Kinetic modelling of the Maillard reaction between proteins and sugars

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Kinetic modelling of the Maillard reaction between proteins and sugars

C. M. J. Brands

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op vrijdag 22 februari 2002
des namiddags om half 2 in de Aula.

ISBN: 90-5808-591-0

Stellingen

1. De uitspraak dat fructose vergeleken met glucose meer of minder reactief is in de Maillardreactie is alleen geldig als ook de daarbij behorende reactiecondities vermeld worden.
   Dit proefschrift

2. Het Heyns product, dat geacht wordt te ontstaan tijdens de reactie van ketose-suikers met aminogroepen (analoog aan de vorming van het Amadori product in de aldose-amino reactie), is geen essentieel intermediair van de Maillardreactie.
   Dit proefschrift

3. Kinetisch modelleren is *het* middel van de toekomst om veranderingen in levensmiddelen te voorspellen en de kwaliteit van producten te optimaliseren.
   Dit proefschrift

4. De schrijfwijze van het woord modelleren geeft al aan dat je een model niet moet eren maar ervan moet leren.

5. Voor een niet erg ervaren blindtypist is het woord complex niet eenvoudig.

6. Na de invoering van de 'Ja / Nee' sticker om de hoeveelheid papier te verminderen, is het aantal huis-aan-huis bladen significant toegenomen.

7. Wat je weet, dat zie je.

Stellingen behorende bij het proefschrift:
Kinetic modelling of the Maillard reaction between proteins and sugars
Carline Brands
Wageningen, 22 februari 2002

Brands, C. M. J. (2002). Kinetic modelling of the Maillard reaction between proteins and sugars. Ph.D. Thesis, Wageningen University, The Netherlands. 127 p.

Keywords: Maillard reaction, sugar isomerisation, kinetics, multiresponse modelling, brown colour formation, lysine damage, mutagenicity, casein, monosaccharides, disaccharides, aldoses, ketoses

The aim of this thesis was to determine the kinetics of the Maillard reaction between proteins and sugars, taking into account other simultaneously occurring sugar reactions. Model systems of foods, consisting of the protein casein and various sugars in a buffered solution, were studied. The reaction conditions were varied, covering relevant heating conditions for foods. The kinetics of the reactions were studied by means of multiresponse modelling.

The main reaction routes that were established in heated sugar-casein systems were (i) isomerisation of the aldose sugars into ketose sugars and vice versa, (ii) degradation of the sugar into carboxylic acids and unidentified products and (iii) the Maillard reaction between the sugar (degradation products) and the protein. In the Maillard reaction, the aldose sugars (like glucose and lactose) reacted with the ε-amino group of lysine residues of the protein to form the Amadori product. Under the conditions studied in this thesis (90-130°C; pH 6.8) it was shown that ketose sugars (like fructose and lactulose) themselves were not reactive in the Maillard reaction. Therefore, the expected Heyns compound could not be detected. The degradation products of the sugars and Amadori products reacted in the Maillard reaction with either the ε-amino group of the lysine residues or the guanidine groups of the arginine residues of the protein to form the brown-coloured melanoidins. The Maillard reactivity of the sugar degradation products was found to be much higher than that of the aldose sugars themselves. The formation of these reaction intermediates was, however, highly temperature dependent. The reaction mechanism of disaccharide sugars was roughly in accordance with that of monosaccharide sugars, with the difference that some sugar degradation pathways were hindered by the glycosidic bound sugar while others were favoured.

The multiresponse modelling approach as used in this thesis appeared to be a very powerful tool to unravel complicated reaction routes as occur in the Maillard reaction. The observed differences in reaction mechanism and reaction rate between the various studied sugars can be used to direct food quality aspects like browning, loss of nutritive value due to lysine damage and formation of mutagenic compounds.

1

General introduction

1.1 Introduction

Many foods are heated while they are processed. This causes many changes and has a large impact on quality. Quality can be defined as 'product performance that results in customer satisfaction and freedom from deficiencies, which avoids customers dissatisfaction' (Juran, 1990). Attributes that influence quality perception of foods involve product safety, nutritional value, sensory properties (taste, flavour, texture and appearance), shelf life, convenience and product integrity. A reaction that is of utmost importance during heating and storage of foods because of its contribution to food quality is the Maillard reaction. The reaction products are significant in foods since they are responsible for the development of flavour and colour, which may be desirable or undesirable, depending on the type of food. The Maillard reaction can also result in loss of nutritive value, the formation of mutagenic compounds and the development of compounds with antioxidant properties (Ames, 1992).

The Maillard reaction is a very complex reaction. To make it even more complex, the reducing sugars that react in the Maillard reaction are also subject to isomerisation and degradation reactions. These reactions affect in turn the Maillard reaction. In order to be able to control the Maillard reaction, and therefore partly the quality of foods, it is necessary to study the reactions of interest quantitatively. Since these reactions are not simple, but interrelated and complex, kinetic modelling is necessary (Van Boekel, 2001).

1.2 The Maillard reaction

The Maillard reaction is a type of non-enzymatic browning which involves the reaction of carbonyl compounds, especially reducing sugars, with compounds that possess a free amino group, such as amino acids and proteins. The first step of the reaction is followed by a cascade of reactions, which make the Maillard reaction a complex network of chemical reactions. The Maillard reaction is traditionally divided into three stages: the early, advanced and final Maillard reaction (Mauron, 1981).

The scheme of the Maillard reaction as proposed by Hodge in 1953 (Figure 1.1) is still widely used. In essence, it states that in the early stage of the Maillard reaction, an aldose sugar, like glucose, condenses with a compound possessing a free amino group to give an N-substituted glycosylamine (A). This condensation product rearranges (B) to form the so-called Amadori product (1-amino-1-deoxy-2-ketose). In many foods, the ε-amino groups of the lysine residues of proteins are the most important source of reactive amino groups. Due to blockage in the Amadori product, these lysine residues are no longer available for digestion

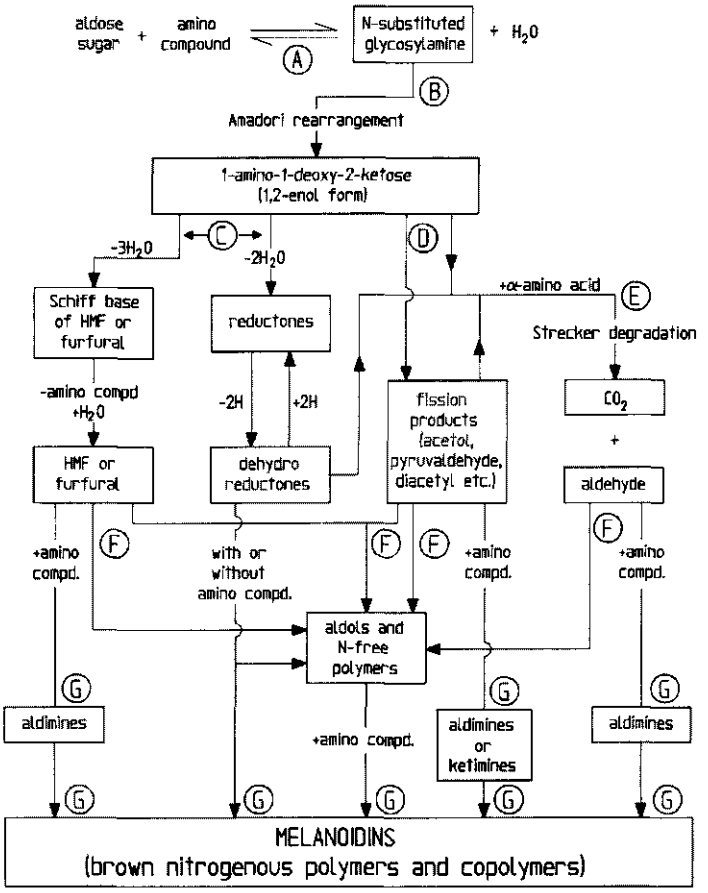

Figure 1.1 Scheme of Maillard reaction pathways according to Hodge (1953).

and consequently the nutritive value decreases. In the advanced stage of the Maillard reaction, the Amadori compound is broken down (C and D). The degradation pathway of the Amadori product is dependent on the pH of the system. At low pH, it undergoes mainly 1,2-enolisation with the formation of 5-hydroxymethylfurfural (HMF). At higher pH, the degradation of Amadori compound is thought to involve mainly 2,3-enolisation, where a variety of fission products, such as short chain carbonyls, dicarbonyls, and reductones are formed. The reaction products include several flavour compounds. Another pathway occurring in the advanced stage of the Maillard reaction is the Strecker degradation (E), which involves the reaction of α-amino acids (but not with proteins) with α-dicarbonyl compounds to yield aldehydes and carbon dioxide. In the final stage of the Maillard reaction, reaction products condense with

amino compounds (G) and form high-molecular weight compounds, the melanoidins, which are mainly responsible for the brown colour. In this stage also, proteins are crosslinked to a considerable degree, which has an impact on structure and functional properties of proteins.

According to the scheme of Hodge, the key step in the early Maillard reaction is the Amadori rearrangement, a step which involves the transition from an aldose to a ketose sugar derivative. It is supposed that in the reaction between ketoses (fructose) and amino groups the formation of ketosylamines is followed by the Heyns rearrangement to form 2-amino-2-deoxyaldoses (Reynolds, 1965).

More recently, Tressl et al. (1994) have given a new perspective to the Maillard reaction mechanism (Figure 1.2). They proposed a mechanism which involves reaction pathways in which not the Amadori compound, but the deoxyhexosuloses (3-deoxyaldoketose, 1-deoxy-2,3-diketose and 4-deoxy-2,3-diketose) are the key intermediates. The reaction of the sugar via the 3-deoxyaldoketose route is favoured at lower pH and leads to formation of brown coloured compounds. At higher pH the 1-deoxy-2,3-diketose and 4-deoxy-2,3-diketose reaction routes are favoured, which lead mainly to flavour formation.

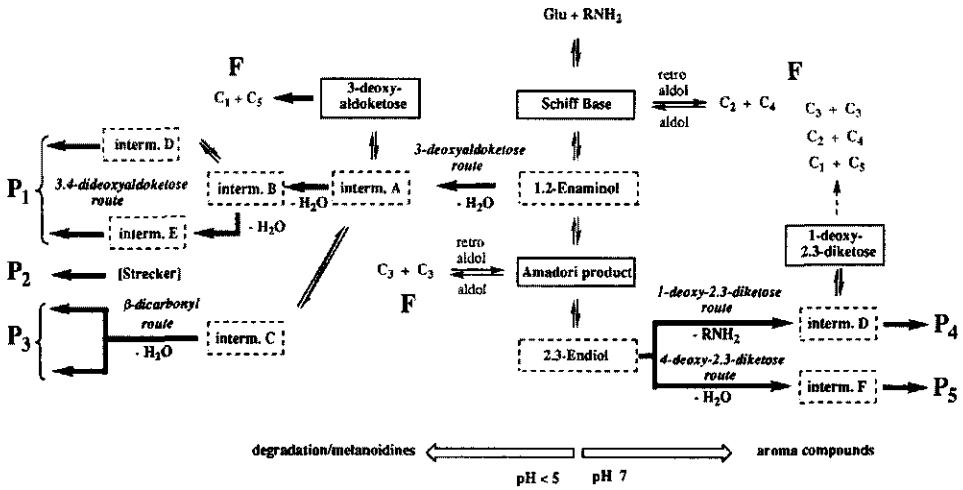

Figure 1.2 Scheme of Maillard reaction pathways according to Tressl et al. (1994).

To facilitate the study of the complex pathways of the Maillard reaction Yaylayan (1997) developed a conceptual representation of the processes occurring during the reaction (Figure 1.3). Propagation of the Maillard reaction was described by the formation and interaction of

the so-called chemical pools generated from specific precursors. The pools that are formed from the decomposition of the sugar, amino acid and Amadori or Heyns products are termed primary fragmentation pools. Further reactions among the populations of these pools lead to interaction pools. These interaction pools generate high and low molecular weight end products. This classification system could also be used to consolidate and categorise the scattered information available in the literature.

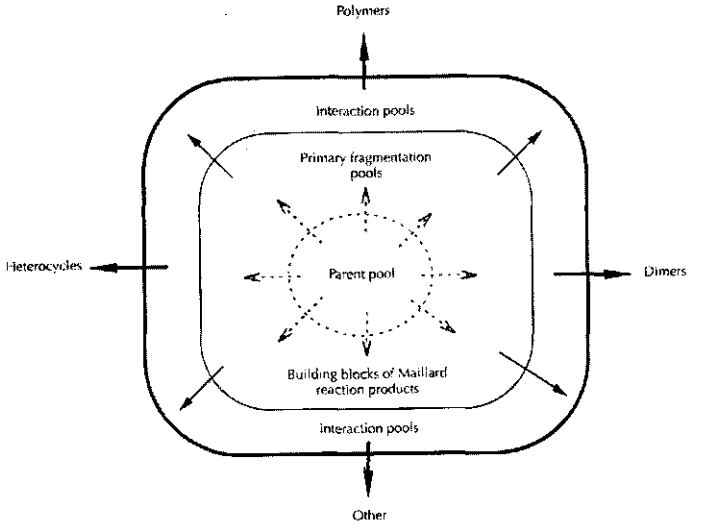

Figure 1.3 Conceptual representation of the Maillard reaction according to Yaylayan (1997).

1.3 Sugar degradation

Another type of non-enzymatic browning occurring during heating is caramelisation, a complex process in which sugar reaction products condense and form brown coloured macromolecules. Although the Maillard reaction as a sugar-amine reaction should be distinguished from the caramelisation reaction occurring when pure sugars are heated, similar reactions can be observed. Many chemical reactions that occur in pure sugars only at very high temperature or strongly alkaline conditions take place at much lower temperature and neutral pH once they have reacted with amino acids. The transformation of an aldose into a ketose via the formation of the N-glycoside during the Maillard reaction is analogous to the Lobry de Bruyn-Alberda van Ekenstein transformation. This isomerisation via the enolisation reaction, which is a reversible reaction, is accompanied by an irreversible transformation of

the sugars into carboxylic acids, generally known as the alkaline degradation reaction (Figure 1.4) (De Bruijn, 1986).

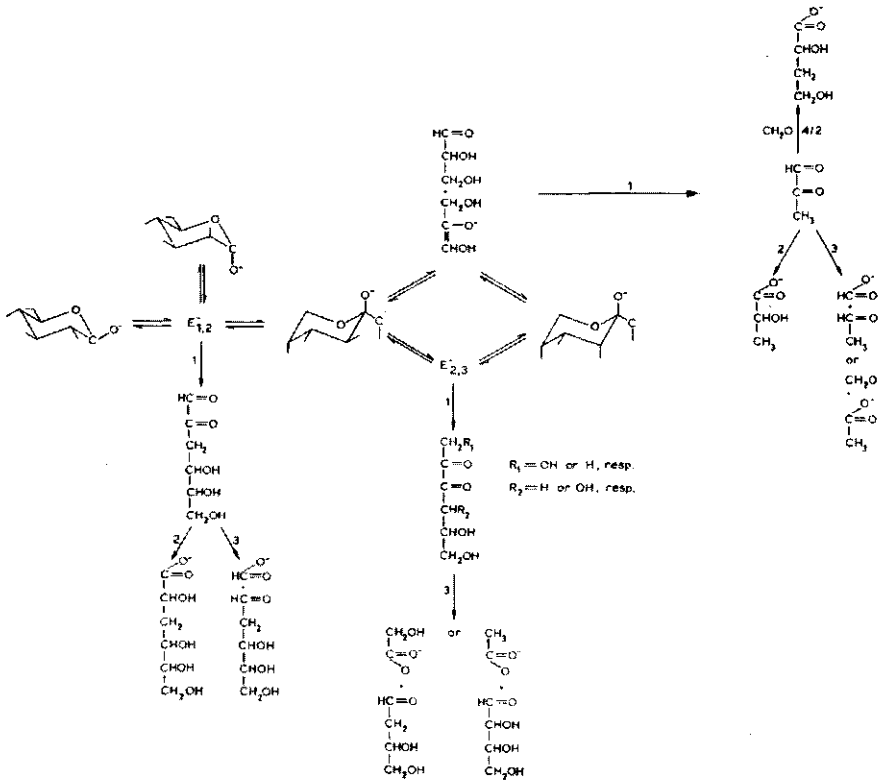

Figure 1.4 Reaction scheme of the alkaline degradation reaction (1=β-elimination; 2=benzilic acid rearrangement; 3=α-dicarbonyl cleavage; 4=aldolisation) according to De Bruijn (1986).

1.4 Kinetic modelling

Since the sugar reactions occur simultaneously with the Maillard reaction and the sugar reaction products take subsequently part in the advanced Maillard reaction, the Maillard reaction becomes very intricate. In order to be able to control the Maillard reaction, and thereby the quality of foods, it is necessary to study the reactions of interest quantitatively as a function of time and temperature.

Trying to describe the changes of a compound (either a reactant or a reaction product) in time with zero-, first- or second-order reactions is too simplistic. The observed reaction rate

constant will reflect a mixture of many elementary rate constants. This simple kinetics approach is only a mathematical fit procedure and does not give any mechanistic insight. For kinetic modelling of complex reactions, a new approach was therefore introduced, called multiresponse modelling. The basic idea is to take into account as many responses as possible at once, as opposed to only one response. By applying multiresponse modelling, more realistic models and more accurate parameter estimates (namely of rate constants and activation energies) will be obtained and this ultimately means better control of food quality (Van Boekel, 1998).

1.5 Aim and outline of thesis

The aim of this thesis was to determine the kinetics of the Maillard reaction between proteins and sugars, taking into account other simultaneously occurring sugar reactions. Because foods may contain various sugars, several sugars were compared in their reaction behaviour. Proteins are usually the most important source of reactive amino groups in foods. Caseins were studied in this thesis because they do not denature and do not cause problems with precipitation during prolonged heating. The temperature range chosen was from 90-130°C, thus covering relevant heating regimes for foods.

In *Chapter 2* of this thesis, the identification and quantification of the main reaction products that were formed during heating of monosaccharide-casein model systems is described, as well as the main reaction pathways that were established. Furthermore, a model for the reaction mechanism is proposed. Since no conventional techniques were available, a relatively new technique was used to quantify the brown-coloured melanoidins. The results of this study are reported in *Chapter 3*. The purpose of *Chapter 4* was to study the kinetics of the reactions occurring during heating of monosaccharide-casein systems, starting with the kinetic model developed in the second chapter and using the multiresponse approach. The effect of heating temperature, pH, reactant concentration and type of sugar were studied and the results were used to test the kinetic model very rigorously. In *Chapter 5* a kinetic model for reactions in disaccharide-casein systems is proposed and tested. In the Maillard reaction mutagenic compounds might be formed. *Chapter 6* reports a study on the mutagenicity of heated sugar-casein model systems. In *Chapter 7* the results of this thesis and their significance for food quality will be discussed in a general discussion.

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2

Reactions of monosaccharides during heating of sugar-casein systems: building of a reaction network model

Abstract

The Maillard reaction is important during heating and processing of foods for its contribution to food quality. To control a reaction as complex as the Maillard reaction it is necessary to study the reactions of interest quantitatively. In this paper the main reaction products in monosaccharide-casein systems, which were heated at 120°C and neutral pH, were identified and quantified, and the reaction pathways were established. The main reaction routes were (i) sugar isomerisation, (ii) degradation of the sugar into carboxylic acids and (iii) the Maillard reaction itself, in which the sugar itself but also its reaction products react with the ε-amino group of lysine residues of the protein. Significant differences in reaction mechanism between aldose and ketose sugars were observed. Ketoses seemed to be more reactive in the sugar degradation reactions than their aldose isomers, and whereas the Amadori product was detected as Maillard reaction intermediate in the aldose-casein system, no such intermediate could be found in the ketose-casein system. The reaction pathways found were put together into a reaction network model, which will be evaluated by kinetic modeling in a subsequent paper.

Carline M. J. Brands and Martinus A. J. S. van Boekel. *Journal of Agricultural and Food Chemistry* **2001**, *49*, 4667-4675.

2.1 Introduction

Heating is a frequently used process in the food industry to obtain safe products with a prolonged shelf life. Heat is also used to improve the sensory properties of food. However, it may also cause changes that decrease food quality. Many desired but also undesired effects of heating are due to the Maillard reaction.

The Maillard reaction is a type of non-enzymatic browning which involves the reaction of carbonyl compounds, especially reducing sugars, with compounds that possess a free amino group, such as amino acids and proteins. The reaction products are significant in foods since they are responsible for flavour and colour, which may be desirable or undesirable depending on the type of food. The Maillard reaction can also result in nutritional damage, the development of components with antioxidant properties (Ames, 1992) and in the formation of mutagenic and antimutagenic compounds (Brands et al., 2000). The Maillard reaction is actually a complex network of chemical reactions, which is traditionally divided into three stages: the early, advanced and final Maillard reaction. The early Maillard reaction between an aldose sugar and an amino group leads to the formation of the Amadori product as a relatively stable intermediate. The Heyns compound is supposed to be formed as the analogous compound when a ketose sugar is the starting sugar. In many foods, the ε-amino groups of the lysine residues of proteins are the most important source of reactive amino groups. Due to blockage in the Amadori product, these lysine residues are no longer available for digestion and consequently the nutritive value decreases. In the advanced Maillard reaction the Amadori product is broken down into numerous fission products. In this stage flavour compounds are formed. In the final stage of the Maillard reaction, reaction products condense with amino compounds and form high-molecular weight compounds, the melanoidins, which are mainly responsible for the brown colour (Mauron, 1981). In this stage also, proteins are crosslinked to a considerable degree (Pellegrino et al., 1999).

Another type of non-enzymatic browning occurring during heating is caramelisation, a complex process in which sugar reaction products condense and form brown coloured macromolecules (without nitrogen). This caramelisation reaction is preceded by sugar isomerisation and sugar degradation reactions. Monosaccharides in aqueous alkaline medium undergo both reversible and irreversible transformations (De Bruijn et al., 1986). The reversible reactions include (i) ionisation, resulting in an equilibrium of neutral and ionised monosaccharides, (ii) mutarotation, resulting in an equilibrium of the different cyclic hemi-acetal structures of monosaccharides and (iii) enolisation, resulting in the transformation of

interconvertible monosaccharides. The isomerisation via the enolisation reaction is known as the 'Lobry de Bruyn-Alberda van Ekenstein transformation' (Speck, 1958) and is accompanied by (iv) irreversible transformation of the monosaccharides into carboxylic acids, generally known as the alkaline degradation reaction. According to Berg and Van Boekel (1994), the degradation products of lactose found in heated milk appeared to be mostly the same as those mentioned in the degradation route of monosaccharides in alkaline medium. This means that the described sugar reactions not only occur in alkaline medium but also take place at neutral pH (the pH of milk is approximately 6.7).

Sugar isomerisation and degradation reactions were reported to be much more important from a quantitative point of view than the Maillard reaction (Berg and Van Boekel, 1994; Van Boekel, 1996). Since these sugar reactions occur simultaneously with the Maillard reaction and the sugar reaction products take subsequently part in the Maillard reaction, the Maillard reaction becomes even more intricate. In order to be able to control the Maillard reaction it is necessary to study the reactions of interest quantitatively.

The objective of this paper was to identify and quantify the main reaction products in heated monosaccharide-casein systems and to establish the main reaction pathways. Glucose and galactose (aldose sugars) and fructose and tagatose (ketose sugars) were the studied monosaccharides. In this paper a model for the reaction mechanism will be proposed, amenable to quantitative purposes. A subsequent paper will deal with the kinetic analysis of the reactions.

2.2 Materials and methods

2.2.1 Chemicals

All chemicals were of analytical grade. Glucose, fructose and galactose were supplied by Merck (Darmstadt, Germany). Fluka Chemie (Buchs, Switzerland) supplied tagatose. Sodium caseinate (a spray-dried powder) was obtained from DMV (Veghel, the Netherlands) containing 90% protein.

2.2.2 Preparation of reaction model systems

Sodium caseinate (3% w/w) and sugar (150 mM monosaccharide) were dissolved in a phosphate buffer (0.1 M; pH 6.8) to give a molar ratio of sugar to lysine residues of 10:1. The samples were heated for various times (0 – 40 min) at 120°C in an oil bath in screw-capped

glass tubes (Schott, 16 x 160 mm). The chosen heating temperature corresponds to sterilisation temperatures usually used in the food industry. The reported heating times include the heating up period of about 2 - 3 minutes. After a given heating time, samples were cooled in ice water, prior to analysis. The reaction mixtures were heat-treated and analysed in at least two-fold.

2.2.3 Analyses of sugars and organic acids

After heating, sugars and organic acids were separated from the protein via Sephadex G25 disposable columns (NAP-25, Pharmacia, Uppsala, Sweden). A sample of 1 ml was brought on the column and was eluted with 10 ml of water. The last 6 ml, containing the sugars and organic acids, were analysed by HPLC using an ion-exchange column (ION-300, Interaction Chromatography Inc., San Jose, CA, USA). The eluent consisted of 0.0025 M sulphuric acid in water, the flow rate was 0.4 ml/min and the column was kept at 85°C. Sugars were detected by monitoring the refractive index and organic acids by their UV absorbance at 210 nm.

2.2.4 Analyses of total acid formation

Titrations were performed to determine total acid formation in heat-treated samples. Samples of 15 ml reaction mixture were titrated with 0.1 N NaOH to pH 8.3. From the difference in added NaOH between the heated and unheated sample the total amount of acid formed was calculated.

2.2.5 Analyses of available lysine residues

Samples of 0.5 ml were diluted with 1.5 ml sodium dodecyl sulphate (SDS; 16% w/w) and refrigerated overnight. Available lysine residues were determined after derivatisation with *ortho*-phthaldialdehyde (Vigo et al., 1992). A fluorescence spectrophotometer (Perkin Elmer, Beaconsfield, England) was used at emission and excitation wavelengths of 430 nm and 340 nm, respectively.

2.2.6 Analyses of Amadori compound

The Amadori compound was determined by means of furosine, using HPLC. Furosine is one of the reaction products of the acidic hydrolysis of the protein-bound Amadori compound. A sample was mixed with hydrochloric acid (end concentration 8 M), sparged with nitrogen for

two minutes and heated in an oven for 23 h at 110°C. The hydrolysed solution was centrifuged and the supernatant was purified by means of a Solid Phase Extraction cartridge (Waters, Milford, Massachusetts, USA). The eluate was injected on a Furosine Dedicated column (Alltech, Breda, the Netherlands) and furosine was detected by its UV absorbance at 280 nm (Resmini et al., 1990). Furosine concentration was recalculated to that of the Amadori compound using a conversion factor of 3.1 (Finot et al., 1981). This factor was confirmed by our own research using the periodate assay (Ahmed and Furth, 1991). In this method formaldehyde is released by periodate oxidation of C1-hydroxyls. The formaldehyde is converted to a chromophore (diacetyldihydrolutidine) by reaction with acetylacetone in ammonia, which can be determined spectrophotometrically at 405 nm.

2.2.7 Analysis of Heyns compound

Methods developed to determine glycated protein are mainly focussed on protein glycated with aldoses, the so-called Amadori compounds. Methods to detect Heyns compounds are hard to find. According to Ruttkat (1996), carboxymethyllysine (CML) is formed after periodate oxidation of both fructosylated and glucosylated proteins and is therefore useful for detection of the Heyns compound. In the present study, heat-treated samples were oxidised and hydrolysed as described by Badoud and co-workers (1996). CML was detected by HPLC after derivatisation with dabsyl (Lin and Chang, 1975). Dr. R. Badoud generously provided the external CML standard.

2.2.8 Analysis of heterocyclic compounds

In the protein-free fraction, obtained via the Sephadex G25 columns, the compounds 5-hydroxymethylfurfural (HMF), furfurylalcohol, 4-hydroxy-2-hydroxymethyl-5-methyl-3(2H)furanone (HHMF) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) were determined by HPLC, using a reversed phase column (Lichrosorb RP-18, Merck). The eluent was 7.5% methanol in water and the flow rate was 0.8 ml/min. Furfurylalcohol was detected by its UV absorbance at 220 nm, the others by their UV absorbance at 280 nm.

2.2.9 Analyses of methylglyoxal

α-Dicarbonyl compounds are highly reactive reaction intermediates but can be trapped with *ortho*-phenylenediamine (OPD). The then formed quinoxaline derivatives can be detected by

HPLC using a C18 column (Lichrosorb RP-18, Merck) and measuring the absorbance at 320 nm (Hollnagel, 2000). The eluents were water (A) and methanol (B) with a gradient of 20-100% in 35 min and a flow rate of 0.5 ml/min.

2.2.10 Analyses of brown compounds

The browning intensity of the heated reaction mixtures was determined by measuring the absorbance at 420 nm with a spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The samples were diluted four times with SDS (16% w/w) to reduce scattering due to protein aggregates. If necessary the samples were diluted once more with water. The browning of the protein-free fraction was measured without dilution. The browning of the protein fraction was calculated by subtracting the browning of the sugar fraction from the browning of the total mixture. The absorbance can be recalculated to the concentration of melanoidins by using the equation of Lambert-Beer. The extinction coefficient needed to solve this equation is a constant and its value depends on the kind of amino acid (Leong and Wedzicha, 2000; Leong, 1999). The extinction coefficient of protein-bound melanoidins formed in glucose-casein and fructose-casein systems was measured to be 500 $l.mol^{-1}.cm^{-1}$ (Brands et al., 2001). The concentration of melanoidins is thus expressed as sugar units incorporated in the brown products.

2.2.11 Mass balance

The total concentration of reactants and reaction products was calculated. To determine whether the main reaction products were identified the mass balance was expressed as percentage of the initial sugar concentration.

2.3 Results and discussion

2.3.1 Identification of reaction products

During heating of the glucose-casein and fructose-casein systems the concentration of reactants decreased and reaction products were formed (Figure 2.1 and 2.2). The main reaction product detected in a heated glucose-casein system was fructose. In the fructose-casein system glucose was formed in considerable amounts. No other sugars were detected. In both sugar-casein systems, formic acid and acetic acid were determined. In the glucose-casein

system protein-bound fructosyllysine was detected as Amadori product. The Heyns compound glucosyllysine was not detected in the heated fructose-casein system while only a very small amount of Amadori compound was found.

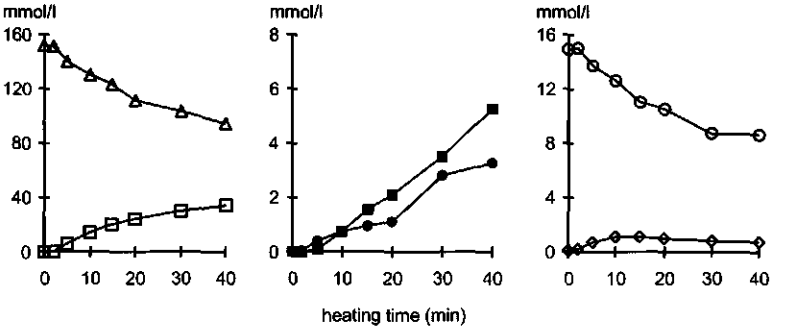

Figure 2.1 Glucose-casein solutions heated at 120°C. Glucose (Δ), fructose (□), formic acid (•), acetic acid (■), lysine residues (O), Amadori compound (◇).

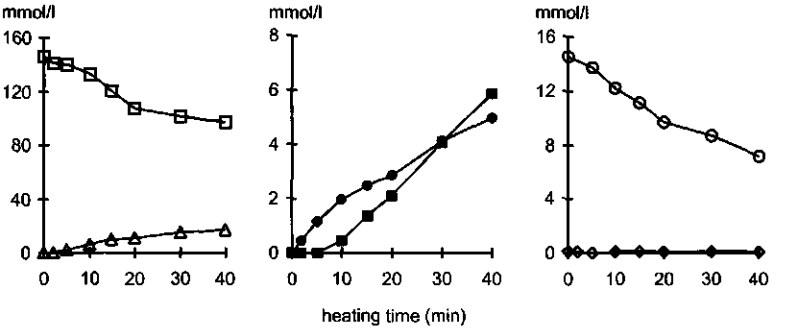

Figure 2.2 Fructose-casein solutions heated at 120°C. Fructose (□), glucose (Δ), formic acid (•), acetic acid (■), lysine residues (O), Amadori compound (◇).

The formation of organic acids caused a pH decrease of 0.3 pH-unit in a glucose-casein system and 0.4 pH-unit in a fructose-casein system after heating for 40 minutes at 120°C (Figure 2.3). The formation of acid, as determined by titration, was in both sugar-casein systems considerably higher than the total amount of acetic and formic acid found by HPLC (Figure 2.3).

