurafhankelijkheid van de reactiesnelheidsconstanten werden

bepaald. Het belang van de omkeerbaarheid van de vorming van het Amdori product werd bestudeerd door middel van model discriminatie (m.b.v. Bayesiaanse statistiek, en het Akaike criterium). De resultaten gaven aan dat kwantitatief gezien het degradatie pad van *N*-(1-deoxy-d-fructos-1-yl)glycine naar de samenstellende componenten glucose en glycine, niet belangrijk was. Bovendien was wat betreft de suiker kinetiek de glucose isomerisatie een van de belangrijkste degradatie paden.

In **Hoofdstuk 6** zijn de belangrijkste reactie condities van de glucose/glycine Maillard reactie gewijzigd. Hierdoor is onderzocht hoe het model presteerde en hoe nauwkeurig het voldeed aan de bepaalde reactiemechanismen. De waargenomen reactiesnelheidsconstanten bleken onafhankelijk van de initiële concentraties en verhoudingen van reactanten. De pH beïnvloedde sterk welke route het reactiemechanisme nam en welk type producten gevormd werd. Voor het eerst is consistente pH afhankelijkheid van de vastgestelde reactiesnelheidsconstanten aangetoond. Bij een pH daling tot ≤ 1 eenheid, werd de pH goed beschreven door een machts functie, zoals ook theoretische analyse aangaf. Bovendien is een gevoeligheidsanalyse van het model uitgevoerd. Hierbij kwamen belangrijke stappen aan het licht, alsmede stappen die achterwege kunnen blijven. Het model bleek goed te funtioneren en was consistent met het vastgestelde reactiemechanisme.

Multirespons kinetische modellering is een zeer goed gereedschap gebleken voor het uiteenrafelen van complexe chemische reacties zoals de Maillard reactie. Behalve het afleiden van kinetische parameters, gaf multiresponse kinetische modellering ook inzicht in het reactie mechanisme zelf. In **Hoofdstuk 7** wordt een overzicht gegeven van de belangrijkste resultaten en hoe na deze studie vervolg zou kunnen woden. Een belangrijke conclusie is dat multirespons kinetisch modelleren verder gaat dan de Maillard reactie: het moet ook toepasbaar zijn bij andere complexe reacties in voedsel.

About the Author

Sara Isabel da Fonseca Selgas Martins was born in Lisbon, Portugal, on the 12th of December 1974. She received her high-school diploma in the area of exact sciences, field of chemistry, at *Escola Secundária de Benfica* (Lisbon) in 1992. In the same year she started her MSc in Agro-Industrial Engineering at the Technical University of Lisbon in *Instituto Superior de Agronomia,* from which she graduated in 1997. Her MSc thesis was done at the department of Agrotechnology and Food Sciences, Wageningen University, The Netherlands. In 1995 she worked in part-time in the analysis of financial viability of industrial projects in Caixa Geral de Depósitos, the Portuguese biggest financial institution. From January till October of 1998, Sara Martins worked as Product Developer at Iglo-Ola, Unilever, Portugal. In November of 1998 she started her Ph.D. at the Agrotechnology and Food Sciences Department, Wageningen University, with a 4-year Ph.D. fellowship from the Portuguese Foundation for Science and Technology. Within this period she has performed, among other things, two research periods of one month each at the department of Food Science and Technology, University of Munich, Germany and at the Procter Department of Food Science, Leeds University, United Kingdom, respectively.

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