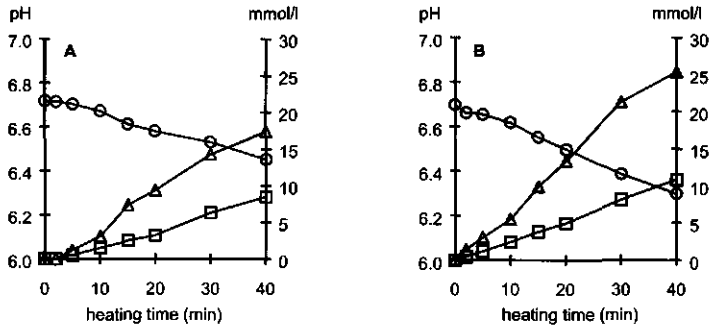

Figure 2.3 pH (O) and total amount of acids as found by titration (Δ) and HPLC (□) in heated glucose-casein (A) and fructose-casein (B) systems.

Other identified compounds were 5-hydroxymethylfurfural (HMF), furfurylalcohol, 4-hydroxy-2-hydroxymethyl-5-methyl-3(2H)furanone (HHMF) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP). However, HMF and furfurylalcohol were formed in very low amounts (0-40 μM). HHMF and DDMP could not be quantified since no reference material was available, but could be identified via their spectra, which were generously provided by Dr. M. Pischetsrieder. These compounds were presumably also formed in low amounts (as judged using the response factor of HMF).

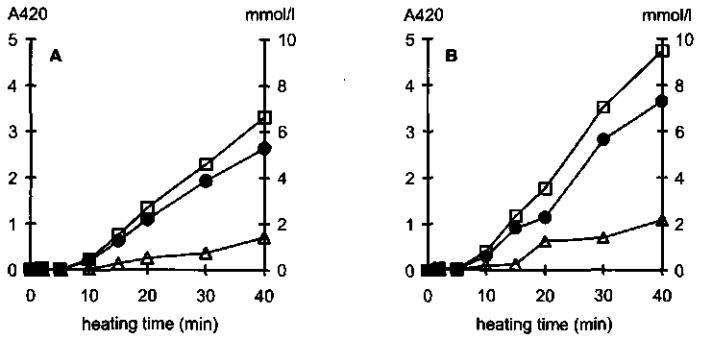

Figure 2.4 Browning of total system (□), protein fraction (•) and sugar fraction (Δ) expressed in absorbance units measured at 420 nm and concentration of protein-bound melanoidins (•) in heated glucose-casein (A) and fructose-casein (B) systems.

The concentration of protein-bound brown compounds (measured as amount of sugar incorporated) was calculated from the absorbance data (Figure 2.4). More browning was observed in the fructose-casein system than in the glucose-casein system.

The results of the mass balance calculations (Figure 2.5) showed an almost negligible amount of missing compounds after 40 minutes of heating at 120°C when all reaction products including brown compounds and unidentified acids were calculated. Of course, many more reaction products were formed but the fact that we come to an almost 100% recovery indicates that the acids formed are stable end products of scission reactions leading to numerous C1-C5 reaction products. Between 10 and 30 minutes more compounds were missing.

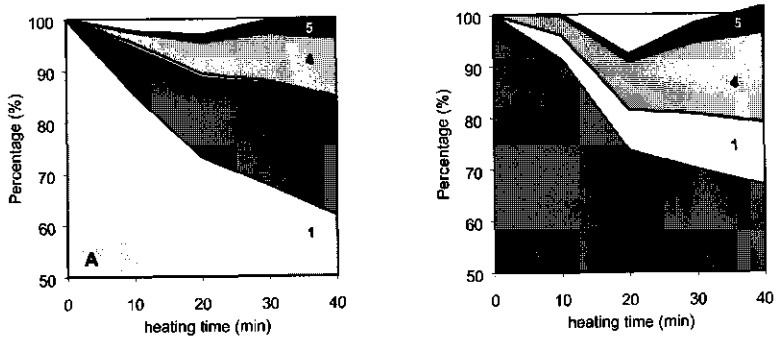

Figure 2.5 Mass balance of reactants and reaction products in heated glucose-casein (A) and fructose-casein (B) systems. Glucose (1), fructose (2), Amadori compound (3), total acids (4) and brown compounds (5).

The reaction products that were detected in the heated galactose- and tagatose-casein systems were comparable with those in the glucose- and fructose-casein systems (results not shown). The decrease of the reactants and increase of reaction products were more rapid for the galactose- and tagatose-casein systems compared to the glucose- and fructose-casein systems, respectively. The more rapid reaction of galactose compared to glucose is in line with literature (Kato et al., 1986).

2.3.2 Identification of reaction pathways

Sugar isomerisation, in which aldoses and ketoses can isomerise into each other, is one of the main reaction pathways in the sugar-casein systems. Another important reaction route was the

Maillard reaction, in which sugars react with the lysine residues. Loss of lysine residues was about equal or somewhat higher in the heated ketose-casein system than in the aldose-casein system. In the aldose-casein system the loss of lysine could partly be explained by the formation of Amadori compound. Partly, because the concentration of Amadori compound did not equal the loss of available lysine. The Amadori compound was subject to substantial breakdown after an initial build up phase, while increasing loss of lysine must have been due to formation of advanced and final Maillard reaction products. It should be noted that advanced Maillard products are to a large extent bound to protein, presumably via lysine. In the heated ketose-casein system no product of the early stage of the Maillard reaction was found. This may mean that it was not formed at all, or that it was quickly degraded again after formation, and that lysine residues were incorporated in advanced and final Maillard reaction products.

An important group of reaction products were the carboxylic acids. It was however not clear whether these acids were formed via the sugar degradation pathway or in the Maillard reaction due to breakdown of the Amadori or Heyns product. To get a better insight in the reaction pathways two additional experiments were performed.

In the first experiment, glucose and fructose solutions were heated in the absence of protein (remaining conditions were kept unchanged) and reaction products were determined. The results are shown in Figure 2.6. Besides a decrease of original sugar and an increase of isomer sugar both formic acid and acetic acid were formed. The amounts of formic and acetic acid formed in the fructose system and in the fructose-casein system were about equal. In the glucose system, on the contrary, the amount of organic acids was much lower than in the glucose-casein system, especially that of acetic acid. In both systems a lag time was observed for the formation of acetic acid, which was evidently longer for the glucose system than for the fructose system. Apparently, an intermediate was formed in the degradation reaction of fructose into acetic acid and an extra intermediate, supposedly fructose, was involved in the degradation reaction of glucose into acetic acid. It was also observed that the glucose system browned only slightly, whereas the fructose system browned much more, although not as intense as the fructose-casein system. This observation is in line with literature. Pilar Buera and co-workers (1987) noticed that caramelisation browning contributed noticeably to total browning in a fructose-glycine solution, whereas it could be neglected in glucose-glycine solutions.

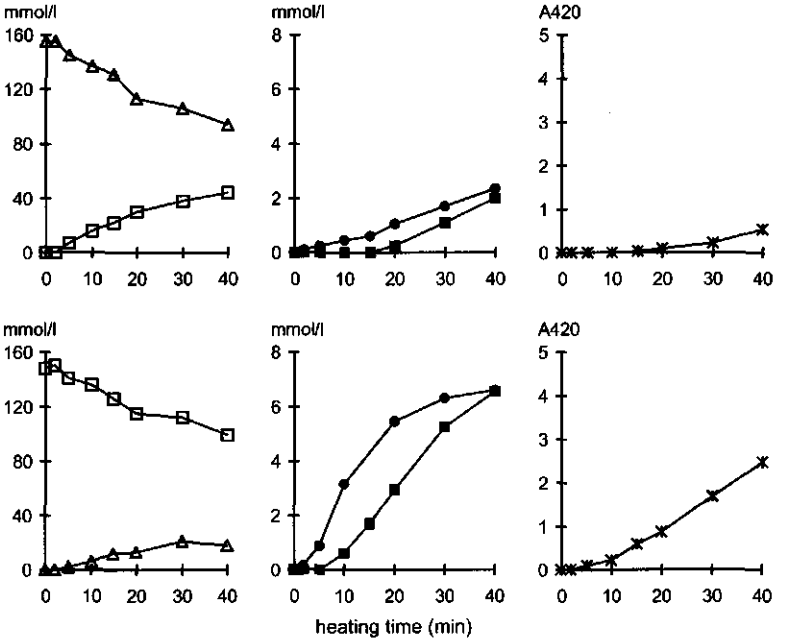

Figure 2.6 Glucose solutions (top) and fructose solutions (bottom) heated without casein at 120°C. Glucose (Δ), fructose (□), formic acid (•), acetic acid (■), absorbance at 420 nm (∗).

In a subsequent experiment the Amadori compound was isolated and heated. Formation of protein-bound Amadori product was induced by incubating 150 mM glucose with 3% casein at 65°C for 15 h. After cooling to room temperature, the glycated protein was separated from sugars and reaction products via the Sephadex G25 columns as described in section 2.2.3. The solution containing protein-bound Amadori product (it was checked that no unbound sugar was present anymore) was heated at 120°C and reaction products were determined (Figure 2.7). Both acetic acid and formic acid were formed, but acetic acid in about 1.5 time higher amounts. No sugars were formed. We tried to isolate protein-bound Heyns compound by the same procedure (incubating fructose with casein at 65°C), but subsequent heating of the incubated protein did not result in formation of organic acids. This confirmed our observation mentioned above that protein-bound Heyns product could not be detected.

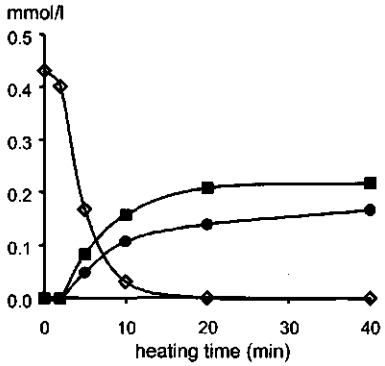

Figure 2.7 Isolated Amadori compound heated at 120°C. Amadori compound (◇), formic acid (•), acetic acid (■).

2.3.3 Reaction mechanism

Glucose and fructose isomerise into one another via the 'Lobry de Bruyn-Alberda van Ekenstein transformation' (Speck, 1958). The 1,2-enediol anion is the key intermediate in this isomerisation reaction in which also mannose is involved. Mannose was, however, not detected in the heated model systems. In addition to the favourable isomerisation of fructose via the 1,2-enediol anion, fructose can also epimerise to psicose via the formation of a 2,3-enediol as intermediate species. However, psicose was not detected in this study. This 'Lobry de Bruyn-Alberda van Ekenstein transformation' is also applicable to the isomerisation of other aldose and ketose sugars among which galactose and tagatose.

As well as being the key intermediate in the isomerisation reactions, the enediol anion species are also considered to be the starting intermediates in degradation reactions (De Bruijn et al., 1986). Via several pathways they lead to carboxylic acids as the final stable degradation products (Figure 2.8). The 1,2-enediol anion can undergo β-elimination to yield 3-deoxyaldoketose. In the same way the dicarbonyl compounds 1-deoxy-2,3-diketose and 4-deoxy-2,3-diketose are formed from the 2,3-enediol anion. These α-dicarbonyl compounds are unstable and undergo either a benzilic acid rearrangement yielding saccharinic acids or a cleavage reaction (cleavage of the C-C bond between the carbonyl groups) towards a carboxylic acid and an aldehyde. Formic acid was determined as one of the organic acids and was likely formed via a C1-C2 cleavage of the 3-deoxyaldoketose (Figure 2.8A). At the same time a C5 compound should be formed. A C5 compound described in literature is 3-deoxypentulose. However, this compound was detected in disaccharide systems, not in

monosaccharide systems (Hollnagel, 2000; Troyano et al., 1992). Another C5 compound is 2-deoxyribose. This compound was not detected, although furfurylalcohol, a cyclisation product of 2-deoxyribose (Rewicki et al., 1994) was identified. The concentration of furfurylalcohol did, however, not equal the amount of formic acid. Possibly, 2-deoxyribose also reacts to other compounds. Berg (1993) observed that it rapidly degraded in similar conditions as in the present study, whereas furfurylalcohol was quite stable during heating.

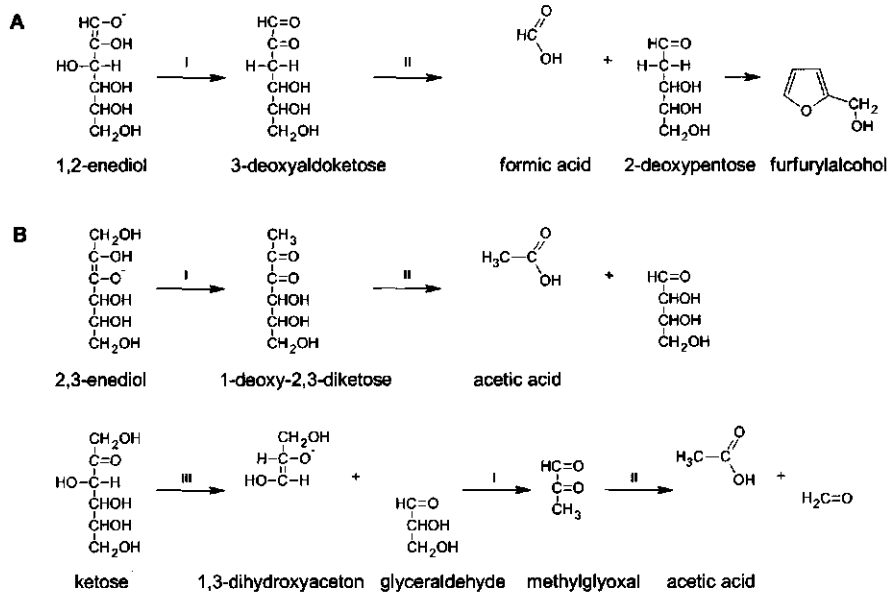

Figure 2.8 Degradation pathways of sugars into carboxylic acids (I = β-elimination, II = α-dicarbonyl cleavage, III = retro-aldolisation) (after De Bruijn (1986)).

The other identified acid was acetic acid, which was either formed by a C2-C3 cleavage reaction of the 1-deoxy-2,3-diketose or via a cleavage reaction of triose intermediates (Figure 2.8B). According to De Bruijn (1986), ketoses may undergo substantial retro-aldolisation towards the important triose intermediates glyceraldehyde and 1,3-dihydroxyaceton. These compounds can react further to the α-dicarbonyl methylglyoxal that can undergo a cleavage reaction and form acetic acid. Experiments in which we trapped reactive α-dicarbonyl compounds with *ortho*-phenylenediamine showed that methylglyoxal was indeed formed. Additional evidence for the formation of an intermediate was the observation of a lag time for the formation of acetic acid.

The titration experiment showed that acid formation was higher than the total amount of acetic and formic acid. Hence, other organic acids were formed but were not identified. Possible acids are lactic acid, which can be formed via benzilic acid rearrangement of methylglyoxal, glycolic acid, which can be formed via α-dicarbonyl cleavage of the 4-deoxy-2,3-diketose, and the already mentioned saccharinic acids (De Bruijn, 1986).

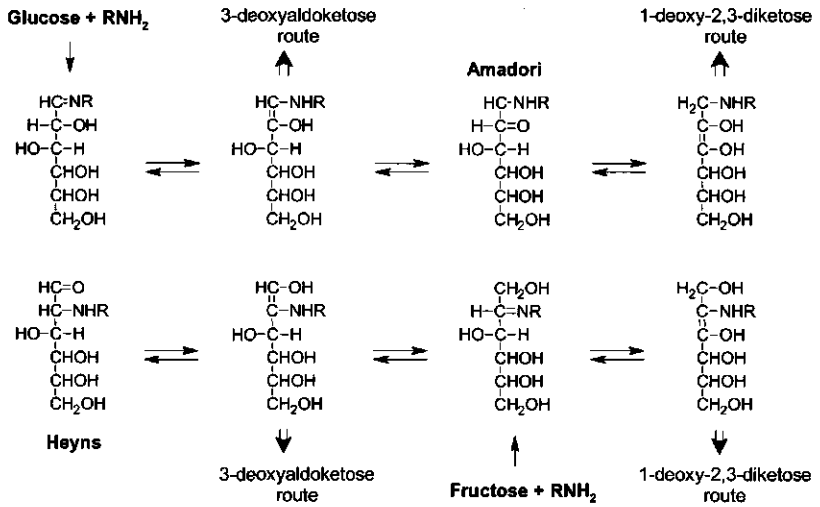

Figure 2.9 Early stage of the Maillard reaction of glucose and fructose (after Rewicki et al. (1994)).

Besides being formed via sugar degradation, organic acids were also formed in the Maillard reaction. In the early stage of the Maillard reaction between free amino groups of lysine residues of the protein and carbonyl groups of a sugar, an N-substituted glycosylamine is formed (Figure 2.9). When the sugar is an aldose, the N-substituted aldosylamine undergoes a rearrangement via a 1,2-enaminol to yield the Amadori compound (1-amino-1-deoxy-2-ketose). Instead of reacting to the Amadori compound, this 1,2-enaminol can react to a 3-deoxyaldoketose. A cleavage reaction of this compound leads subsequently to the formation of formic acid (Figure 2.8A). The Amadori compound can react via 2,3-enolisation to a 1-deoxy-2,3-diketose. Subsequent cleavage of this compound results in the formation of acetic acid (Figure 2.8B). When the sugar is a ketose in stead of an aldose, an N-substituted ketosylamine is formed in the early stage of the Maillard reaction and can react via a 1,2-enaminol to a 3-deoxyaldoketose or via a 2,3-enaminol to a 1-deoxy-2,3-diketose (Figure 2.9).

Via these deoxyosones formic and acetic acid can be formed (Figure 2.8). Since acid formation in the ketose system was about the same in the absence or presence of lysine residues, acid formation via the Maillard reaction is apparently not significant. Via the 1,2-enaminol the Heyns compound (2-amino-2-deoxy-1-aldose) can be formed. If this reaction route is only a side reaction, the Heyns compound is only formed as a by-product of the Maillard reaction. This would explain why we could not detect any Heyns. In foods the Heyns compound has never been detected (Ruttkat, 1996), it has only been found in systems that were heated at physiological temperatures (McPherson et al., 1988).

Apart from the organic acids, several compounds can be formed in the advanced Maillard reaction (Rewicki et al., 1994; Tressl et al., 1994). These compounds were not analysed in the present study. Because of their significance in the advanced Maillard reaction, they were grouped among the advanced Maillard reaction products (AMP). In contrast with the Amadori compound, the Heyns compound is not necessarily involved in these reaction routes (Rewicki et al., 1994). AMP can also be formed via reaction of sugar reaction products (Cn) with lysine residues. The AMP eventually lead to the brown coloured melanoidins in the final stage of the Maillard reaction.

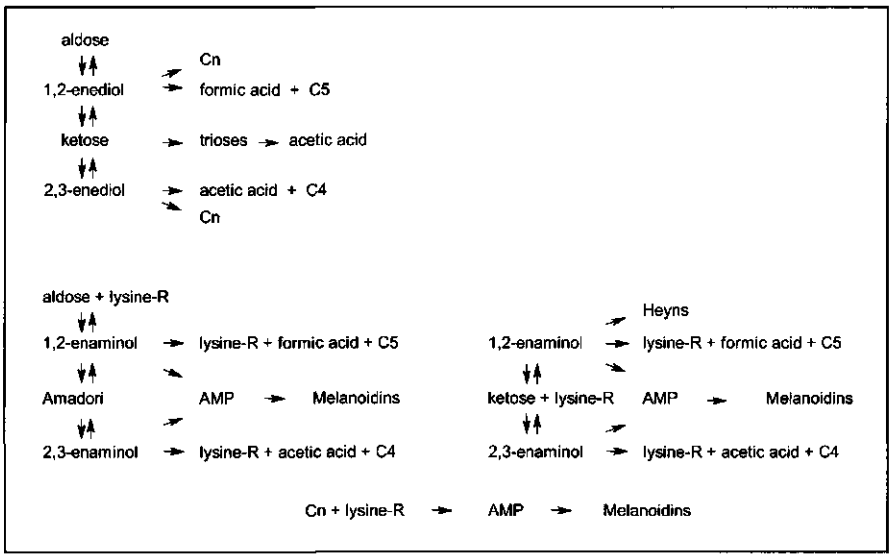

Figure 2.10 Reaction network model for sugar-casein reactions (Cn: unidentified sugar reaction compounds with n carbon atoms ($1 \leq n \leq 6$), AMP: advanced Maillard reaction products, lysine-R: protein bound lysine residues).

The reactions described in this article are summarised in Figure 2.10. The model is divided into two parts, one containing the sugar reactions (isomerisation and degradation) and the other describing the sugar-casein reactions (Maillard reaction) for both the aldose and ketose sugars. In a subsequent paper the proposed reaction network model will be analysed in kinetic terms.

2.4 Conclusion

In this paper reaction mechanisms were proposed to explain the observed reaction products for the reactions of the aldose sugars glucose and galactose and the ketose sugars fructose and tagatose in the presence of the protein casein at neutral pH and 120°C. Ketoses seemed to be more reactive in the sugar degradation reactions than their aldose isomers and also the reaction of ketoses and aldoses in the Maillard reaction differed. Due to this difference in reaction mechanism both sugars contribute to a different extent to quality factors like nutritional damage, colour and flavour. In line with this, remarkable differences in mutagenicity were observed for aldoses and ketoses heated under circumstances corresponding to those in the present study (Brands et al., 2000).

In a subsequent paper, the proposed model will be kinetically analysed and tested by varying temperature, pH and concentration of reactants.

Acknowledgements

We are grateful to Lennard Muilwijk and Barry de Wit for performing some of the experimental work.

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3

Quantification of melanoidin concentration in sugar-casein systems

Abstract

Melanoidins are the final, brown-coloured, high molecular weight products of the Maillard reaction. The aim of the present study was to determine the average molar extinction coefficient of melanoidins formed in heated glucose-casein and fructose-casein systems. The value of the extinction coefficient can be used to translate spectrophotometrically measured browning (absorbance values) into melanoidin concentration. In the present study the melanoidins were quantified by measuring the concentration of sugar incorporated into the melanoidins, using ^{14}C-labelled sugar. The extinction coefficient of the melanoidins remained constant during the observation period as the absorbance at 420 nm increased to ≈ 8 units, and it was calculated to be 477 (± 50) $l.mol^{-1}.cm^{-1}$ in the glucose-casein reaction and 527 (± 35) $l.mol^{-1}.cm^{-1}$ in the fructose-casein reaction. This difference is not significant. An increase of the number of sugar molecules per reactive amino group during the heating of glucose-casein and the fructose-casein mixtures was observed by the radiochemical method as well as by microanalysis of the high molecular weight fraction.

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3.1 Introduction

In the final stage of the Maillard reaction between sugars and compounds possessing a free amino group, such as amino acids and proteins, melanoidins (also referred to as advanced glycation end products 'AGE' in literature concerning in-vivo glycation) can be formed (Hodge, 1953). The brown-coloured melanoidins are a heterogeneous mixture of high molecular weight compounds (O'Brien and Morrissey, 1989). Besides being caused by the high molecular weight melanoidins, browning can also be due to low molecular weight coloured compounds, sometimes referred to as low molecular weight melanoidins (Leong and Wedzicha, 2000). The brown compounds have a high impact on the quality of foods. Colour is an important food quality characteristic and a key factor in consumer acceptance (Rizzi, 1997).

The mechanism of the formation of colour is not fully understood and the structure of melanoidins is largely unknown. Up to now, only hypotheses are available regarding the structures of melanoidins formed in sugar-amino acid systems (Cämmerer and Kroh, 1995; Kato and Tsuchida, 1981; Tressl et al., 1998; Yaylayan and Kaminsky, 1998). In sugar-casein systems, it is established that colour formation is mainly due to the formation of protein oligomers cross-linked by low molecular weight coloured Maillard reaction products (Hofmann, 1998a). The structures of some chromophores present in protein-based melanoidins have been elucidated (Hofmann, 1998b). From the point of view of colour, melanoidins can be built up of sub-units in two contrasting ways. One possibility is that melanoidins are formed by more or less random reactions of low molecular weight reaction intermediates (which may inherently be coloured or not). Alternatively, a repeating unit (which may be colourless or contribute little to colour) may form the backbone of melanoidins, with chromogenic low molecular weight structures attaching themselves to this backbone, resulting in high molecular weight coloured structures. The results of the studies by Hofmann (1998a; 1998b) indicate that proteins can act as the colourless backbone of melanoidins.

The missing information on melanoidin formation and melanoidin structure makes it very difficult to quantify melanoidins. This quantification is necessary when trying to predict or optimise browning in processed foods. Both Davies and co-workers (1997) and Brands and Van Boekel (2001) developed a kinetic model for the formation of brown colour in glucose-glycine mixtures and sugar-casein systems, respectively. Browning has usually been measured spectrophotometrically and expressed in absorbance units. If colour can be expressed in concentration units, it is possible to relate colour directly to the rates of

formation of intermediates in the kinetic model and hence to predict it. Assuming Lambert-Beer's law is obeyed, absorbance data can, in principle, be related to melanoidin concentration. Apart from concentration, the absorbance depends on the molar extinction coefficient of these melanoidins. Leong determined the average extinction coefficient of melanoidins derived from glucose and glycine and various other amino acids using ^{14}C-labelled glucose (Leong and Wedzicha, 2000; Leong, 1999). A benefit of this approach is that the molar extinction coefficient of melanoidins can be expressed simply 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. When defined in this way, it was observed that the extinction coefficient remained constant with time. Once the extinction coefficient is known, melanoidins can be quantified and used to validate proposed kinetic models.

In the present study, the average extinction coefficient of melanoidins obtained from glucose and the protein casein was determined, following the method of Leong and Wedzicha (2000). Leong (1999) observed that the molar extinction coefficient was dependent of the kind of amino acid that was heated in the presence of glucose. Although in many foods, proteins instead of amino acids are the greatest source of free amino groups, the molar extinction coefficient of melanoidins obtained from the reaction between glucose and (the lysine residues of) a protein has however not been determined before. To study the effect of the type of sugar on the value of the extinction coefficient, the same experiments were carried out with fructose, the ketose isomer of glucose, as the reacting sugar. Furthermore, a microanalysis of the melanoidins was carried out to investigate the number of sugar molecules incorporated per protein molecule.

3.2 Materials and methods

3.2.1 Chemicals

All chemicals were of analytical grade. D-glucose and D-fructose were supplied by Sigma chemicals (United Kingdom). D-[U-^{14}C]-glucose and D-[U-^{14}C]-fructose were obtained from Amersham Life Science Ltd. (United Kingdom). Sodium caseinate (a spray-dried powder) was obtained from DMV (the Netherlands) containing 90% protein. Casein is a protein with almost no secondary or tertiary structure and has shown to be extremely stable when heated (Guo et al., 1989).

3.2.2 Preparation of reaction model systems

Sodium caseinate (3% w/w) was dissolved in a phosphate buffer (0.1 M, pH 6.8) and kept refrigerated overnight. Sugar (150 mM of glucose or fructose) was dissolved in the casein solution. Before making up to a volume of 100 ml, 1 MBq of radiolabelled sugar (U-^{14}C glucose or U-^{14}C fructose) was added to the sugar-casein system (amount of added labelled sugar was ≈ 0.1 μmol and therefore negligible with respect to the unlabelled sugar). This solution was distributed over 8 screw-capped glass tubes (Schott, 16 x 160 mm) and heated for various times (0, 10, 20, 30, 40, 50, 60 and 90 min) at 120°C in an oil bath. After being heated, the samples were cooled in ice-water. The glucose-casein reaction mixtures were heat-treated and analysed in three-fold, the fructose-casein systems in two-fold.

3.2.3 Dialysis

To separate unreacted radiolabelled substances and low molecular weight products from the high molecular weight products, the samples were dialysed. An aliquot (10 ml) of each reaction mixture was dialysed in Visking tubing with a cut-off value of 12000 Daltons. The samples were dialysed against distilled water for 4 or 5 days (8 water replacements). After dialysis, the retentate was removed to a volumetric flask containing 20 ml of 20% (w/w) sodium dodecyl sulphate (SDS) solution and made up to 100 ml with distilled water. SDS was added to dissolve any flocculated protein.

3.2.4 Scintillation counting

Aliquots (1 ml) of the diluted dialysed fraction were pipetted into a scintillation vial containing 10 ml of scintillation fluid. The vial was shaken vigorously and counted for 1 minute using a Packard Tri-Carb 1900TR scintillation counter. The count due to ^{14}C was corrected for quenching by the internal standard method. The specific activity of ^{14}C-glucose or ^{14}C-fructose in the reaction mixture was calculated from the counts obtained from 1 ml of a 50-fold diluted unheated reaction mixture and was expressed as number of disintegrations per minute (dpm) per mol of glucose or fructose. 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.

3.2.5 Spectrophotometric analysis

In a parallel experiment, in which no radiolabelled sugar was added to the reaction mixture, the browning of reaction mixtures and of corresponding retentates after dialysis was determined. Browning was measured spectrophotometrically as the absorbance at 420 nm. The undialysed samples were diluted 5-fold in SDS (final concentration 4% w/w) to reduce light scattering. The dilution of the dialysed samples was as described before for the experiments with labelled sugar.

3.2.6 Microanalysis

The sugar-casein systems that were heated for 10, 30 and 60 minutes (without addition of radiolabelled sugar) were dialysed and the retentates were freeze-dried. Microanalysis was carried out using a Carlo Erba Elemental Analyser. The weight of each sample was about 2 mg.

3.2.7 Analyses of Amadori compound

The Amadori compound was determined by means of furosine, using HPLC (Resmini et al., 1990). Furosine concentration was converted to that of the Amadori compound using a conversion factor of 3.1 (Brands and Van Boekel, 2001).

3.2.8 Analyses of available lysine residues

Available lysine residues were determined after derivatisation with *ortho*-phthaldialdehyde, as described previously (Brands and Van Boekel, 2001).

3.3 Results and discussion

3.3.1 Browning

Considerable browning was observed in both the glucose-casein and fructose-casein reactions during heating (Figure 3.1). After an induction time during which no browning was detected, fructose browned faster than glucose. Due to differences in reaction behaviour between glucose and fructose (Brands and Van Boekel, 2001) it is difficult to compare the results for browning with literature data. Besides the pH, water activity, sugar to amino group ratio, the rate of browning depends strongly on the temperature.

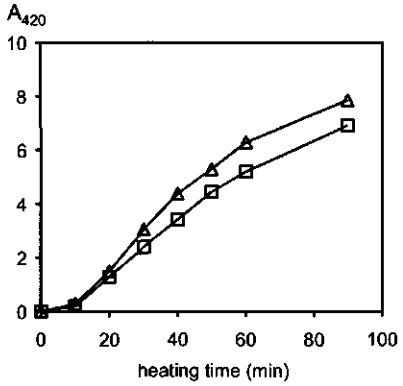

Figure 3.1 Browning (measured as absorbance at 420 nm) of glucose-casein (□) and fructose-casein (Δ) reactions at 120°C with time.

During dialysis about 30% of the absorbing compounds (at 420 nm) passed into the dialysate (Figure 3.2). Consequently, the majority of the coloured compounds was retained in the high molecular weight fraction. This result is in line with literature describing browning in sugar-protein systems. Morales and Van Boekel (1998) observed that the pigments causing browning in heated glucose-casein and lactose-casein reactions were mainly bound to the protein. Hofmann (1998a) reported that the predominant part of the reaction products formed in a glucose-casein reaction was of high molecular weight (> 50000 Daltons) and this increase of the molecular weight ran in parallel with the intensity of browning.

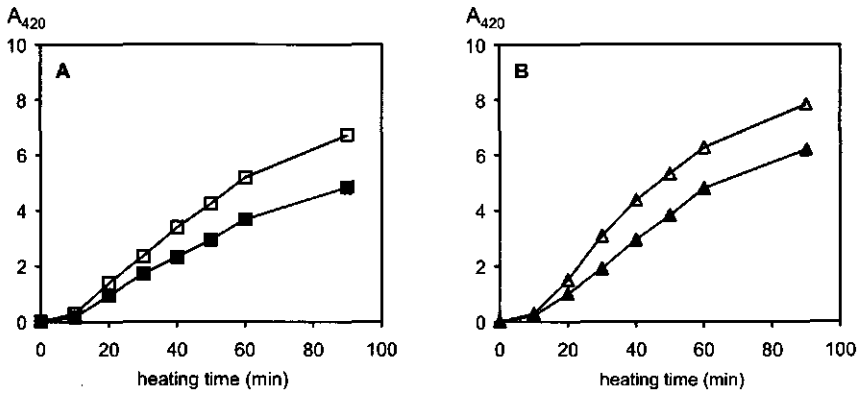

Figure 3.2 Browning (measured as absorbance at 420 nm) of glucose-casein (A) and fructose-casein (B) reactions at 120°C before (□, Δ) and after (■, ▲) dialysis.

These results are in contrast with sugar-amino acid reactions in which a much lower percentage of colour was detected in the high molecular weight fraction. In a study of Leong and Wedzicha (2000) the high molecular weight melanoidin fraction (> 3500 Dalton) contributed only up to 10% of the absorbance of the glucose-glycine reaction mixtures. Hofmann (1998a) reported that only trace amounts of compounds with molecular weights greater than 3000 Daltons were formed in glucose-glycine and glucose-alanine systems and that colour was therefore almost exclusively due to the low molecular weight fraction.

These differences between amino acids and proteins in the character of the coloured products is likely to be due to the fact that the protein is a high molecular weight compound itself. As a consequence, reaction products will be of high molecular weight *per se*. On the other hand, Monti and co-workers (2000) showed that the formation of high molecular weight coloured compounds is not exclusive for protein-bound chromophores. They studied the formation of colour in two model systems consisting of lactose and lysine or N^{α}-acetyllysine. In the system containing N^{α}-acetyllysine the main contribution to colour was due to the high molecular weight fraction (> 10000 Daltons), while in the reaction with free lysine the contribution of the high molecular weight fraction was negligible. It was concluded that the formation of melanoidins proceeded faster in a model system containing blocked α-amino groups because less material was converted in other reaction pathways, mainly the Strecker degradation, that leads to low molecular weight products.

Heating sugars in the absence of protein also leads to browning. When these samples were dialysed, almost no coloured compounds were retained in the high molecular weight fraction. Brown-coloured sugar degradation products can therefore only contribute to the melanoidins once they have reacted with the protein.

3.3.2 Melanoidin formation

The experiments used to obtain the absorbance-time data given in Figure 3.2 were repeated with the sugar uniformly labelled with ^{14}C. In Figure 3.3 the concentration of the high molecular weight compounds, measured as the concentration of sugar incorporated in this fraction, is plotted against time. The high molecular weight fraction did, however, not only consist of the brown coloured melanoidins but is expected to have contained also non-coloured products of the early stages of the Maillard reaction when these are bound to the protein. However, with the exception of the Amadori compounds, most of them can not be determined because little is known of their identity. In the present study it was assumed that only the Amadori compound was formed in significant amounts. On the basis of this

assumption, the melanoidin concentration was obtained by subtracting the concentration of Amadori compound from the concentration of sugar incorporated into the high molecular weight fraction (see Figure 3.3).

As can be seen from Figure 3.3, ^{14}C-sugar in the retentate was also detected for the unheated samples and the count rates were somewhat higher than the samples heated for a short time. Since we corrected for the formation of Amadori compound and because unreacted sugar and non-covalently bound sugar and sugar fragments were supposedly washed away during dialysis, we can not offer an explanation for this observation.

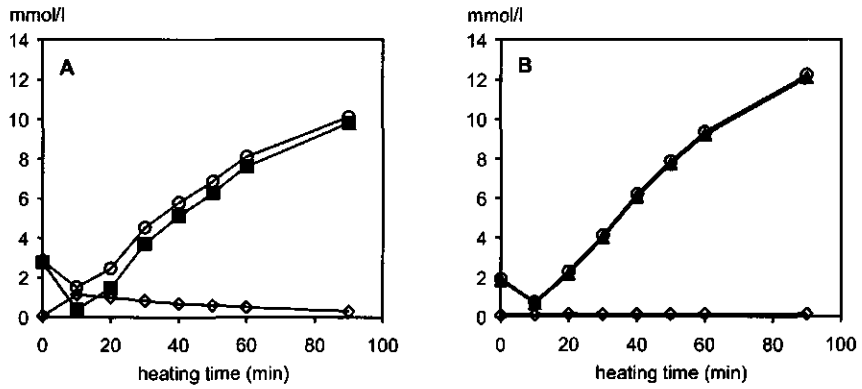

Figure 3.3 Formation of high molecular weight compounds measured as incorporated sugar (O), Amadori compound (◇) and melanoidins (■, ▲) in glucose-casein (A) and fructose-casein (B) reactions at 120°C.

3.3.3 Extinction coefficient of melanoidins

The relationship between the absorbance of the melanoidin fraction and the concentration of melanoidins as determined using ^{14}C-labelled sugar is shown in Figure 3.4, where a change in melanoidin concentration is the result of a corresponding change in the extent of reaction. The relationship between browning and amount of labelled sugar incorporated into the melanoidins is clearly linear, and the value of the extinction coefficient can be deduced from the slope of this line. The results indicate that, over the observation period, the extinction coefficient of the melanoidins remains constant. This is most likely when a repeating unit or, alternatively, the protein forms the backbone of melanoidins with chromogenic low molecular weight structures attaching themselves to this backbone. In the glucose-casein system the average molar extinction coefficient (± standard deviation) was calculated to be 477 (± 50)

$l.mol^{-1}.cm^{-1}$. In the fructose-casein system it was 527 (± 35) $l.mol^{-1}.cm^{-1}$. This difference between the extinction coefficients of melanoidins formed in glucose-casein and fructose-casein reactions was not significant at the 5% level. This indicates that in the glucose-casein and fructose-casein systems similar reaction intermediates might be formed, giving rise to melanoidins with the same average extinction coefficient.

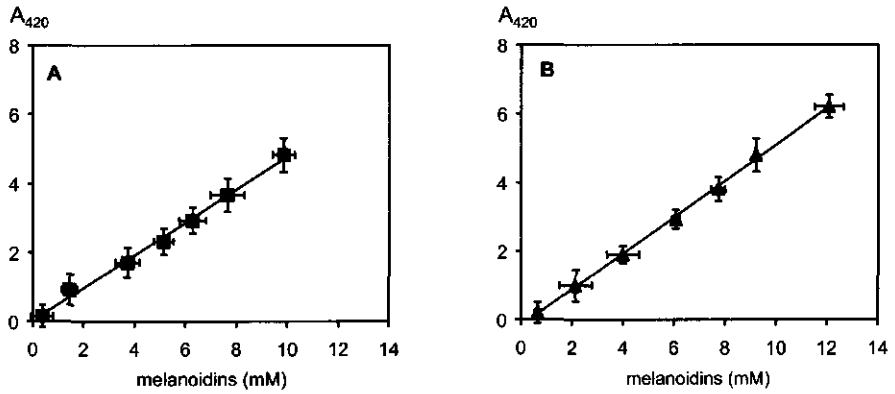

Figure 3.4 Browning (measured as absorbance at 420 nm ± standard deviation) as function of the melanoidin concentration (measured as incorporated sugar ± standard deviation) detected in glucose-casein (A) and fructose-casein (B) reactions after heating at 120°C.

The value of the average extinction coefficient of melanoidins formed in the glucose-casein reaction was compared with the ones obtained from melanoidins formed in glucose-amino acid reactions. Leong (1999) estimated values of ε at 470 nm to range from 695 $l.mol^{-1}.cm^{-1}$ for valine to 940 $l.mol^{-1}.cm^{-1}$ for glycine. Since A_{470} / A_{420} ≈ 0.57, the extinction coefficients of melanoidins obtained from glucose-amino acid systems were calculated to be about 2.5 to 3.5 times higher than the extinction coefficient of glucose-casein melanoidins. This means that, in sugar-amino acid reactions, less glucose molecules (or glucose fragments) have to be incorporated into the melanoidins than in sugar-casein systems to increase the absorbance by one unit.

Leong and Wedzicha (2000) observed that the extinction coefficient obtained for high molecular weight melanoidins formed in a glucose-glycine reaction mixture was in remarkable agreement with the value obtained for the whole reaction mixture using kinetic considerations. Since the high molecular weight melanoidin fraction contributed only up to 10% of the total absorbance, it could be concluded that the extinction coefficient of the low

molecular weight fraction must have been the same. The same value for both low and high molecular weight coloured compounds is only possible when high molecular weight material is formed from coloured substructures and the extent of conjugation does not change as the polymer grows.

3.3.4 Microanalysis

For the evaluation of the microanalysis data of the melanoidins, the results have to be fitted to a model of reaction stoichiometry. Such a model for the casein-sugar reaction is based on the model used by Wedzicha and Kaputo (1992) but modified so that the only products are melanoidins and water. Carbon dioxide is assumed not to be released because Strecker degradation does not occur. Thus, the overall reaction for the formation of the melanoidin is a combination of a molecules of sugar (glucose or fructose) consisting of l, m and n atoms of C, H and O, respectively, and b molecules of protein consisting of p, q, r and s atoms of C, H, N and O, respectively, to give a melanoidin with the following formula:

$$C_{la+pb}H_{ma+qb-2y}N_{rb}O_{na+sb-y}$$

where y is the amount of H_2O liberated. When glucose or fructose is the reacting sugar, $l = 6$, $m = 12$ and $n = 6$. The formula of the protein was determined by microanalysis and, considering that the molecular weight of casein is 23000 g.mol^{-1}, it was calculated that $p = 951$, $q = 1553$ and $r = 240$ (parameter s was not calculated since the percentage of oxygen could not be determined by microanalysis). The theoretical formula of the protein is $C_{1029}H_{1600}N_{262}O_{308}S_5P_6$. Whilst the experimental values differ from the theoretical values, the C/N ratios are 3.97 and 3.93 (experimental and theoretical values respectively) and, therefore, in excellent agreement with each other. This confirms the accuracy of the microanalysis with respect to carbon and nitrogen. The unknowns a and b may be found by solving the following equations:

$$C = 6a + 951b$$
$$N = 240b$$

where C and N represent the number of the corresponding atoms in the formula of the glycated protein. Since the C/N ratio is insensitive to the presence of moisture, which could be a significant "impurity" in the glycated protein sample, the values of a/b calculated in this way are expected to be reliable. Thus, Table 3.1 gives the measured C/N ratios and the corresponding values of a/b, the number of sugar molecules incorporated into the polymer per protein molecule. The number of sugar equivalents per protein molecule increased with heating time in both the glucose-casein and the fructose-casein system. After 60 minutes of

heating, the *a/b* ratio in the fructose-casein system was somewhat, although not significantly, higher than the *a/b* ratio in the glucose-casein system, corresponding to a slightly higher degree of browning (also apparent from Figure 3.2).

Table 3.1 C/N ratio and calculated number of molecules of sugars (*a*) incorporated per molecule of casein (*b*) or lysine residue (*b'*).

Sugar	Heating time (min)	C/N	*a/b*	*a/b'*	*a/b'* radiochem.
-	-	3.97			
Glucose	10	4.01	1.85 ± 0.75	0.14 ± 0.06	0.10 ± 0.03
Glucose	30	4.10	5.39 ± 0.15	0.41 ± 0.01	0.30 ± 0.03
Glucose	60	4.22	10.00 ± 1.32	0.77 ± 0.10	0.54 ± 0.04
Fructose	10	4.01	1.65	0.13	0.05 ± 0.01
Fructose	30	4.15	7.34	0.56	0.27 ± 0.04
Fructose	60	4.30	13.26 ± 0.14	1.02 ± 0.01	0.62 ± 0.01

The ε-amino groups of the lysine residues are the most susceptible amino groups of the protein towards glycation (Tagami et al., 2000). One molecule of casein contains 13 lysine residues on average (since casein consists of 4 different casein types, an average has to be used (Walstra and Jenness, 1984)). In Table 3.1 the calculated number of sugar molecules per lysine residue (*a/b'*) is shown (*b'* is the number of lysine residues per casein molecule). From the results we can conclude that after 60 minutes of heating about three-quarters of the lysine residues present in the glucose-casein system have reacted with glucose (or glucose equivalents with 6 carbon atoms) and all lysine residues present in the fructose-casein system. This does, however, not correspond with results observed before (Brands and Van Boekel, 2001) saying that in both sugar-casein systems the loss of available lysine residues was not more than 60% after 60 minutes of heating at 120°C (see Figure 3.5).

Except for the ε-amino group of lysine residues, other potential glycation sites of casein are the N-terminal α-amino group and the guanidino group of arginine residues (Tagami et al., 2000). During heating, more reactive reaction products, such as various dicarbonyl compounds, will be generated in the reaction system. These compounds have a higher affinity for the guanidino groups of arginine residues and hence increase the involvement of arginine residues in the glycation reaction (Yeboah et al., 2000). One molecule of casein contains 5 arginine residues on average (Walstra and Jenness, 1984). If we take the reaction of sugar degradation compounds with arginine residues into account, the number of sugar equivalents per amino group will decrease.

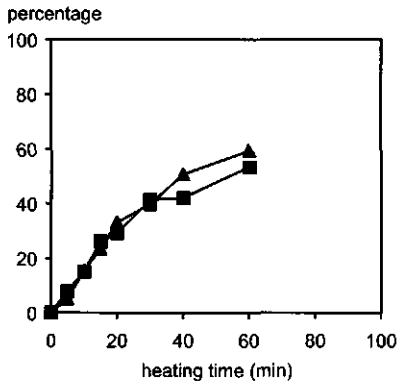

Figure 3.5 Loss of available lysine residues in glucose-casein (■) and fructose-casein (▲) systems after heating at 120°C.

In order to be able to compare the results of the microanalysis with the results of the radiochemical experiment, the concentration of sugar incorporated in the high molecular weight fraction was divided by the concentration of lysine residues (see Table 3.1). It can be concluded that the trend is similar but that the calculated results of the microanalysis are significantly higher ($P < 0.05$) than those from the radiochemical experiment.

3.4 Conclusions

Lately, more and more studies are focussing on melanoidin formation. In the present study, the average extinction coefficients of melanoidins formed in sugar-casein systems were determined, using ^{14}C-labelled sugar. No significant difference was found between the extinction coefficient of melanoidins formed in glucose-casein systems on the one hand and fructose-casein systems on the other hand. The number of sugar molecules per molecule of protein, as determined by microanalysis, was higher in the fructose-casein system, which was in line with the higher browning rate.

Acknowledgements

We thank Elbert van der Klift of the Organic Chemistry group, Wageningen University, for the microanalysis measurements.

The results reported in this paper are from research carried out with financial support from the European Commission, within the framework of a short-term scientific mission for COST (Co-operation in Science and Technology) Action 919, 'Melanoidins in Food and Health'.

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4

Kinetic modelling of reactions in heated monosaccharide-casein systems

Abstract

In the present study a kinetic model of the Maillard reaction occurring in heated monosaccharide-casein systems was proposed. Its parameters, the reaction rate constants, were estimated via multiresponse modelling. The determinant criterion was used as the statistical fit criterion instead of the familiar least squares to avoid statistical problems. The kinetic model was extensively tested by varying the reaction conditions. Different sugars (glucose, fructose, galactose and tagatose) were studied regarding their effect on the reaction kinetics. This study has shown the power of multiresponse modelling for the unravelling of complicated reaction routes as occur in the Maillard reaction. The iterative process - proposing a model, confronting it with experiments, criticising the model - was passed through 4 times to arrive at a model that was largely consistent with all results obtained. A striking difference was found between aldose and ketose sugars as suggested by the modelling results: not the ketoses themselves, but only their reaction products were found to be reactive in the Maillard reaction.

Carline M. J. Brands and Martinus A. J. S. van Boekel. *Submitted for publication.*

4.1 Introduction

In order to be able to control chemical reactions in foods, the reactions of interest need to be studied in a quantitative way. The reactions occurring in a monosaccharide-protein system were subject of study in a previous paper (Brands and Van Boekel, 2001). Three main reaction routes were found: (a) isomerisation of the sugar, (b) degradation of the sugar, and (c) the Maillard reaction in which the sugar (and/or its breakdown products) reacts with the ε-amino group of lysine residues of the protein. The second and third reaction routes have reaction products in common. In Figure 4.1 the proposed reaction network model is shown, which summarises the main findings. The chemistry behind the reaction scheme was extensively discussed in the previous paper (Brands and Van Boekel, 2001). The present paper will deal with the kinetic analysis of the reaction network model. With knowledge of kinetics, it becomes possible to describe the changes in a quantitative way, and to predict changes from certain time-temperature combinations. In addition, kinetics is a tool for understanding reaction mechanisms.

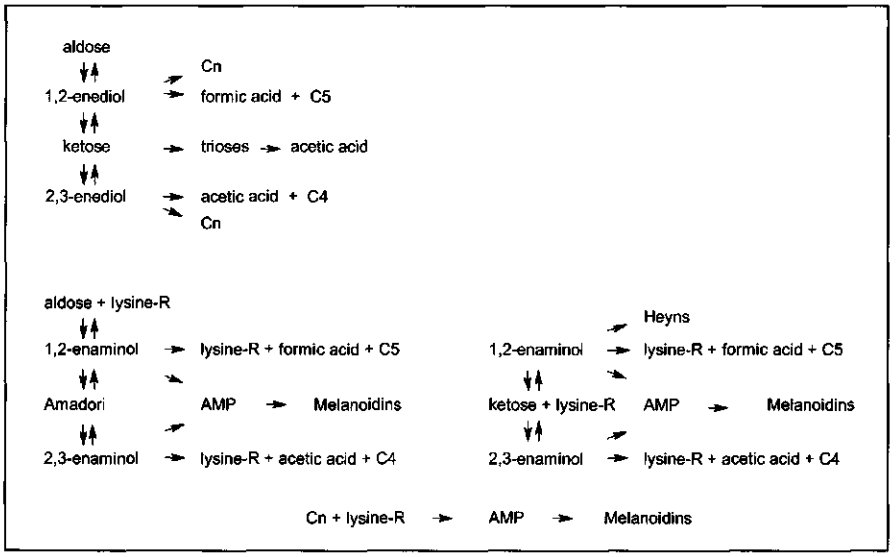


Figure 4.1 Proposed reaction network model for monosaccharide sugars in the presence of casein (Cn: unidentified sugar reaction compounds with n carbon atoms ($1 \leq n \leq 6$), AMP: advanced Maillard reaction products, lysine-R: protein bound lysine residues).

Some useful steps to be considered in elucidating complex reaction networks, such as the one in Figure 4.1, were listed in literature (Antal et al., 1990; Berg, 1993; Van Boekel, 1996b):

1. *Identification of all stable reaction products and determination of the mass balance.* A mass balance should give insight into the question whether indeed the main products have been identified, or that perhaps reaction products are missing. This has been done as described in the previous paper (Brands and Van Boekel, 2001). The main reaction products were the sugar isomer, the organic acids formic and acetic acid as stable sugar breakdown products, the protein-bound Amadori compounds and the brown-coloured melanoidins. Unidentified products were characterised as advanced Maillard reaction products (AMP) and sugar degradation products, including unidentified organic acids (Cn with $1 \leq n \leq 6$).
2. *Identification of primary and secondary reaction routes.* The primary reaction routes in the sugar-casein systems were sugar isomerisation, sugar degradation and the reaction of the protein with the sugar and/or its breakdown products in the Maillard reaction, in the end leading to the formation of protein-bound brown-coloured compounds. The formation of the Heyns compound, HMF and other heterocyclic compounds as HHMF and DDMP were the products of secondary reaction routes.
3. *Propose a mechanism for the reaction network.* This was the subject of the previous paper and is summarised in Figure 4.1. In this article the reaction network model will be further refined by the modelling exercise.
4. *Determine effect of temperature.* The reactions described here are of a chemical nature. It should therefore be possible to model the effect of temperature quantitatively by the Arrhenius equation or Eyring equation. This will also be addressed in this paper.
5. *Determine effect of pH.* This will be subject of study in the present paper. During heating the pH decreases due to the formation of organic acids and therefore we need to study the effect of pH. The pH can have an effect both on the reaction rate and the reaction mechanism.
6. *Determine influence of reactant concentration.* The kinetic model of Figure 4.1 should not be dependent on the initial concentration of the reactants. This can be tested by varying the concentrations of the reacting sugars and protein.
7. *Test whether the model is able to quantitatively fit the experimental data.* This step, together with the three preceding ones, will be the subject of the present paper and needs some further consideration.

To fit the model to the experimental data, the reaction network (Figure 4.1) needs to be translated into a mathematical model. This can be done by setting up differential equations for

each reaction step. These non-linear coupled differential equations are difficult to solve analytically, if possible at all, but can be solved by numerical integration. The mathematical model then needs to be fitted to the experimental data. The question how well the proposed model describes the experimental data must be addressed from a statistical point of view. If reactants and products involved in a reaction are measured at the same time, it is possible to take all such responses into account, which is called multiresponse modelling. The advantage of multiresponse modelling is the use of the information in various responses so that more precise parameter estimates and more realistic models can be determined (Van Boekel, 1996a). It gives at the same time more insight into the reaction mechanism. However, such an approach requires a special statistical treatment. The most simple (but mostly incorrect) approach to fit mathematical models to data and to estimate rate constants is to minimise the overall residual sum of squares (RSS) from all the responses. However, Hunter (1967) showed that this criterion is only valid under the restrictions that (a) each of the responses has a normally distributed uncertainty, (b) the data on each response have the same variance and (c) there is no correlation between the variances of the individual measurements of the responses. These restrictions are mostly not met when dealing with multiresponse modelling. For instance, samples will be analysed simultaneously for different compounds (restriction c is not met) and some responses will be measured more precisely than others (restriction b is not met). Box and Draper (1965) provided a solution for this problem following a Bayesian approach, assuming normally distributed errors. It is possible to form the so-called dispersion matrix from the responses. If the determinant of the dispersion matrix is minimised, the most probable estimates of the parameters will be found. Software is now available to use this approach (www.athenavisual.com).

The purpose of the present paper was to study the kinetics of the reactions occurring during heating of sugar-casein systems, starting with the kinetic model developed in our previous paper (Brands and Van Boekel, 2001) and using the above mentioned multiresponse approach. Kinetic modelling is an iterative process: proposing a model, confronting it with experiments, criticising the model, adjusting the model, and confronting the adapted model with experiments again. In the present study, we will go through this iterative process several times. New results, obtained when studying the effect of heating temperature, pH, reactant concentration and type of sugar, were used to test the kinetic model very rigorously.

4.2 Materials and methods

4.2.1 Chemicals

All chemicals were of analytical grade. Glucose, fructose and galactose were supplied by Merck (Darmstadt, Germany). Fluka Chemie (Buchs, Switzerland) supplied tagatose. Sodium caseinate (a spray-dried powder) was obtained from DMV (Veghel, the Netherlands) containing 90% protein.

4.2.2 Preparation of reaction model systems

The standard model system consisted of a reducing sugar (150 mM glucose, fructose, galactose or tagatose) and sodium caseinate (3% w/w) dissolved in a phosphate buffer (0.1 M; pH 6.8) to give a molar ratio of sugar to lysine residues of about 10:1. The samples were heated at 120°C in an oil bath in screw-capped glass tubes (Schott, 16 x 160 mm). When the concentration, heating temperature or pH deviated from the standard, this will be mentioned. The reported heating times include the heating up period of about 2 - 3 minutes. After a given heating time, samples were cooled in ice water, prior to analysis. The reaction mixtures were heat-treated in at least two-fold.

4.2.3 Analyses of reactants and products

The reaction mixtures were analysed as described previously (Brands and Van Boekel, 2001). Available lysine residues were determined after derivatization with *ortho*-phthaldialdehyde (Vigo et al., 1992) and subsequent fluorescence detection (emission wavelength 430 nm; excitation wavelength 340 nm). The samples were therefore diluted 4 times in sodium dodecyl sulphate (SDS 16%). These samples were also used to determine the browning intensity by measuring the absorbance at 420 nm spectrophotometrically. The low molecular weight (sugars and sugar breakdown products) fraction was separated from high molecular weight (protein) fraction via Sephadex G25 disposable columns and analysed for sugars and organic acids by HPLC (Brands and Van Boekel, 2001) and spectrophotometrically for browning. The browning of the protein fraction was calculated by subtracting the browning of the sugar fraction from the browning of the total mixture. This absorbance was recalculated to the concentration of protein-bound melanoidins by dividing by the extinction coefficient according to Lambert-Beer's law. The extinction coefficient of protein-bound melanoidins formed in glucose-casein and fructose-casein systems was measured to be 500 $l.mol^{-1}.cm^{-1}$ (Brands et al., 2001). The concentration of melanoidins is thus expressed as sugar units

incorporated in the brown products. The Amadori compound was determined by means of furosine, using HPLC (Resmini et al., 1990). Furosine concentration was converted to that of the Amadori compound using a conversion factor of 3.1 (Brands and Van Boekel, 2001).

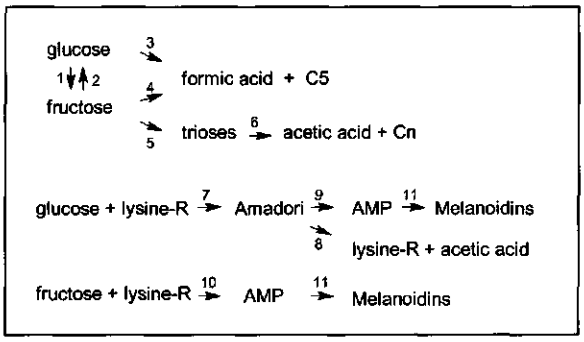
4.2.4 Kinetic modelling

Computer simulations of reactions were done by numerical integration of differential equations that were set up for a particular reaction scheme. The parameters of the model, the rate constants, were estimated by non-linear regression using the determinant criterion (Stewart et al., 1992). The software package Gregpak/Athena Visual Workbench was used for numerical integration as well as to minimise the determinant (www.athenavisual.com). To discriminate between various models, the posterior probability was calculated (Stewart et al., 1998). The model with the highest posterior probability was defined as the most likely one. In the example in which we used the least-squares fitting, the software package DynaFit (Kuzmic, 1996) was used.

4.3 Results and discussion

The reaction network model of Figure 4.1 is quite complex but can be simplified for modelling purposes. On the assumption of steady state behaviour of the enediols, it can be shown that the concentration of enediols is directly proportional to the monosaccharide concentration (De Bruijn, 1986). This kinetic model still has many parameters, namely a rate constant for every reaction step. Estimation of all these parameters at once requires a large number of data points. For multiresponse modelling with the determinant criterion the following constraints are in order: the number of responses r cannot exceed the number of runs n and the number of parameters p must be less than the number of runs n (Van Boekel, 1996a). Therefore the kinetic model was further simplified. As we mentioned in the previous paper (Brands and Van Boekel, 2001), acid formation in the ketose system was about equal in the absence and presence of casein and therefore acid formation via the Maillard reaction will be neglected. The Heyns compound could not be detected and therefore this reaction step will be omitted. It is assumed that the ketose reacts directly with lysine residues to advanced Maillard reaction products (AMP). The formation of formic acid in the aldose systems was only slightly catalysed by amino groups and therefore it was assumed that formic acid was only formed via sugar degradation. Acetic acid can be formed via the Amadori compound and via the degradation of ketoses in triose intermediates. The formation of acetic acid via the

ketoses directly was neglected. Furthermore, the degradation of the sugars in unidentified reaction products (Cn) and the reaction of the sugar degradation compounds with the lysine residues of the protein will first be neglected. This all leads to the simplified kinetic model of Scheme 4.1, including 11 parameters.



Scheme 4.1 Kinetic model for monosaccharide-casein reactions

This reaction scheme gives the following differential equations for the concentrations:

$$\frac{d[glu]}{dt} = -k_1[glu] + k_2[fru] - k_3[glu] - k_7[glu][lys]$$

$$\frac{d[fru]}{dt} = k_1[glu] - k_2[fru] - k_4[fru] - k_5[fru] - k_{10}[fru][lys]$$

$$\frac{d[formic]}{dt} = k_3[glu] + k_4[fru]$$

$$\frac{d[acetic]}{dt} = k_6[triose] + k_8[Amadori]$$

$$\frac{d[triose]}{dt} = 2k_5[fru] - k_6[triose]$$

$$\frac{d[lys]}{dt} = -k_7[glu][lys] + k_8[Amadori] - k_{10}[fru][lys]$$

$$\frac{d[Amadori]}{dt} = k_7[glu][lys] - k_8[Amadori] - k_9[Amadori]$$

$$\frac{d[AMP]}{dt} = k_9[Amadori] + k_{10}[fru][lys] - k_{11}[AMP]$$

$$\frac{d[M]}{dt} = k_{11}[AMP]$$

In the previous paper (Brands and Van Boekel, 2001) we studied isolated reactions (for instance, the reaction of glucose or fructose in the absence of casein and the reaction of the

isolated protein-bound Amadori compound fructosyllysine) to make it possible to construct a reaction network model that describes the reactions in a chemically justified way. These isolated reactions could also be used to make an independent estimate of the reaction rate constants for the reactions concerned. Sugar isomerisation and degradation reactions could be studied independently of the Maillard reaction by omitting protein. The reactions of glucose could, however, not be studied independently from the reactions of fructose since considerable amounts of fructose were formed when heating glucose, and *vice versa*, although less. Therefore, the reaction rate constants were estimated by modelling the data obtained from experiments with glucose and fructose simultaneously, thus using the data of both sugar-casein systems. This means that the number of responses increased by a factor of two. Another way to estimate the reaction rate constants is to fix the rate constants of the reactions in the fructose system while estimating the rate constants in the glucose system, and *vice versa*. This method is very time consuming since it is an iterative process. Comparable results, but much easier, were obtained by jointly modelling the reactions of glucose and fructose. The double number of differential equations were numerically integrated and fitted to the data.

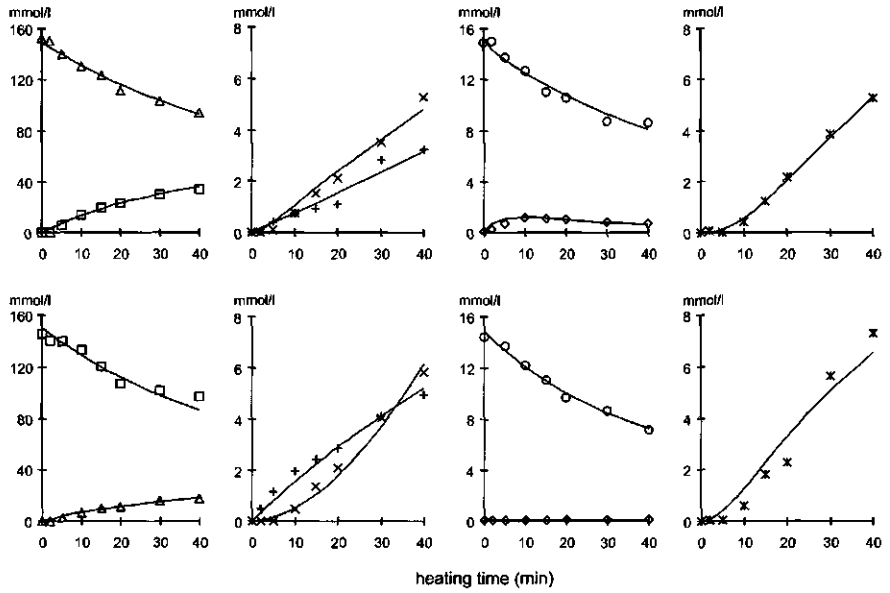


Figure 4.2 Simulation (drawn lines) based on the kinetic model of Scheme 4.1 for glucose-casein (upper part) and fructose-casein systems (lower part) heated at 120°C. Glucose (Δ), fructose (□), formic acid (+), acetic acid (x), lysine residues (○), Amadori compound (◇), melanoidins (*).

In Figure 4.2 the results are shown of the fit of the kinetic model of Scheme 4.1 to the experimental data. For the clarity of the graphs the averages of the experimental data for every response are shown, but the actual modelling was done using all experimental data. In general, the fits were deemed very good as judged from the residuals. Only a minor lack of fit could be observed in the fructose-casein system: browning was overestimated at the beginning and underestimated at the end of heating. Overall, it seemed that the model was able to describe the reactions occurring in heated sugar-casein systems very well. The logarithm of the posterior probability was -31.992. The 95%-confidence intervals of the rate constants for reaction steps 5 and 6 (degradation of ketoses into triose intermediates and subsequent reaction to acetic acid) and reaction step 11 (formation of melanoidins) were somewhat wide (Table 4.1). This means that these reaction steps were not very important or, more likely, that the data obtained do not contain enough information to estimate them. From a mechanistically point of view these reaction steps can not be omitted.

Table 4.1 Rate constants and their 95%-confidence interval as found by kinetic modelling for the model displayed in Scheme 4.1 to the data of glucose-casein and fructose-casein systems (120°C; initial pH 6.8), using the determinant criterion and the least-squares criterion.

Rate constant	determinant	least-squares
k_1 (min^{-1})	0.01028 ± 0.00046	0.01020 ± 0.00021
k_2 (min^{-1})	0.00509 ± 0.00023	0.00508 ± 0.00022
k_3 (min^{-1})	0.00047 ± 0.00004	0.00066 ± 0.00017
k_4 (min^{-1})	0.00110 ± 0.00006	0.00105 ± 0.00018
k_5 (min^{-1})	0.00712 ± 0.00214	0.00461 ± 0.00046
k_6 (min^{-1})	0.00439 ± 0.00143	0.00673 ± 0.00152
k_7 ($l.mmol^{-1}.min^{-1}$)	0.00018 ± 0.00001	0.00024 ± 0.00002
k_8 (min^{-1})	0.11134 ± 0.01146	0.11224 ± 0.04683
k_9 (min^{-1})	0.14359 ± 0.01710	0.16831 ± 0.07595
k_{10} ($l.mmol^{-1}.min^{-1}$)	0.00015 ± 0.00001	0.00017 ± 0.00003
k_{11} (min^{-1})	0.12514 ± 0.03048	0.07302 ± 0.02621

It was mentioned in the introduction that it could be incorrect to use the least squares criterion for estimating the reaction rate constants in multiresponse modelling and that therefore the determinant criterion was used. To show that it indeed matters which fit criterion is used, the model was also fitted to the experimental data by minimising the residual sum of squares. Although the fit of the model to the data did not differ that much, much larger 95%-confidence intervals were obtained than when the determinant was minimised (Table 4.1). Taking into account differences in variances and covariances, as is implicitly done when

using the determinant criterion, clearly leads to a much more precise estimation of the kinetic parameters. Obviously, precision of parameter estimates is an important issue, and therefore it is essential to use the determinant criterion.

Under the above-mentioned conditions (heating temperature 120°C; pH 6.8; sugar concentration 150 mM; protein concentration 3%), the proposed kinetic model fitted the experimental data very well. To strain the model, the reaction conditions were altered.

4.3.1 Effect of heating temperature

Generally, the rate of chemical reactions increases with increasing temperature. Since the Maillard reaction consists of several reaction steps, each with a possibly different temperature sensitivity, it strongly depends on temperature which reaction route prevails and what pattern of intermediates and end products is formed. Furthermore, temperature affects the activities of the reactants. The active form of the sugar is considered to be the open chain, the concentration of which increases with temperature. The percentage of fructose in its acyclic form at neutral pH is about 0.7% at room temperature and 13.1% at 80°C (Yaylayan et al., 1993). The concentration of acyclic form of glucose is much lower and therefore more difficult to detect. In literature the reported percentage of glucose in its acyclic form varies from 0.002% (Hayward and Angyal, 1977) to 0.022% (Cantor and Peniston, 1940) at neutral pH and room temperature. The percentage of acyclic form at higher temperatures is not known.

In order to be able to predict the reactions at various temperatures, the temperature dependence has to be determined. A consistent temperature dependence is an additional indication that a model is acceptable. The relationship between the rate constant (k) and temperature (T) is frequently indicated by the well-known Arrhenius equation:

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

k_0 is the so-called pre-exponential factor, R is the gas constant (8.31 $J.mol^{-1}.K^{-1}$) and E_a is 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 an empirical equation and appears to fit many reactions and is therefore frequently used. It is, however, an oversimplification. Eyring developed a theoretical basis for the relation between k and T in the so-called transition-state theory:

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

where k_B is Boltzmann's constant ($1.38*10^{-23}$ JK^{-1}), h is Planck's constant ($6.62*10^{-34}$ Js^{-1}). $\Delta S^{\ddagger}$ is the activation entropy, and $\Delta H^{\ddagger}$ the activation enthalpy. Direct estimation of the kinetic parameters k_0 and E_a or $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ from these equations is to be preferred over the usual stepwise estimation, where reaction rate constants at constant temperature are determined first and subsequently E_a or $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are estimated from the rate constants so obtained. The stepwise procedure generally results in a relatively large confidence interval of the kinetic parameters due to a large standard deviation resulting from propagation of errors and a small number of degrees of freedom (Van Boekel, 1996a).

Generally, when estimating activation energies or activation enthalpies and entropies a high correlation is found between the parameters, because the experimental range of temperatures studied is narrow compared to the absolute temperature range over which the Arrhenius or Eyring equation would apply. Therefore the equations should be reparameterised. The reparameterised Arrhenius equation was used to model the reactions at various heating temperatures simultaneously and appears as follows (Schokker, 1997):

$$k = X\exp(-YE_a)$$

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

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

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

Glucose-casein and fructose-casein systems were heated at 90, 100, 110, 120 and 130°C. The model of Scheme 4.1 was fitted to the data. In Figure 4.3 the results of the fit for the sugar-casein systems heated at 100 and 120°C are given as an example. An increase of temperature leads to a higher loss of the reactants and an increased formation of the reaction products. Formation of Amadori compound was faster, but its degradation was also faster, which resulted in the same maximum concentration for all heating temperatures. The estimates of the activation energies and their 95%-confidence intervals are shown in Table 4.2.

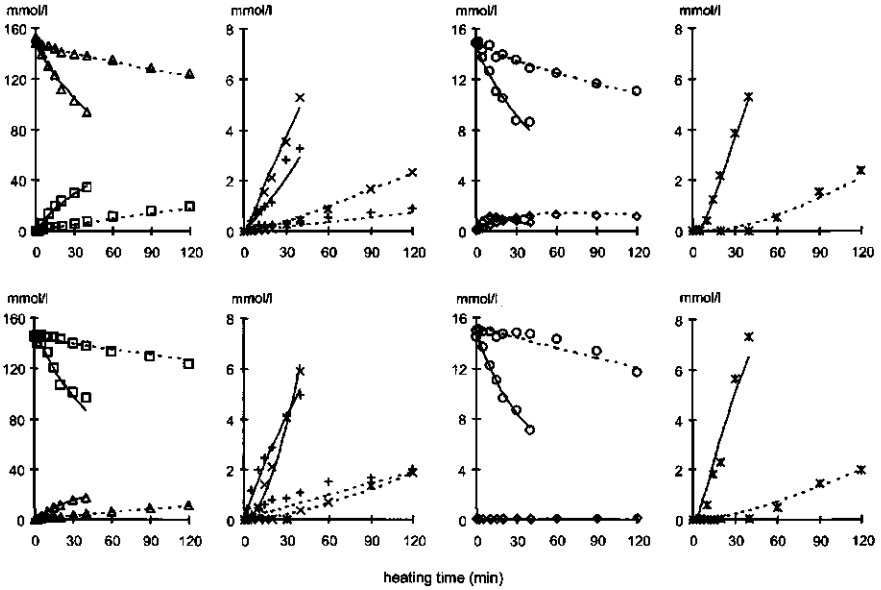


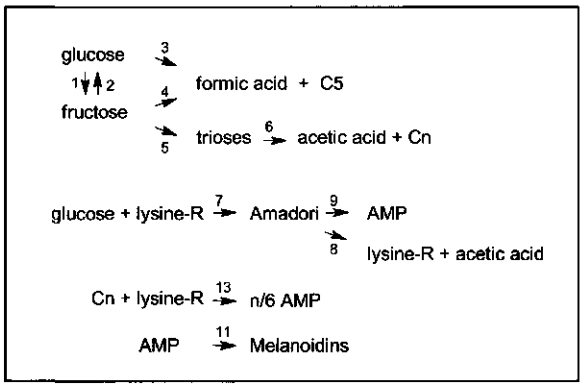
Figure 4.3 Simulation (drawn lines) based on the kinetic model of Scheme 4.1 for glucose-casein (upper part) and fructose-casein systems (lower part) heated at 100 (dotted line) and 120°C (solid line). Glucose (Δ), fructose (□), formic acid (+), acetic acid (x), lysine residues (○), Amadori compound (◇), melanoidins (*).

Table 4.2 Rate constants and activation energies of glucose-casein and fructose-casein systems heated in the range from 90 - 130°C as found by kinetic modelling of the model displayed in Scheme 4.1.

Rate constants	Heating temperature					Activation energy
	90°C	100°C	110°C	120°C	130°C	(kJ/mol)
k_1 (min^{-1})	0.00039	0.00118	0.00342	0.00934	0.02431	126 ± 2
k_2 (min^{-1})	0.00026	0.00072	0.00193	0.00491	0.01194	117 ± 2
k_3 (min^{-1})	0.00001	0.00004	0.00012	0.00036	0.00103	137 ± 7
k_4 (min^{-1})	0.00003	0.00011	0.00042	0.00149	0.00498	159 ± 5
k_5 (min^{-1})	0.00012	0.00037	0.00109	0.00306	0.00816	129 ± 17
k_6 (min^{-1})	0.00071	0.00187	0.00466	0.01110	0.02531	109 ± 19
k_7 ($l.mmol^{-1}.min^{-1}$)	0.00001	0.00003	0.00008	0.00020	0.00046	114 ± 2
k_8 (min^{-1})	0.00510	0.01528	0.04323	0.11601	0.29647	124 ± 4
k_9 (min^{-1})	0.00616	0.01914	0.05607	0.15554	0.41017	128 ± 4
k_{10} ($l.mmol^{-1}.min^{-1}$)	0.00000	0.00001	0.00004	0.00012	0.00034	138 ± 4
k_{11} (min^{-1})	0.01631	0.03172	0.05958	0.10838	0.19138	75 ± 11

The activation energies of most reactions were in the order of 120 kJ/mol, which was as expected for chemical reactions (Van Boekel, 1998). This consistent temperature dependence is an indication that the model is acceptable. How well the model fits to the data is another indication for the acceptability of the model (see Figure 4.3). The kinetic model fitted the data of the glucose-casein systems heated at various temperatures very well. The fit for the heated fructose-casein systems was less, especially for the formation of formic acid, loss of lysine and browning. At 90, 100 and 110°C formic acid formation was underestimated by the model in the beginning of heating, while at 120 and 130°C it was estimated correctly at the beginning of heating but overestimated at the end. This might be due to a pH effect (see next section). Browning in the fructose-casein system was underestimated at the end of heating at 110, 120 and 130°C and overestimated at the beginning of heating at 90, 100 and 110°C. Another lack of fit was the loss of lysine residues in fructose-casein systems heated at 90, 100 or 110°C. At 100°C for instance, the lysine residues did not decrease until 60 minutes, whereas the model predicts lysine to decrease immediately. Obviously, there is something wrong in the model for the fructose-casein system.

Therefore a new model (Scheme 4.2) was proposed in which not fructose, but unidentified reaction compounds (sugar degradation products (Cn) formed via reaction step 3, 4, 5, 6, 8 and the reintroduced reaction step 12) react with lysine residues to form advanced Maillard reaction products (AMP).



Scheme 4.2 Kinetic model for monosaccharide-casein reactions

The differential equation for the concentration of Cn (including the trioses) and AMP were then as follows:

$$\frac{d[triose]}{dt} = 2k_5[fru] - k_6[triose] - k_{13}[triose][lys]$$

$$\frac{d[C_n]}{dt} = k_3[glu] + k_4[fru] + k_{12}[fru] + k_6[trioses] + k_8[Amadori] - k_{13}[C_n][lys]$$

$$\frac{d[AMP]}{dt} = k_9[Amadori] + \frac{3}{6}k_{13}[triose][lys] + \frac{n}{6}k_{13}[C_n][lys] - k_{11}[AMP]$$

In the differential equation of AMP the parameter n is introduced. AMPs are the precursors of the melanoidins. Melanoidins have been defined as the number of C6-equivalents incorporated in the brown high molecular weight compounds. A Cn compound reacts in a 1:1 ratio with the lysine residues of the protein. If 1 mmol Cn reacts with 1 mmol lysine, n/6 * 1 mmol AMP and subsequently melanoidins are formed. Parameter n is expected to be smaller or equal to 6, because Cn is defined as a sugar fragment with $1 \leq n \leq 6$ carbon atoms.

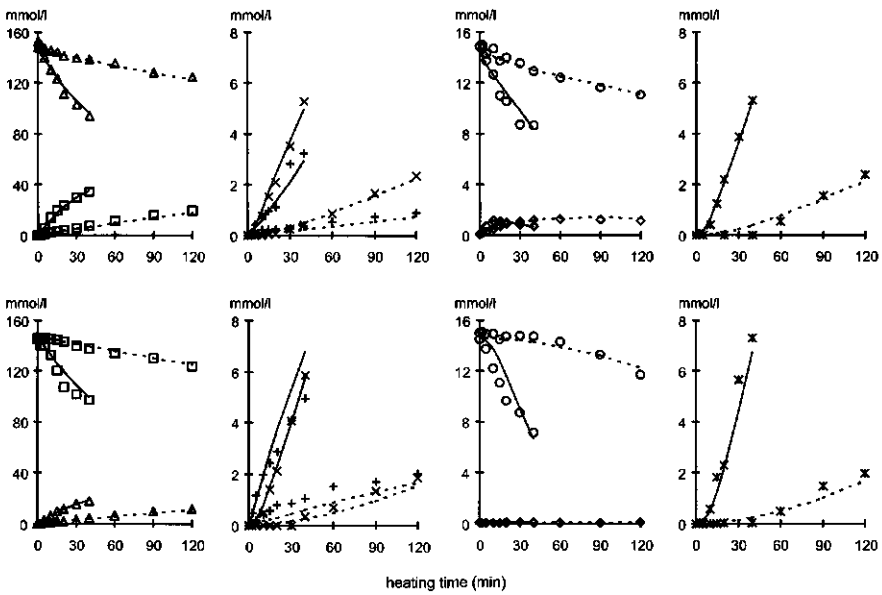


Figure 4.4 Simulation (drawn lines) based on the kinetic model of Scheme 4.2 for glucose-casein (upper part) and fructose-casein systems (lower part) heated at 100 (A) and 120°C (B). Glucose (Δ), fructose (□), formic acid (+), acetic acid (x), lysine residues (○), Amadori compound (◇), melanoidins (*).

The adapted model was able to fit the data, although an inconsistent temperature dependence was observed for reaction step 11 since the activation energy approaches zero (not shown). The parameter n was estimated to be 6.2 (± 0.5). The adapted model did not show an obvious change in fit for the glucose-casein system. In the fructose-casein system the fit for the loss of lysine was improved at lower temperatures and browning at higher temperatures, but the fit for lysine loss at higher temperature and browning at lower temperature was worsened (Figure 4.4). We assumed that the rate constant of the reaction between the intermediate compounds and lysine residues was the same for the various Cn compounds. Furthermore, we assumed that the average number of carbon atoms (n) per Cn compound did not depend on the reaction temperature. These assumptions might have led to the improper fit of the model to the lysine and melanoidin data. Another reason for the not perfect fit is the drop of pH during heating. This will be the topic of study in the next section.

4.3.2 Effect of pH

During heating of sugar-casein systems the pH decreases due to the formation of organic acids, among which formic acid and acetic acid. To study the effect of pH on the reaction kinetics a glucose-casein and a fructose-casein system with an initial pH of 5.9 were heated at 120°C and kinetic modelling was carried out, using the kinetic model of Scheme 4.1. The results are shown in Figure 4.5 and these were compared with the results of systems with an initial pH of 6.8 (Figure 4.2). In both sugar-casein systems the loss of reactants was less and the formation of reaction products was lower when the initial pH was decreased. Whereas the loss of lysine was about equal in both sugar-casein systems heated at pH 6.8, it was less in the fructose-casein system than in the glucose-casein system at pH 5.9. This difference was also observed for the degree of browning. From the reaction rate constants (compare Table 4.3 and 4.1) it can be observed that the rate of all reactions decreased with decreasing pH. Obviously, the pH has an effect both on the sugar reactions (isomerisation and degradation) and on the Maillard reaction.

Except for the fact that the pH has an influence on the rates of the reactions, the reaction mechanism could also be influenced by the pH. It is claimed that certain reaction pathways are favoured at lower pH while others at higher pH. For instance, 5-hydroxymethylfurfural (HMF) formation increased with decreasing pH (Figure 4.6), but still its amounts are an order of magnitude lower than other reaction products. This change in reaction mechanism might be an explanation for the poor fit of formic acid in the fructose-casein system. Formic acid and HMF are both considered to be formed via the 1,2-enolisation route. As the pH lowers during

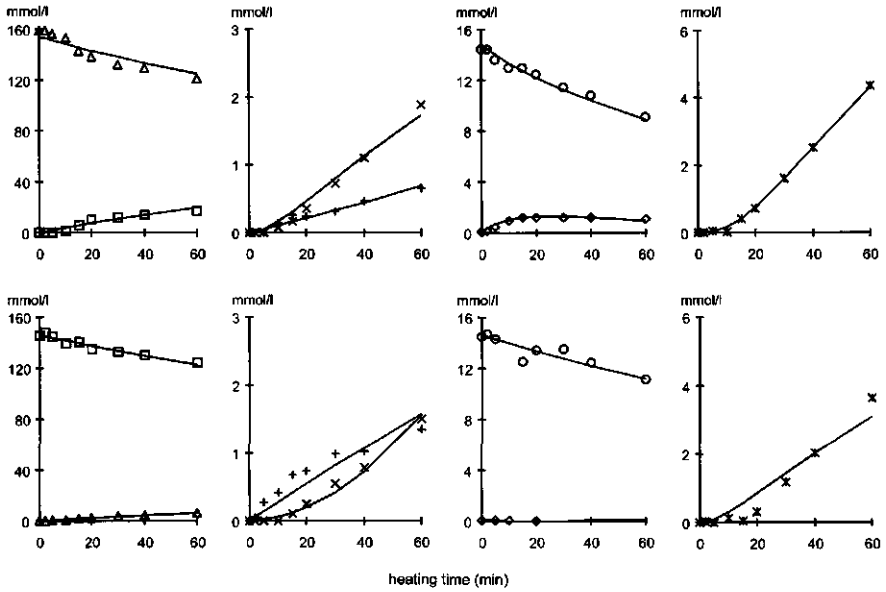


Figure 4.5 Simulation (drawn lines) based on the kinetic model of Scheme 4.1 for glucose-casein (upper part) and fructose-casein systems (lower part) heated at 120°C, initial pH 5.9. Glucose (Δ), fructose (□), formic acid (+), acetic acid (x), lysine residues (○), Amadori compound (◇), melanoidins (*).

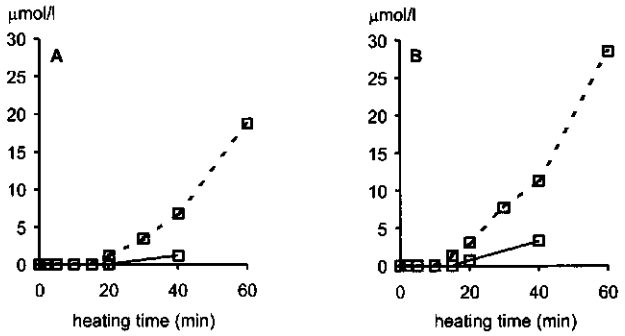


Figure 4.6 Formation of HMF in glucose-casein (A) and fructose-casein systems (B) heated at 120°C. Dotted line: initial pH 5.9; solid line: initial pH 6.8.

heating, HMF starts to be formed at the expense of formic acid and therefore the formation of formic acid levels off.

Generally it has been stated that the rate of browning and substrate loss increases with increasing pH, up to a pH of about 10 (Ashoor and Zent, 1984; Dworschak and Oersi, 1977; Labuza and Baisier, 1992). An explanation for this pH effect is that protonation of the amino

group of the lysine residues of the protein increases with decreasing pH. Due to this protonation the nucleophilicity and therefore the reactivity of the amino group is lost. The higher the pH, the larger the percent of amino groups in the unprotonated form and, therefore, more lysine residues can react with reducing sugars. The pK_a (the pH where 50% of the amino group is protonated) of the ε-amino group of lysine residues is 10.53 at room temperature (Labuza and Baisier, 1992). It can be calculated that at 25°C at pH 6.8 0.0186% of the lysine residues is in its unprotonated form, whereas it is 8 times less (0.0023%) at pH 5.9. Since some subsequent steps in the Maillard reaction are proton catalysed, both effects will result in an optimum pH for the reaction rate of the Maillard reaction browning. Another factor causing pH rate dependence is related to the amount of acyclic form of the reducing sugars. The acyclic form is the reactive form and it is considered to decrease with decreasing pH (Labuza and Baisier, 1992). A pH decrease will therefore slow down both the Maillard reaction and the isomerisation and degradation reaction of the sugars. The amount of acyclic form of glucose was found to increase about threefold in the pH range from 6.5 to 7.5 at 25°C (Cantor and Peniston, 1940). The acyclic form of fructose, however, was not found to increase as a function of pH at 25°C, while it only increased with a factor of 1.1 in the pH range from 2 to 9 at 80°C (Yaylayan et al., 1993). The observed difference between glucose and fructose in Maillard reaction reactivity due to lowering of the pH can therefore not be explained by a relatively higher concentration decrease of the acyclic form of fructose. According to Hayashi and Namiki (Hayashi and Namiki, 1986), formation of C2 and C3 sugar fragments increased with increasing pH. These fragments are much more reactive towards amino groups than the original sugars. This can explain why browning and loss of lysine are less in the fructose-casein system compared to the glucose-casein system at pH 5.9, while at pH 6.8 the opposite pattern was observed. If fructose itself is not reactive in the Maillard reaction but only its degradation products (as proposed in the previous section) less lysine will react and less browning will occur when the pH is lowered. This is another indication that reaction step 13 (Scheme 4.2) can not be neglected.

In an additional experiment we heated glucose-casein and fructose-casein systems at 100°C with an initial pH of 6.8 and the pH was kept constant during heating, using a pH Stat Controller. The reaction rate constants of Table 4.2 for the reaction at 100°C were used to predict the loss of reactants and formation of reaction products. The results are shown in Figure 4.7. In the glucose-casein system the decrease of glucose and formation of fructose were predicted very well but the formation of acids and browning and the loss of lysine were somewhat underestimated. Apparently, the drop in pH during heating slows down the

Maillard reaction mainly and the effect on isomerisation is less. In the fructose-casein system, the prediction of loss of lysine and formation of melanoidins is even worse. The loss of fructose is also underestimated, which is an indication that fructose is decreased via an additional reaction route (reaction step 12) and that these degradation products react in the Maillard reaction with lysine residues (reaction step 13).

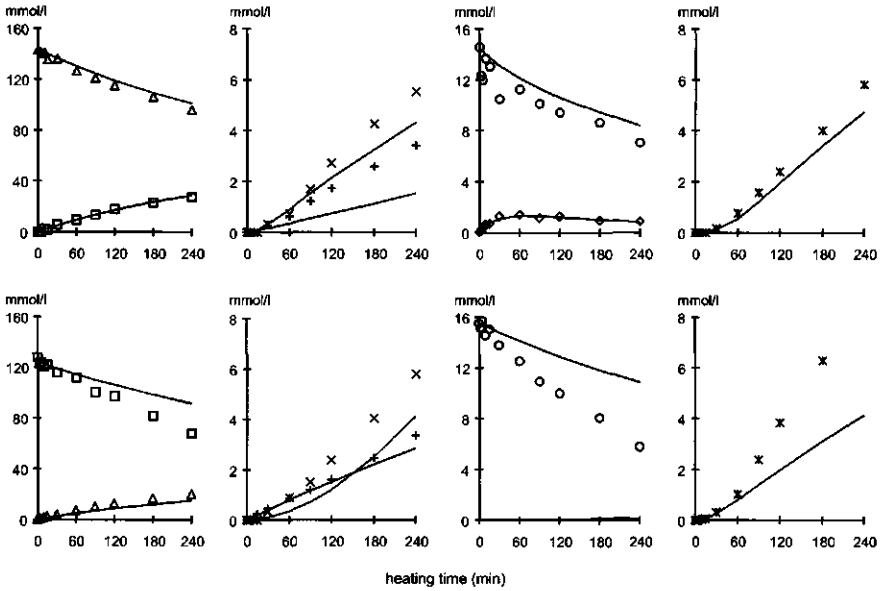


Figure 4.7 Predictions (drawn lines) based on the reaction rate constants of Table 4.1 for glucose-casein (upper part) and fructose-casein reactions (lower part) at 100°C and constant pH 6.8. Glucose (Δ), fructose (□), formic acid (+), acetic acid (x), lysine residues (○), Amadori compound (◇), melanoidins (*).

In this part of our study, the kinetic model will be optimised for pH effects. The conversion between the protonated and the unprotonated lysine residues is very fast, just as the reaction between the cyclic and acyclic form of the sugars. The available reactant concentration is therefore in direct proportion to the total concentration, at least, when the pH is constant during heating. A difference in pH is then taken into account in the reaction rate constants. If the pH is not constant during heating, the reaction rate constants change during heating as a function of pH. The model of Scheme 4.1 was fitted to the data of the sugar-casein systems heated at 120°C (initial pH 6.8), taking the effect of the pH drop on the loss of lysine into account in the differential equations. This was done by multiplying the lysine

concentration with the factor $10^{-\Delta pH}$. When the pH is decreased with 1 unit the concentration of unprotonated lysine residues, the reactive form, decreases 10^1 times. The effect of pH was less for the sugars and was therefore neglected. In Figure 4.8 the results of the fit are shown.

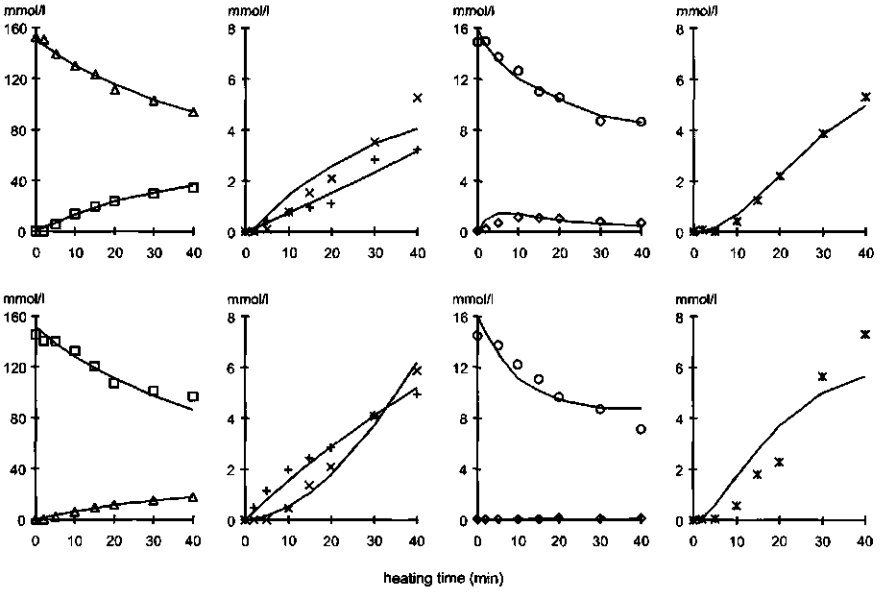
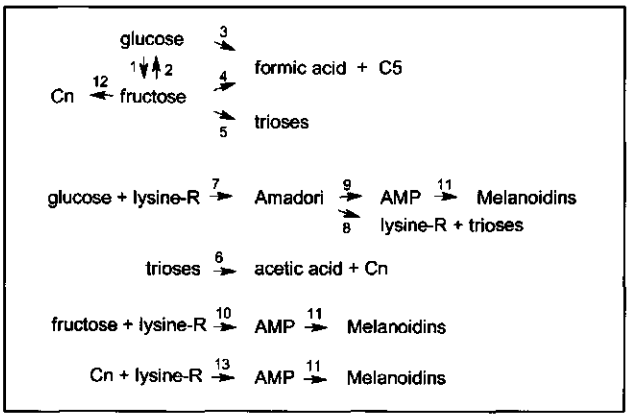


Figure 4.8 Simulation (drawn lines) based on the kinetic model of Scheme 4.1 for glucose-casein (upper part) and fructose-casein systems (lower part) heated at 120°C, taking into account effect of pH drop on reactivity of lysine residues. Glucose (Δ), fructose (□), formic acid (+), acetic acid (x), lysine residues (○), Amadori compound (◇), melanoidins (*).

As can be observed, a maximum loss of lysine residues was predicted for the fructose-casein system, while, according to the data, lysine should further decrease. This can be effectuated by taking into account the reaction of lysine residues with sugar degradation products (reaction step 13). Furthermore, the fit of the model to the acetic acid data of the glucose-casein system flattened off, while it had to increase according to the data. In the fructose system (a ketose) it was assumed that acetic acid is formed via triose intermediates. Logically, it can be expected that formation of acetic acid via the Amadori compound (an aminoketose) also arises from these triose intermediates. All these changes are shown in the adapted kinetic model of Scheme 4.3. The model was fitted to the data of the glucose-casein and fructose-casein systems heated at 120°C. Scheme 4.3 gave a very good fit (see Figure 4.9) as judged from the residuals. The formation of acetic acid in the glucose-casein system was better



Scheme 4.3 Kinetic model for monosaccharide-casein reactions

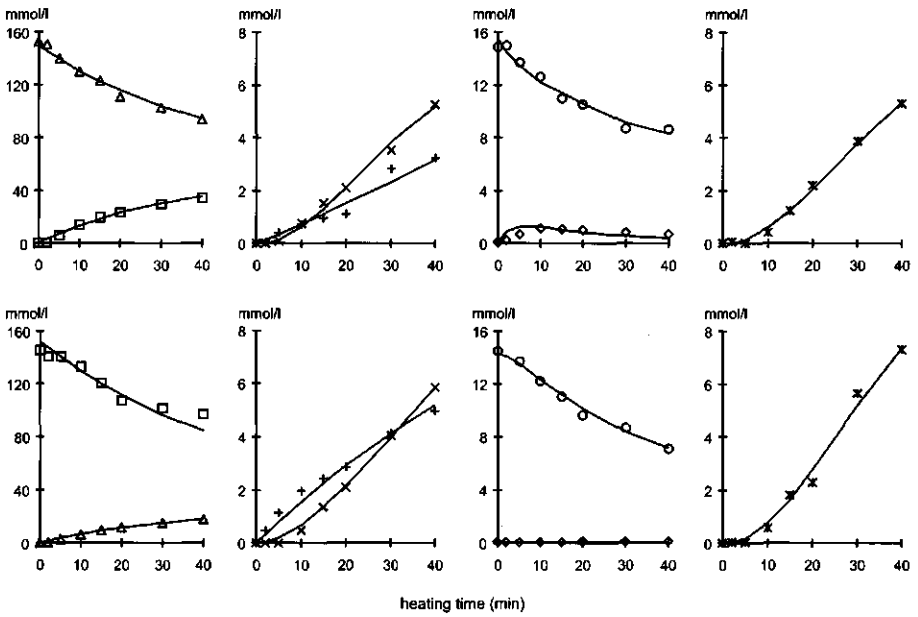


Figure 4.9 Simulation (drawn lines) based on the kinetic model of Scheme 4.3 for glucose-casein (upper part) and fructose-casein systems (lower part) heated at 120°C, taking into account effect of pH drop on reactivity of lysine residues. Glucose (Δ), fructose (□), formic acid (+), acetic acid (x), lysine residues (○), Amadori compound (◇), melanoidins (*).

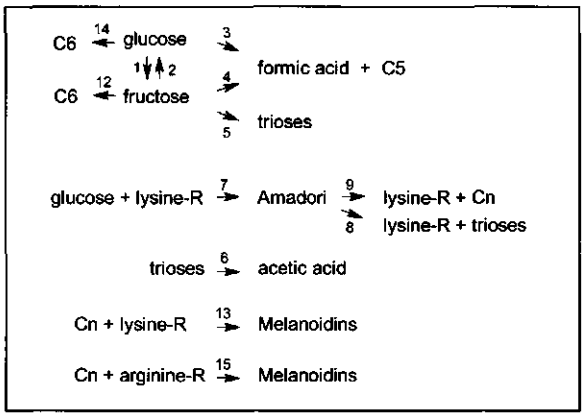
predicted and both the loss of lysine and browning were better described when reaction step 13 was included. A minor lack of fit in the glucose-casein system is the fit for the Amadori compound, which is somewhat overestimated in the beginning of heating and underestimated after 20 minutes of heating. In Table 4.4 the reaction rate constants are given. It was observed that the 95%-confidence interval of k_{10} was very wide and that the rate constant itself was very small. The fit of the model to the data did not change when reaction step 10, the reaction of fructose with lysine residues, was neglected. This is yet another indication that not fructose itself, but its degradation products are reactive in the Maillard reaction. Furthermore, the 95%-confidence interval of k_{11} was very wide. The parameter n (number of carbon atoms per Cn compound) was estimated to be 10.8 ± 2.1. A value higher than 6 was not expected. To predict enough browning this was apparently necessary. Despite these imperfections, Scheme 4.3 described the reactions occurring in a monosaccharide-casein system better than Scheme 4.1, when taking the effect of pH into account. This visual observation was supported by the objective criterion of the software program Athena: the logarithm of the posterior probability increased from -39.871 to -34.965.

Table 4.4 Rate constants and their 95%-confidence intervals as found by kinetic modelling for the model displayed in Scheme 4.3 to the data of glucose-casein and fructose-casein systems (120°C; initial pH 6.8).

Rate constant	
k_1 (min^{-1})	0.01040 ± 0.00048
k_2 (min^{-1})	0.00503 ± 0.00024
k_3 (min^{-1})	0.00047 ± 0.00004
k_4 (min^{-1})	0.00109 ± 0.00006
k_5 (min^{-1})	0.00103 ± 0.00019
k_6 (min^{-1})	0.05428 ± 0.01850
k_7 ($l.mmol^{-1}.min^{-1}$)	0.00032 ± 0.00004
k_8 (min^{-1})	0.15338 ± 0.03749
k_9 (min^{-1})	0.16341 ± 0.03773
k_{10} ($l.mmol^{-1}.min^{-1}$)	0.00006 ± 0.00004
k_{11} (min^{-1})	0.09345 ± 0.02972
k_{12} (min^{-1})	0.00847 ± 0.00231
k_{13} ($l.mmol^{-1}.min^{-1}$)	0.00173 ± 0.00044

To eliminate the imperfections of Scheme 4.3 the model was further improved (Scheme 4.4). In this scheme the reaction of fructose with lysine (reaction step 10) and the reaction via AMP to browning (reaction step 11) were neglected. It was proposed that the Amadori compound reacted toward Cn compounds and that the protein was released (reaction step 9).

These Cn compounds were expected to react with lysine toward melanoidins (reaction step 13). Furthermore, it was proposed that browning in the glucose-casein system could also occur without involvement of the Amadori compound. Glucose can degrade into Cn compounds (reaction step 14), which subsequently can react with lysine residues to form brown compounds (reaction step 13). Up till now, we neglected the roll of arginine in the Maillard reaction. The guanidine group of arginine is not very reactive towards sugars, but can be very reactive in the presence of some sugar degradation products, mainly deoxyosones and other dicarbonyls (Yeboah et al., 2000). When the lysine concentration becomes limiting or when considerable amounts of degradation products are formed, the reaction of arginine can no longer be neglected. The concentration of the arginine residues in our model systems is about 6 mmol/L and this reaction step (reaction step 15) will be included in the model of Scheme 4.4.



Scheme 4.4 Kinetic model for monosaccharide-casein reactions

The following differential equations were numerically integrated and fitted to the data:

$$\frac{d[glu]}{dt} = -k_1[glu] + k_2[fru] - k_3[glu] - k_7[glu][lys] \times 10^{-\Delta pH} - k_{14}[glu]$$

$$\frac{d[fru]}{dt} = k_1[glu] - k_2[fru] - k_4[fru] - k_5[fru] - k_{12}[fru]$$

$$\frac{d[formic]}{dt} = k_3[glu] + k_4[fru]$$

$$\frac{d[acetic]}{dt} = k_6[triose]$$

$$\frac{d[triose]}{dt} = 2k_5[fru] + 2k_8[Amadori] - k_6[triose] - k_{13}[triose][lys]\times 10^{-\Delta pH} - k_{15}[triose][arg]$$

$$\frac{d[lys]}{dt} = -k_7[glu][lys]\times 10^{-\Delta pH} + k_8[Amadori] + k_9[Amadori] - k_{13}[triose][lys]\times 10^{-\Delta pH}$$

$$- k_{13}[C_5][lys]\times 10^{-\Delta pH} - k_{13}[C_6][lys]\times 10^{-\Delta pH}$$

$$\frac{d[Amadori]}{dt} = k_7[glu][lys]\times 10^{-\Delta pH} - k_8[Amadori] - k_9[Amadori]$$

$$\frac{d[C_5]}{dt} = k_3[glu] + k_4[fru] - k_{13}[C_5][lys]\times 10^{-\Delta pH}$$

$$\frac{d[C_6]}{dt} = k_{12}[fru] + k_{14}[glu] + k_9[Amadori] - k_{13}[C_6][lys]\times 10^{-\Delta pH} - k_{15}[C_6][arg]$$

$$\frac{d[arg]}{dt} = -k_{15}[triose][arg] - k_{15}[C_6][arg]$$

$$\frac{d[M]}{dt} = \frac{3}{6}k_{13}[triose][lys] + \frac{5}{6}k_{13}[C_5][lys] + \frac{6}{6}k_{13}[C_6][lys] + \frac{3}{6}k_{14}[triose][arg] + \frac{6}{6}k_{14}[C_6][arg]$$

The fit of this model to the data is shown in Figure 4.10. The reaction rate constants are given in Table 4.5. A reasonable fit for all reactants and products was obtained as judged from the residuals. The logarithm of the posterior probability increased to –31.394. Scheme 4.4 was therefore the best model obtained so far. The rate of reaction step 9 approached to zero. This means that the Amadori compound is only involved in the browning reaction of the glucose-casein system via the formation of triose intermediates.

Table 4.5 Rate constants and their 95%-confidence intervals as found by kinetic modelling for the model displayed in Scheme 4.4 to the data of glucose-casein and fructose-casein systems (120°C; initial pH 6.8).

Rate constant	
k_1 (min^{-1})	0.00985 ± 0.00038
k_2 (min^{-1})	0.00513 ± 0.00023
k_3 (min^{-1})	0.00047 ± 0.00004
k_4 (min^{-1})	0.00109 ± 0.00006
k_5 (min^{-1})	0.00090 ± 0.00019
k_6 (min^{-1})	0.08256 ± 0.03575
k_7 ($l.mmol^{-1}.min^{-1}$)	0.00016 ± 0.00003
k_8 (min^{-1})	0.13630 ± 0.03143
k_9 (min^{-1})	-
k_{12} (min^{-1})	0.00569 ± 0.00131
k_{13} ($l.mmol^{-1}.min^{-1}$)	0.00335 ± 0.00075
k_{14} (min^{-1})	0.00269 ± 0.00057
k_{15} ($l.mmol^{-1}.min^{-1}$)	0.00081 ± 0.00059

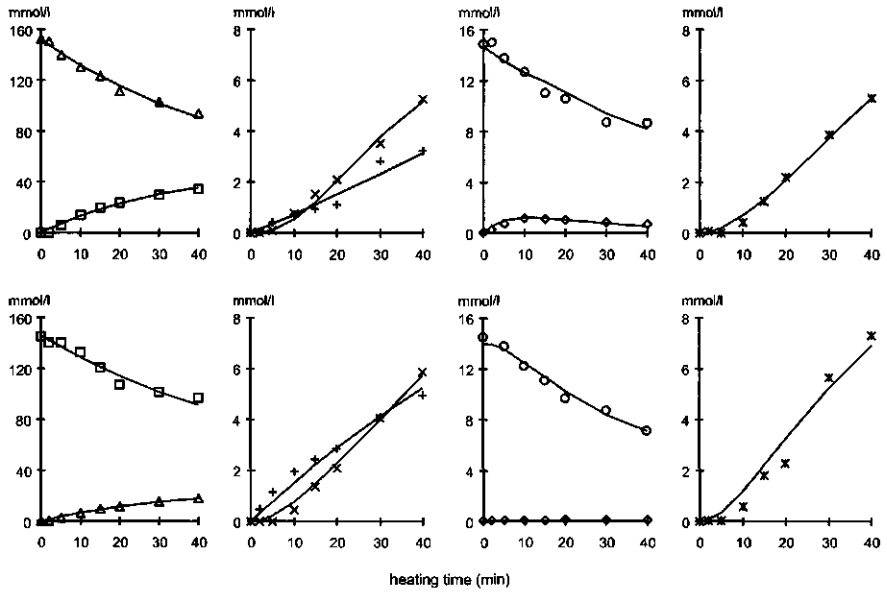


Figure 4.10 Simulation (drawn lines) based on the kinetic model of Scheme 4.4 for glucose-casein (upper part) and fructose-casein systems (lower part) heated at 120°C. Glucose (Δ), fructose (□), formic acid (+), acetic acid (x), lysine residues (O), Amadori compound (◇), melanoidins (*).

Table 4.6 Rate constants and activation energies of glucose-casein and fructose-casein systems heated in the range from 90 - 130°C as found by kinetic modelling of the model displayed in Scheme 4.4.

Rate constants	Heating temperature					Activation energy
	90°C	100°C	110°C	120°C	130°C	(kJ/mol)
k_1 (min^{-1})	0.00038	0.00116	0.00336	0.00924	0.02413	126 ± 2
k_2 (min^{-1})	0.00025	0.00071	0.00191	0.00486	0.01181	117 ± 2
k_3 (min^{-1})	0.00001	0.00004	0.00013	0.00039	0.00112	140 ± 7
k_4 (min^{-1})	0.00002	0.00010	0.00038	0.00138	0.00463	160 ± 6
k_5 (min^{-1})	0.00005	0.00013	0.00034	0.00086	0.00204	114 ± 5
k_6 (min^{-1})	0.00791	0.01963	0.04643	0.10511	0.22852	102 ± 13
k_7 ($l.mmol^{-1}.min^{-1}$)	0.00001	0.00003	0.00007	0.00015	0.00032	102 ± 4
k_8 (min^{-1})	0.00812	0.02161	0.05461	0.13167	0.30386	110 ± 6
k_9 (min^{-1})						
k_{12} (min^{-1})	0.00005	0.00021	0.00083	0.00307	0.01065	164 ± 6
k_{13} ($l.mmol^{-1}.min^{-1}$)	0.00069	0.00135	0.00253	0.00460	0.00811	75 ± 9
k_{14} (min^{-1})	0.00006	0.00021	0.00066	0.00199	0.00567	138 ± 4
k_{15} ($l.mmol^{-1}.min^{-1}$)	0.00211	0.00211	0.00211	0.00211	0.00211	0

The model of Scheme 4.4 was also fitted to data of glucose-casein and fructose-casein heated at 90, 100, 110, 120 and 130°C using the reparameterised Arrhenius equation. Reasonable fits were obtained. The activation energies of the reactions are shown in Table 4.6. The reaction between arginine residues and sugar degradation products Cn (k_{15}) was not found to be temperature dependent. This was presumably caused by the fact that we did not have any information on the concentrations of arginine residues and the Cn compounds.

4.3.3 Effect of sugar concentration

To study the effect of sugar concentration, 75 mM in stead of 150 mM glucose or fructose were heated at 120°C in the presence of casein. If a kinetic model is consistent, the reaction rate constants should be independent of the concentration of the reactants. The reaction rate constants of Table 4.5 (using Scheme 4.4, including pH effect) were used to predict the reactions. In Figure 4.11 the simulations are shown.

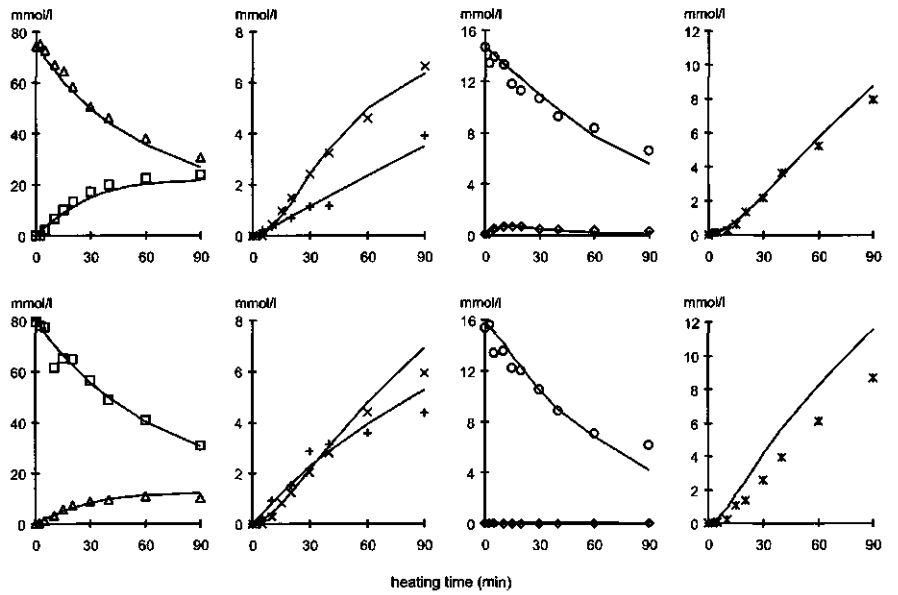


Figure 4.11 Effect of sugar concentration: Prediction (drawn lines) based on the reaction rate constants of Table 4.5 for glucose-casein (upper part) and fructose-casein systems (lower part) heated at 120°C with an initial sugar concentration of 75 mM. Glucose (Δ), fructose (□), formic acid (+), acetic acid (x), lysine residues (○), Amadori compound (◇), melanoidins (*).

The prediction of the sugars and acids was perfect, the prediction of the loss of lysine was somewhat less but reasonable. Amadori and browning in the glucose-casein system were predicted very well. Browning in the fructose-casein system was, however, somewhat overestimated. All in all, it seems that the model performed reasonably well.

4.3.4 Effect of protein concentration

Sugar-casein systems with a concentration of 1.5% sodium caseinate in stead of the standard used 3% were heated at 120°C with 150 mM of sugar. As mentioned before, the reaction rate constants should be independent of the concentration of the reactants if the kinetic model is consistent. The reaction rate constants of Table 4.5 were used to predict the reactions. In Figure 4.12 the simulations are shown. The predictions of the reactants and reaction products were quite good. The major lack of fit was the formation of brown components. Since there is a lack of data in formation of Cn and loss of arginine, browning can only be predicted with less certainty. Again, we conclude that the model holds well.

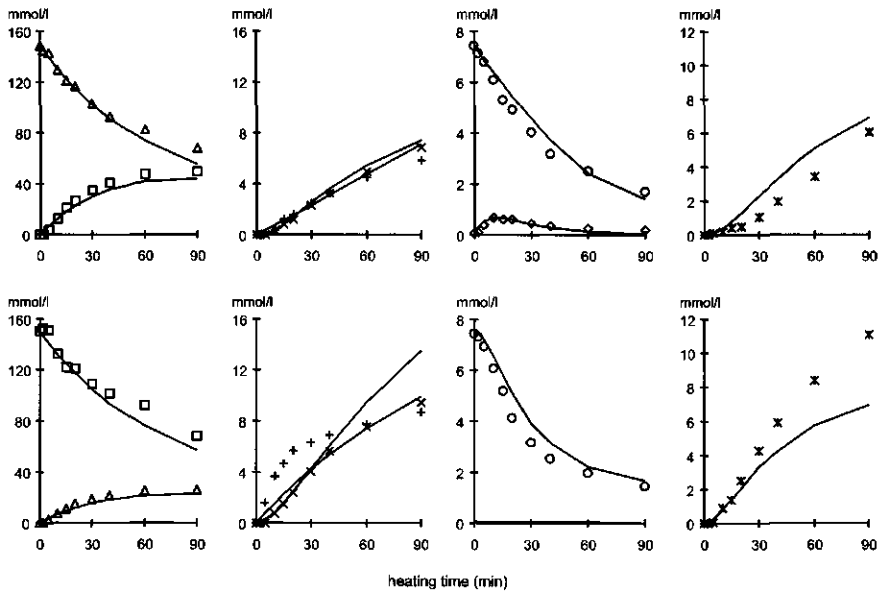
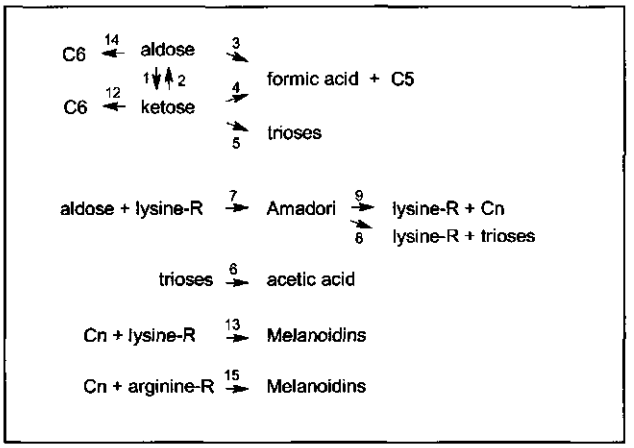


Figure 4.12 Effect protein concentration: Prediction (drawn lines) based on the reaction rate constants of Table 4.5 for glucose-casein (upper part) and fructose-casein systems (lower part) heated at 120°C with an initial sugar concentration of 75 mM. Glucose (Δ), fructose (□), formic acid (+), acetic acid (x), lysine residues (○), Amadori compound (◇), melanoidins (*).

4.3.5 Effect of type of sugar

In this study big differences were observed between the reaction behaviour of glucose and fructose when heated in the presence of a protein. These differences are mainly due to differences in reaction mechanism between aldose and ketose sugars. In Scheme 4.5, the reactions occurring in monosaccharide-casein systems are given for aldose sugars and ketose sugars in general. The aldose sugar and the ketose sugar can isomerise into each other via the Lobry de Bruyn-Alberda van Ekenstein transformation. Both the aldose and the ketose can degrade into formic acid. Consequently, a C5 compound, possibly 2-deoxyribose, is formed (Brands and Van Boekel, 2001). The ketose can also react to a 3-deoxyaldoketose via its 1,2-enediol and to a 1-deoxy-2,3-diketose or 4-deoxy-2,3-diketose via its 2,3-enediol or it can degrade into triose intermediates like glyceraldehyde and 1,3-dihydroxyaceton, both fast reacting toward methylglyoxal. The aldose can, whether or not catalysed by the protein, react to a 3-deoxyaldoketose or can react with lysine residues of the protein to the Amadori compound. This Amadori compound (an aminoketose) can react further to 1-deoxy-2,3-diketose or 4-deoxy-2,3-diketose or degrade into trioses (like a ketose) whereby the protein is released. The C5 (2-deoxyribose), C3 (trioses) and C6 (deoxyosones) can react with the lysine residues of the protein to form the brown-coloured protein-bound melanoidins. The C3 and C6 compounds can also react with the arginine residues of the protein to form melanoidins.



Scheme 4.5 Kinetic model for monosaccharide-casein reactions

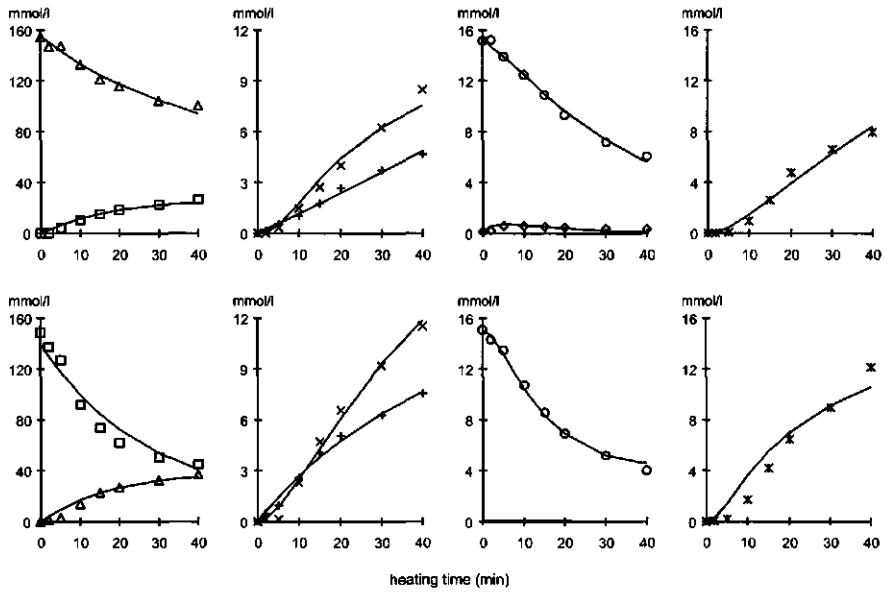


Figure 4.13 Simulation (drawn lines) based on the kinetic model of Scheme 4.5 for galactose-casein (upper part) and tagatose-casein systems (lower part) heated at 120°C, taking into account effect of pH drop. Glucose (Δ), fructose (□), formic acid (+), acetic acid (x), lysine residues (○), Amadori compound (◇), melanoidins (*).

Table 4.7 Rate constants and their 95%-confidence intervals as found by kinetic modelling for the model displayed in Scheme 4.5 to the data of galactose-casein and tagatose-casein systems (120°C; initial pH 6.8).

Rate constant	
k_1 (min^{-1})	0.00965 ± 0.00115
k_2 (min^{-1})	0.01556 ± 0.00146
k_3 (min^{-1})	0.00070 ± 0.00008
k_4 (min^{-1})	0.00224 ± 0.00019
k_5 (min^{-1})	0.00216 ± 0.00026
k_6 (min^{-1})	0.15480 ± 0.05621
k_7 ($l.mmol^{-1}.min^{-1}$)	0.00018 ± 0.00002
k_8 (min^{-1})	0.37286 ± 0.05844
k_9 (min^{-1})	-
k_{12} (min^{-1})	0.01453 ± 0.00380
k_{13} ($l.mmol^{-1}.min^{-1}$)	0.00539 ± 0.00146
k_{14} (min^{-1})	0.00371 ± 0.00091
k_{15} ($l.mmol^{-1}.min^{-1}$)	0.00050 ± 0.00049

For comparison, also the reactions occurring in a galactose-casein and tagatose-casein system heated at 120°C were studied. The reaction mechanism of glucose and galactose (both aldoses) and fructose and tagatose (both ketoses) were assumed to be comparable (Brands and Van Boekel, 2001). The kinetic model of Scheme 4.5, which is basically the same as that in Scheme 4.4, was used to fit the data. In Figure 4.13 the fit of the model to the galactose and tagatose data is shown and in Table 4.7 the reaction rate constants are given. The percentage of galactose in its acyclic form is about 10 times higher than that of glucose at neutral pH at room temperature (Hayward and Angyal, 1977), which is generally believed to be the reason for a higher reactivity of galactose than glucose. The isomerisation rate of galactose into tagatose (k_1) was not influenced by the higher percentage of acyclic form. Apart from that, the formation of acids (via k_3 and k_8), loss of lysine (k_7), degradation of Amadori compound (k_8) were indeed faster in the galactose-casein system compared to the glucose-casein system. Furthermore, more sugar degradation products were formed (k_3, k_8, k_{15}) and therefore browning was also faster. The percentage of acyclic tagatose is 15% lower than the percentage of fructose in its open-chain form at neutral pH at room temperature (Bunn and Higgins, 1981). This did not automatically lead to a lower reactivity of tagatose compared to fructose when heated at 120°C. As well as the isomerisation reaction of the ketose into the aldose (k_2) and the degradation (k_4, k_5, and k_{12}) were significantly higher in the tagatose-casein system than in the fructose-casein system. Since more reaction products were formed, browning was also higher.

Except for the formation of melanoidins in the tagatose-casein system, the fit of the model to the data was very good. This lack of fit for the melanoidins was also observed when fitting the model to the fructose-casein data with different sugar or protein concentrations. Apparently the model needs some minor adjustments. The determination of the concentrations of the reaction intermediates, together with the arginine concentration, will certainly contribute to a better understanding of the reactions.

4.4 Conclusions

The main conclusion from this work is that it is possible to model a complex reaction like the Maillard reaction. The multiresponse modelling approach as used in this study appears to be a very powerful tool to unravel complicated reaction routes. This is so because use is made of all the information in the observed responses. In the present study, the iterative process of modelling - proposing a model, confronting it with experiments, criticising the model - was passed through 4 times to arrive at a model that was able to describe the reactions in

monosaccharide-casein systems under different reaction conditions rather well. The results obtained in this study show remarkable differences in reaction mechanism between ketose and aldose sugars. The differences found may offer possibilities to optimise food quality with respect to the Maillard reaction.

Acknowledgements

We are grateful to Ruud van Gorp and Anton de Bruin for performing some of the experimental work.

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5

Kinetic modelling of reactions in heated disaccharide-casein systems

Abstract

The reactions occurring in disaccharide-casein reaction mixtures during heating at 120°C and pH 6.8 were studied. Two main degradation routes were established: (i) isomerisation of the aldose sugars lactose and maltose in their ketose isomers lactulose and maltulose, respectively, and subsequent degradation into galactose and glucose, respectively, and formic acid among other unidentified compounds, and (ii) the Maillard reaction, in which the aldose sugars themselves but also degradation products react with the protein, eventually leading to the formation of brown compounds. These reactions were put together into a kinetic model and the proposed model was extensively tested using multiresponse modelling. The model was adapted several times. The final kinetic model was able to explain the observed changes in reactants and products and allowed a quantitative prediction of reactions in heated disaccharide-casein systems.

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5.1 Introduction

The Maillard reaction of various carbohydrates with amino acids or proteins has been thoroughly investigated. This reaction, which is of importance in many heated, dried and stored foods, leads to the formation of flavouring ingredients, browning products, the loss of nutritive value (Ledl, 1990), formation of antioxidants (Lignert and Hall, 1986), protein cross-linking (Pellegrino et al., 1999), while mutagenic but also antimutagenic compounds might be formed (Brands et al., 2000). In order to be able to control complex chemical reactions like the Maillard reaction, the reactions of interest need to be studied in a quantitative way. In previous papers (Brands and Van Boekel, 2001a; Brands and Van Boekel, 2001b) reactions in monosaccharide-casein systems were studied quantitatively. Due to the complexity of the reaction network it could not be described by simple kinetics but rather by multiresponse kinetic modelling. Kinetic modelling has shown to be a powerful tool to unravel complicated reaction networks. Once a kinetic model is selected and validated, values of rate constants can be estimated so that changes can be predicted in a quantitative way.

Some investigations have shown that the reaction mechanisms of monosaccharides and disaccharides are different. Kato and co-workers (1988) compared glucose and several disaccharides (having glucose at the reducing end) in their amino-carbonyl reaction behaviour in the presence of ovalbumin at accelerated storage conditions. Although they did not find a major difference in the decrease of the free amino group, browning differed strongly and was dependent on sugar structure. It appeared that sugars with a glycosidic bond at the C4 hydroxyl group of the glucopyranose (like lactose, cellobiose and maltose) were difficult to cleave and browned therefore slowly. When sugars were bonded at the C6 hydroxyl group (like in melibiose and isomaltose) further degradation was not blocked and the rate of browning was therefore comparable to that of glucose.

Other investigations have shown that the reaction products obtained from monosaccharides and disaccharides differ. The reaction products β-pyranone and 3-furanone that were obtained from the 1,4-glycosidic linked disaccharides lactose and maltose, have never been detected in glucose reaction mixtures (Kramhöller et al., 1993; Pischetsrieder and Severin, 1996). Troyano and co-workers (1992b) showed that 3-deoxypentulose was formed as a reaction product of lactose during heating of milk, whereas it was not formed from glucose or galactose. The glycosidic linked sugar gives, apparently, rise to typical disaccharide degradation products, while typical monosaccharide degradation products were prevented to be formed.

Due to the differences in reaction mechanism of monosaccharides and disaccharides, the kinetic model developed previously for monosaccharide-protein reactions (Brands and Van Boekel, 2001b) had to be modified for prediction of reaction compounds formed in disaccharide-casein systems. The aim of the present study was, therefore, to develop a kinetic model for disaccharide-casein systems heated at temperatures corresponding to sterilisation conditions. Lactose and maltose, both 1,4-glycosidic linked disaccharides, were studied. These sugars are of considerable relevance for foods. Maltose is an important degradation product of starch and occurs as such particularly in malted food, whereas the milk sugar lactose is found in dairy products. Maltose consists of two glucose units. Lactose consists of a glucose and a galactose unit, with the reactive carbonyl group on the glucose unit. We also studied the disaccharides lactulose and maltulose, the ketose isomers of lactose and maltose, respectively. Maltulose consists of a fructose and a glucose moiety and lactulose of a fructose and a galactose moiety, both having fructose at the reducing end. In the present study, the main reaction products were identified and the main reaction pathways will be established. Based on the previously derived kinetic model for monosaccharides, an adapted model will be proposed and tested whether it is able to fit the experimental data quantitatively.

5.2 Materials and methods

5.2.1 Chemicals

All chemicals were of analytical grade. Lactose and maltose were supplied by Merck. Lactulose was supplied by Fluka Chemie and maltulose by Aldrich Chemie. Sodium caseinate (a spray-dried powder) was obtained from DMV (Veghel, The Netherlands) containing 90% protein.

5.2.2 Preparation of reaction model systems

A disaccharide sugar (150 mM lactose, lactulose, maltose or maltulose) and sodium caseinate (3% w/w) were dissolved in a phosphate buffer (0.1 M; pH 6.8) to give a molar ratio of sugar to lysine residues of about 10:1 or 5:1. Reaction model mixtures without casein were also prepared. The samples were heated for various times (0 – 60 min) at 120°C in an oil bath in screw-capped glass tubes (Schott, 16 x 160 mm). The reported heating times include the heating-up period of about 2 - 3 minutes. After a given heating time, samples were cooled in ice water, prior to analysis.

5.2.3 Analyses of sugars and organic acids

After heating, sugars and organic acids were separated from the protein via Sephadex G25 disposable columns (NAP-25, Pharmacia), as described previously (Brands and Van Boekel, 2001a). The protein-free fraction was analysed by HPLC using an ion-exchange column. The ION-300 column (Interaction Chromatography Inc., San Jose, CA, USA) was used to separate galactose and tagatose or glucose and fructose, and organic acids. The eluent consisted of 0.0025 M sulphuric acid in water and the flow rate was 0.4 ml/min. The Benson Carbohydrate Lead column (Alltech, Breda, The Netherlands) was used to separate lactose and lactulose or maltose and maltulose. The eluent was water with a flow rate of 0.5 ml/min. The HPLC columns were kept at 85°C. The sugars were detected by monitoring the refractive index. The organic acids were separated with the ION-300 column and detected by their UV absorbance at 210 nm.

5.2.4 Analyses of total acid formation

Titrations were performed to determine total acid formation in heat-treated samples. Samples of 15 ml reaction mixture were titrated with 0.1 N NaOH to pH 8.3. From the difference in added NaOH between the heated and unheated sample the total amount of acid formed was calculated.

5.2.5 Analyses of available lysine residues

Available lysine residues were determined after derivatisation with *ortho*-phthaldialdehyde, as described previously (Brands and Van Boekel, 2001a).

5.2.6 Analyses of Amadori compound

The Amadori compound was determined by means of furosine, using HPLC, as described previously (Brands and Van Boekel, 2001a).

5.2.7 Analyses of brown compounds

The browning intensity of the heated reaction mixtures and the protein-free fraction was determined by measuring the absorbance at 420 nm with a spectrophotometer (Pharmacia Biotech) as described previously (Brands and Van Boekel, 2001a). The browning of the protein fraction was calculated by subtracting the browning of the sugar fraction from the browning of the total mixture. The absorbance can be recalculated to the concentration of

melanoidins by using the equation of Lambert-Beer. The extinction coefficient of protein-bound melanoidins formed in disaccharide-casein systems is not known. In glucose-casein and fructose-casein systems the effective extinction coefficient was estimated to be 500 l. $mol^{-1}.cm^{-1}$ (Brands et al., 2001). Since the extinction coefficient is expected to be more dependent on the type of amino group than on the type of sugar, we assume that the obtained values can be used in this study. The concentration of melanoidins is thus expressed as sugar units (C12) incorporated in the brown products.

5.2.8 Kinetic modelling

Computer simulations of reactions were done by numerical integration of differential equations that were set up for a particular reaction scheme. The parameters of the model, the rate constants, were estimated by non-linear regression using the determinant criterion (Stewart et al., 1992). The software package Gregpak/Athena Visual Workbench was used to minimise the determinant (www.athenavisual.com). To discriminate between various models, the posterior probability was calculated (Stewart et al., 1998). The model with the highest posterior probability was defined as the most likely one.

5.3 Results and discussion

5.3.1 Identification and quantification of reactants and products

In the lactose-casein and lactulose-casein system, the detected reaction products were the corresponding isomer (lactulose and lactose, respectively), galactose and its isomer tagatose, and formic acid. In the maltose-casein and maltulose-casein system the monosaccharides glucose and its isomer fructose were formed instead of galactose and tagatose, as well as the corresponding isomers (maltulose and maltose, respectively) and formic acid. In contrast with the monosaccharide-casein systems (Brands and Van Boekel, 2001a) no acetic acid was formed. In the lactose-casein and maltose-casein system also the protein-bound Amadori compounds lactulosyllysine and maltulosyllysine, respectively, were detected. In all systems brown compounds were formed.

In Figure 5.1 the decrease of reactants and formation of reaction products is shown as averages of duplicates. No significant differences were observed between the lactose-casein and maltose-casein system. When comparing the lactulose-casein system with the maltulose-casein system the same conclusion could be drawn. The differences between the aldoses and the ketoses, on the other hand, were much more apparent. Although the decrease of initial

sugar was about the same, the decrease of lysine was much faster in the ketose systems than in the aldose systems. Also the increase of all the reaction products, including browning, was much faster in the ketose systems than in the aldose systems. The course of degradation of reactants and formation of reaction products also differed strongly. After 20 minutes of heating, the curves obtained from the ketose-casein systems (except browning) started to flatten off, whereas the reaction products of the aldose-casein systems tended to increase linearly. This was likely caused by a faster decrease in pH in the ketose-casein systems (see Figure 5.2). The rate of the Maillard reaction, sugar isomerisation and subsequent degradation decreases with decreasing pH. The drop in pH was partially (about half) caused by the formation of formic acid (compare Figure 5.1 and 5.2). According to literature other acids such as lactic, levulinic, propionic, butyric, pyruvic, citric and saccharinic acids might have been formed (Berg, 1993), but they were rather difficult to identify. Compared to monosaccharide systems, about 4 times more formic acid was formed.

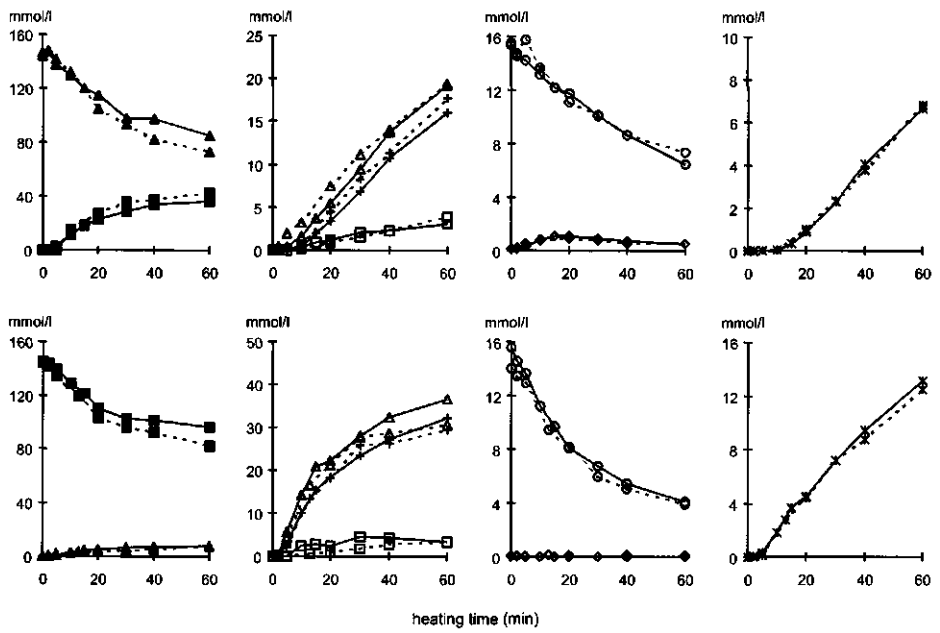


Figure 5.1 Lactose-casein (solid lines) and maltose-casein (dotted lines) systems (top) and lactulose-casein (solid lines) and maltulose-casein (dotted lines) systems (bottom) heated at 120°C. Lactose/maltose (▲), lactulose/maltulose (■), galactose/glucose (Δ), tagatose/fructose (□), formic acid (+), lysine residues (○), Amadori compound (◇), melanoidins (*).

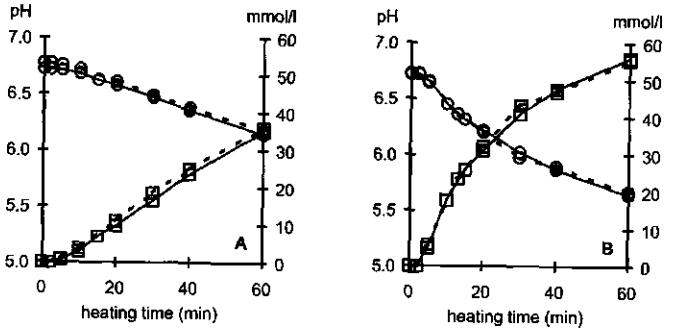


Figure 5.2 pH (O) and total amount of acids as found by titration (□) in heated lactose-casein (solid line) and maltose-casein (dotted line) systems (A) and lactulose-casein (solid lines) and maltulose-casein (dotted lines) systems (B).

A mass balance was established for the lactose-casein and lactulose-casein system to see whether the main reaction compounds were identified (Figure 5.3). The amounts of sugar degradation products have been expressed as percentage mol-C12 from the starting C12-disaccharide according to the following definition:

$$\text{mol Cn} = \frac{n}{12} \times \text{mol C12}$$

in which Cn represents a sugar degradation product with n carbon atoms. The melanoidin concentration was calculated from the browning of the protein fraction as described in the section 5.2.7. It appeared that only less than 10% of the initial amount of sugar could not be identified in this way.

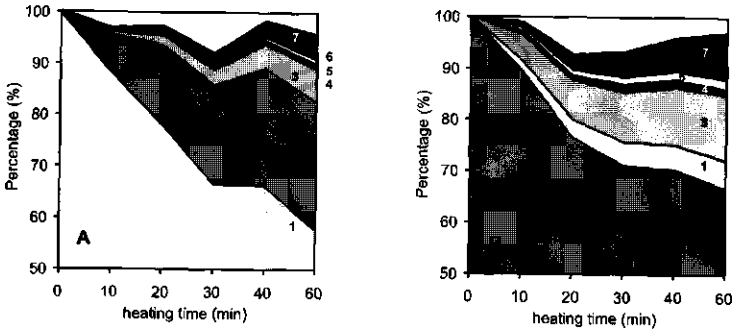


Figure 5.3 Mass balance of reactants and reaction products in heated lactose-casein (A) and lactulose-casein (B) systems. Lactose (1), lactulose (2), galactose (3), tagatose (4), formic acid (5), Amadori compound (6) and melanoidins (7).

In Figure 5.4 the results of lactose and lactulose heated in the absence of casein are given. These graphs, when compared with the graphs from Figure 5.1, show that the formation of sugars and formic acid did not depend on the presence of casein. Browning, on the contrary, did depend on the presence of casein and was about 3 times lower when no casein was present. Furthermore, it can be observed that the amounts of galactose and formic acid were much higher in the lactulose systems than in the lactose systems.

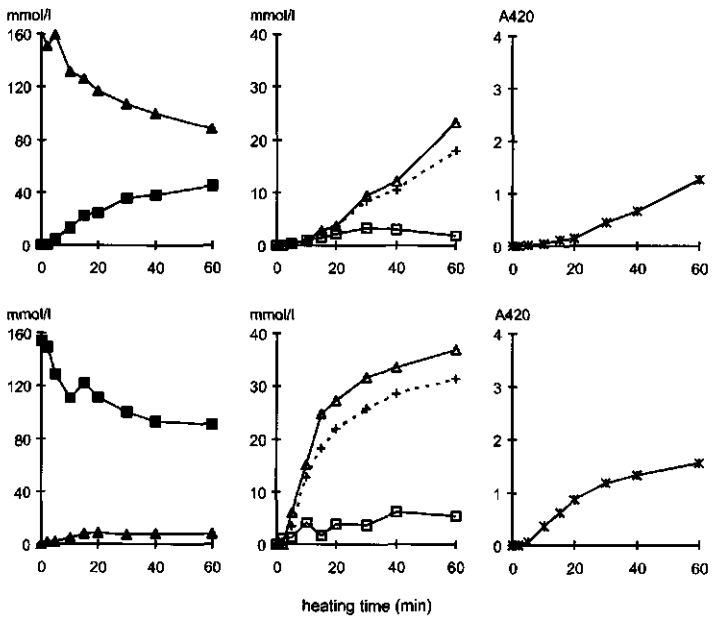


Figure 5.4 Lactose solutions (top) and lactulose solutions (bottom) heated without casein at 120°C. Lactose (▲), lactulose (■), galactose (Δ), tagatose (□), formic acid (+), absorbance at 420 nm (*).

Berg and Van Boekel (Berg and Van Boekel, 1994; Van Boekel, 1998) studied the reactions of lactose in heated milk. They identified the same reaction compounds as in the present study in the lactose-casein system, except tagatose. Tagatose was however found in heated milk by Troyano and co-workers (1992a). In milk lower concentrations of reaction compounds were formed and the decrease of reactants was also slower than in the lactose-casein model system we used. This might be due to the phosphate buffer used in the present study. Except for a buffering effect, phosphate is reported to have a catalytic effect on the sugar-protein reactions (Bell, 1997).

5.3.2 Reaction network model

Lactose and maltose can undergo two types of transformation: (i) the Lobry de Bruyn-Alberda van Ekenstein (LA) rearrangement and (ii) the Maillard reaction (Troyano et al., 1992b). Via the LA transformation lactose can isomerise into lactulose and maltose into maltulose. A 1,2-enediol anion is the key intermediate in this reaction, and is also considered to be the starting intermediate in degradation reactions. The 1,2-enediol anion can undergo β-elimination to yield 3-deoxyaldoketose. Neither the formation of this reactive dicarbonyl compound nor the further decomposition is influenced by the presence of the glycosidic linked sugar (Pischetsrieder and Severin, 1996), which is easily split off. The 3-deoxyaldoketose can undergo α-dicarbonyl cleavage to yield formic acid and 2-deoxyribose, or it can undergo a retroaldolisation reaction to form glyoxal, or it can cyclise to form HMF. This 3-deoxyaldoketose reaction route is favoured under slightly acidic conditions. Under the conditions used in this study formation of HMF was therefore very low (0-10 μM).

In the Maillard reaction lactose and maltose can react with available amino groups of the protein to form an N-substituted glycosyl amine, which is in equilibrium with its 1,2-enaminol. This 1,2-enaminol can undergo the same reactions as the 1,2-enediol as described before. Furthermore, the 1,2-eneaminol can react to an aminoketose, the Amadori product, which is subject to further degradation via its 2,3-eneaminol. Hollnagel and Kroh (2000) proposed a peeling off mechanism for the formation of 1,4-deoxyglucosone from oligosaccharides, whereby the glycosidic residue at C4 and the amino residue at C1 are eliminated. 1,4-Deoxyglucosone can degrade into formic acid and 3-deoxypentulose (Hollnagel and Kroh, 2000; Troyano et al., 1992b). This reaction pathway is specific for di- and oligosaccharide sugars and therefore 3-deoxypentulose was never found in monosaccharide systems. The 2,3-eneaminol can also lead to the formation of 1-deoxyglucosones (1-DG). Cyclisation and enolisation of 1-deoxyglucosone result in 5- or 6-membered rings. Starting from these intermediates, the degradation of lactose and maltose differs from that of glucose. For the case of glucose, the pyranoid product eliminates water to give a γ-pyranone and the furanoid dehydrates to a 4-furanone. This reaction is not facilitated if a sugar is bound to position 4. Therefore, lactose and maltose favour pathways toward compounds that still possess the glycosidic substituent, such as glycosyl β-pyranone [4-(glycopyranosyloxy)-2-hydroxy-2-methyl-2*H*-pyran-3(6*H*)-one] and glycosyl 3-furanone [4-(glycopyranosyloxy)-5-hydroxymethyl-2-methyl-3(2)-furanone] (Pellegrino et al., 2000; Pischetsrieder and Severin, 1996).

Lactulose and maltulose, both ketose sugars, can be transformed via the 1,2-enolisation route, but they can also degrade via their 2,3-enediol anion in the same way as described for the Amadori compound. From previous research it is known that ketoses themselves are not reactive in the Maillard reaction and that they can degrade into fragments without any involvement of an amino group (Brands and Van Boekel, 2001a).

Galactose and glucose are formed via the 3-deoxyglucosone (3-DG) and 1,4-deoxyglucosone (1,4-DG) pathways starting from lactose or lactulose and maltose or maltulose, respectively, as the initial sugar. Galactose and glucose can react in the LA transformation and Maillard reaction as described previously (Brands and Van Boekel, 2001b). The sugars tagatose and fructose were identified as the isomer sugars of galactose and glucose, respectively.

The sugar degradation products β-pyranone and 3-furanone (Pischetsrieder and Severin, 1996), 3-deoxypentulose (Troyano et al., 1992b), 2-deoxyribose (Berg and Van Boekel, 1994) and fragmentation products with dicarbonyl structure (Hofmann, 1999) are not very stable. Sugar degradation products might easily condense with available amino groups and form brown-coloured melanoidins.

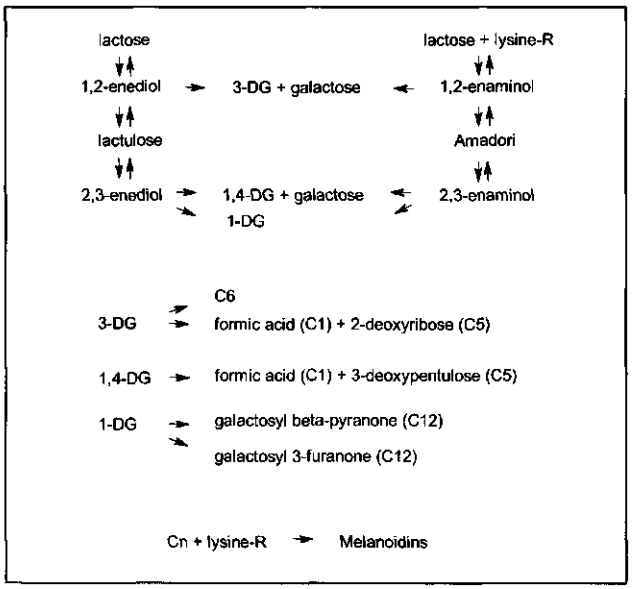
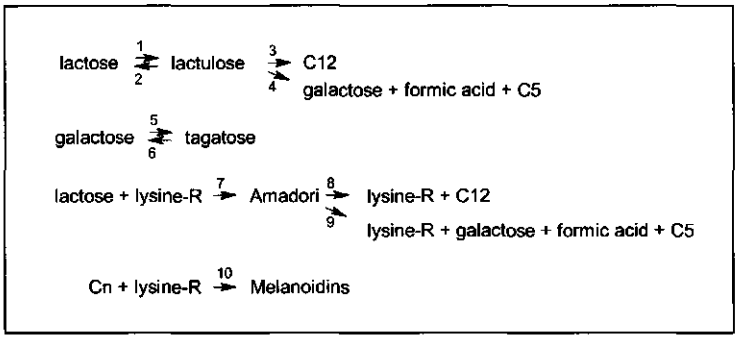


Figure 5.5 Reaction network model for disaccharide-casein reactions (lysine-R: protein bound lysine residues, Cn: unidentified sugar reaction compounds with n carbon atoms ($1 \leq n \leq 6$)).

The reactions described above are put together into a model for lactose and lactulose reactions (see Figure 5.5). This reaction network model is quite complex but can be simplified for modelling purposes.

5.3.3 Kinetic modelling of lactose and lactulose reactions

The reaction network model of Figure 5.5 was first simplified to obtain a more workable kinetic model with less parameters. The degradation reactions via the 1,2-enediol or 1,2-eneaminol of the disaccharides were neglected. This reaction step is favoured under acidic conditions, whereas the initial pH of the systems used in this study is near neutral. The 3-deoxyglucosone route is not essential to explain the formation to explain certain reaction compounds. In disaccharide systems formic acid can also be formed via the 1,4-deoxyglucosone route. Furthermore, it can be shown that, on the assumption of steady state behaviour of the enediols, the concentration of enediols is directly proportional to the sugar concentration (De Bruijn, 1986). Neglecting the reactions via galactose (except its isomerisation into tagatose) simplifies the model further. The simplified kinetic model is shown in Scheme 5.1.



Scheme 5.1 Kinetic model for disaccharide-casein reactions

The kinetic model was translated into a mathematical model by setting up the following differential equations:

$$\frac{\mathrm{d}[lac]}{\mathrm{d}t} = -k_1[lac] + k_2[lu] - k_7[lac][lys]$$

$$\frac{\mathrm{d}[lu]}{\mathrm{d}t} = k_1[lac] - k_2[lu] - k_3[lu] - k_4[lu]$$

$$\frac{d[gal]}{dt} = k_4[lu] - k_5[gal] + k_6[tag] + k_9[Ama]$$

$$\frac{d[tag]}{dt} = k_5[gal] - k_6[tag]$$

$$\frac{d[formic]}{dt} = k_4[lu] + k_9[Ama]$$

$$\frac{d[lys]}{dt} = -k_7[lac][lys] + k_8[Ama] + k_9[Ama] - k_{10}[C_{12}][lys] - k_{10}[C_5][lys]$$

$$\frac{d[Ama]}{dt} = k_7[lac][lys] - k_8[Ama] - k_9[Ama]$$

$$\frac{d[M]}{dt} = k_{10}[C_{12}][lys] + \frac{5}{12}k_{10}[C_5][lys]$$

$$\frac{d[C_{12}]}{dt} = k_3[lu] + k_8[Ama] - k_{10}[C_{12}][lys]$$

$$\frac{d[C_5]}{dt} = k_4[lu] + k_9[Ama] - k_{10}[C_5][lys]$$

These differential equations were numerically solved and fitted to the data. Because the data of the lactose-casein and lactulose-casein system were fitted simultaneously, the number of responses and therefore also the number of differential equations had to be doubled. The results of the fits are shown in Figure 5.6. For the clarity of the graphs the averages of the experimental data for every response are shown, but the actual modelling was done using all experimental data.

The model of Scheme 5.1 did not describe all reactions in the sugar-casein system very well (Figure 5.6). In the lactose-casein system the decrease of lactose and its isomerisation into lactulose were described quite well. Also the formation of formic acid was well described. The formation of galactose, and therefore also that of tagatose, was underestimated by the model. Whereas the Amadori compound and the melanoidins were described very well, the model underestimated the loss of lysine in the beginning of heating and overestimated it at the end. In the lactulose-casein system, the isomerisation of lactulose into lactose was predicted very well, but the decrease of lactulose was strongly overestimated. Galactose was clearly underestimated by the model and formic acid was initially underestimated, but overestimated at the end of heating. Like in the lactose system, loss of lysine was somewhat overestimated in the beginning of heating but strongly overestimated at the end. Since the predicted lysine concentration approaches zero, browning flattens off after 40 minutes of heating. The lack of fit in the lactulose-casein system might partially be due to the very rapid pH drop, which is much faster than in the lactose-casein system (see Figure 5.2).

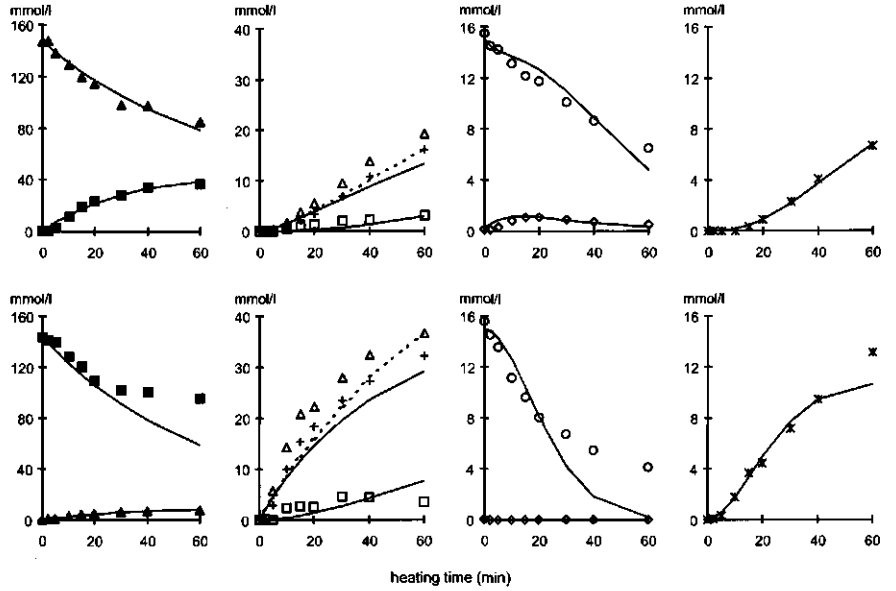


Figure 5.6 Simulations (drawn lines) based on the kinetic model of Scheme 5.1 for lactose-casein and lactulose-casein systems heated at 120°C. Lactose (▲), lactulose (■), galactose (Δ), tagatose (□), formic acid (+), lysine-residues (○), Amadori compound (◇), melanoidins (*).

To reduce the effect of difference in pH decrease between the lactose-casein system and the lactulose-casein system, a system with half the amount of lactulose (75 mM) was heated. It was observed that the decrease in pH in this system was very similar to the pH decrease in the lactose-casein system (only slightly higher). The data of these two model systems were modelled simultaneously (except for tagatose data which was not quantified).

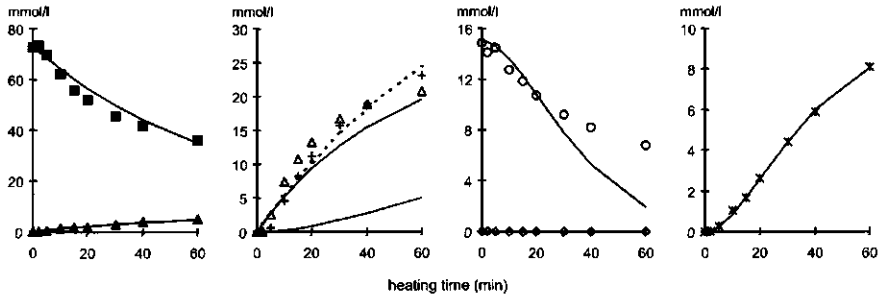


Figure 5.7 Simulations (drawn lines) based on the kinetic model of Scheme 5.1 for lactulose-casein systems heated at 120°C with an initial sugar concentration of 75 mM. For legends see Figure 5.6.

The fit for the lactose-casein system did not change (results not shown). The fits for lactulose and formic acid in the lactulose-casein system were indeed improved, but galactose formation was still underestimated (Figure 5.7). The fit for lysine loss was not improved. The logarithm of the posterior probability was –11.550.

In a previous paper (Brands and Van Boekel, 2001b), we described the effect of pH drop on the reactivity of lysine residues. During heating, the pH of the sugar-casein systems drops and due to protonation of the lysine residues, the reactivity of the protein decreases. The concentration of reactive lysine residues will decrease 10^1 times when the pH drops 1 unit. To improve the fit of the model to the data, the effect of pH on reactivity of lysine residues was taken into account in the differential equations. This was done by multiplying the lysine concentration with the factor $10^{-\Delta pH}$. The results are shown in Figure 5.8. The logarithm of the posterior probability was increased to –9.587, which means that the overall fit of the model was noticeable improved.

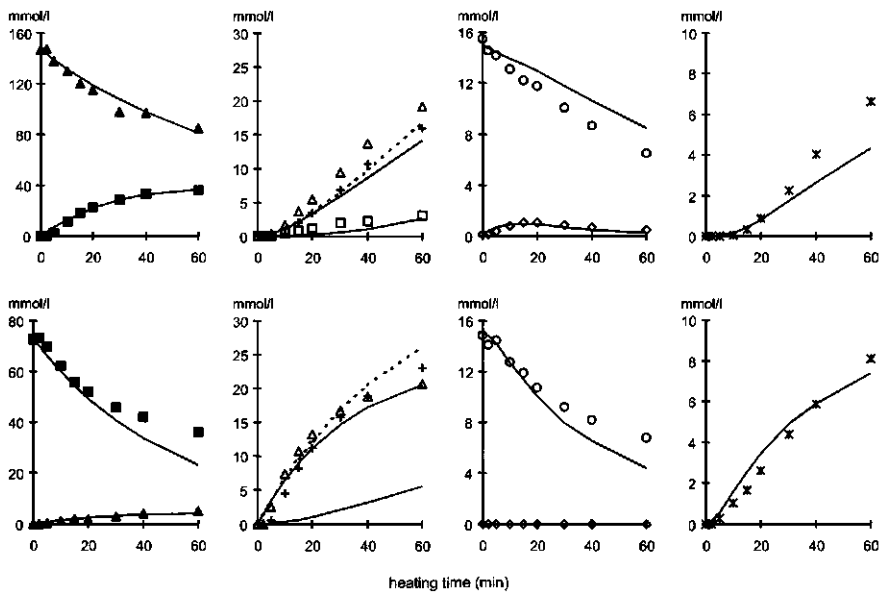
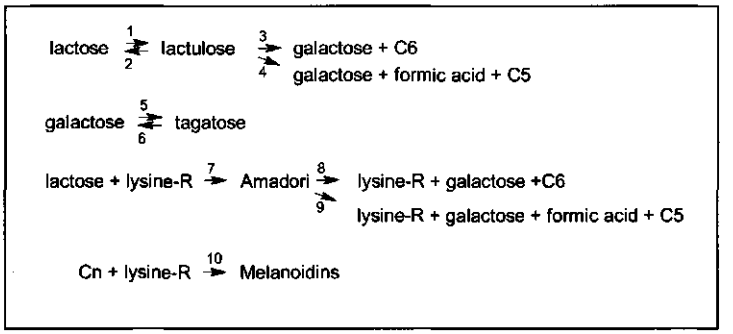


Figure 5.8 Simulations (drawn lines) based on the kinetic model of Scheme 5.1, taking into account the effect of pH-decrease on the activity of lysine, for lactose-casein and lactulose-casein systems heated at 120°C. Lactose (▲), lactulose (■), galactose (Δ), tagatose (□), formic acid (+), lysine-residues (○), Amadori compound (◇), melanoidins (*).

Although the loss of lysine was overestimated in the lactulose-casein system and underestimated in the lactose-casein system, the course of the loss of lysine was improved. Since galactose was still underestimated and lactulose loss was overestimated by the model, we will first focus on the fit of the sugars.

Berg and Van Boekel (Berg and Van Boekel, 1994) developed a model in which lactulose and the Amadori compound react towards a C6 compound (splitting off galactose) in stead of a C12 compound (as depicted in Scheme 5.1). In an adapted scheme (Scheme 5.2), we assumed that in stead of galactosyl 3-furanone and galactosyl β-pyranone unglycosylated furanones and pyranones were formed. We tested this model and it improved the fit for lactulose degradation in the lactulose-casein system and the galactose fit in the lactose-casein system, but galactose formation in the lactulose system was now strongly overestimated (Figure 5.9). The logarithm of the posterior probability was –11.273.



Scheme 5.2 Kinetic model for disaccharide-casein reactions

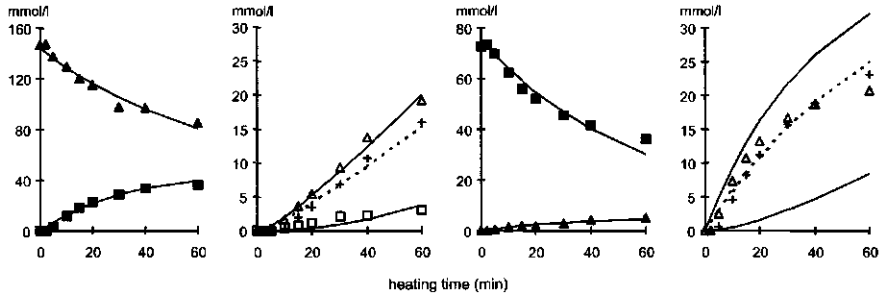
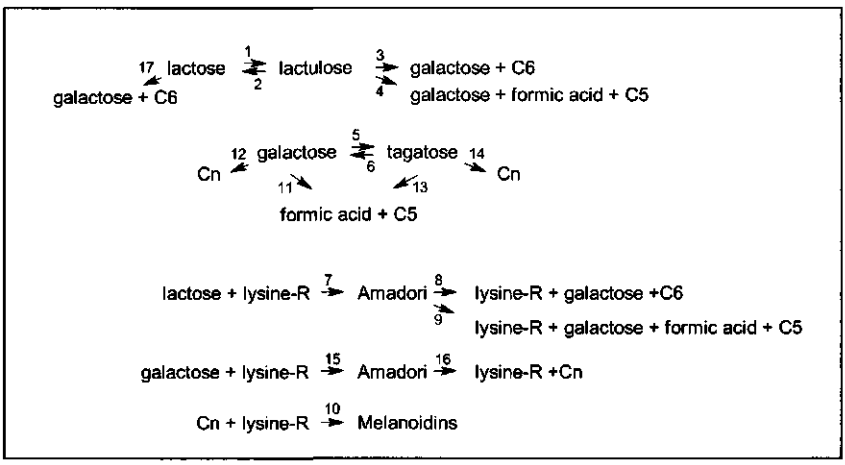


Figure 5.9 Simulations (drawn lines) based on the kinetic model of Scheme 5.2 for lactose-casein and lactulose-casein systems heated at 120°C. Lactose (▲), lactulose (■), galactose (Δ), tagatose (□), formic acid (+).

We tried to improve the fit for galactose by taking the reactions of galactose into account. The reaction rate constants for the galactose reactions were estimated in a former paper (Brands and Van Boekel, 2001b) and were used here as fixed values. The Maillard reaction of lactose was neglected in the lactulose-casein system and replaced by the reaction of galactose with the lysine-residues of the protein. This adaptation improved the fit slightly (the logarithm of the posterior probability was –11.014): the formation of galactose tended to flatten off when heating proceeded, but was still estimated too high (results not shown). Apparently, the galactose concentration is estimated too high in the lactulose-casein system in order to predict enough galactose in the lactose-casein system. Therefore, we adapted our model once again.



Scheme 5.3 Kinetic model for disaccharide-casein reactions

The reaction of lactose via the 3-DG route was taken into account (Scheme 5.3). It is assumed that this route does not occur at neutral pH (Pischetsrieder and Severin, 1996), but since the pH decreases during heating it can become important. We assumed that via this route galactose is split off and that another C6 component is formed. The results are shown in Figure 5.10. The fit of the model to the data was indeed improved (the logarithm of the posterior probability was increased to –6.342). The rate of the reaction from Amadori to C6 compounds (via 1-DG) approached zero. Still the fit of lysine and browning is not right. Since the loss of lysine flattens off whereas browning keeps increasing, a reaction is probably overseen.

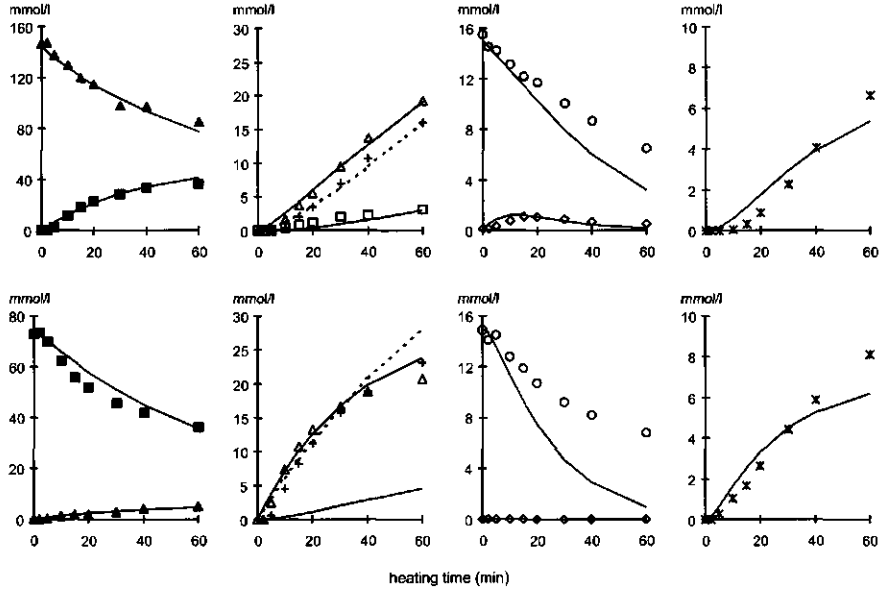
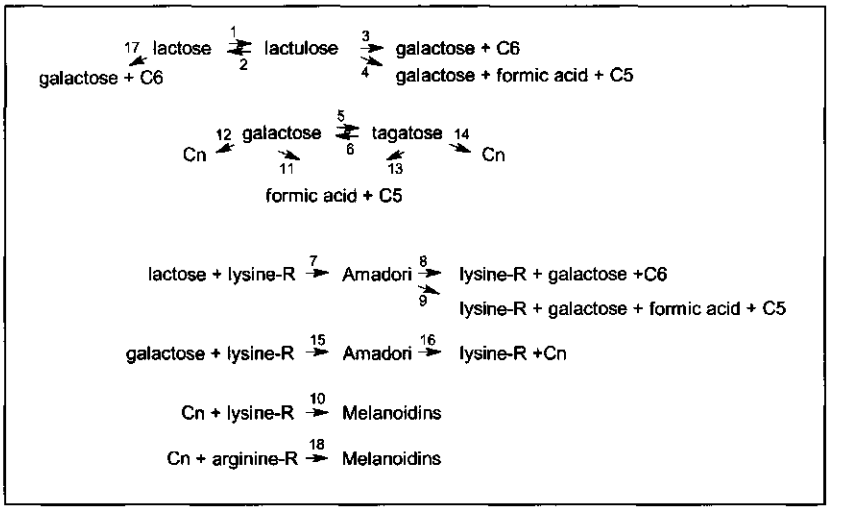


Figure 5.10 Simulations (drawn lines) based on the kinetic model of Scheme 5.3 for lactose-casein and lactulose-casein systems heated at 120°C. Lactose (▲), lactulose (■), galactose (Δ), tagatose (□), formic acid (+), lysine-residues (○), Amadori compound (◇), melanoidins (*).



Scheme 5.4 Kinetic model for disaccharide-casein reactions

In our previous paper on monosaccharides (Brands and Van Boekel, 2001b) we explained this ongoing browning by reaction of sugar fragments with guanidine groups of the arginine residues. Taking the reaction with arginine residues into account (Scheme 5.4) improves the fit enormously, at least, visually (Figure 5.11). According to goodness of fit criterion of Athena the fit is not improved: log posterior probability is –7.078. This is apparently due to the loss of degrees of freedom because more parameters are taken into account. The reaction rate constants (± 95% confidence intervals) are shown in Table 5.1.

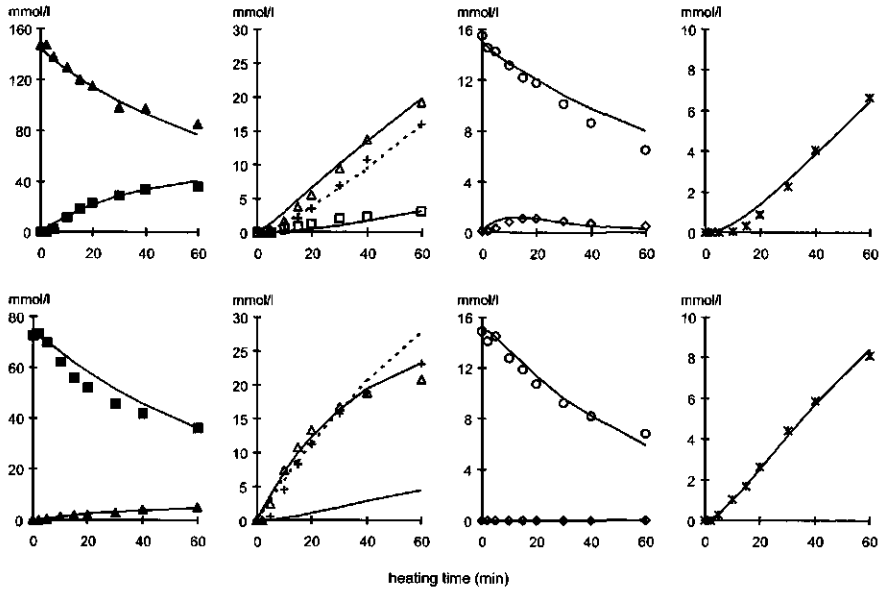


Figure 5.11 Simulations (drawn lines) based on the kinetic model of Scheme 5.4 for lactose-casein and lactulose-casein systems heated at 120°C. Lactose (▲), lactulose (■), galactose (Δ), tagatose (□), formic acid (+), lysine-residues (○), Amadori compound (◇), melanoidins (*).

If a kinetic model is consistent, the reaction rate constants should be independent of the concentration of the reactants. Therefore, we tried to describe the change in reactant concentration of lactose-casein systems that were heated with only half the initial sugar concentration, using the reaction rate constants from Table 5.1. In Figure 5.12 the simulations are shown. This model fits the data extremely well, thus supporting the validity of the model depicted in Scheme 5.4.

Table 5.1 Rate constants and their 95% high posterior density intervals as found by kinetic modelling for the model displayed in Scheme 5.4 to the data of lactose-casein and lactulose-casein systems (120°C; initial pH 6.8).

Rate constant	lactose/lactulose reactions	galactose/tagatose reactions
k_1 (min^{-1})	0.00947 ± 0.00054	
k_2 (min^{-1})	0.00207 ± 0.00012	
k_3 (min^{-1})	0.00219 ± 0.00079	
k_4 (min^{-1})	0.00850 ± 0.00062	
k_5 (min^{-1})		0.00965
k_6 (min^{-1})		0.01556
k_7 ($l.mmol^{-1}.min^{-1}$)	0.00013 ± 0.00003	
k_8 (min^{-1})	0.00000	
k_9 (min^{-1})	0.10478 ± 0.02812	
k_{10} ($l.mmol^{-1}.min^{-1}$)	0.00517 ± 0.00051	
k_{11} (min^{-1})		0.00070
k_{12} (min^{-1})		0.00371
k_{13} (min^{-1})		0.00224
k_{14} (min^{-1})		0.01669
k_{15} ($l.mmol^{-1}.min^{-1}$)		0.00018
k_{16} (min^{-1})		0.37286
k_{17} (min^{-1})	0.00123 ± 0.00022	
k_{18} ($l.mmol^{-1}.min^{-1}$)	0.00143 ± 0.00017	

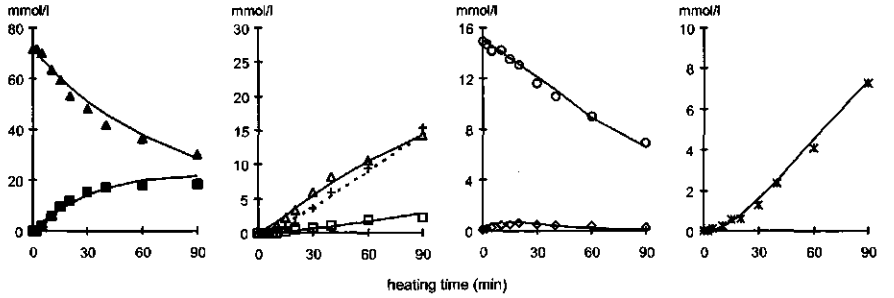


Figure 5.12 Predictions (drawn lines) based on the reaction rate constants of Table 5.1 for lactose-casein systems heated at 120°C with an initial sugar concentration of 75 mM. Lactose (▲), lactulose (■), galactose (Δ), tagatose (□), formic acid (+), lysine-residues (○), Amadori compound (◇), melanoidins (*).

5.3.4 Maltose and maltulose reactions

The reactions occurring in a maltose-casein and maltulose-casein system were simulated using the reaction rate constants of Table 5.1. In stead of the reaction rate constants of galactose the reactions rate constants of glucose, obtained from previous work (Brands and Van Boekel, 2001b), were used to describe the reactions of the breakdown products of maltose. The results of the prediction are shown in Figure 5.13. The model describes the reactions very good. Apparently it was not important which sugar, either glucose (like in maltose) or galactose (as in lactose), was glycosylated at the C4 position of the reducing part of the disaccharide sugar. The reactions in the maltulose reaction mixture were not very well described (results not shown) which was most likely due to the much faster pH decrease in that system.

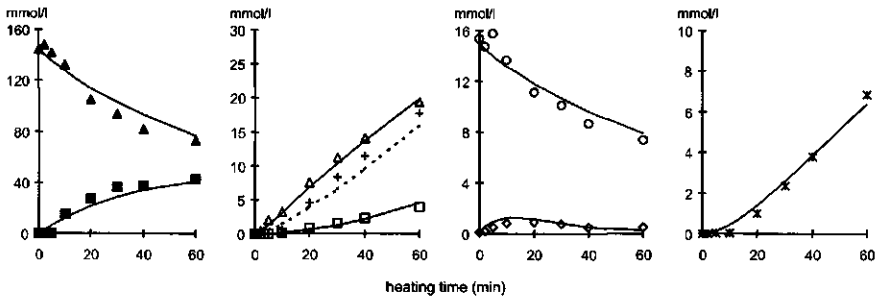


Figure 5.13 Predictions (drawn lines) based on the reaction rate constants of Table 5.1 for maltose-casein systems heated at 120°C. Maltose (▲), maltulose (■), glucose (Δ), fructose (□), formic acid (+), lysine-residues (○), Amadori compound (◇), melanoidins (*).

5.4 Conclusions

In this paper a reaction model was proposed and optimised for the reactions of the disaccharide sugars lactose and maltose and their ketose isomers in the presence of the protein casein at neutral pH and 120°C. If we compare the kinetic model for disaccharide sugars with the one developed for monosaccharide reactions previously (Brands and Van Boekel, 2001b), we can conclude that some degradation reactions are hindered by the glycosidic linked sugar in disaccharides (formation of acetic acid since apparently no triose intermediates can be formed and formation of formic acid via the 3-DG route) but that other routes are favoured (formation of formic acid via 1,4-DG route). Kinetic modelling now approves the observation which was described in literature before (Pischetsrieder and Severin, 1996). The

multiresponse modelling approach as used in this study appears to be a very powerful tool to unravel complicated reaction routes. The kinetic model derived in this study can be used to predict and optimise the quality of foods containing the disaccharides sugars maltose or lactose. For the reaction mechanism of the disaccharides it was not important whether galactose or glucose was glycosylated to the reducing end of the sugar.

Acknowledgements

We are grateful to Korina Pieters for performing some of the experimental work.

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6

Mutagenicity of heated sugar-casein systems: effect of the Maillard reaction

Abstract

The formation of mutagens after heating sugar-casein model systems at 120°C was examined by the Ames test, using *Salmonella typhimurium* strain TA100. Several sugars (glucose, fructose, galactose, tagatose, lactose and lactulose) were compared in their mutagenicity. Mutagenicity could be fully ascribed to Maillard reaction products and strongly varied with the kind of sugar. The differences in mutagenicity between the sugar-casein systems were caused by a difference in reaction rate and a difference in reaction mechanism. Sugars with a comparable reaction mechanism (glucose and galactose) showed a higher mutagenic activity corresponding with a higher Maillard reactivity. Disaccharides showed a lower mutagenic activity (lactulose) or no mutagenic activity (lactose) than their corresponding monosaccharides. Ketose sugars (fructose and tagatose) showed a remarkable higher mutagenicity compared with their aldose isomers (glucose and galactose), which was due to a difference in reaction mechanism.

Carline M. J. Brands, Gerrit M. Alink, Martinus A. J. S. van Boekel and Wim M. F. Jongen. *Journal of Agricultural and Food Chemistry* **2000**, *48*, 2271-2275.

6.1 Introduction

The Maillard reaction of amino acids or proteins with reducing sugars takes place during processing, cooking and storage of foods. The first step of the reaction, that is the condensation of free amino groups with carbonyl compounds, is followed by a series of other chemical reactions. These reactions cause changes in flavour and taste, formation of a brown colour and loss of nutritive value (Ames, 1992).

Another reported result of the Maillard reaction is the formation of mutagenic compounds. Maillard reaction mixtures are found to induce chromosome aberrations in Chinese hamster ovary cells (Powrie et al., 1981), to induce gene conversion in yeast (Powrie et al., 1981; Rosin et al., 1982) and to be mutagenic to *Salmonellae* (Kitts et al., 1993; Powrie et al., 1981; Shinohara et al., 1980; Shinohara et al., 1983). Shinohara et al. (1980) demonstrated that reaction products in a glucose-lysine solution heated at 100°C for 10 hrs induced reverse mutations in *Salmonella typhimurium* TA100 without S9 activation. Kitts et al. (1993) observed that glucose-lysine mixtures heated for 1 hr at 121°C showed mutagenic activity in *Salmonella typhimurium* TA98 and TA100 strains without S9 pretreatment. Mutagenicity was reduced in TA100 and eliminated totally in TA98 with added S9. Heated solutions of glucose with other amino acids also showed mutagenic activity in the *Salmonella typhimurium* TA100 strain, although the mutagenic response was less than with lysine (Powrie et al., 1981; Shinohara et al., 1983).

Most mutagenicity research of Maillard reaction products is performed with glucose as source of carbonyl group and amino acids as source of amino group. Less is known about the mutagenicity of reaction mixtures consisting of other sugars and other sources of amino groups. In most foods, the ε-amino groups of the lysine residues of proteins are the most important source of reactive amino groups. Since proteins are well-known for their antimutagenic activity (Vis et al., 1998), it might be possible that sugar-protein systems show no mutagenicity at all.

This paper reports a study on the mutagenicity of heated sugar-protein model systems. The protein casein was used as the source of amino groups. Several sugars were compared in their mutagenicity. Glucose, an aldose sugar, is the most studied sugar in Maillard reaction research and also the most abundant sugar in nature. Fructose is the ketose isomer of glucose. During heating of glucose, substantial amounts of fructose are formed due to the Lobry de Bruyn-Alberda van Ekenstein rearrangement (Speck, 1958). The reaction mechanism of fructose is known to be considerably different from that of glucose (Reynolds, 1965). Both glucose and fructose were selected as sugar reactants in this study presuming that each sugar

may produce different amounts and perhaps types of mutagens. These sugars were compared with other aldose and ketose sugars, galactose and tagatose, and the disaccharides lactose and lactulose. Mutagenicity was examined by the Ames test, using *Salmonella typhimurium* strain TA100 (Maron and Ames, 1983).

6.2 Materials and methods

6.2.1 Chemicals

All chemicals were analytical grade. Glucose, fructose, galactose and lactose were supplied by Merck (Darmstadt, Germany); tagatose and lactulose by Fluka Chemie (Buchs, Switzerland). Sodium caseinate (spray-dried) was obtained from DMV (Veghel, the Netherlands).

6.2.2 Preparation of reaction mixtures

Sodium caseinate (3% w/w) and sugar (150 mM) were dissolved in a phosphate buffer (0.1 M; pH 6.8) and heated for 20, 40 and 60 minutes at 120°C in screw-capped glass tubes in an oil bath.

6.2.3 Browning intensity

The browning intensity of the heated reaction mixtures was determined by measuring the absorbance at 420 nm against water with a spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The samples were diluted 4 times with sodium dodecyl sulphate (SDS; 16% w/w) to reduce scattering due to protein aggregates. If necessary the samples were diluted once more with water.

6.2.4 Mutagenicity assay

The mutagenicity was examined in the Ames assay (Maron and Ames, 1983). Histidine-requiring strains of *Salmonella typhimurium* with mutations in the histidine operons can be reverted to histidine prototrophy by active mutagens (Ames et al., 1975). In this study, mutagenicity was investigated using strain TA100, which is susceptible to base-pair substitution. The test was performed with and without metabolic activation by S9, a liver homogenate from Aroclor 1254 pretreated rats. In the test, 0.1 ml of an overnight culture of TA100 was mixed with 0.4 ml S9 mix or 0.4 ml phosphate buffer (0.1 M; pH 7.4) and 0.5 ml of the reaction mixture. In the negative control (spontaneous mutations), phosphate buffer

(0.1 M; pH 6.8) was used in stead of the reaction mixture. After a preincubation at 37°C for 30 minutes, 2.5 ml of top agar was added and the entire liquid was poured onto an agar plate. Histidine-revertant colonies were counted after a 48-hr incubation at 37°C. Each assay was performed in triplicate. As a positive control, 0.1 μg 4-nitroquinoline-N-oxide (4-NQO) per plate without S9 and 5 μg benzo[a]pyrene (B[a]P) with S9 were used. The mutagenic activity was corrected for spontaneous mutations by subtracting the number of revertants in the negative control. The result of the assay was considered mutagenic if the total number of revertants per plate was at least twice as high as the number of spontaneous revertants per plate.

6.2.5 Statistical analysis

All data are expressed as mean ± SEM. Different mutagenic responses observed from different sugars heated for the same time, were analysed by the Student's t-test. Statistical differences at $P \leq 0.05$ were considered to be significant.

6.3 Results

First, heated glucose-casein and fructose-casein systems were tested for their mutagenicity. A dose response of mutagenic activity was observed in both systems heated for 60 minutes at 120°C (Figure 6.1). The samples were preincubated for 30 minutes at 37°C in the absence of S9 mix. Preincubation was a necessity to get a good response. The best response was obtained with 0.5 ml sample per plate.

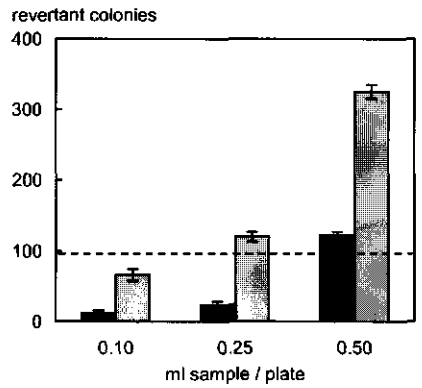

Figure 6.1 Mutagenicity response of glucose-casein (black bar) and fructose-casein samples (shaded bar) heated for 60 minutes tested without S9 (dotted line: spontaneous mutagenic activity = 94 ± 2).

Table 6.1 Mutagenicity of heated sugar-casein systems[a]

sugar	heating time		
	20 min	40 min	60 min
glucose	16 ± 5	62 ± 7	123 ± 4
fructose	128 ± 11	272 ± 10	325 ± 10
galactose	63 ± 9	139 ± 14	198 ± 10
tagatose	135 ± 11	360 ± 18	406 ± 4
lactose	21 ± 3	63 ± 19	93 ± 13
lactulose	67 ± 11	111 ± 12	134 ± 11

[a] Mutagenic activity is corrected for spontaneous mutations (= 94 ± 2 revertants / plate); positive control: 862 ± 45 revertants / plate.

In Table 6.1 the heating time response of mutagenicity of the glucose-casein and fructose-casein systems tested without S9 treatment is shown. Both sugar-casein systems showed a significant increase of revertants in time. The glucose-casein samples were mutagenic after 60 minutes heating while the fructose-casein samples were already mutagenic after 20 minutes. For the three heating times tested, mutagenic activity of the fructose-casein samples was significantly higher than that of the glucose-casein samples. Mutagenic activity was absent when the systems were incubated with S9 mix (Figure 6.2).

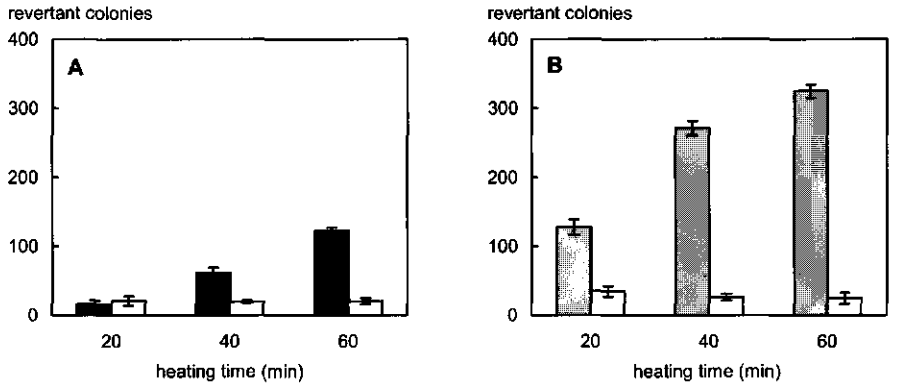

Figure 6.2 Mutagenic activity of 0.5 ml glucose-casein (A) and fructose-casein samples (B). (black/shaded bars) tested without S9 (spontaneous mutagenic activity = 94 ± 2); (open bars) tested with S9 (spontaneous mutagenic activity = 118 ± 7).

When either casein or any of the sugars in solution were heated separately under the same conditions as the sugar-casein systems, no mutagenic activity was noted (Figure 6.3). Consequently, mutagenicity can be fully ascribed to Maillard reaction products.

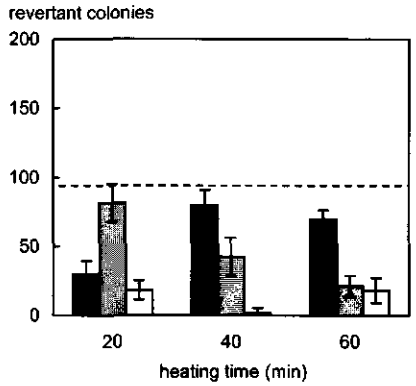

Figure 6.3 Mutagenic activity of the separate heated reactants. (Black bar) glucose; (shaded bar) fructose; and (open bar) casein (dotted line: spontaneous mutagenic activity = 94 ± 2).

Another aldose, galactose, and its ketose isomer, tagatose, were also tested in this study (Table 6.1). Both sugar-casein systems showed an increase of revertants in time. The tagatose-casein system was mutagenic after 20 minutes of heating and had a significantly higher mutagenic activity than the galactose-casein system at the tested heating times. The galactose-casein system was mutagenic after 40 minutes of heating.

The disaccharides lactose and lactulose were also studied. Lactose consists of a glucose and a galactose unit. The glucose unit contains the reactive carbonyl group. Lactulose is the ketose isomer of lactose consisting of fructose and galactose with the reactive group on the fructose moiety. After 60 minutes of heating no mutagenic activity could be found in the lactose-casein system and only slight mutagenic activity was observed in the lactulose-casein system (Table 6.1).

To study the possible antimutagenic effect of the protein, glucose and fructose were heated with glycine. Glycine is the simplest amino acid and behaves similar to lysine in the Ames test using tester strain TA100 (Shinohara et al., 1983). The glycine concentration was equal to the concentration of ε-lysine residues of the protein in the sugar-casein systems (15 mM). Comparison of the heated sugar-glycine systems with the sugar-casein systems (Figure 6.4) showed a significant increase of mutagenicity in the glucose-glycine system with regard to the glucose-casein system. Only a slight significant difference between the fructose systems was observed. Addition of 0.4 ml 3% sodium caseinate dispersion (instead of 0.4 ml phosphate buffer) to the sugar-amino acid mixture, just before preincubation, decreased the

mutagenicity of the glucose-glycine system (Figure 6.4A). In the fructose system no significant effect of addition of casein was observed (Figure 6.4B).

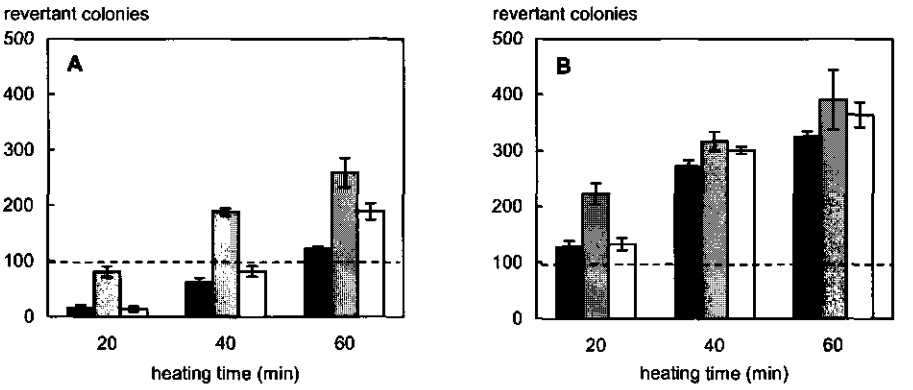

Figure 6.4 Mutagenic activity of glucose (A) and fructose (B) heated with (black bar) casein; (shaded bar) glycine; or (open bar) heated with glycine and afterwards addition of casein (dotted line: spontaneous mutagenic activity = 94 ± 2).

The browning of all the sugar-casein systems investigated in this study is shown in Table 6.2. The colour development of aldose sugars was in the descending order: galactose > glucose > lactose. The ketose sugars browned faster than their aldose isomers.

Table 6.2 Absorbance (420 nm) of sugar-casein systems.

sugar	heating time		
	20 min	40 min	60 min
glucose	1.76	3.95	5.84
fructose	2.75	5.07	7.66
galactose	3.27	6.51	8.86
tagatose	4.84	9.08	12.24
lactose	1.01	2.67	3.84
lactulose	2.98	5.52	7.34

6.3 Discussion

This study showed that mutagenic substances can be formed in the Maillard reaction between sugars and casein heated at 120°C. The mutagenicity of the model systems strongly varied with the kind of sugar. After 60 minutes of heating the mutagenic activity was in the

descending order: tagatose > fructose > galactose > lactulose > glucose > lactose. Mutagenicity could be fully ascribed to Maillard reaction products, a result which is in line with the observations of Shinohara et al. (1980) and Powrie et al. (1981). On this basis, it is to be expected that sugars which are more reactive in the Maillard reaction form higher amounts of mutagenic compounds. An indicator which is often used for Maillard reactivity is browning.

A faster browning (Table 6.2) was observed in the galactose-casein system as compared to the glucose-casein system, indicating that galactose was more reactive in the Maillard reaction. The mutagenic activity of the galactose-casein system was higher in the same order of magnitude (about 50%) than that of the glucose-casein system. The increased reactivity of galactose can be explained by its higher amount present in the acyclic form (Hayward and Angyal, 1977). The acyclic form of the sugar is the form in which the sugar reacts with the lysine-residues in the Maillard reaction.

Lactose showed a lower reactivity in the Maillard reaction than glucose, i.e. a slower browning was observed (Table 6.2). This lower reactivity was in agreement with the lower number of revertants after 60 minutes of heating. Although the number of revertants increased in time, no mutagenic activity could be observed in the lactose-casein system. This observation is a confirmation of the results of Rogers and Shibamoto (1982) and Berg et al. (1990), who could not detect mutagenic activity in milk and model systems containing lactose and casein. Disaccharides are considered to have another reaction mechanism than monosaccharides due to the glycosidic linked sugar. The bound sugar prevents typical monosaccharide degradation products to be formed and gives rise to typical disaccharide products (Kramhöller et al., 1993). These compounds might have less or no mutagenic activity.

The ketose fructose browned somewhat faster than its aldose isomer glucose (Table 6.2). The degree of browning is, however, not a good indicator to compare ketoses with aldoses in Maillard reactivity. Besides the Maillard reaction another reaction mechanism leads to browning, namely caramelisation of the sugar. Ketoses contribute noticeably to browning via caramelisation reactions while caramelisation in aldose systems may be neglected as a significant browning mechanism (Pilar Buera et al., 1987). Loss of available lysine-residues is another indicator which can be used for comparing sugars in their Maillard reactivity (Kato et al., 1986; Naranjo et al., 1998; Van Boekel and Brands, 1998). However, an almost three times higher mutagenicity was observed in the fructose-casein system as compared to the glucose-casein system (Table 6.1) while only a minor difference in lysine loss was observed (Van Boekel and Brands, 1998). Apparently, the difference in mutagenicity between the

ketose and aldose-casein systems was not caused by a difference in Maillard reactivity, but was due to a difference in reaction mechanism. Other compounds with higher mutagenic activity might be formed in the ketose-casein systems compared with the aldose-casein system, or, alternatively, the amount of antimutagenic compounds formed in the ketose-casein system was much lower than in the aldose-casein system. Numerous compounds are formed in the Maillard reaction. Some may be mutagenic while others may be antimutagenic. In our study, we could not differentiate between those compounds. The test provides information on the net influence of combined mutagens and antimutagens.

Mutagenic activity was found to be much higher in the tagatose-casein system than in galactose-casein system (Table 6.1). This observation confirmed our hypothesis that the difference in mutagenicity was caused by a difference in reaction mechanism between ketoses and aldoses.

Although we found differences between mono-aldoses and mono-ketoses, Powrie et al. (1981) and Omura et al. (1983) did not find any difference in mutagenicity between glucose and fructose in their studies. The fact that they used amino acids as the source of amino groups might explain this difference. Heating glycine in stead of casein decreased the difference in mutagenicity between the glucose and fructose system (Figure 6.4). Casein apparently suppresses the mutagenic activity of some specific Maillard compounds in the glucose system. This assumption was confirmed when adding casein to the glucose-glycine system (Figure 6.4). It is interesting to see that this antimutagenic activity of casein was not effective in fructose-casein systems, which is yet another indication of different reaction products in the case of fructose.

Only a slight significant difference in mutagenicity could be observed between the ketose and aldose disaccharide lactulose and lactose (Table 6.1), in contrast with the ketose and aldose monosaccharides. Evidently, less mutagenic compounds (in amount or in activity) are formed in the disaccharide systems, due to a change in reaction mechanism.

It should be stressed that mutagenic activity of the sugar-casein systems was weak compared to chemical mutagens like NQO. Moreover, mutagenic activity of the sugar-casein systems incubated with S9 was absent, which means that no indirect active mutagens were formed and, even more importantly, that direct mutagens were detoxified by S9. This result was also observed by Kitts et al. (Kitts et al., 1993).

It is not yet known which substances are responsible for the slight mutagenic activity. Possible mutagens, which can be formed in the model systems are, according to literature, methylglyoxal (Nagao et al., 1979), 5-hydroxymethylfurfural (HMF), ε-(2-formyl-5-

(hydroxymethyl)pyrrol-1-yl)norleucine (LPA) (Omura et al., 1983), 4-hydroxy-2-hydroxymethyl-5-methyl-3(2H)furanone (HHMF) (Hiramoto et al., 1995) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) (Hiramoto et al., 1997). In the model systems of the present study, HMF as mutagen can be omitted since it is an indirect mutagen which does not show mutagenicity in the absence of S9. Like our sugar-casein systems, methylglyoxal, HHMF and DDMP showed mutagenicity with TA 100 which was significantly decreased in the presence of S9 (Hiramoto et al., 1995; Hiramoto et al., 1997; Nagao et al., 1979). HHMF and DDMP are formed in monosaccharide systems and not in disaccharide systems (Kramhöller et al., 1993), which might be an explanation for the lower mutagenicity of disaccharide systems. Omura et al. (1983) suggested that LPA is one of the main mutagens formed by the Maillard reaction between glucose and lysine. In glucose-casein systems, LPA is formed in higher amounts than in lactose-casein systems (Morales and Van Boekel, 1996), which might be another explanation for the lower mutagenicity of disaccharide systems. However, LPA is bound to protein and it is not known whether LPA is also mutagenic if bound. No explanation from literature could be found for the higher mutagenicity of ketose-casein systems. Most likely, other compounds than mentioned above are responsible for this.

The results obtained in this study show remarkable differences in mutagenicity, due to the Maillard reaction, between ketoses and aldoses, and mono- and disaccharides, under conditions that correspond to sterilisation conditions in the food industry. The differences found may offer possibilities to optimise food quality with respect to mutagenicity due to the Maillard reaction.

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7

General discussion

7.1 Introduction

Controlling the Maillard reaction is of utmost importance during heating and storage of foods because of its impact on food quality. As was reviewed in the general introduction, food quality aspects can be divided in safety and nutritional aspects, shelf life and sensory properties, convenience and product integrity. The Maillard reaction has an effect mainly on the sensory properties (colour and flavour), nutritional aspects (loss of essential amino acids) and safety aspects (the formation of mutagenic compounds).

To be able to control these food quality aspects, mechanistic insight of the underlying reactions that are responsible for these quality changes is needed. Therefore, the research described in this thesis focused on the kinetics of the chemical reactions in sugar-casein systems. A kinetic approach was used to determine the rates of the reactions and their dependence on reaction conditions, but also to understand the mechanism of the reactions. The multiresponse modelling technique has been used to develop a kinetic model for sugar-casein reactions and has shown to be a powerful tool in unravelling the complicated reaction routes. The developed models (Chapter 4 for monosaccharide-casein reactions and Chapter 5 for disaccharide-casein reaction) were able to describe the reactant degradation and product formation as a function of various reaction conditions quantitatively. With these kinetic models we are now able to predict changes during heating of sugar-casein systems.

In the present chapter an attempt is made to integrate the results obtained in the various chapters of this thesis and to correlate the results to quality attributes. The studied compounds that are important for food quality were available lysine (for its contribution to nutritive value), melanoidin formation (for its contribution to colour) and mutagenic compounds (for its effect on food safety). The effect of reaction conditions like heating temperature and time, pH, reactant concentration and type of reactants on the quality aspects will be discussed.

7.2 Nutritional aspects

Foods containing proteins or amino acids are subject to nutritional damage during heating. The amino groups of the amino acids (whether or not bound to the protein) can react in the Maillard reaction and once they are bound in Amadori compounds, in advanced glycation products or in melanoidins, the modified amino acids are no longer available for the human body. If the affected amino acids are essential amino acids, this can lead to loss of nutritive value. In the model systems used for the research described in this thesis the protein casein was studied. The reactive amino groups are the ε-amino groups of the lysine residues and, to a

lesser extent, the guanidine groups of the arginine residues. Lysine is an essential amino acid, whereas arginine is not. A kinetic model was developed, which gives mechanistic insight in the reaction of the lysine residues. These models can also be used to predict the loss of available lysine as a function of heating temperature and time, pH, reactant concentration and type of sugar.

7.2.1 Effect of pH

When the initial pH of the sugar-casein systems was lowered from pH 6.8 to 5.9 it was found that the loss of lysine was less (Chapter 4). Generally it has been stated that the rate of substrate loss in the Maillard reaction increases with increasing pH, up to a pH of about 10 (Labuza and Baisier, 1992). An explanation for this pH effect is that the reactive form of the reducing sugar, the acyclic form, is considered to decrease with decreasing pH. Furthermore, protonation of the amino group of the lysine residues increases with decreasing pH. Due to this protonation the nucleophilicity and therefore the reactivity of the amino group is lost. The higher the pH, the larger the percentage of amino groups in the unprotonated form and, therefore, more lysine residues can react with reducing sugars. In this thesis it was shown that the effect of pH on the reactivity of lysine residues was far more important than the effect of pH on sugar reactivity.

During heating of sugar-casein systems the pH decreases due to the formation of organic acids. This means that the amount of reactive amino groups decreases with progressing heating resulting in a decreasing reaction rate.

7.2.2 Effect of heating temperature

Generally, the rate of chemical reactions increases with increasing temperature. Since the Maillard reaction consists of several reaction steps, each with a possibly different temperature sensitivity, it strongly depends on temperature which reaction route prevails and what pattern of intermediates and end products is formed.

To predict the reactions at various temperatures, the temperature dependencies were determined (Chapter 4). The reaction between glucose and lysine residues had an activation energy of about 100 kJ/mol. The reaction between reaction intermediates (originating from glucose, fructose or Amadori) and lysine residues was less temperature dependent (about 75 kJ/mol). The reactivity towards lysine residues was much higher for reaction intermediates than for glucose at the lowest temperature studied (90°C). Due to the lower activation energy of the latter this discrepancy decreased as the temperature was increased (till 130°C), but still

the reactivity of the intermediates was considerably higher than that of glucose. The activation energy needed for the formation of these reaction intermediates was however very high (140 kJ/mol for reaction products from glucose and 160 kJ/mol for reaction products derived from fructose) or an extra reaction step was involved (Amadori product formation before its degradation into fragments). This means that although the reaction rate between intermediates and lysine residues might be high, the reaction will not occur when the temperature is too low for the formation of noticeable concentrations of intermediates.

To prevent lysine damage it is thus better to heat at lower temperatures and consequently a longer time.

7.2.3 Effect of heating time

Most chemical reaction rates increase with a factor 2 to 3 when the temperature increases 10°C (corresponding to an activation energy of 100-120 kJ/mol). This factor is referred to as Q_{10}. This means that products should be heated twice or three times as long when the temperature is decreased with 10°C to obtain the same result for a certain reaction (for example, inactivation of microorganisms). If another, undesirable, reaction has a Q_{10} of 4 (the formation of reactive intermediates from fructose, for instance) it means that only half or three quarters of the unwanted compounds are formed when the temperature is lowered by 10°C.

Although the heating time has to be extended if the reaction temperature is decreased, quality loss can be reduced.

7.2.4 Effect of sugar concentration

The reactant concentration should not have an effect on the reaction rate constants when a consistent model is developed. The reactant concentration does, of course, have an effect on the concentration of reaction products that are formed. If the sugar concentration was halved, lysine damage was less than halved (Chapter 4). This was due to a lower pH decrease, so that relatively more sugar fragments are formed which subsequently can react with the lysine residues of the protein.

Lysine damage is thus directly related to the sugar concentration.

7.2.5 Effect of protein concentration

Increasing the protein concentration has only a small effect on the pH decrease during heating and therefore lysine damage is almost doubled when the protein concentration is doubled.

Thus, relatively seen, protein concentration does not have an effect on the lysine damage if the sugar concentration remains constant.

7.2.6 Effect of type of sugar

In this thesis several sugars were compared in their reaction behaviour towards lysine. The higher loss of lysine in galactose-casein systems compared to glucose-casein systems could be explained by a higher percentage of galactose in its acyclic form, which is generally believed to be the reactive form (Chapter 4). A larger part is therefore able to react in the Maillard reaction, but also more reactive intermediates can be formed which will react with the lysine residues.

Lysine damage was about equal in the glucose-casein and fructose-casein systems heated at 120°C with an initial pH of 6.8 (Chapter 2). When the heating temperature or the initial pH was lowered the loss of lysine was less in the fructose-casein system than in the glucose-casein system (Chapter 4). This difference could not be explained by a difference in acyclic form between the sugars. Although it is always assumed in literature that fructose reacts in the same way as glucose with lysine residues in the Maillard reaction (formation of Heyns compound instead of Amadori product), multiresponse modelling showed that fructose itself does not react with lysine residues; only its degradation products react in the Maillard reaction. Since the formation of Maillard reactive intermediates from fructose appeared to be strongly pH and temperature dependent, this explains why at lower pH and lower temperatures the damage of lysine in fructose-casein systems was limited.

Compared to the loss in the glucose-casein system, the loss of lysine in the lactose-casein and maltose-casein was somewhat lower (Chapter 5). For short heating times this can be explained by the somewhat lower reaction rate of the disaccharides compared to glucose in the early stage of the Maillard reaction. With prolonged heating, the reaction rate of disaccharide degradation products with lysine residues in the advanced stage of the Maillard reaction was on the other hand higher than that of glucose degradation products. Thus, it depends on the amount of reaction intermediates that are formed if the loss of lysine in the disaccharide systems exceeds that from the monosaccharide systems. In the aldose-disaccharide systems, heated in the presence of casein at 120°C, the formation of degradation product was not very high, but in the ketose-disaccharide systems far more degradation products were formed which led to a higher loss of lysine.

It could be concluded that the type of sugar has an eminent effect on the rate and extent of quality loss.

7.3 Sensory aspects

The Maillard reaction has two important sensory consequences, namely flavour and colour. In this thesis the sensory aspect colour was studied. The Maillard reaction is also known as the nonenzymatic browning reaction and is responsible for brown colour formation in many heated foods. Some examples are the brown crust of bread, brown colour of condensed milk, deep-fried potatoes, roasted meat and coffee beans.

Chapter 3 of this thesis described how the melanoidins, the high-molecular weight compounds that are mainly responsible for colour formation in sugar-protein systems, can be quantified. Once colour could be quantified in concentration units, it could be taken into account in the kinetic model. In Chapter 4 the model proposed in Chapter 2 was rigorously tested and adapted to come to a model that was able to predict browning as a function of heating temperature and time, pH, reactant concentration and type of sugar.

The use of kinetic modelling gave us the mechanistic insight we needed to explain melanoidin formation. It showed us that besides lysine also arginine must be involved in melanoidin formation.

7.3.1 Effect of pH

As for lysine damage, it has been stated that the rate of browning increases with increasing pH (up to a pH of about 10) (Labuza and Baisier, 1992). Therefore less browning was observed when systems with an initial pH of 5.9 in stead of 6.8 were heated (Chapter 4). During heating of sugar-casein systems the pH decreases due to the formation of organic acids, which has an effect on the reactivity of the amino groups. Although lysine damage flattened off after a certain heating time, this effect was less apparent for browning. This ongoing browning was explained by the reaction of sugar fragments with arginine residues.

Thus, pH decrease slows down the browning. Depending on the type of food this might be desirable or undesirable.

7.3.2 Effect of heating temperature

Generally, the rate of chemical reactions increases with increasing temperature and therefore more browning was observed when the reaction mixtures were heated at higher temperatures. Browning cannot occur before any reaction intermediates are formed that can react with the protein. Therefore, an induction time for browning was observed, which was temperature dependent. The temperature dependence of the reaction of lysine residues with intermediates

to form brown compounds was already discussed in section 7.2.2. The temperature dependence of the reaction of arginine residues towards brown compounds was estimated, but probably because no experimental data were obtained, no temperature dependence could be established (Chapter 4).

A certain temperature is thus needed to get browning within a certain heating time. Once browning occurs, it increases with temperature.

7.3.3 Effect of reactant concentration

The effect of reactant concentration on browning and lysine damage was the same (see section 7.2.4 and 7.2.5). When the protein concentration was halved, brown colour formation was also halved. However, when the sugar concentration was halved, browning was less than halved due to the lower pH decrease.

Therefore, it depends on which quality aspect has to be optimised (nutritive value or colour) what reactant concentrations are favoured.

7.3.4 Effect of type of sugar

In Chapter 4 it was concluded that the reaction between glucose and lysine residues does not lead to browning immediately and the reaction between fructose and lysine residues does not occur. Thus a certain heating time is needed to obtain sugar fragments that can react with the protein and can lead to browning. Once browning occurred a clear difference was found in browning between the ketose fructose and the aldose glucose. Although the loss of lysine in the fructose-casein system was only slightly higher, browning was clearly higher compared to the glucose-casein system. This is partly due to the fact that in the fructose system more fragments are formed that can react with the protein (either the lysine or the arginine residues), but also because in the glucose-casein system some of the lysine residues were bound in the Amadori compound and not in the melanoidins.

In the aldose-disaccharide systems, heated at 120°C in the presence of casein, browning was somewhat less than in the glucose-casein system although the rate constants for the reaction between the protein and sugar fragments were estimated to be higher. This can be ascribed to a smaller formation of reactive intermediates. In the ketose-disaccharide system higher concentrations of reactive intermediates were formed, which led to a higher browning rate.

7.4 Safety aspects

The Maillard reaction is also known for the formation of mutagenic as well as antimutagenic compounds. Most of the literature about Maillard-related mutagenicity in foods is about cooking of meat. It was suggested that creati(ni)ne, free amino acids and hexoses, present in raw meat, react in the Maillard reaction to form heterocyclic amines. In the absence of creati(ni)ne heterocyclic amines can be formed during pyrolysis of amino acids and proteins. Pyrolysis occurs at temperatures above 300°C. Heterocyclic amines have shown to be highly mutagenic. A carcinogenic effect of the heterocyclic amines was suggested for humans who are genetically sensitive and/or moderately to highly exposed to them (Jägerstad et al., 1998).

In Chapter 6 of this thesis it was shown that mutagenic substances can also be formed in reaction model mixtures containing sugars and casein, but lacking creati(ni)ne under conditions that correspond to sterilisation conditions in the food industry. It should however be stressed that mutagenicity was rather low. In the Maillard reaction also antimutagenic compounds can be formed. It is known, for instance, that melanoidins can bind the highly mutagenic heterocyclic amines (Lee et al., 1994).

7.4.1 Effect of heating temperature and pH

The effect of pH and heating temperature on the mutagenicity was not studied in this thesis. It can, however, be assumed that mutagenicity will increase with increasing Maillard reactivity, thus with increasing heating temperature and increasing pH.

7.4.2 Effect of type of sugar

Various sugars were compared in their mutagenic behaviour (Chapter 6). The results are summarised in Figure 7.1. The differences between the various aldose systems could be explained by their difference in Maillard reactivity. The difference between the ketose-casein and aldose-casein systems (heated at 120°C and an initial pH of 6.8) was remarkably and could not be explained by their difference in Maillard reactivity, but rather by a difference in reaction mechanism. An explanation for the higher mutagenic activity of the ketose-casein systems might be that compounds with higher mutagenic activity were formed or, alternatively, fewer compounds with antimutagenic activity were formed.

It could be concluded that the mutagenicity of the model systems varied strongly with the kind of sugar.

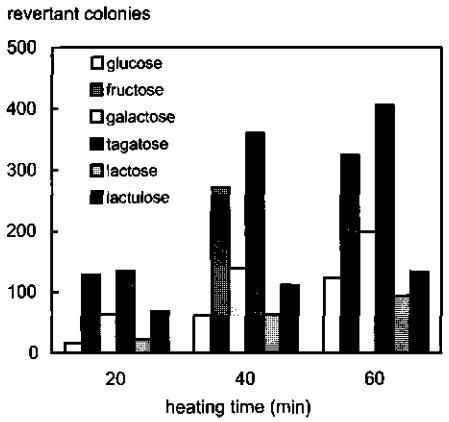

Figure 7.1 Mutagenic activity of various sugar-casein systems after heating at 120°C.

7.4.3 Effect of protein

Mutagenicity in sugar-casein systems could be fully ascribed to Maillard reaction products (Chapter 6). Therefore, it can be expected that more mutagenic compounds will be formed when the protein concentration, and therefore the rate of the Maillard reaction, is increased. On the other hand it is known that proteins, including casein, can bind mutagenic compounds (Vis et al., 1998). Casein can therefore lower the overall mutagenicity of heated Maillard reaction mixtures. In this thesis it was shown that casein had an antimutagenic effect on the compounds formed in glucose-glycine systems, although it had no effect on the mutagenic activity of fructose-glycine systems.

The effect of casein on mutagenicity is thus twofold due to its reactivity in the Maillard reaction and its antimutagenic activity.

7.5 Conclusions

In this study it was attempted to use multiresponse modelling in order to follow quality changes in time. In Figure 7.2 the effect of heating temperature, pH, reactant concentration and type on the nutritive value and colour are summarised. The kinetic model derived in this thesis is optimised for several reaction conditions and can be used to predict quality changes for combinations of reaction conditions that are not studied. Figures such as Figure 7.2 can be used to choose optimum conditions for certain quality aspects. Should the Maillard reaction have been studied using simple kinetics and only one response was followed in time, no

conclusions could have been drawn about the combination of reaction conditions that were not studied, because no mechanistic insight in the reaction would be obtained.

In this study, multiresponse modelling has shown to be a very powerful tool to follow quality changes in time quantitatively.

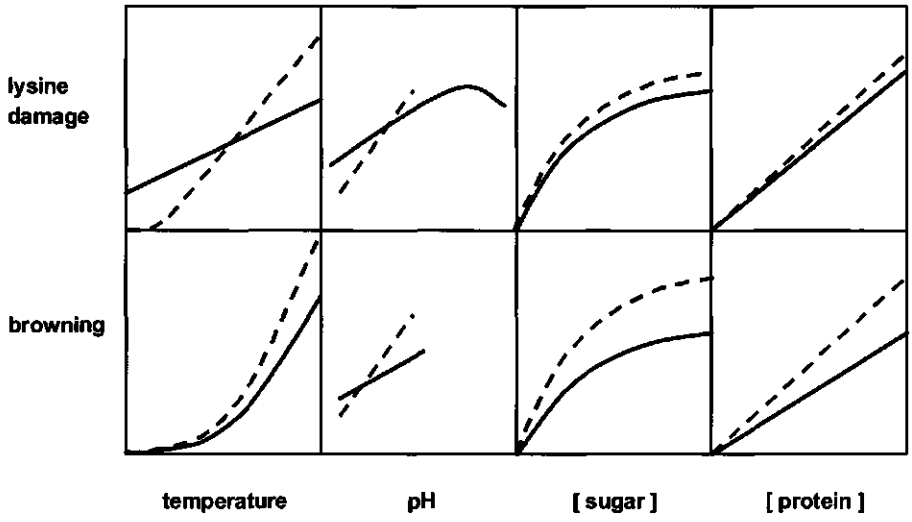

Figure 7.2 Effect of reaction conditions on two quality aspects: lysine damage and browning (glucose: solid line, fructose: dotted line).

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Summary

Samenvatting

Summary

The Maillard reaction is of utmost importance during heating and storage of foods because of its contribution to food quality. Maillard reaction products are responsible for the development of flavour and colour. The Maillard reaction can result in loss of nutritive value, and mutagenic compounds but also antimutagenic compounds might be formed. In order to be able to control the Maillard reaction, and therefore partly the quality of foods, it is necessary to study the reactions of interest quantitatively.

The aim of this thesis was to determine the kinetics of the Maillard reaction between proteins and sugars. With knowledge of kinetics, it becomes possible to describe the changes in a quantitative way, and to predict changes from certain time-temperature combinations. In addition, kinetics is a tool for understanding reaction mechanisms. Since the Maillard reaction is a very complex reaction, kinetic modelling was necessary.

In this thesis no real foods but model systems thereof were studied. These model systems consisted of the protein casein and various sugars in a buffered solution. The reaction conditions, such as pH, temperature, reactant concentrations were varied, covering relevant heating conditions for foods.

In Chapter 2 reaction mechanisms were proposed to explain the observed reaction products for the reactions of the aldose sugars glucose and galactose and the ketose sugars fructose and tagatose in the presence of casein at neutral pH and 120°C. The main reaction routes were (i) sugar isomerisation, (ii) degradation of the sugar into carboxylic acids resulting in considerable pH decrease, and (iii) the Maillard reaction, in which the sugar but also its reaction products react with the ε-amino group of lysine residues of the protein. The reaction pathways found were put together into a reaction network model. Significant differences in reaction mechanism between aldose and ketose sugars were observed. Ketoses seemed to be more reactive in the sugar degradation reactions than their aldose isomers, and whereas the Amadori product was detected as Maillard reaction intermediate in the aldose-casein system, no such intermediate could be found in the ketose-casein system.

Chapter 3 focused on the quantification of melanoidins. Melanoidins are the final high-molecular weight compounds of the Maillard reaction that are mainly responsible for colour formation in heated sugar-protein systems. The aim of the study was to determine the average molar extinction coefficient of melanoidins formed in heated glucose-casein and fructose-casein systems, using ^{14}C-labelled sugar. The estimated extinction coefficients were used in the rest of the thesis to translate spectrophotometrically measured browning into melanoidin concentration (expressed as amount of sugar incorporated).

In Chapter 4 the kinetics of the reactions occurring during heating of monosaccharide-casein systems was studied using multiresponse modelling. As a starting-point the reaction network model developed in the second chapter was used. The effect of heating temperature, pH, reactant concentration and type of sugar were studied and the results were used to test the kinetic model very rigorously. The iterative process of kinetic modelling - proposing a model, confronting it with experiments, criticising the model - was passed through several times to arrive at a model that was largely consistent with all results obtained. This study has shown the power of multiresponse modelling for the unravelling of complicated reaction routes as occur in the Maillard reaction. A striking difference was found between aldose and ketose sugars as suggested by the modelling results: not the ketoses themselves, but only their reaction products were found to be reactive in the Maillard reaction under the conditions studied.

In Chapter 5 the reactions occurring in disaccharide-casein reaction mixtures at neutral pH and 120°C were studied. Two main degradation routes were established: (i) isomerisation of the aldose sugars lactose and maltose in their ketose isomers lactulose and maltulose, respectively, and subsequent degradation into galactose and glucose, respectively, and formic acid among other unidentified compounds, and (ii) the Maillard reaction, in which the aldose sugars themselves but also sugar degradation products react with the protein, eventually leading to the formation of brown compounds. These reactions were put together into a kinetic model and the proposed model was extensively tested using multiresponse modelling. The model was adapted several times. The final kinetic model was able to explain the observed changes in reactants and products and allowed a quantitative prediction of reactions in heated disaccharide-casein systems. The reaction mechanism of disaccharide sugars differed slightly from that of monosaccharide sugars: some sugar degradation pathways were hindered by the glycosidic bound sugar while others were favoured.

Chapter 6 reports a study on the mutagenicity of heated sugar-casein model systems. The formation of mutagens after heating sugar-casein model systems at 120°C was examined in the in-vitro Ames test. Several sugars (glucose, fructose, galactose, tagatose, lactose and lactulose) were compared in their mutagenicity. Although mutagenic activity was weak, it could be fully ascribed to Maillard reaction products and strongly varied with the kind of sugar. The differences in mutagenicity between the sugar-casein systems were caused by a difference in reaction rate and a difference in reaction mechanism. Sugars with a comparable reaction mechanism (glucose and galactose) showed a higher mutagenic activity corresponding with a higher Maillard reactivity. Disaccharides showed a lower mutagenic activity (lactulose) or no mutagenic activity (lactose) than their corresponding

monosaccharides. Ketose sugars (fructose and tagatose) showed a remarkably higher mutagenicity compared with their aldose isomers (glucose and galactose), which was ascribed to a difference in reaction mechanism, as described in the previous chapters.

In Chapter 7 the results of this thesis and their significance for food quality were discussed. The effect of reaction conditions on quality aspects like colour and loss of nutritive value were summarised in a figure, which can be used to choose optimum conditions for certain quality aspects. This thesis has shown that by making use of multiresponse modelling one is able to predict quality changes caused by the Maillard reaction in time quantitatively.

Samenvatting

De Maillardreactie speelt een zeer belangrijke rol tijdens verhitten en bewaren van levensmiddelen door de bijdrage die zij levert aan de kwaliteit van levensmiddelen. De producten van de Maillardreactie zijn van belang in levensmiddelen, omdat zij verantwoordelijk zijn voor de ontwikkeling van kleur, geur en smaak. De Maillardreactie kan daarnaast leiden tot verlies van voedingswaarde en tot de vorming van mutagene maar ook antimutagene componenten. Om de Maillardreactie te kunnen beheersen, en daardoor gedeeltelijk ook de kwaliteit van levensmiddelen, is het noodzakelijk om de Maillardreactie en andere gelijktijdig optredende suikerreacties kwantitatief te bestuderen.

Het doel van dit proefschrift was het bepalen van de kinetiek van de Maillardreactie zoals die plaatsvindt tussen eiwitten en suikers. Wanneer de kinetiek bekend is, kunnen veranderingen kwantitatief beschreven worden en kunnen deze veranderingen ook voorspeld worden voor bepaalde tijd-temperatuur combinaties. Bovendien is kinetiek een middel om reactiemechanismen te begrijpen. Omdat de Maillardreactie een zeer ingewikkelde reactie is, kon niet worden volstaan met eenvoudige kinetiek, maar was kinetisch modelleren een vereiste.

In de experimenten beschreven in dit proefschrift werden geen echte levensmiddelen, maar modelsystemen bestudeerd. Deze modelsystemen bestonden uit het eiwit caseïne en verschillende soorten suikers, in een gebufferde oplossing. De reactiecondities zoals temperatuur, pH en concentratie van de reactanten werden gevarieerd, waarbij de condities die relevant zijn voor de levensmiddelenindustrie bestreken werden.

In hoofdstuk 2 van dit proefschrift werden reactiemechanismen voorgesteld die de geïdentificeerde reactieproducten voor de reacties van de aldose-suikers glucose en galactose en de ketose-suikers fructose en tagatose in de aanwezigheid van caseïne bij neutrale pH en een temperatuur van 120°C kunnen verklaren. De belangrijkste reactieroutes waren (i) isomerisatie van de aldose-suiker in de ketose-suiker en vice versa, (ii) afbraak van de suiker in organische zuren wat resulteerde in een aanzienlijke pH-daling en (iii) de Maillardreactie tussen de suikers en zijn afbraakproducten met de ε-aminogroep van de lysineresiduen van het eiwit. De gevonden reactiewegen werden in een reactienetwerkmodel samengevoegd. Belangrijke verschillen in reactiemechanismen werden gevonden tussen aldose- en ketose-suikers. Onder de bestudeerde omstandigheden leken de ketosen bijvoorbeeld veel reactiever in de suikerafbraakreacties dan hun aldose-isomeren. En in tegenstelling tot de aldose-caseïne reactie waar het Amadoriproduct als tussenproduct van de Maillardreactie werd

geïdentificeerd, kon in de ketose-caseïne reactie een dergelijke component niet worden aangetoond.

Hoofdstuk 3 richt zich op het kwantificeren van de melanoïdinen. Melanoïdinen zijn de hoogmoleculaire componenten die aan het eind van de Maillardreactie gevormd worden. Deze componenten zorgen voornamelijk voor de bruine kleur zoals deze gevormd wordt in suiker-eiwit systemen. Het doel van het onderzoek beschreven in dit hoofdstuk was het bepalen van de extinctiecoëfficiënt van melanoïdinen gevormd in glucose-caseïne en fructose-caseïne systemen. Hierbij werd gebruik gemaakt van radioactief gemerkte suiker. De gevonden waarden voor de extinctiecoëfficiënten werden in de rest van het proefschrift gebruikt om de bruinkleuring die met behulp van de spectrofotometer bepaald was om te rekenen naar melanoïdinenconcentratie (uitgedrukt als hoeveelheid suiker ingebouwd in de melanoïdinen.

In hoofdstuk 4 werd de kinetiek van de reacties die plaatsvinden tijdens verhitten van monosacharide-caseïne systemen bestudeerd. Dit werd gedaan door middel van kinetisch modelleren, waarbij rekening werd gehouden met zoveel mogelijk responsies (multirespons modelleren). Als startpunt werd het reactienetwerkmodel uit hoofdstuk 2 genomen. De invloed van verhittingstemperatuur, pH, concentratie van de reactanten en het type suiker werden bestudeerd. De resultaten hiervan werden gebruikt om het kinetische model uitgebreid te testen. Het iteratieve proces van kinetisch modelleren – een model voorstellen, het confronteren met experimenten, het bekritiseren van het model – werd een aantal keer doorlopen, om uiteindelijk bij een model uit te komen dat grotendeels consistent was met de gevonden resultaten. Deze studie heeft de kracht van multirespons modelleren laten zien voor het ontrafelen van complexe reactiemechanismen zoals die plaatsvinden in de Maillardreactie. Hiermee werd aangetoond dat een opvallend verschil bestaat tussen aldose- en ketose-suikers: niet de ketosen zelf, maar alleen hun reactieproducten zijn reactief in de Maillardreactie onder de bestudeerde omstandigheden.

In hoofdstuk 5 zijn de reacties die optreden in disacharide-caseïne systemen bij neutrale pH en 120°C verhitting onderzocht. De twee belangrijkste reactieroutes werden vastgesteld: (i) isomerisatie van de aldose-suikers lactose en maltose in hun respectievelijke ketose-isomeren lactulose en maltulose, en daaropvolgende degradatie in respectievelijk galactose en glucose, en mierezuur naast andere niet-geïdentificeerde componenten, en (ii) de Maillardreactie, waarin de aldose-suikers en de suikerafbraakproducten reageren met het eiwit wat uiteindelijk leidt tot bruine componenten. Deze reacties werden in een kinetisch model samengebracht en dit model werd uitgebreid getest door middel van multirespons modelleren. Het model werd enkele malen aangepast. Het uiteindelijke model was in staat om de waargenomen veranderingen in reactanten en producten te verklaren en om een kwantitatieve

voorspelling te doen van de reacties in verhitte disacharide-caseïne systemen. Het reactiemechanisme van disachariden week enigszins af van dat van monosachariden: sommige suikerafbraakreacties werden gehinderd door de gebonden suiker, terwijl andere reactieroutes juist de voorkeur hadden.

In hoofdstuk 6 is de mutageniteit van verhitte suiker-caseïne systemen onderzocht met behulp van de Ames-test. Verschillende suikers (glucose, fructose, galactose, tagatose, lactose en lactulose) werden vergeleken in hun mutageniteit. Hoewel de mutageniteit zwak was, kon deze volledig toegeschreven worden aan Maillardreactieproducten en varieerde sterk met de soort suiker. De verschillen in mutageniteit tussen de suiker-caseïne systemen werden veroorzaakt door een verschil in reactiesnelheid en een verschil in reactiemechanisme. Suikers met een vergelijkbaar reactiemechanisme (glucose en galactose) toonden een hogere mutagene activiteit naarmate de Maillardreactiviteit hoger was. Disaccharides lieten een lagere mutagene activiteit zien (lactulose) of helemaal geen mutagene activiteit (lactose) vergeleken met de overeenkomstige monosaccharides. Ketose suikers (fructose en tagatose) toonden een opvallend hogere mutageniteit vergeleken met hun aldose isomeren (glucose en galactose), wat toegeschreven werd aan het verschil in reactiemechanisme, zoals beschreven in eerdere hoofdstukken.

In hoofdstuk 7 worden de resultaten van dit proefschrift en hun betekenis voor de kwaliteit van levensmiddelen bediscussieerd. De invloed van reactiecondities op kwaliteitsaspecten als kleur en verlies van voedingswaarden werden samengevat in een figuur die gebruikt kan worden om optimale condities voor bepaalde kwaliteitsaspecten te kiezen. Dit proefschrift heeft laten zien dat het met behulp van multirespons modelleren mogelijk is om kwaliteitsveranderingen die veroorzaakt worden door de Maillardreactie kwantitatief te voorspellen.

Nawoord

Het is zover, mijn proefschrift is af! Na ruim 5 jaar experimenteren, modelleren, lezen, schrijven en vele leuke uitstapjes is het einde van mijn promotieonderzoek in zicht. Veel mensen hebben mij tijdens deze periode bijgestaan. Graag wil ik hen op deze plaats bedanken.

Allereerst mijn promotoren Wim Jongen en Tiny van Boekel. Wim en Tiny, jullie vormen een mooi koppel. Dit werd echt goed duidelijk vanaf het moment dat jullie samen de duobaan als hoogleraar vervulden. Tiny, jou wil ik speciaal bedanken voor het vertrouwen dat jij altijd in mij hebt gehad. Vanaf het moment dat je belde om te vragen of ik geïnteresseerd was in een AIO-baan tot de deadline voor het inleveren van de leesversie wist ik me altijd gesteund door de gedachte dat jij vertrouwen had in een goede afloop. Door je uiterst efficiënte manier van werken had je altijd tijd voor me op momenten dat ik het echt nodig had. Wim, van jou heb ik geleerd het onderzoek in een breder kader te kunnen plaatsen. Jouw enthousiasme tijdens onze besprekingen heeft mij enorm gestimuleerd.

I am very grateful to all the participants involved in the EU project 'Optimisation of the Maillard Reaction. A Way to Improve Quality and Safety of Thermally Processed Foods'. I learned a lot from you during our six-monthly meetings, each time organised by another university. Thank you for the great concern with which these meetings (including the social events) were organised.

Toen het EU-project afgelopen was werden de halfjaarlijkse uitstapjes voortgezet in de vorm van COST Action 919 'Melanoidins in Food and Health'. In het kader van dit project kreeg ik de mogelijkheid om eind 2000 gedurende een maand onderzoek te doen bij het Procter Department of Food Science, University of Leeds. I am grateful to Professor Bronek Wedzicha for hosting me and learning me more interesting details about the Maillard reaction. I also would like to thank Sandra Mundt for making my stay in Leeds such a pleasant time, both at work and at home. Sandra, thank you for your help in the lab and the nice room that you and Rob offered me. This period has lead to a special friendship. I really enjoy that you want to be my 'paranimf'.

Tijdens de zomer van 1998 heb ik een deel van mijn promotieonderzoek uitgevoerd bij de leerstoelgroep Toxicologie. Ik wil met name Gerrit Alink en Eric Vis bedanken voor hun inbreng van kennis en hulp tijdens deze periode. Eric, bedankt dat je me wegwijs hebt gemaakt in de Ames-test. Gerrit, bedankt voor de vruchtbare discussies die geleid hebben tot mijn eerste wetenschappelijke publicatie.

Voor de praktische hulp bij alle andere experimenten wil ik Maria Baltussen graag bedanken. Maria, door jouw praktische kennis heb ik me vlot op het onderwerp in kunnen

werken en konden alle apparatuurproblemen snel opgelost worden. Verder wil ik Henk Jansen graag bedanken voor de computertechnische hulp.

In het kader van hun afstudeervak of stage hebben Lennard Muilwijk, Korina Pieters, Kirsten van der Goot, Barry de Wit, Anton de Bruin en Ruud van Gorp bijgedragen aan dit proefschrift. Bedankt voor jullie werk en de leuke samenwerking!

Door alle collega's werd de promotietijd ook een erg gezellige tijd met leuke labuitjes, borrels, barbecues en AIO-reizen. Speciaal wil ik ook mijn labgenoten van 202 en kamergenoten van 156, 208 en 211b bedanken voor hun gezelligheid en de leuke discussies.

Ook wil ik mijn volleybalteamgenoten van WaHo niet vergeten. Het was en is heerlijk ontspannen met jullie tijdens inspannende trainingen en spannende wedstrijden. Bedankt!

De familie Bokkers wil ik graag bedanken voor hun gezelligheid en de rust die ik vond tijdens onze bezoekjes aan de Achterhoek. Inge, samen behoren wij tot de 'kouwe kant' van de familie, ik ben blij dat je mijn paranimf wilt zijn.

Pap, mam, Jan-Willem, bedankt voor jullie betrokkenheid en steun. Ondanks dat jullie misschien niet altijd precies begrepen waar ik mee bezig was, bleven jullie vragen en belangstelling tonen. Dank, dat jullie trots op me zijn.

En natuurlijk Eddie. Da ge bedankt zed, da witte!

Carline

Curriculum Vitae

Carline Brands werd geboren op 9 maart 1971 te Schaijk. In 1989 behaalde zij het VWO-diploma aan het Maasland College te Oss. In datzelfde jaar begon ze met de studie Levensmiddelentechnologie aan de toenmalige Landbouwuniversiteit te Wageningen. Onderdelen van deze studie waren de afstudeervakken Zuivelkunde en Fysische- en Kolloïdchemie en een stage uitgevoerd bij Coberco Dairies te Deventer. In 1995 studeerde zij af. Van april tot en met augustus 1996 was zij werkzaam als toegevoegd onderzoeker bij Stichting Hout Research (SHR) te Wageningen. Van september 1996 tot en met juni 2001 was zij aangesteld als assistent in opleiding (AIO) bij de leerstoelgroep Geïntegreerde Levensmiddelentechnologie, thans Productontwerpen en Kwaliteitskunde, van Wageningen Universiteit. Sinds juli 2001 is zij werkzaam als postdoc bij dezelfde leerstoelgroep op een project van Friesland Coberco Dairy Foods, getiteld 'Kinetic modelling of heat- and storage-induced chemical changes in orange juice'.

This project was financially supported by the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD program, CT96-1080, 'Optimisation of the Maillard Reaction. A Way to Improve Quality and Safety of Thermally Processed Foods'. The work was carried out at the Department of Agrotechnology and Food Sciences, Product Design and Quality Management Group, Wageningen University, The Netherlands.

Cover: Japanese maple leaf adapted by Eddie Bokkers en Carline Brands
Printing: Ponsen en Looijen, Wageningen